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**Invited Paper**

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# EFFECT OF *TRANS*-2-HEXENAL VAPOR PRETREATMENT ON ALLEVIATTION OF HEAT SHOCK IN TOMATO SEEDLINGS (MICRO TOM)

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## ABSTRACT

*Trans*-2-hexenal is a plant natural compound exhibiting a very safe, eco-friendly and strong antifungal activity. We investigated the effect of *trans*-2-hexenal in alleviating heat stress on tomato seedlings in trials conducted from November 28, 2016 to January 4, 2017 at the Tokyo University of Agriculture, Japan. Wilting in seedlings was alleviated through exposure to 0.001 or 0.010 ppm *trans*-2-hexenal, compared to untreated seedlings at 48°C. On the other hand, seedlings treated with 1.000 or 10.000 ppm *trans*-2-hexenal showed severe wilting. Electrolyte leakage and malondialdehyde (MDA) levels of heat-treated tomato leaves also showed alleviation from heat stress upon exposure to 0.001 ppm or 0.010 ppm *trans*-2-hexenal vapor. Results suggest that pre-treatment of tomato seedlings with *trans*-2-hexenal protected tomato seedlings from stress due to heat shock, indicating a possible practical application in tropical developing countries, as a cheap, energy-saving and environment friendly technique.

**Key words:** green leaf volatiles (GLVs), heat stress alleviation, natural compound

## INTRODUCTION

Tomato, considered as one of the most important vegetables in the world, is conserved daily as a source of nutrients, like sugars, organic and amino acids, polyphenols, folic acid, lycopene (carotenoid), vitamin C, as well as minerals (Toor et al. 2005, Vinson et al. 2001 Giovanelli et al. 1999, Van Duyn and Pivonka, 2000). World tomato production has reached up to 170 million tons (FAOSTAT, 2014).

In developing countries, there is positive correlation between vegetable consumption and price, and a negative correlation between vegetable price and morbidity rate (Bouis 1991). Accordingly, an insufficient vegetable supply might affect negatively human health, leading to increased infant mortality and abortion rate (Bouis 1991). In many Southeast Asian countries, productivity of main crops has increased recently in a drastic manner, shifting the focus of food problems from quantity to quality, such as the intake of micronutrients to promote health (Imai 1998). Thus, fresh vegetables, including tomato, attract much attention and a year round stable supply is desirable. In addition, a low cost and simple technique for tomato production under high temperature is particularly required.

High temperature is one of the serious causes for decreased productivity of a variety of crops (Boyer 1982, Hall and Anthony 2001). To counteract the effects of increased temperature, agricultural equipment, such as air control facilities, are used. However, these require high investments for their introduction, and/or running costs (Hara 2016). Thus, there is a need for a simple and low-cost technology to alleviate the effects of stress due to high temperature.

Recently, carbonyl compounds derived from peroxidized linolenic acid are recognized as important signals involved in the environmental stress response (Yamauchi et al. 2008, Mueller and Berger 2009). There is therefore a need for new agricultural technologies which could alleviate the environmental stress caused by such chemicals. Thus, we focused on the possibility of using *trans*-2-hexenal to alleviate the stress caused by heat on tomato. *trans*-2-Hexenal belongs to a group of C6 carbonyl compounds, known as green leaf volatiles (GLVs), which are generated from linolenic and linoleic acid. Generally occurring in nature, *trans*-2-hexenal is well-known in the signaling of plant stress response (Hatanaka and Harada 1973; Mano 2012). This study sought to investigate the effects of *trans*-2-hexenal in alleviating stress in tomato seedlings due to heat by focusing on ethylene production.

## MATERIALS AND METHODS

### Plant material, pre-treatment with *trans*-2-hexenal vapor, and heat treatment

Experiments were conducted on tomato seeds (*Solanum lycopersicum* L. cv. Micro tom) grown at the Tokyo University of Agriculture from November 28, 2016 to January 4 2017. These were sown from a moist urethane sponge and then transferred to an incubator (MIR-253; SANYO, Japan) set at 25°C in dark conditions. After germination, the seedlings were transferred to a greenhouse at the Tokyo University of Agriculture and grown until reaching its 4th true leaf stage. Upon reaching this stage, the seedlings were transplanted into rockwool (10 × 10 × 5 cm) and grown for 6 weeks. The plants were grown hydroponically in a commercial nutrient solution (OAT house, OAT Agrio Co., Ltd, Japan). The seedlings were then placed inside a 38-L desiccator and exposed to 0.001, 0.010, 0.100, 1.000 and 10.000 ppm of *trans*-2-hexenal vapor which was vaporized by dropping on a filter paper for 1 h prior to heat treatment (untreated seedlings served as controls). After which, the seedlings were incubated at 48°C for 90 mins, and indicators were investigated as described below.

### Ethylene production

To monitor the effect of the pre-treatment of *trans*-2-hexenal vapor on the time-dependent change of ethylene production upon heat treatment, a portable ethylene gas analyzer (CI-900FK, CID Bio-Science Inc., United State) was used. Heat-treated seedlings were placed in a 2L chamber set at 48°C and then ethylene concentration from the chamber was monitored every 20 minutes for 3 hours.

### Stomatal conductivity and leaf temperature

Stomatal conductivity and leaf temperature were measured from the leaf abaxial surface using a leaf porometer (SC-1, Decagon, United States) after heat treatment (48°C) for 2 h.

### Electrolyte leakage

Electrolyte leakage was determined by a conductivity method based on Lafuente et al. (1991). Tomato leaves were cut into discs (8 mm diam.) using a cork borer. The discs were soaked in a 2.0-mL tube filled with 1 mL de-ionized water. After incubation for 1 h at ambient temperature, the solution conductivity was measured using a portable electric conductivity meter (B-771, Horiba, Japan). The sample was then boiled at 100°C for 1h to allow maximum elution. Percentage electrolytes that originally diffused was calculated as follows:

$$\% \text{ electrolyte} = \frac{C1}{C2} \times 100,$$

where, C1 and C2 are solution conductivities before and after boiling, respectively.

### MDA concentration

MDA concentration was measured based on the method of Zhang et al. (2010). A tomato leaf sample (0.2 g) was soaked in 6 mL 15% trichloroacetic acid (TCA) containing 0.25% thiobarbituric acid (2 TBA) and then incubated at 30°C for 24 h in the dark. The soaked extraction solution was

measured the absorbance (A) at 532, 600, and 450 nm using a spectrophotometer (U-1100, Hitachi, Japan). The concentration of diffused MDA was calculated as follows:

$$\text{MDA concentration (nmol g-1FW)} = 6.45 \times (A_{532} \times A_{600}) \times 0.56 \times A_{450}$$

## RESULTS and DISCUSSION

### Visual observation

A concentration-dependent response was observed for tomato seedlings exposed to 0.001, 0.010, 0.100, 1.000 and 10.000 ppm of *trans*-2-hexenal vapor for 1 h and incubated at 48°C for 90 minutes. A concentration-dependent response was observed, Wilting of heat-treated seedlings was rather slight in seedlings pre-exposed to 0.001 and 0.010 ppm *trans*-2-hexenal vapor, compared to the control (no *trans*-2-hexenal vapor treatment 0 ppm). In contrast, seedlings exposed to 1 or 10 ppm *trans*-2-hexenal vapor were severely wilted.

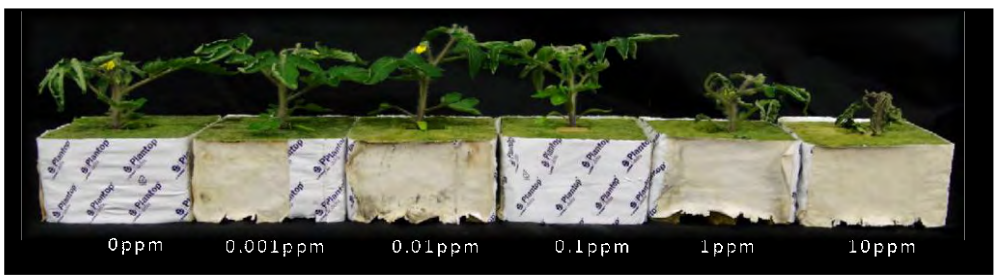


**Fig. 1.** *Trans*-2-hexenal vapor treatment on tomato 'Micro tom' seedlings. Tomato seedlings were exposed to 0.001, 0.010, 0.100, 1.000 and 10.000 ppm *trans*-2-hexenal vapor for 1 h and then incubated at 48°C for 90 mins.

### Ethylene production

Ethylene production of tomato seedlings treated with *trans*-2-hexenal vapor and incubated at 48°C are shown in Fig. 2. An increase in ethylene production was observed for all treatments, with seedlings exposed to 1 and 10 ppm *trans*-2-hexenal vapor produced a remarkable amount of ethylene compared to the control.

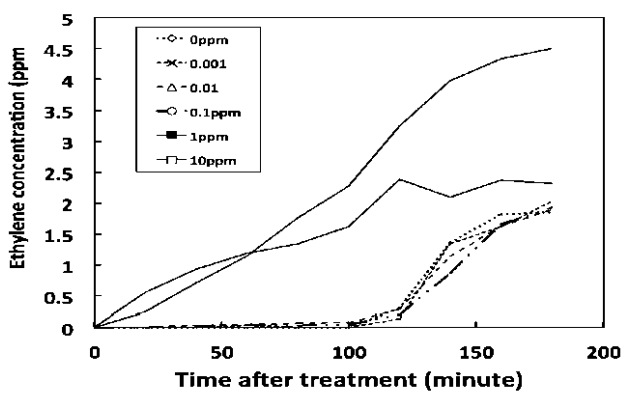


**Fig. 2.** Ethylene production from tomato 'Micro tom' seedlings pre-treated with *trans*-2-hexanal vapor at 48°C. Tomato seedlings were exposed to 0.001, 0.010, 0.100, 1.000 and 10 .000 ppm *trans*-2-hexenal vapor for 1 h and incubated at 48°C for 90 min. Tomato seedlings were kept in a 1-L plastic vessel and ethylene concentration was monitored every 20 min using a portable ethylene analyzer.

Ethylene is a gaseous plant hormone which sometimes has an important role in heat shock signaling (Abeles et al. 1992, Larkindale and Huang 2004, Jenks and Hasegawa 2005). In our case, the higher ethylene production from the severely-damaged tomato seedlings suggest a more sensitive response to strong heat shock.

**Leaf stomatal conductance and leaf temperature**

There was a variation in leaf stomatal conductance among seedlings exposed to *trans*-2-hexenal, depending on *trans*-2-hexenal concentration (Fig. 3). Seedlings exposed to 0.001 and 0.010 ppm *trans*-2-hexenal vapor exhibited a significantly greater stomatal conductance, while a significantly lower stomatal conductance was observed for seedlings treated with 10.000 ppm *trans*-2-hexenal. The temperature of seedling leaves for each treatment had no significant difference (data not shown). On peroxidase over produced tobacco, which wilt easily compared to a wild type, high level of stomatal conductance was reported (Lagrimini et al. 1990). Because our previous study showed that treatment with *trans*-2-hexenal reduces GSH levels in tomato fruit (Data not shown), wilting in tomato seedlings might be well attributed to a reduced cell turgor pressure (Bartling et al. 1993. *trans*-2-Hexenal induces abiotic stress tolerance, which is induced by several heat shock factors (HSFs) and heat shock proteins (HSPs) (Yamauchi et al. 2015).

**Leaf electrolyte leakage and MDA**

Electrolyte leakage of seedlings exposed to either 0.001 or 0.010 ppm *trans*-2-hexenal vapor was significantly lower than the control (Fig. 4). In contrast, seedlings exposed to 10.000 ppm *trans*-2-hexenal vapor had the highest electrolyte leakage, indicating the most severe cell membrane damage due to heat shock (Fig. 3). Heat stress was reported to cause cell membrane injury and membrane lipid peroxidation in many plant species (Van Rensburg and Krüger 1994; Gong et al. 1998; Liu and Huang 2000; Jiang and Huang 2001).

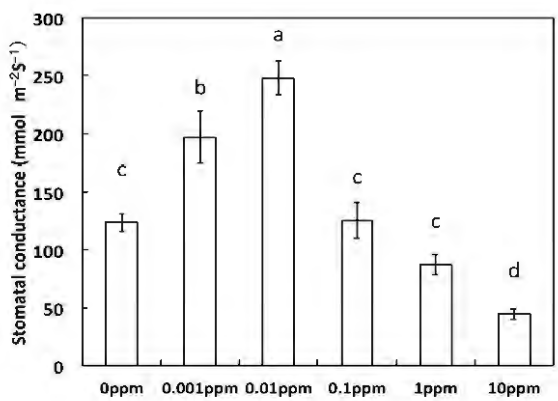


**Fig. 3.** Stomatal conductance of tomato 'Micro tom' seedlings pre-treated with *trans*-2-hexenal vapor at 48°C. Tomato seedlings were exposed to 0.001, 0.010, 0.100, 1.000 and 10.000 ppm *trans*-2-hexenal vapor for 1 h and then incubated for 90 min at 48°C. Stomatal conductance was measured with a leaf porometer. Bars represent means ± SE of three replicates. Treatments not sharing the same letter are significantly different based on Fisher's LSD test ($P < 0.05$)

The MDA content of seedlings exposed to either 0.001 or 0.010 ppm *trans*-2-hexenal vapor was significantly lower than the control (Fig. 5). On the other hand, MDA content of seedlings exposed to either 1.000 or 10.000 ppm *trans*-2-hexenal vapor was remarkably higher than the other treatments, showing the most severe level of membrane peroxidation (Fig. 4). MDA is a product derived from the

peroxidation of unsaturated fatty acids, such as phospholipids, and causes cell membrane damage (Halliwell and Gutteridge 2015).

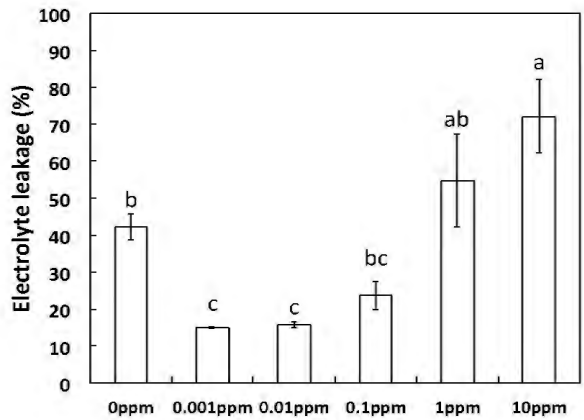


**Fig. 4.** Electrolyte leakage from tomato 'Micro tom' leaf discs pre-treated with *trans*-2-hexenal vapor at 48°C. Tomato seedlings were exposed to 0.001, 0.010, 0.100, 1.000 and 10.000 ppm *trans*-2-hexenal vapor for 1 h and then incubated at 48°C for 90 min. Leaf discs with a diameter of 1 cm were placed in 1 ml de-ionized water and electrolyte leakage was checked. Bars represent means ± SE of three replicates expressed as % electrolyte leakage against dead leaves. Treatments not sharing the same letter are significantly different based on Fisher's LSD test ($P < 0.05$).

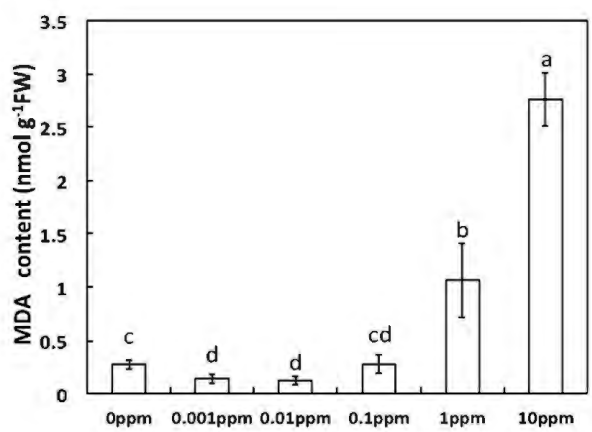


**Fig. 5.** MDA content of tomato 'Micro tom' seedlings pre-treated with *trans*-2-hexenal vapor at 48°C. Tomato seedlings were exposed to 0.001, 0.010, 0.100, 1.000 and 10.000 ppm *trans*-2-hexenal vapor for 1 h and then incubated at 48°C for 90 min. Bars represent means ± SE of three replicates expressed as % against dead leaves. Treatments not sharing the same letter are significantly different based Fisher's LSD test ($P < 0.05$).

## CONCLUSION

The pre-exposure treatment of tomato seedlings with either 0.001 or 0.010 ppm *trans*-2-hexenal could alleviate damage caused by heat shock. It can be useful for producers and consumers in

tropical countries, as pre-treatment could be applied as an energy-saving, environmentally friendly, and easy method of coping with high temperature conditions.

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# ISOLATION, CHARACTERIZATION AND RAPID SCREENING OF COPPER-TOLERANT CYANOBACTERIA CONSORTIA FROM MINING SITES AND A STRAWBERRY FARM IN BENGUET PROVINCE, PHILIPPINES

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## ABSTRACT

Cyanobacteria are one of the most diverse photosynthetic prokaryotes in aquatic and terrestrial habitats. In this study, cyanobacterial consortia from soil and rock samples of mining sites, a strawberry farm and the Wangal forest in Benguet province were collected in April 2016 and evaluated for their tolerance to different concentrations of copper ($Cu^{2+}$) at the Plant Genetics and Cyanobacterial Biotechnology Laboratory, Institute of Biology, University of the Philippines Diliman. Upon microscopic examination, the consortia consisted of 23 cyanobacteria, putatively identified as *Anacystis, Chroococcus, Gloeocapsa, Gloeothece, Microcystis*, *Pleurocapsa*, *Synechococcus, Synechocystis, Rhabdoderma, Chamaesiphon, Stichosiphon, Borzia, Oscillatoria, Phormidium, Romeria, Nostoc, Anabaena, Anabaenopsis, Calothrix, Cylindrospermum, Dolichospermum, Raphidiopsis* and *Rivularia*, and were found to thrive at 24°C to 34°C and pH from 5.4 to 8.7. The growth response of cyanobacteria consortia to increasing concentrations of $Cu^{2+}$ metal was investigated using microtiter plate readings as a rapid screening method. The cyanobacteria consortia from Philex mines exhibited maximum tolerance at 5.0 mg/L $Cu^{2+}$ concentration ($M_{5.0}$), while those from Balatoc mines, Antamok mines, a strawberry farm and Wangal forest were able to tolerate up to 6.0 mg/L $Cu^{2+}$ concentration ($M_{6.0}$). The cyanobacteria consortia may be used for bioremediation of wastewaters from industries where effluents have a high toxic metal load.

**Key words:** blue-green algae, copper ($Cu^{2+}$), heavy metal, metal tolerance, bioremediation

## INTRODUCTION

Cyanobacteria are free-living, primary producers that are found in both aquatic and terrestrial habitats, and are diverse in terms of morphology and physiology, with a superficial resemblance to green algae due to chlorophyll a pigments and phycocyanin. These occupy all possible ecological niches, due to their capability to adapt to a wide range of environmental conditions (Tandeau De Marsac and Houmard 1993). Their combined efficiency in capturing $CO_2$ and suitability for wastewater treatments and other industrial plants make them one of the most promising renewable sources for a fully sustainable environment (Singh and Singh 2014). Blue-green algae are important agricultural assets and significant fixers of atmospheric nitrogen (Moreira et al. 2013) and phosphorus. Some of them are proven agents for bioremediation (Ananya and Ahmad 2014). For instance, a cyanobacterial mat containing *Chlorella*, *Phormidium*, and *Oscillatoria* was designed to absorb hexavalent chromium (Balaji et al. 2016) and copper ($Cu^{2+}$) from wastewater (Chaturvedi et al.

2013). Jao (2004) isolated cadmium-tolerant and cadmium-absorbing cyanobacteria *Oscillatoria* sp. and *Chroococcus minutus*.

This study aimed to identify and examine cyanobacterial diversity in terrestrial ecosystems in the mining areas, agricultural farm and forest of Benguet province. This is also a pilot study to isolate metal-tolerant cyanobacteria upon exposure to copper and together with gold, which is also mined in the region. There is a need to screen the local cyanobacteria consortia that have great potential for sequestering heavy metals as these may be used as agents for bioremediation applications.

## MATERIALS AND METHODS

### Collection and sampling sites

Sampling was conducted in Tuba, Itogon, and Wangal, La Trinidad, Benguet Province (Table 1). The choice of mining areas was based on Soriano (2001), who reported a relatively high presence of heavy metal in soil and water, and Jao (2004) who screened cyanobacteria and microalgae isolated from the areas for bioremediation purposes. Both studies had implicated an extreme environment for cyanobacterial growth. Soil samples were also collected from a strawberry farm and a pristine forest in Wangal, La Trinidad, Benguet, which served as the positive control for cyanobacteria diversity. Cyanobacterial consortia were collected from rock crevices and soil in five areas within 100 m to 500 m range of the mining sites. Each study area was divided into three sampling sites. Around five rock crevices and 250 g soil particles were collected from each sampling site.

The physicochemical properties of soil, such as pH and temperature, were measured during daytime using a pH meter (Exstik®Extech PH100) and a temperature probe (Exstik®Extech EC400), respectively. Nitrogen, phosphorus, potassium (NPK) testing was also conducted as per the standard protocol of a Soil Test Kit (provided by the Bureau of Soils and Water Management, Department of Agriculture, Republic of the Philippines). The temperature of the soil at a depth of 8 to 12 cm was measured using soil thermometer (Taylor 5976N) in triplicate.

**Table 1.** Coordinates of sampling sites in Benguet Province, Philippines.

| Sampling Site | Global Positioning System (GPS) | |
|---|---|---|
| | North (N) | East (E) |
| Philex Mines, Tailing Pond System 1 (TPS1), Tuba | 16°28'15".3 | 120°65'82".9 |
| Philex Mines, Tailing Pond System 3 (TPS3), Tuba | 16°23'60".7 | 120°67'52".5 |
| Antamok Mines, Itogon | 16°40’13”.2 | 120°65’81”.8 |
| Balatoc Mines, Itogon | 16°37’09”.4 | 120°63’90”.2 |
| Strawberry Farm, La Trinidad | 16°45’34”.1 | 120°58’16”.0 |
| Wangal Forest, La Trinidad | 16°45’42”.3 | 120°56’40”.7 |

### Isolation and purification of cyanobacteria

Soil samples (10 g), with surface scrapings from rock samples, were collected from each sampling site and serially diluted using a standard dilution technique (Allen and Stein 1973). Soil sample was dissolved in an Erlenmeyer flask containing 95 ml 0.85% saline water, and was marked as $10^{-1}$dilution. A 10-ml sample was transferred from $10^{-1}$dilution to the next conical flask containing 90 ml saline water, marked as $10^{-2}$ dilution. This $10^{-2}$ dilution was used to prepare triplicates of 1 mL samples, which were transferred to three tubes containing 9 mL of commercially-mixed BG-11 medium (HIMEDIA Laboratories) each to produce 10 ml of culture. The three replicate tubes were

incubated at 30°C ± 2°C in a continuously illuminated chamber, 3000 lux, for two weeks (Kumar et al. 2013). The culture vessels were shaken manually at least twice a day to enhance aeration. Upon occurrence of growth, each was spread plated on sterile BG-11 plates. Several attempts were made to purify cyanobacteria consortia by subsequent streaking on BG-11 plates in order to separate each other.

The consortia were characterized morphologically under a student medical microscope RM-3 (Radical Scientific Equipments, Pvt. Ltd.) and cyanobacterial images were captured at 400× and 1000× total magnification. Morphological characters observed include presence or absence of sheath, shape and size of the vegetative cells, heterocysts, akinetes (if present), position, and branching pattern of the axenic cultures of cyanobacterial strains, as described by Desikachary (1959), Komarek et al. (2014), and Singh et al. (2013). Putative identification based on morphological characteristics was done using Bergey's Manual of Systematic Bacteriology (Garrity et al. 2001).

**Growth response of cyanobacteria consortia to different $Cu^{2+}$ concentrations**

A liquid-nutrient BG-11 medium was prepared by dissolving 1.627 g of commercially available BG-11 powder (HIMEDIA Laboratories) in 1 L distilled water, using 1 M NaOH or HCl to adjust the pH to 7.1. This BG-11 medium was used as a diluent for preparing varying concentrations of $Cu^{2+}$. A stock solution of 10 mg/L of copper was prepared from $CuSO_4 \cdot 5H_2O$, and were further diluted into increasing concentrations of $Cu^{2+}$ metal with 0 (control), 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg/L viz. $M_0$, $M_{0.1}$, $M_{0.5}$, $M_{1.0}$, $M_{2.0}$, $M_{3.0}$, $M_{4.0}$, $M_{5.0}$ and $M_{6.0}$.

The cyanobacteria consortia were transferred into liquid nutrient BG-11 medium treated with different $Cu^{2+}$ concentrations prepared in three (3) replicates. A rapid screening procedure was conducted by introducing 2000 µL of the consortia from each sampling site into 24-well microtiter plates. These were incubated at 30°C ± 2°C in a continuously illuminated chamber, 3000 lux and the optical densities (OD) were directly read from the plates using Epoch™ Multi-Volume Spectrophotometer System at 665 nm. The initial reading for all treatments was recorded and daily measurements were obtained every five days thereafter for a total of 40 days. Optical Density (OD) readings were converted to percentage change in growth and recorded as growth curves starting at the $Cu^{2+}$ concentrations of $M_{3.0}$ since preliminary experiments showed that the cyanobacteria consortia from mining sites, the strawberry farm and Wangal forest were able to tolerate $Cu^{2+}$ concentrations of up to $M_{2.0}$ (LRS Sanchez, unpublished data).

$$\text{Percentage change in growth} = (\text{current OD}/\ \text{initial OD}) - 1 \times 100$$

Growth data were analyzed statistically using SPSS (Statistical Package for Social Sciences) v20. One-way repeated-measures analysis of variance (ANOVA) and post-hoc Tukey's HSD test were used in determining significant differences between and among the variable factors used in this study.

## RESULTS AND DISCUSSION

**Physicochemical properties of soil**

The soil environment in Benguet Province was slightly acidic, as well as alkaline, with pH ranging from 5.4 to 8.7. The soil temperature ranged from 24°C to 34°C. NPK testing revealed that the pristine forest in Wangal site had the highest NPK content (Table 2). The high N content in the forest floor of this highly steep pristine forest is an indicator of effective N mineralization in the area, and that the soil microorganisms present are important regulators of biogeochemical cycles in forest soils (Ishizuka 1992, Rennenberg and Dannenmann 2015).

On the other hand, the strawberry farm in La Trinidad, Benguet has high N, sufficient K and

deficient P content. The usual N fertilizer used includes urea (46% N), ammonium nitrate (34% N), ammonium sulfate (21% N, 24% S), potassium nitrate (13% N), and calcium nitrate (15% N) (Ullio 2010). Urea is needed in large amounts because it is applied during the early flowering stage to improve the size of fruit. Farm growers had already applied nitrogen fertilizer during testing, since it was already summer post-harvest when growth for new leaf and runner is needed to reestablish planting vigor for the next year's crop. The high N soil content may also be attributed to a pre-plant plastic multi-application that might have helped in the preservation of N that was mineralized in the soil when strawberry roots were not fully developed (Muramoto et al. 2004). Unlike N, strawberries do not have a high P requirement. Phosphorus is essential for energy management from new root formation up to fruit development. Phosphorous was deficient for plant roots, as a function of the pH of soil solution at 5.4, since the testing was done after the harvest season. K is sufficiently found in the soils, which is required in relatively high amounts to prevent excessive transpiration, and for resistance against microbial diseases.

All six sites had sufficient K content; however, the mining sites had varying amounts from low to relatively high N and P. The deficiency of these minerals in soil accounts for a non-conducive growth for plants considering that these are regularly disturbed areas.

**Table 2.** Physicochemical properties of soil samples collected in Benguet Province, Philippines.

| Sampling Site | Description | Temp | Nutrients | | | pH |
|---|---|---|---|---|---|---|
| | | (°C) | N | P | K | |
| Philex Mines, Tailing Pond System 1 (TPS1) | Re-vegetated site | 29 | Low | Low | Sufficient | 8.1 |
| Philex Mines, Tailing Pond System 3 (TPS3) | Active site | 34 | Low | Deficient | Sufficient | 8.7 |
| Antamok Mines | Small-scale mining site | 29 | Low | Low | Sufficient | 7.1 |
| Balatoc Mines | Abandoned site | 32 | Low | High | Sufficient | 6.8 |
| Strawberry Farm | Agricultural site | 26 | High | Deficient | Sufficient | 5.4 |
| Wangal Forest | Pristine forest | 24 | High | High | Sufficient | 5.4 |

**Isolation and morphological characterization of cyanobacteria**

Microscopic examination revealed the composition and morphology of the consortia consisting of 23 putatively identified cyanobacteria belonging to Subsections Chroococcales, Oscillatoriales, and Nostocales. The number of genera comprising a consortium for each sampling site is shown in Table 3.

Subsection Chroococcales was found in all sampling sites. The unicellular, colonial, non-heterocystous cells appeared to be hemispherical to spherical, spherical to ovoid. Cells appear singly, in 2's or in 3's, blue-green in color and were putatively identified as *Anacystis, Chroococcus, Gloeocapsa, Gloeothece, Microcystis, Pleurocapsa, Synechococcus, Synechocystis, Rhabdoderma, Chamaesiphon,* and *Stichosiphon* (Desikachary 1959, Rippka et al. 1979, Castenholz 1989) (Fig. 1, A to L). *Chroococcus, Gloeocapsa,* and *Gloeothece* were found to be present in all sampling sites, while *Rhabdoderma* was found to be present only in mining sites probably due to a higher temperature requirement (Wehr et al. 2015) ranging from 29°C to 32°C. There were fewer Chroococcales in the Philex mining sites that may be attributed to a deficient or lower P content in the soil.

**Table 3.** Putatively identified cyanobacteria from consortia in each sampling site.

| Sampling Site | Total number of putatively identified genera | Putative Identification of Cyanobacteria in Consortia Subsection | | |
|---|---|---|---|---|
| | | **Chroococcales** | **Oscillatoriales** | **Nostocales** |
| Philex Mines, TPS1 | 10 | *Chroococcus, Gloeocapsa, Gloethece, Rhabdoderma* | *Oscillatoria, Phormidium* | *Nostoc, Anabaena, Anabaenopsis, Dolichospermum* |
| Philex Mines, TPS3 | 9 | *Chroococcus, Gloeocapsa, Gloethece, Anacystis* | *Oscillatoria, Phormidium* | *Nostoc, Anabaena, Anabaenopsis* |
| Antamok Mines | 15 | *Chroococcus, ChamaesiphonRhabdoderma, Stichosiphon, Synechococcus, Synechocystis* | *Oscillatoria, Phormidium, Borzia, Romeria* | *Nostoc, Anabaena, Anabaenopsis, Calothrix, Rivularia* |
| Balatoc Mines | 15 | *Chroococcus, Gloeocapsa, Gloethece, Rhabdoderma, Chamaesiphon, Stichosiphon* | *Oscillatoria, Phormidium, Borzia, Romeria* | *Nostoc, Anabaena, Anabaenopsis, Dolichospermum, Raphidiopsis* |
| Strawberry Farm | 14 | *Chroococcus, Gloeocapsa, Gloethece, Anacystis, Pleurocapsa, Stichosiphon, Synechococcus, Synechocystis* | *Oscillatoria, Borzia, Romeria* | *Nostoc, Anabaena, Anabaenopsis* |
| Wangal Forest | 18 | *Chroococcus, Gloeocapsa, Gloethece, Chamaesiphon, Microcystis, Pleurocapsa, Stichosiphon, Synechococcus, Synechocystis* | *Oscillatoria, Borzia* | *Nostoc, Anabaena, Anabaenopsis, Dolichospermum, Cylindrospermum ,Calothrix, Rivularia* |

Subsection Oscillatoriales was also observed in all sampling sites (Fig. 1, M to P). Four (4) representative cyanobacteria from consortia were putatively identified as *Borzia, Oscillatoria, Phormidium* and *Romeria*. *Oscillatoria* was found in all sites, while *Phormidium* is found only in mining sites probably due to its higher soil pH requirements ranging from 6.8 to 8.1 (Wehr et al. 2015). Subsection Nostocales revealed eight representative cyanobacteria from consortia (Fig. 1, Q to X). The putatively identified *Nostoc* has a thallus body composed of vegetative cells of uniform diameter, subspherical to spherical, blue-green in color with intercalary heterocysts. The akinetes are spherical and are formed in between heterocysts. The other filamentous cyanobacteria from the consortia have trichomes that are uniformly broad throughout, tapered or untapered with conspicuous constrictions. The trichomes may be straight or curved with elongated or spherical blue-green vegetative cells. The akinetes are spherical and green or yellowish in color. Intercalary heterocysts are spherical, while the terminal heterocysts are oval. These isolates, putatively identified as *Anabaena, Anabaenopsis, Calothrix, Cylidrospermum, Dolichospermum, Raphidiopsis*, and *Rivularia*. *Nostoc*, *Anabaen*a, and *Anabaenopsis*, were found in all sites. There were fewer Nostocales in Philex mines probably due to the higher alkaline condition of the soil ranging from pH 8.1 to 8.7.

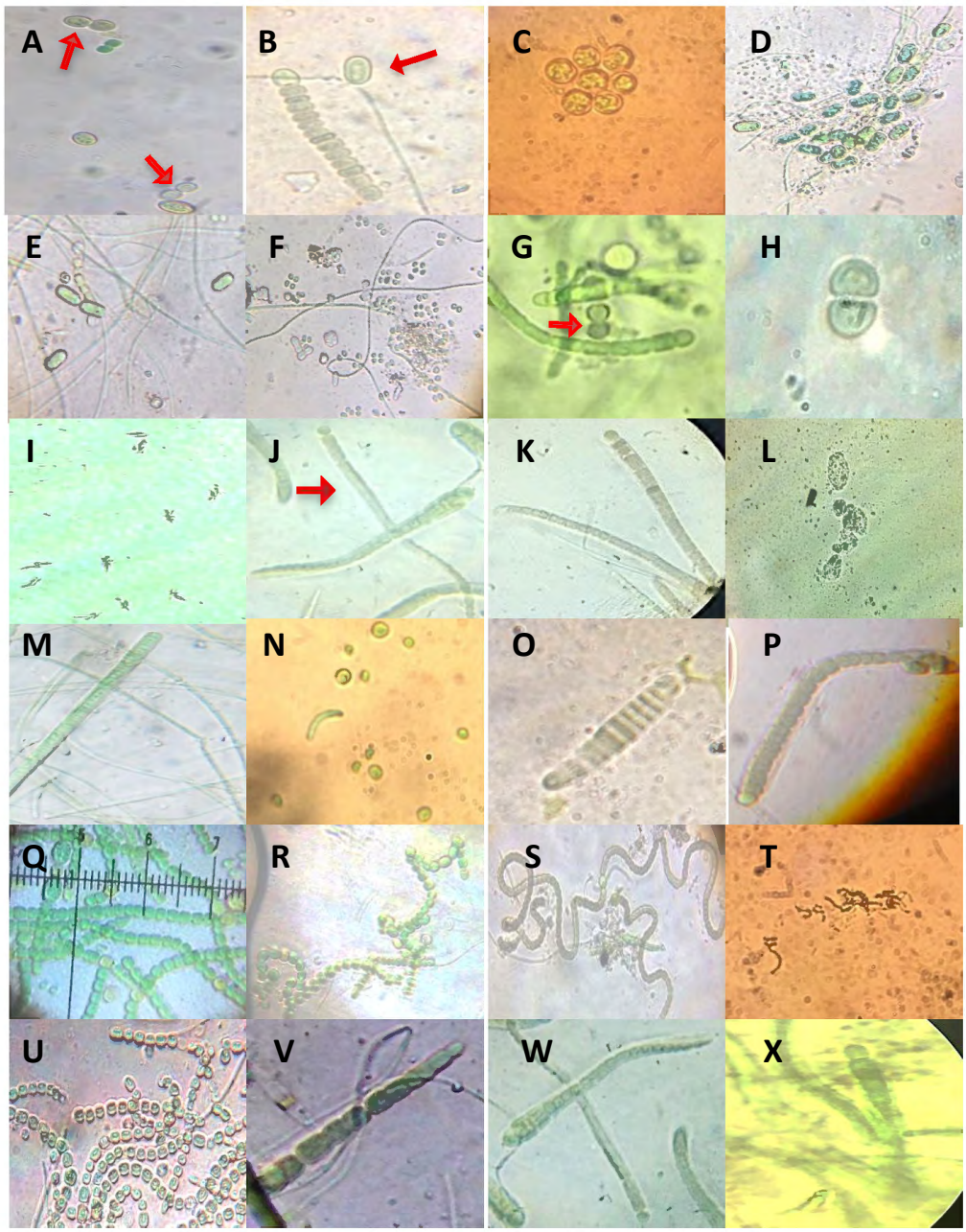


**Fig. 1.** Cyanobacteria consortia isolated in extreme soils of Benguet putatively identified as A. *Chroococcus* (red arrows), B. *Anacystis* (red arrow), C. *Microcystis*, D. *Gloeocapsa*, E. *Gloethece*, F. *Pleurocapsa*. G. *Synechococcus*, (red arrow), H. *Synechocystis*, I. *Rhabdoderma* J. *Stichosiphon* (red arrow), K. *Chamaesiphon*, L. unidentified colonies, M. *Oscillatoria*, N. *Romeria*, O. *Borzia*, P. *Phormidium*, Q. *Nostoc*, R. *Anabaena*, S. *Anabaenopsis*, T. *Raphidiopsis* U. D*olichospermum*, V. *Calothrix*, W. Rivularia, X. *Cylindrospermum*. Scale: 20 µm.

**Growth response of terrestrial cyanobacterial consortia to different copper concentrations**

A total of 23 distinct putatively identified cyanobacteria comprising consortia were exposed to increasing $Cu^{2+}$ metal concentrations. Both $Cu^{2+}$-treated and untreated consortia ($M_0$) had a percentage increase in growth throughout the 40-day observation period. The growth behavior of cyanobacteria consortia from Philex mines Tailing Pond System 1 and Tailing Pond System 3 showed the highest growth percentage increase after 35 and 30 days of incubation in all $Cu^{2+}$ concentrations, respectively (Fig. 2, A and B). All consortia showed a lag of about 5 days and seemed to continue until day 10 for $Cu^{2+}$-treated consortia, with an exponential growth rate at 15 days and the stationary phase starting at day 21. The non-$Cu^{2+}$treated consortia ($M_0$) remained fairly constant, with very slow growth. The consortia were able to survive up to $M_{5.0}$ $Cu^{2+}$ concentrations for both TPS1 and TPS3. However, a faster growth in the latter may be attributed to the presence of more unicellular genera comprising the consortia, hence capable of faster reproduction via cell division or colony reproduction. The percentage growth of the consortia at $M_{6.0}$ $Cu^{2+}$ concentrations started to decline from day 21, but minimal growth can be observed from day 31 onwards. This may be attributed to the next cycle or generation of cyanobacteria that were still capable of undergoing reproduction.

The growth behavior of cyanobacteria consortia from Antamok and Balatoc mines for all $Cu^{2+}$ concentrations showed the highest growth percentage increase at 25 days (Fig. 2C and 3A). All consortia showed a lag at about 3-5 days for $Cu^{2+}$-treated consortia, with exponential growth rate at 10-15 days, and the stationary phase starting at day 21. The growth of consortia from Antamok mines at $M_0Cu^{2+}$ concentration was fairly constant compared to a relatively slow growth of consortia from Balatoc mines. Consortia from Antamok and Balatoc mines were able to withstand $M_{6.0}$ $Cu^{2+}$ concentrations and exhibited maximum tolerance to $M_{5.0}$ and $M_{3.0}$ $Cu^{2+}$ concentrations, respectively.

Theoretically, the chances of finding metal-tolerant cyanobacteria are higher when populations are exposed to metals, such as in mining areas. The extraction process employed by mines is not 100% efficient, leaving some of the metals within the water contained in the tailing ponds. Therefore, it is expected that populations of cyanobacteria from and near the tailing pond systems have adapted to high metal concentrations, such as *Microcoleus vaginatus* (Phormidiaceae) and *Phormidium* sp. isolated in the tailings of a gold mine in South Africa (Orlekowsky et al. 2013).

The growth behavior of cyanobacteria consortia from the strawberry farm in all $Cu^{2+}$ concentrations exhibited a lag of about 5 days (Fig. 2B). It seemed to continue until day 12 for $Cu^{2+}$-treated consortia, with an exponential growth rate at 15 days and the stationary phase starting at day 21. The growth response of the consortia at $M_{5.0}$ $Cu^{2+}$ concentration showed the highest increase in percentage growth similar to that of $M_0$. However, consortia were also able to withstand up to $M_{6.0}$ $Cu^{2+}$ concentration.

Cyanobacteria consortia from Wangal forest showed a growth response with the usual lag phase of 5 days, exponential growth rate at 15 days and the stationary phase, which usually starts on the 17$^{th}$ day (Fig. 3C). Tukey's HSD test confirmed that the consortia at $M_{6.0}$, $M_{5.0}$ and $M_{4.0}$ $Cu^{2+}$ concentrations showed the highest increase in percentage growth. These were able to tolerate increasing $Cu^{2+}$ concentrations evidently every 20 days and exhibited a higher maximum tolerance at $M_{6.0}$. The growth response was significantly different from the mining sites and strawberry farm since Wangal forest is a pristine area where there were more indigenous cyanobacteria, around 3 to 6 genera comprising the consortia. Cyanobacteria consortia from Wangal forest were composed of several unicellular genera and a few filamentous strains that follow the usual 21-day life cycle of the blue-green algae. This could probably account for a mechanism of tolerance depending on the effect of the initial strength of copper to various consortia and the extent of heterogeneity of the initial cyanobacterial population (Shavyrina et al. 2001).

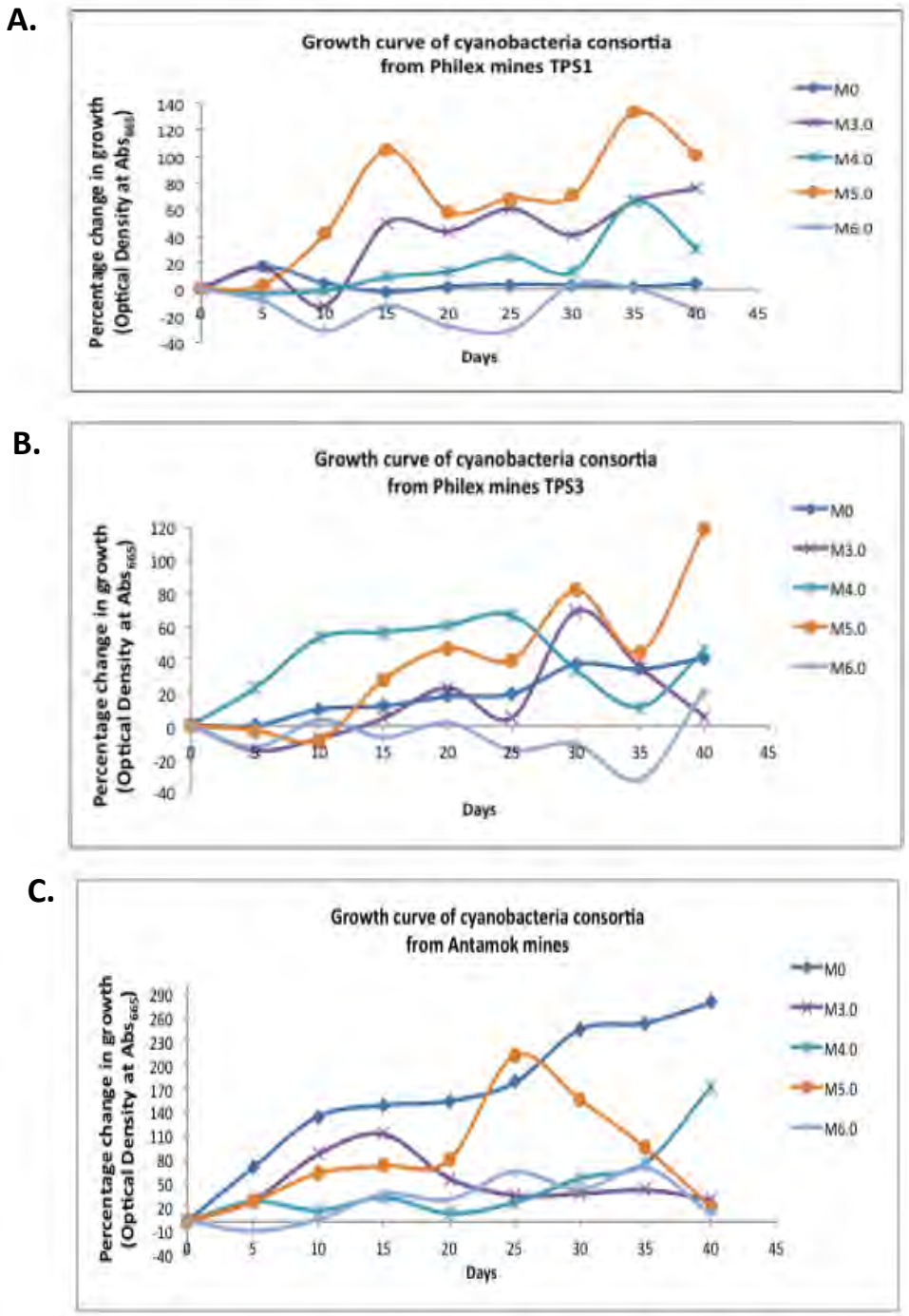


**Fig. 2.** Percentage change in the growth of cyanobacteria consortia from (A) Philex mines TPS1, (B) Philex mines TPS3, and (C) Antamok mines in terms of optical density at Absorbance 665 nm. Growth curves represent the response of cyanobacteria consortia at $M_0$, $M_{3.0}$, $M_{4.0}$, $M_{5.0}$ and $M_{6.0}$ copper concentrations.

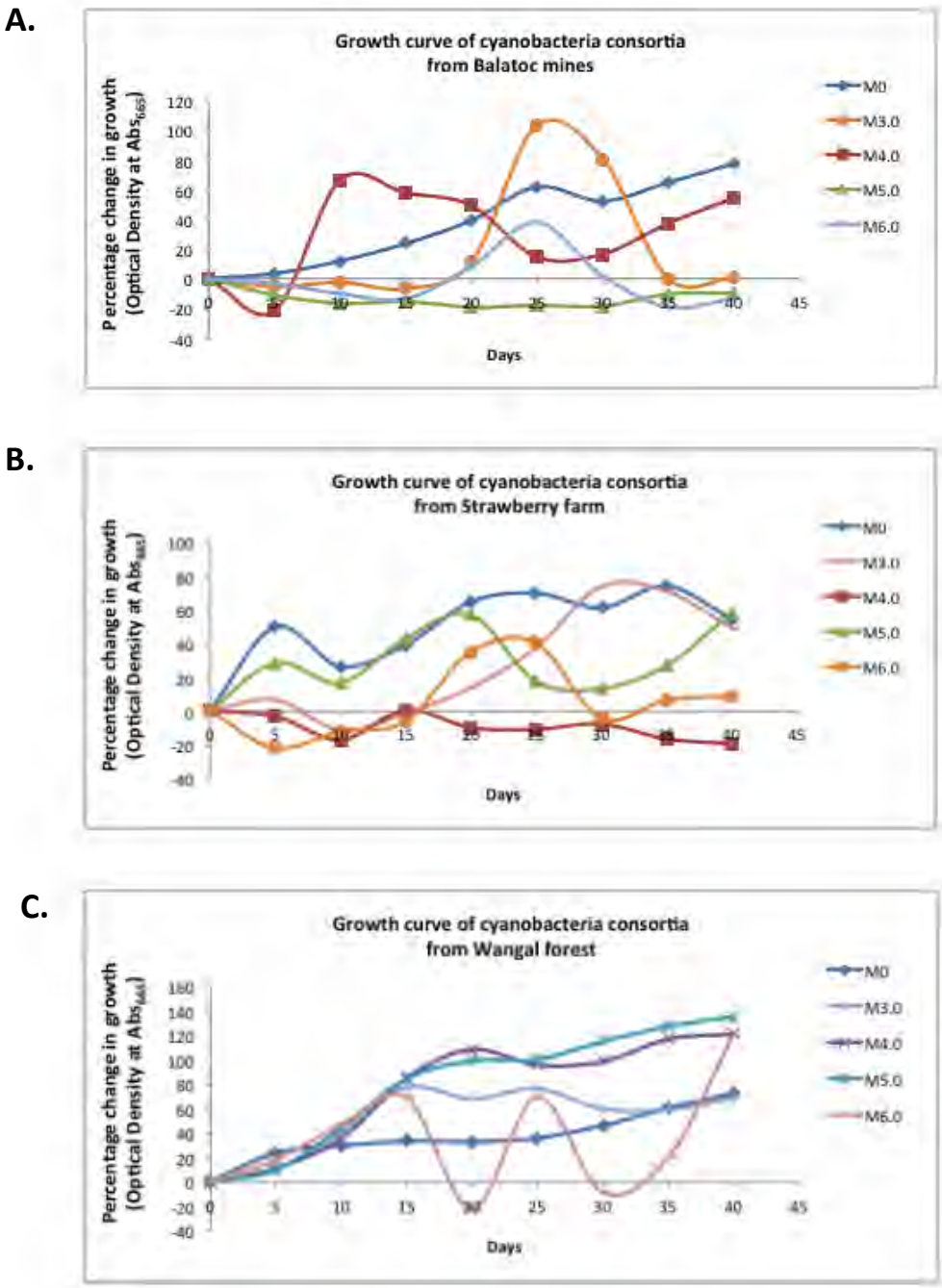


**Fig. 3.** Graph showing percentage change in the growth of cyanobacteria consortia from (A) Balatoc mines, (B) Strawberry farm, and (C) Wangal forest in terms of optical density at Absorbance 665 nm. Growth curves represent the response of cyanobacteria consortia at $M_0$, $M_{3.0}$, $M_{4.0}$, $M_{5.0}$ and $M_{6.0}$ copper concentrations.

All cyanobacteria consortia observed had a wide range of tolerance to increasing $Cu^{2+}$ concentrations. The cultures remained alive even beyond the 40-day observation with microscopic examination revealing very bright green cells. This likely resistance to $Cu^{2+}$ can be attributed to

several factors. The presence of $Ca^{2+}$ and $Zn^{2+}$ found in BG-11 may have contributed to the reduction of $Cu^{2+}$ uptake since these ions have been reported to stabilize cell wall integrity (Loveria 1999), and binding to proteins (Waldron and Robinson 2009). $Cu^{2+}$ also has a stimulatory effect on the consortia that probably stimulated tolerance to $Cu^{2+}$, particularly, in the case of representative cyanobacteria putatively identified as *Chroococcus*, *Synechococcus*, *Synechocystis*, and *Microcystis* which have been reported to show increased tolerance to $Cu^{2+}$ (Gupta 1992, Jao 2004).

*Oscillatoria* has been shown to rapidly adsorb $Cu^{2+}$ from aqueous solution and is effective in the removal of the metal from mine water (Ahuja et al. 1997). It cannot be ruled out that some metal ion binding may take place inside the cell in the same way as to the exterior of the cell (Khummongkol et al. 1982). When metals like $Cu^{2+}$ are taken inside the cells, it may be converted to nontoxic forms by binding or precipitation. Once inside the cells, $Cu^{2+}$ may be removed by efflux or compartmentalized within specific membrane-bound organelles (Jao 2004). A study on *Lyngbya putealis* isolated from a metal-contaminated site revealed an increase in photosynthetic pigments, biomass production, starch, and carbohydrates, with increasing cobalt and $Cu^{2+}$ concentration up to 0.5 mg/L in single metal systems (Kiran and Thanasekara 2011). Heterocyst-forming cyanobacteria, like *Anabaena*, is on high demand for $Cu^{2+}$ as a cofactor for oxygenic photosynthesis and nitrogen fixation (Nicolaisen et al. 2010). Several *Gloethece* were also observed in the soils of Benguet province that were previously reported to produce essential attributes for removing heavy metals, such as $Cu^{2+}$ and $Pb^{2+}$. The simultaneous presence of these two metals caused a mutual inhibition in the adsorption of each metal (Pereira et al. 2011).

The abovementioned homeostatic mechanisms would probably explain the wide tolerance of all cyanobacteria isolates to different $Cu^{2+}$ concentrations.

## CONCLUSION

Twenty-three putatively identified genera were observed comprising the cyanobacteria consortia through morphological characterization. This study reveals that cyanobacteria consortia from soils of mining sites, a strawberry farm and a pristine forest of Benguet province exhibit wide tolerance to varying $Cu^{2+}$ metal concentrations and are able to grow up to 40 days in normal culture condition. Cyanobacteria consortia from Antamok mines, Balatoc mines, strawberry farm and Wangal forest were able to tolerate up to $M_{6.0}$ $Cu^{2+}$ concentration indicating high tolerance compared to consortia found in Philex mining sites that can only tolerate up to $M_{5.0}$ $Cu^{2+}$ concentration. Copper metal tolerance of cyanobacteria strains present in Benguet province provides a tremendous potential for bioremediation of wastewaters of different industries, where the effluents have a high load of toxic metals. These cyanobacteria can be cultured as putative candidate sources for bioremediation.

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# MORPHOLOGICAL CHARACTERIZATION OF WILD *Rhynchostylis gigantea* IN THAILAND

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## ABSTRACT

The diversity of 49 living wild *Rhynchostylis gigantea* accessions collected from 22 locations from six regions throughout Thailand were morphologically characterized. The 42 characters, consisting of 12 quantitative and 30 qualitative characters were analyzed at Kasetsart University, Kamphaeng Saen Campus, Thailand, from 2012-2015. The quantitative characters mainly consisted of flower characters; i.e. size of flower, sepals and petals. Thirty qualitative characters were phenotypic characters of the whole plant that were characterized individually by the modified DOA Test Guidelines of Vanda and Vanda Hybrid Descriptor. Only 34 characters (22 qualitative and 12 quantitative traits) could be used for multivariate and clustering analysis. The orchid accessions could be grouped into two groups based on the geography of Thailand, with the members of the first group located in the low-land and the second group in the mountainous area.

**Key words:** orchid, diversity, characterization, morphology, clustering analysis

## INTRODUCTION

Inhabitant orchid diversity is now a crisis worldwide due to several constraints, such as a lack of social awareness, over-exploitation, deforestation and urbanization (Koopowitz et al. 2003). In Thailand, Thitiprasert et al. (2007) reported that at least 175 orchid species are endangered, including orchid species of the genus *Rhynchostylis*. Under the tribe Vandeae, sub tribe Aeridinae, and family Orchidaceae (Dressler 1993), the genus *Rhynchostylis* is a small genus consisting of only four species. Three species, *R. gigantea*, *R. retusa* and *R. coelestis* are reported as endemic in southeast Asian countries, such as Thailand, Laos PDR, and Myanmar. Among the three species, *R. gigantea* is the best known. Being considered as an inhabitant endangered species, the commercially grown genus *Rhynchostylis;* however, has a high commercial value used for orchid export in Thailand due to available *in vitro* seed germination. In terms of the number of orchids exported from Thailand, as reported by Plant Varieties Protection Division, Ministry of Agriculture, Thailand, commercial hybrids of *R. gigantea* is in the highest demand compared with the other native orchid species. Artificial pollination of the rare alba, rubra forms and the regular form of this species, together with the success of *in vitro* seed germination, gave a wide array of flower colors that catch the eyes of consumers, thus bringing out a high exports per year. To maintain a balance between in-habitat protection and commercialization of this orchid species, knowledge of inhabitant diversity is necessary.

To determine *R. gigantea* diversity, morphological characterization has always been the first attempt due to its simplicity. Examples of orchids investigated on morphological variability include populations of *Epipactis helleborin* (Ehlers et al. 2002), epiphytic orchid species on a small oceanic island (Mallet et al. 2014), and the variance among populations of tropical orchid with a restricted gene flow (Tremblay 1997). Evolutionary studies based on morphological characterization were also reported in the subtribe Aeridina (Hidayat et al. 2006) and in *Liparis resupinata* (Tetsana et al. 2014).

However, for wild *R. gigantea*, a diversity study has never been reported. In this study, we investigated the morphological variation of 49 living wild *R. gigantea* collected throughout the floristic regions of Thailand. The number of twelve quantitative and twenty-two qualitative characters were characterized in order to understand the correlation and distribution of this species in Thai natural habitats.

## MATERIALS AND METHODS

### Plant samples

Forty-nine living wild *R. gigantea* were collected mainly from their primary natural habitat in Thailand, together with a secondary collection from plants grown in the Horticultural Research Center, Department of Agriculture, Ministry of Agriculture (DOA, Thailand), wherein their natural habitats were confirmed. The collected samples were grouped into 5 populations (Table 1), based on the floristic region of Thailand, as described by Smitinand (1958). These populations were the North (N), North-eastern (NE), East (E), South-western (SW), and Peninsular (PEN) populations. A group collected from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) rescue centers under DOA, Thailand was also included, and thus denoted as a group with 'uncertain origin'. The outgroup samples were *R. retusa* and *R. coelestis*, since they belong to the same genus. All collected plants were maintained under a 50% shaded greenhouse at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand, from 2012-2015.

### Morphological characterization

The number of 42 qualitative and quantitative characters, including 8 characters from stems and leaves, 2 characters of inflorescence, and 32 characters of flowers, were characterized. Each accession was evaluated individually by the modified DOA Test Guidelines of Vanda and Vanda Hybrid Descriptor (http://www.doa.go.th/ pvp/images/stories/ form_dus/27%20rb_vanda.pdf).

### Qualitative traits characterization

The 30 qualitative characters from both vegetative and reproductive parts were characterized.

***Vegetative characters*:** Vegetative characters were evaluated during the first year the sample was collected. These characters included leaf shape (assessed by measuring the ratio of the length and width of the leaf), leaf apices, leaf cross section, angle at the top of leaf, twisting of the leaf apex, leaf margins, leaf variegation on the abaxial side, and color of the leaf base.

***Floral characters:*** Due to the habit of 'once a year' flowering during December and February for this species, the floral characters, hence, were obtained during 2 flowering seasons, one from 2012-2013 and another from 2013-2014. Three flowers from the top, center, and bottom positions of an inflorescence indicated by arrows in the Fig.1A were characterized. The qualitative traits characterized included flower arrangement, inflorescence orientation, flower form, shape of dorsal sepal, shape of dorsal sepal apiece, cross section of dorsal sepal, twisting of dorsal sepal, wave in dorsal sepal, blushing on dorsal sepal, shape of lateral sepal, shape of lateral sepal apiece, cross section of lateral sepal, twisting of lateral sepal, wave in lateral sepal, blushing on lateral sepal, shape of petal, shape of petal apiece, cross section of petal, twisting of petal, wave in petal, blushing on petal, and lip character.

## RESULTS AND DISCUSSION

Morphological variance of wild type *R. gigantea* in Thailand was determined and characterized through the modified Vanda descriptor. However, as the plant samples were collected from various natural habitats (Table 1), they were not only varied in age and size, but also some other phenotypes.

**Table 1.** Samples used for this study and the climatic conditions of their origin.

| Provincial code | Region[1/] / Province | | Sample size | Climatic zone [2/] |
|---|---|---|---|---|
| 1 | N | Chiang Rai | 2 | T1-H2 |
| 2 | N | Chiang Mai | 1 | T1-H1 |
| 3 | N | Lamphun | 6 | T1-H1 |
| 4 | N | Tak | 3 | T1-H1 |
| 5 | N | Sukothai | 1 | T2-H1 |
| 6 | N | Phitsanulok | 3 | T2-H1 |
| 7 | N | Kamphaeng Phet | 2 | T2-H1 |
| 8 | NE | Loei | 3 | T1-H1 |
| 9 | NE | Khon Kaen | 3 | T2-H1 |
| 10 | NE | Sakon Nakhon | 6 | T2-H2 |
| 11 | NE | Nakhon Phanom | 2* | T1-H2 |
| 12 | NE | Mukdahan | 1 | T2-H2 |
| 14 | NE | Ubon Ratchathani | 3* | T2-H2 |
| 15 | E | Sri Sa Ket | 2 | T2-H2 |
| 17 | E | Nakhon Ratchasima | 1 | T2-H1 |
| 18 | SW | Kanchanaburi | 3** | T2-H2 |
| 19 | SW | Ratchaburi | 1 | T3-H2 |
| 20 | SW | Petchaburi | 1 | T3-H3 |
| 21 | SW | Prachuap Kriri Khan | 1* | T3-H3 |
| 22 | PEN | Chumphon | 4 | T3-H3 |

[1/] The abbreviation of region: North (N), North-eastern (NE), East (E), South-western (SW), and Peninsular (PEN).

[2/] The climatic zone of Thailand according to Khedari et al. (2002) was divided into 3 zones based on temperature, with, T1=12-38 ºC, T2=16-38 ºC, and T3=20-38ºC, and into 4 zones based on relative humidity with, H1=30-100%, H2=41=100%, H3=50-100% and H4=59-100%.

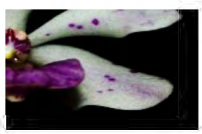
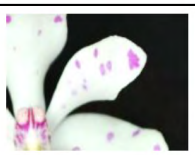
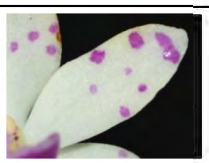
* The sample from CITES rescue center that may have another origin elsewhere.

** Two samples from Kanchanaburi and one sample from CITES rescue center.

Phenotypes that were highly correlated with physiological parameters were explained by Zotz et al. (2001), including plant height, leaf color, leaf texture, length of inflorescence, and number of flowers in an inflorescence. Therefore, in this report, these characters were disregarded and thus, 42 characters (8 characters from stems and leaves, 2 characters based on inflorescence, and 32 floral characters) were characterized. Among these 42 characters, 30 were qualitative while 12 were quantitative. The selected qualitative characters are listed in Table 2.

**Table 2.** Vegetative and floral characteristics of *Rhynchostylis gigantea* modified from DOA's Vanda descriptor.

| Characteristics | Code | Scales of Characterization |
|---|---|---|
| Leaf shape | T1 | (1) average of L/W ratio =3-6:1 (2) average of L/W ratio =7-10:1 (3) average of L/W ratio =>12:1 (4) average of L/W ratio = 21:1 |
| Leaf apices | T2 |  (0) Acute (1) Oblique (2) Emerginate (3) Praemorse (4) Tridenlate |
| Cross section of leaf | T3 |  (0) Flat (1) Carinate |
| Angle at the top of leaf | T4 |  (0) <90º (1) 90º (2) >90º |
| Twisting of leaf apex | T5 |   (0) Absent (1) Present |
| Leaf margins | T6 |   (0) Entire (1) Undulate/Wavy |
| Inflorescence orientation | T10 |    (1) Erect (1) Horizontal (2) Pendulous |
| Flower form | T11 |  Incurved  (1) Semi-incurved  (2) Flat |

| | | | | | |
|---|---|---|---|---|---|
| Shape of sepal and petal apices | T13, T19, T25 |  Acute | (1) Obtuse  | (2) Cuspidate  | (3) Orbicular  |
| Cross section of sepal and petal | T14, T26 |  Margin incurved | |  (1) Margin | |
| Blushing on dorsal sepal | T17 | (1) Absent  | | (1) Present  | |
| Cross section of sepal | T20 |  Assemetry | |  (1) Opposite | |
| Wavy lateral sepal | Absent T22 |  | (1) Present |  | |
| Blushing on lateral sepal | T23 (0) Absent |  | (1) Present |  | |
| Twisting of petal | (0) Absent T27 |  | (1) Present |  | |
| Wave in petal | T28 (0) Absent |  | (1) Present |  | |

***Variance analysis of quantitative characters***

All quantitative characters fell in the size of flower. Flowers were sampled from 3 different positions; namely from the top, middle and bottom, of the inflorescence (Fig. 1A). Twelve quantitative characters of wild *R. gigantea* flower and floral component were measured. The characters of each flower that were measured are in Fig.1B. Multivariate Analysis of Variance (MANOVA) analysis showed highly significant differences among regions ($P< 0.01$) (data not

shown). Morphological variance is assumed to be the result of diverse environmental conditions and/or habitat heterogeneity (Wagner et al. 2015).

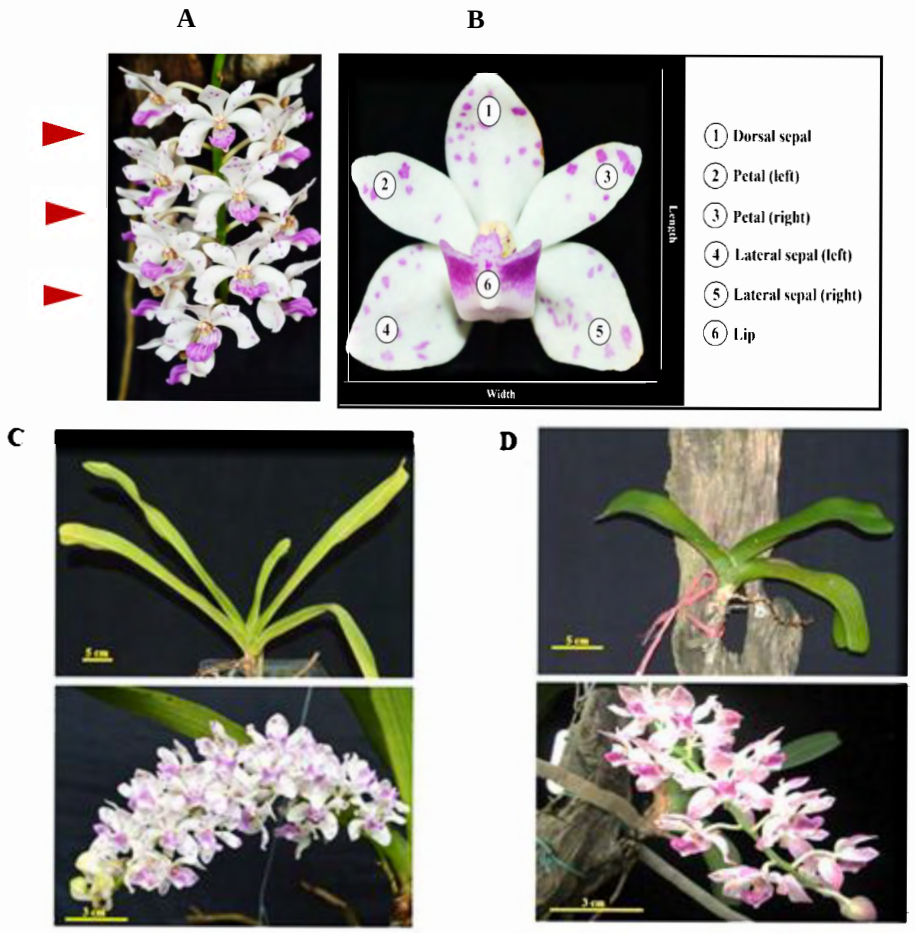



**Fig. 1.** Wild *Rhynchostylis gigantea* distributed in Thailand. The position of an inflorescence of collected flowers for morphological characterization (A) and flower structure (B). The characteristic of the stem, inflorescence and floral representative of group I (C) and group II (D) according to UPGMA analysis in Fig. 3.

Analysis of variance for each trait according to natural habitat showed high significant difference in floral size and dorsal sepal width ($P<0.05$) (Table 3). Sample from northern and south-western regions had the largest floral size and dorsal sepal width relative to samples from other regions. Whilst, smaller floral size in width was present from samples from the peninsular region. A narrow dorsal sepal was present in accessions collected from the Eastern and peninsular regions. This indicated that *R. gigantea* accessions, based on the population studied, had a low amount of variability in terms of size of floral-part characteristics.

The low variability in these samples may be due to a narrow genetic base of *R. gigantea* samples collected, based on morphological characteristics. Variation among *R. gigantea* accessions, however, was highly observed from 22 qualitatively floral-characteristics out of the 30 morphological

floral traits (flower arrangement, inflorescence orientation, flower form, shape of dorsal sepal apices, cross section of dorsal sepal, blushing on dorsal sepal, shape of lateral sepal apices, cross section of lateral sepal, wave in lateral sepal, blushing on lateral sepal, shape of petal apices, twisting of petal, wave in petal, blushing on petal, and lip characteristics). A similar finding in terms of the high variation of floral characteristics was also reported by Wongsa et al. (2013) in a *R. gigantea* population.

**Table 3.** Mean value (mm), standard error, and *P-value* of ANOVA based on 12 quantitative traits from 49 *Rhynchostylis gigantea* accessions.

| Character[2/] | | Location of Origin[1/] | | | | | *P-value* |
|---|---|---|---|---|---|---|---|
| | | **N** | **NE** | **E** | **SW** | **PEN** | |
| FS | W | 28.5±0.4 [a] | 28.±0.5 [a] | 27.4±0.9 [a] | 29.0±0.7 [a] | 24.4±0.6 [b] | 0.0004 |
| | L | 24.6±0.4 [a] | 23.7±0.4 [ab] | 21.9±0.2 [bc] | 24.0±0.7 [a] | 21.4±0.9 [c] | 0.0028 |
| DS | W | 9.5±0.1 [a] | 9.0±0.2 [ab] | 8.7±0.2 [b] | 9.5±0.2 [a] | 8.9±0.4 [ab] | 0.0307 |
| | L | 14.8±0.3 | 14.7±0.3 | 13.7±0.4 | 14.2±0.5 | 13.6±0.6 | 0.1515 |
| LSL | W | 9.3±0.1 | 9.1±0.2 | 9.0±0.2 | 9.2±0.2 | 8.5±0.3 | 0.2543 |
| | L | 14.8±0.2 | 14.8±0.3 | 13.8±0.4 | 14.8±0.4 | 13.7±0.4 | 0.1853 |
| LSR | W | 9.3±0.2 | 9.1±0.2 | 9.0±0.2 | 9.0±0.5 | 8.5±0.4 | 0.3392 |
| | L | 14.7±0.2 | 14.7±0.3 | 13.6±0.4 | 14.6±0.5 | 13.6±0.5 | 0.0739 |
| PL | W | 5.3±0.1 | 5.4±0.2 | 5.1±0.2 | 5.4±0.1 | 4.9±0.3 | 0.3506 |
| | L | 13.6±0.2 | 14.1±0.3 | 12.9±0.3 | 13.7±0.5 | 13.5±0.5 | 0.3292 |
| PR | W | 5.1±0.1 | 5.3±0.2 | 5.0±0.2 | 5.5±0.2 | 4.7±0.3 | 0.2310 |
| | L | 13.7±0.2 | 14.3±0.3 | 13.2±0.2 | 13.9±0.4 | 13.5±0.5 | 0.2442 |

[1/] Abbreviations of location of origin: N=North, NE = North-eastern, E=East, SW=South-western, PEN=Peninsular, and U=Uncertain origin.

[2/] Abbreviations of floral characters: FS=Flower size, DS=Dorsal sepal, LSL=Lateral sepal (left), LSR=Lateral sepal (right), PL=Petal (left), PR=Petal (right), W=Width of flower size, and L=Length of flower size.

***Evaluation of quantitative characters***

Characterization of 30 qualitative characters revealed that 22 of them were polymorphic while the rest were monomorphic. The number of 8 qualitative characters that were monomorphic consisted of 1) shape of dorsal sepal, 2) twisting of dorsal sepal, 3) wave in dorsal sepal, 4) shape of lateral sepal, 5) twisting of lateral sepal, 6) shape of petal, 7) cross section of petal and 8) blushing on petal. Therefore, only 22 qualitative traits and 12 quantitative traits from 49 *R. gigantea* samples were subjected to principal component analysis and cluster analysis.

***Principle Component Analysis (PCA)***

PCA is a technique that identifies the traits contributing most on the variation within a group of varieties or genotypes. Complex data are transformed from a number of relative traits into a smaller number of variable as PCs. The PCA shows that the first four components with an eigenvalue greater than 1.0 contributed about to 80.75% of the total variation (Table 4). PC1, accounted for 59.56% of the total variation, was mostly determined by the negative value of all quantitative characters and a positive value for leaf shape, leaf apices, and cross section of dorsal sepal. The highest loadings in PC1 indicated the importance of this component. These traits carried the largest portion of its variability. PC2, contributed 10.60% of the total variation, was mostly determined by a positive value of quantitative traits and a qualitative trait, such as twisting of leaf apex, leaf margin, shape of lateral sepal, petal and cross section of dorsal sepal. PC3 accounted for 5.65%, and was mostly determined by a positive value of floral sepal length, lip character, twisting of petal, and a negative value of sepal and petal width, leaf apex, and leaf margin characteristics.

The correlation coefficient between any two traits is approximated by a cosine of the angle between their vectors (Dehghani et al. 2008). A strong positive association among LSLW, LSRW, DSW, SLL, SLW and SRW; among LSRL, DSL, and LSLL among T3, T10, T11, T17, T22, T23, T27, T28 and T30; between T5 and T6, and among T1, T2, T8, T9, T14 and T19 (Table 2.) was shown in Fig. 2, as indicated by the small obtuse angles between their vectors. There was a negative correlation between groups T3 and T1, as indicated by the angle of approximately 180 degrees.

**Table 4.** The Eigenvalues, proportion of variation, and cumulative variations across the axis of the first ten principal components.

| Principal component | Eigen value | Variation (%) | Cumulative variation (%) |
|---|---|---|---|
| 1 | 20.24 | 59.56 | 59.56 |
| 2 | 3.60 | 10.60 | 70.17 |
| 3 | 1.92 | 5.65 | 75.82 |
| 4 | 1.68 | 4.93 | 80.75 |
| 5 | 0.99 | 2.91 | 83.66 |
| 6 | 0.77 | 2.25 | 85.91 |
| 7 | 0.60 | 1.77 | 87.69 |
| 8 | 0.47 | 1.39 | 89.07 |
| 9 | 0.45 | 1.33 | 90.40 |
| 10 | 0.39 | 1.16 | 91.56 |

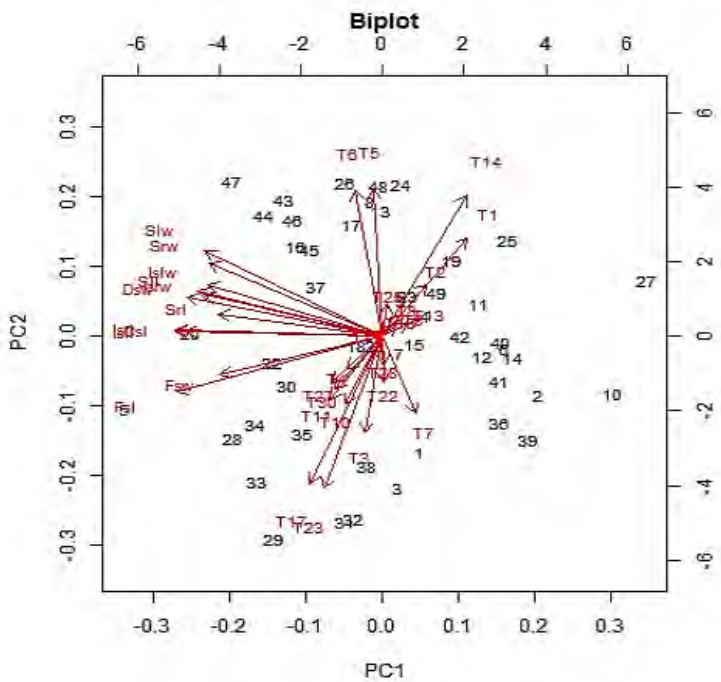



**Fig. 2.** Two dimensions of PCA analysis showing a relation among 49 accessions of wild *Rhynchostylis gigantea* collected in Thailand. Data from 22 qualitative and 12 quantitative characters were analyzed. The symbols (T..) are qualitative traits as explained in Table 2.

***Clustering analysis using Unweighted Pair Group Method with Arithmetic Mean (UPGMA)***

Clustering analysis was performed on 49 wild *R. gigantea* accessions through an UPGMA approach. *R. retusa* and *R. colestis* were used as the outgroup samples. *R. retusa* and *R. colestis* clearly separated from the ingroup samples of *R. gigantea* (Fig. 3). This was in agreement with the morphological classification of this genus by Seidenfaden and Wood (1992) and Eng-Soon (2005).

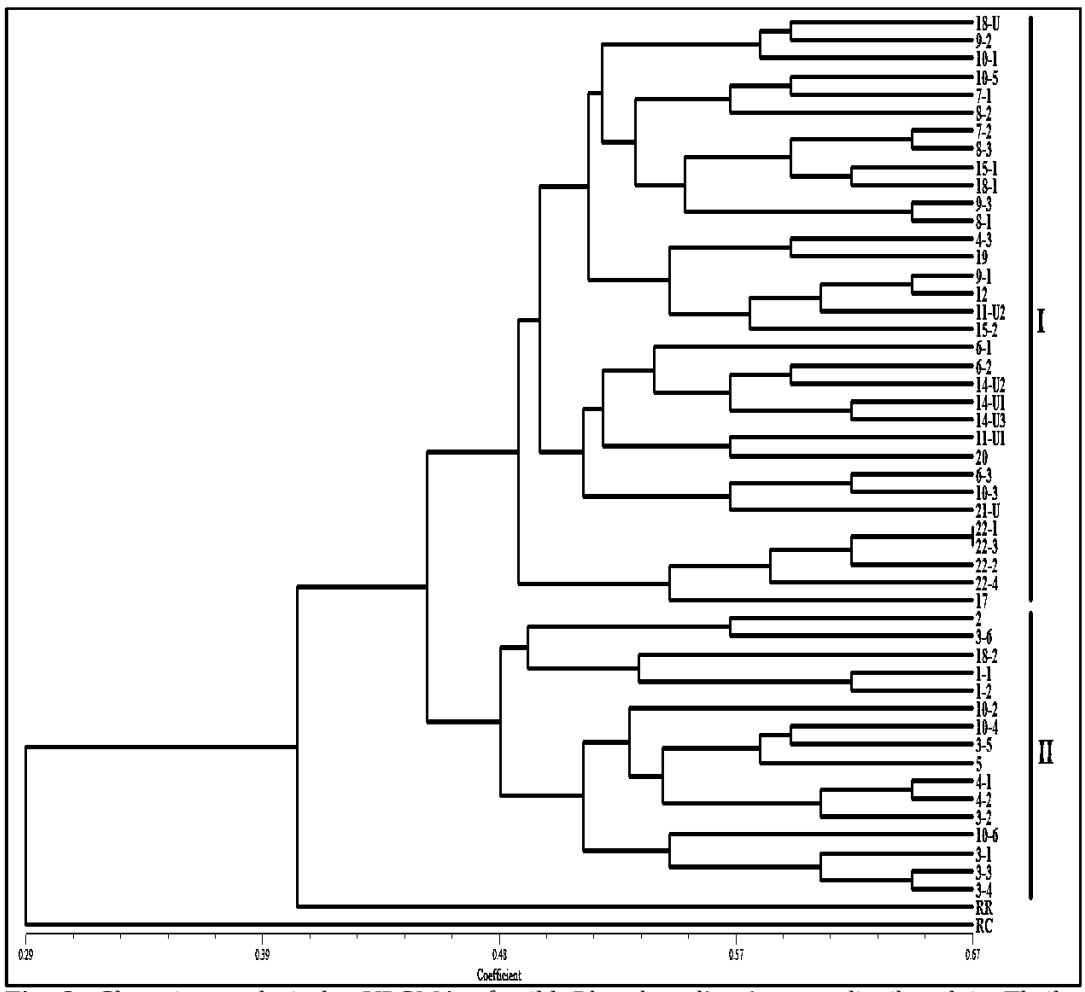



**Fig. 3.** Clustering analysis by UPGMA of wild *Rhynchostylis gigantea* distributed in Thailand generated by all characters.

For the ingroup accessions of wild *R. gigantea*, results revealed that the coefficient similarity was between 0.286 and 0.667. They were divided into two groups (Fig. 2A), corresponding to the geography of Thailand (Fig. 4), rather than the provincial location of their origin. The first group (Fig.1C) consisted of 33 accessions from different geographic regions, except the Northern region. The random pattern of distribution of accessions indicated no association between diversity of morphological trait and geography. The characteristics of this group included a leaf ratio of greater than 7:1 (97%) and a flat leaf cross section (76%). Inflorescence orientation observed were pendulous (82%) and horizontal (18%). No pink blushing was observed on the dorsal sepal (85%), lateral sepal (94%), and petals (100%). This group is widely distributed in the low-land areas of Thailand (Fig. 4).

The second group (Fig. 1D) consisted of 16 accessions from the Northern mountainous area of Thailand, including Chiang Rai, Chiang Mai, Lamphun, and Sukhothai provinces. The characteristics of this group, distinguished from the first group, include a leaf ratio of 3-7:1 (100%), craniate shape of leaf cross section (81.3%), non-twisting leaf apex (87.5%), pendulous inflorescence orientation (97.7%), pink blushing on the dorsal sepal (93.7%) and lateral sepal (87.5%) (Fig. 4).

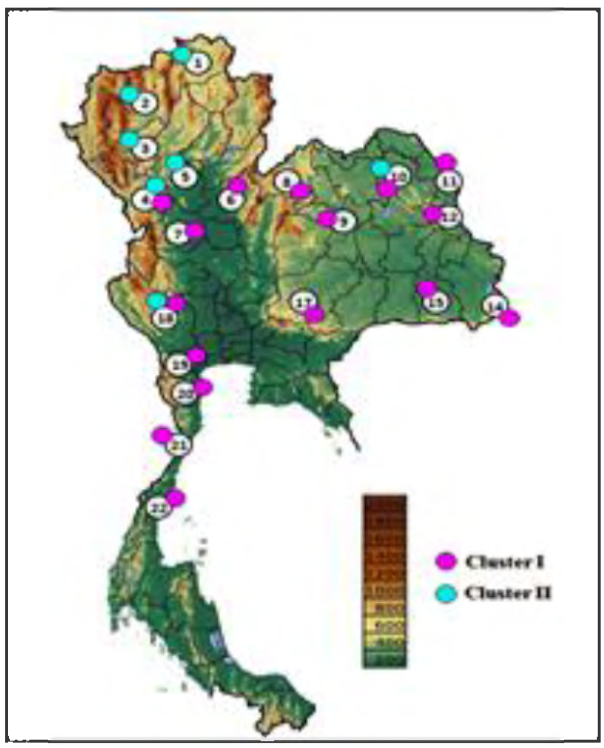



**Fig. 4.** The geographic maps of Thailand in correlation with the grouping of accessions.

In 2002, Khedari et al. discussed the climatic area of Thailand, which associated the altitude gradient and climate condition. The correlation revealed that the mountainous area has a lower ambient temperature and relative humidity than the lowland area (Table 1 and Fig. 4). Differences in environmental conditions, temperature, relative humidity, light, and precipitation are important factors influencing plant physiological performance (Zotz et al. 2001), which affects morphological variation in orchid species (Morales et al. 2010; Blinova 2012). In many orchid species, the environmental gradient was reported as a contributor to phenotypic variation, such as *Dactylorhiza hatagirea* (Warghat et al. 2012) and *Tolumnia variegate* (Morales et al. 2010). In their natural habitat, epiphytic plants encounter severe environmental stress, such as intense light and water shortage. Some orchid species utilize the Crassulacean Acid Metabolism (CAM) as a system for survival under severe environmental conditions. Some species have evolved to change their physiological process in order to adapt to their environment (Adiban and Ainuddin 2011). As a result, structures, such as shoots, leaf number and area, have been altered for survival (Blinova 2012; Willems and Ellers 1996).

Tremblay (1997) and Morales et al. (2010) reported that not only the macroclimate, but also the microclimate, influence morphological variation. In terms of natural distribution, *R. gigantea* is a vascular epiphyte which grows on their host trees, and are distributed throughout deciduous forests at an average of 200-1000 m above sea level and in coastal forests on limestone habitat (Pridgeon et al. 2014). Hoverer, an epiphyte-host interaction was also reported as associated with morphological variation (Callaway et al. 2002). Host trees facilitate not only to support epiphytes but also provide

habitat. Host structure mainly influences variability in epiphyte performance. Different host traits, such as whole tree architecture, bark texture, bark chemical, leaf density, and host age, as well as substrate stability, microclimate, nutrition support, and toxicity (Wagner et al. 2015) contribute to the morphological variation of several orchid species, such as *Liparis resupinata* (Tetsana et al. 2014), *Caladenia catenata* (Morrison and Weston 1985), *Epidendrum* and *Tolumnia variegate* (Pinheiro and Cozzolino 2013). Moreover, season is another factor affecting morphological variation in epiphytes (Einzmann et al. 2014; Ackerman et al. 2011). These factors could therefore explain differences in *R. gigantea* population distribution in Thailand.

Genetic diversity associated with altitude gradient has been described in many plant species (Ohsawa and Ide 2008), such as *Cattley liliputana,* (Leles et al. 2015). In the contrast, Mallet et al. (2014) reported that genetic variation of an epiphytic orchid species, *Jumellea rossii*, was affected by habitat heterogeneity rather than by geographic distance. However, a diversity study on *R. retusa* in India revealed genetic similarity with altitude gradient (Parab and Krishnan 2008).

Considering the accessions obtained from CITES rescue centers, morphological characters were not grouped according to the locations of CITES rescue centers. For example, the accession 18-U collected from the rescue center at Kachanaburi (SW region) clustered with the same subgroup of *R. gigantea* accessions from Khon Kaen (9-2) and Sakon Nakorn (10-1), which were collected from the provinces in the North-eastern region of Thailand with a distance of 500-700 kilometers. Similar results were also found for other accessions collected from other CITES rescue centers as well. This may be due mainly to the illegal relocation of wild species by the vendors, which were reported were from neighboring countries (Phelps 2015). The trade network route between point of harvest and final market place was complex and might be transferred through several intermediary areas.

## CONCLUSION

The diversity of *Rhynchostylis gigantea* accessions collected from 22 locations across five regions of Thailand was investigated. From a total of 42 morphological characters (30 qualitative and 12 quantitative traits), only 34 characters (22 qualitative and 12 quantitative traits) were used for multivariate and clustering analysis. The orchid accessions were grouped into two based on the geographical area of Thailand. The first group is found in the low-land while the second group is found on the mountainous area. These results suggest that diverse environmental conditions and/or habitat heterogeneity cause phenotypic variation. However, based on these morphological characteristics genetic diversity of *R. gigantea* was low, therefore, molecular analysis should be employed to obtain further information.

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# CURRENT CONDITIONS AND PROFITABILITY OF THE NUTMEG INDUSTRY IN BOGOR REGENCY, INDONESIA

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## ABSTRACT

The Indonesian nutmeg industry has developed extensively from the processing of nutmeg fruit to various types of products. Despite of its growing demand, the nutmeg industry is facing severe quality problems in both raw material and oil products. This paper aimed to clarify and analyze the current conditions and profitability of the nutmeg industry, to examine the importance of nutmeg processing and the prospects of nutmeg industry for rural development in Indonesia. Primary data was collected in 2012 and 2015 through an interview survey of nutmeg producers in Bogor Regency, an important production, processing and distribution market of nutmeg products located near Jakarta, Indonesia. Data analysis focused on 18 selected respondents from three groups of producers: farmers, sweets and oil producers. Profitability analysis revealed that nutmeg cultivation enabled farmers to earn a family income of 10 million IDR per person on an average, despite conducting neither cultivation management nor processing. Processing nutmeg products has brought a higher income and profit. The values added per one-kilogram nutmeg fruit increased by as much as two and by 23 times, when processed into nutmeg sweets and nutmeg oil, respectively. However, the survey revealed that a lack of management during every stage of cultivation and processing, which has led to serious problems, such as shortage of raw material, product rejection, and extreme price fluctuation. Therefore, an awareness of product quality and safety must be emphasized to all concerned parties in order to sustain the nutmeg industry in Bogor Regency.

**Key words**: processing, production cost, quality management

## INTRODUCTION

Since its emergence in late 1512 (Budavari et al. 1996), nutmeg (*Myristica fragrans* Houtt.) production in Indonesia has developed widely from nutmeg fruit production to the processing of various nutmeg products. Nutmeg spices, sweets, and oil are the most common products across the country. In the perspective of a home industry business, nutmeg production has provided job opportunities for locals, particularly for women or housewives working in nutmeg cultivation and/or processing of nutmeg food products. Therefore, the nutmeg industry has become an important source of income for locals. In 2014, Indonesia has exported 11,469 tons of nutmeg, with a total value of more than 80 million USD (UN Data 2014). Moreover, Indonesia supplied 80% of the world's total demand for nutmeg oil in 2015, making the country the largest producer and exporter of nutmeg in the world. Nutmeg oil has been widely used as an essential ingredient in many manufacturing industries related to food, beverages, cosmetics, and pharmaceuticals. With a high demand from these industries, nutmeg oil is currently the country's most expensive nutmeg product, with a price increase of at least 5 times from 2006 to 2014 (Kumar 2016).

However, despite the increasing price and growing demand, the nutmeg industry in Indonesia is facing severe quality control problems for both raw material and oil products. The Netherlands' Centre for the Promotion of Imports from developing countries stated that an import restriction has been issued towards Indonesian nutmeg, due to the occurrence of fungal infection and aflatoxin contamination, mainly on spices and oil products, which are the most traded nutmeg commodities to the international market (CBI 2013). With tons of rejected nutmeg spices, producers and exporters are required to provide a proof of quality check. This issue has made the domestic production and sales of nutmeg unstable, and has created a significant impact on producers. Therefore, it is important to clarify the current state of nutmeg production, as well as its impact on the nutmeg producers in Indonesia. The lack of development in quality control may lead to a loss in competitiveness of Indonesian nutmeg (Besar 2003). Thus, studies were conducted on the identification of nutmeg phytochemical and contaminant content, and on developing methods for improving nutmeg quality (Muchtaridi et al. 2010, Dharmaputra et al. 2015). Studies focusing on the social and economic aspects of the nutmeg industry in Indonesia are limited. Wahyudi and Indrawanto (1995) studied the price of Indonesian nutmeg in the international market, and showed that price was determined by the amount of its export and stock quantities. Moreover, its bargaining position was rather weak due to a surplus in export quantities. The size of cropping land and number of productive trees are the most important factors influencing profitability (Indrawanto and Yuliono 1997). In the past 20 years, nutmeg production and sales in Indonesia have changed completely. The recent development of nutmeg oil has affected its current production and price. The growth of the nutmeg processing industry seems to provide better options for increasing farmer's income.

This study sought to clarify the current conditions and potential of the nutmeg industry, in consideration of recent quality problems and extreme fluctuations in price and sales of nutmeg products in Indonesia. Specifically, this paper aimed to analyze the profitability of the nutmeg industry, to examine the economic importance of nutmeg processing, and to determine the prospects for rural development involving nutmeg production in Indonesia.

## METHODOLOGY

### Study area

In 2014, nutmeg production in Indonesia covers several locations throughout the archipelago, including a total cultivated area of 158,326 hectares (Agricultural Information System and Data Center 2016). One of the important production sites for nutmeg is Bogor Regency, West Java Province. Cultivation areas are centered mainly in the surrounding mountainous areas of Mt. Salak, Mt. Gede, and Mt. Pangrango. With its good proximity and direct access to Jakarta, Bogor Regency is not only is a key production area, but also plays a strategic role as the center for processing and distribution market for many nutmeg products. Despite this, nutmeg production in Bogor Regency is facing severe problems regarding its sustainability. Therefore, Bogor Regency was chosen as the study area for this paper.

### Data collection and selection

Primary data was collected in 2012 and 2015 by interview surveys of several different groups of nutmeg producers in Bogor Regency. The interview survey in 2012 was conducted on 26 producers, through the assistance of the Indonesian Agency for Agricultural Research and Development, while the rest were introduced by those who had been interviewed. It focused on the following: (1) production characteristics, (2) cost and sales conditions, (3) income and profitability, and (4) current problems and future prospects. Due to the recent problems and changes in the industry, the second interview survey was carried out in 2015 on selected respondents in order to further analyze and determine the current condition of nutmeg cultivation and processing in Bogor Regency.

**Data analysis**

Based on the 26 respondents, five groups of nutmeg producers were identified, namely Group 1 (cultivators only), Group 2 (sweets processors only), Group 3 (cultivators and sweets processors), Group 4 (oil processors), and Group 5 (cultivators and oil processors). Out of the five groups, a number of farmers were selected from Group 1 (nine farmers), Group 3 (four sweets producers), and Group 5 (five oil producers), based on the background of a typical nutmeg farmer.

## PROFILE OF THE SELECTED RESPONDENTS

Generally, the important characteristics of the selected respondents were their educational background and level of personal skills (Table 1). Three of the five nutmeg oil producers have at least graduated from high school, while only two nutmeg farmers and no nutmeg sweets producer has graduated from high school. Nutmeg oil producers tend to have a higher educational background than the others. The nutmeg oil producers had more skills and abilities in production management and processing technology. This highlights the need for oil producers to have a certain level of skill and education to operate distillation machines and use modern technology during the production process.

**Table 1.** Profile of the selected nutmeg producers.

| Items | Nutmeg Farmers | Nutmeg Sweets Producers | Nutmeg Oil Producers |
|---|---|---|---|
| Number of respondents [persons] | 9 | 4 | 5 |
| Sex (M : F) [persons] | 5 : 4 | 1 : 3 | 4 : 1 |
| Average age [years] | 44.1 | 59.0 | 47.2 |
| Average number of years engaged in the nutmeg industry [years] | 17.2 | 36.5 | 11.6 |
| High school graduate [%] | 22.2 | 0 | 60.0 |
| Personal skills score [points] | 5.2 | 6.8 | 8.6 |

Source: Survey data in 2012

Note: The survey respondents were questioned about following personal skills and abilities, and given 1 point for each skill and ability of the respondents: a) Able to write and read, b) Able to speak Indonesian, c) Owns a radio, d) Owns a television, e) Owns a phone or a cellphone, f) Owns a computer, g) Able to use internet, h) Able to use a type-writer, i) Able to use computer software, j) Has some managerial planning skill, k) Has accounting skills, l) Understands the plantation system, m) Understands harvesting skills, n) Understands processing skills, o) Has attended technological training.

## PROFITABILITY OF THE NUTMEG INDUSTRY

**Nutmeg cultivation**

Nutmeg cultivation in Bogor Regency is conducted mostly in family farms. Farmers usually cultivate nutmeg trees in a small land area nearby their homes. The nutmeg cultivation process starts with seed germination and propagation to produce a nutmeg seedling with 3-5 branches (Sunanto 1993) which is usually sold at around 10,000 IDR (1 USD = 13,324 IDR) per tree. Since Bogor Regency has heavy rainfall even during the dry season, nutmeg trees are provided naturally with enough water and humidity and can grow easily without too much maintenance. Thus, most nutmeg farmers do not irrigate trees and barely give fertilizer during cultivation. The harvesting period of nutmeg fruit differs depending on the use for the fruits. Initial fruiting starts at about six years after planting and reaches full production after 25 years (Sunanto 1993**)**. Nutmeg sweets require fruits at the age of 5-6 months, when the fruit is usually at its maximum size. On the other hand, young fruits at an age of three or four months are the best ingredients for nutmeg oil due to its high oil content. Harvesting is usually done using a pole with a hook to cut off fruits one by one. To make

harvesting faster, some farmers shake the tree. However, this random harvesting method requires additional sorting (selection by age and size) in accordance with the requirements for the succeeding processing stage. Meanwhile, it is also important to note that nutmeg harvesting in Bogor Regency is usually done directly by buyers, who are mostly rural assemblers or other nutmeg producers, including producers of nutmeg sweets and oil, because farmers mainly sell nutmeg fruit as their end-product. Post-harvesting processes, such as sorting and drying of fruits for raw material for the processing of sweets or oil, are mostly conducted by the buyers themselves.

Nutmeg farmers in Bogor Regency earned 10 million IDR per year on average. On the other hand, production costs of 31,000 IDR were for seedlings, labor, transportation, and taxes (Table 2). Since nutmeg cultivation can be easily done, and farmers only need 23,000 IDR to start planting a tree, nutmeg farming can be a steady source of income. Although the amount of profit can only be considered as an additional source of income, farmers in Bogor Regency still favor nutmeg cultivation because of their lack in skill or because of the funds needed for nutmeg processing.

**Nutmeg sweets processing**

Overall, nutmeg producers who cultivated and processed fruits into sweets earned a higher income and profit than nutmeg farmers (Table 3). Making nutmeg sweets requires the use of sugar and nutmeg fruit, and the cost of sugar accounted for most of the production costs. Production also required more laborers than other means of nutmeg production. The production cost of one kg of nutmeg sweets of four producers ranged from 17,200 and 22,100 IDR, and had no decreasing trend following scale expansion. In other words, the economies of scale did not appear in the processing of nutmeg sweets. However, the family income per member increased with an increase in sales. The value added on one kg of nutmeg fruit processed into sweets ranged from 6,600 to 8,700 IDR, which is about two times as much as one kg nutmeg fruits sold just as is (Table 2 and Table 3). Although the development of the nutmeg sweets processing industry needs more funds and labor input than nutmeg cultivation only, it can provide a higher added value, as well as job opportunities for locals, which are mostly women and housewives from surrounding areas.

**Nutmeg oil processing**

Unlike nutmeg sweets, material for processing nutmeg oil is only the fruit, particularly its seeds and mace. Since most producers from Bogor Regency only possess large distillation machines,

**Table 2.** Profitability of nutmeg farmers (1 USD = 13,324 IDR (Central Bank of Indonesia as of 9 August 2017).

| Items | | | A | B | C | D | E | F | G | H | I | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Laborer** | **Number of Family Laborers [persons]** | | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** |
| Sales | Annual Sales Quantity (A) [kg] | Seeds | 15 | 20 | 45 | - | 120 | 150 | 200 | - | - | 61 |
| | | Fruits | - | - | - | 750 | - | - | - | 5,000 | 12,000 | 1,972 |
| | Year 2012 Selling Price [1,000 IDR/kg] | Seeds | 16.0 | 17.0 | 24.0 | - | 25.0 | 25.0 | 25.0 | - | - | 24.4 |
| | | Fruits | - | - | - | 3.0 | - | - | - | 3.5 | 4.8 | 4.4 |
| | Total Sales (B) [1,000 IDR/year] | | 240.0 | 340.0 | 1,080.0 | 2,250.0 | 3,000.0 | 3,750.0 | 5,000.0 | 17,500.0 | 57,600.0 | 10,084.4 |
| Production Cost | Material Cost [1,000 IDR/year] | | 4.7 | 0.3 | 1.3 | 2.7 | 0.7 | 1.0 | 10.7 | 0.7 | 5.7 | 3.1 |
| | Family Labor Cost (C) [1,000 IDR/year] | | 0.7 | 0.1 | 0.2 | 0.4 | 0.1 | 0.2 | 1.6 | 0.1 | 0.9 | 0.5 |
| | Transportation Cost [1,000 IDR/year] | | 40.0 | 40.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8.9 |
| | Taxes [1,000 IDR/year] | | 49.5 | 3.5 | 8.9 | 15.8 | 8.5 | 3.5 | 59.3 | 6.4 | 11.8 | 18.6 |
| | Total Production Cost (D) [1,000 IDR/year] | | 94.9 | 43.9 | 10.4 | 18.9 | 9.3 | 4.7 | 71.6 | 7.2 | 18.3 | 31.0 |
| Net Profit (B-D) [1,000 IDR/year] | | | 145.1 | 296.1 | 1,069.6 | 2,231.1 | 2,990.7 | 3,745.4 | 4,928.4 | 17,492.8 | 57,581.7 | 10,053.4 |
| Family Income (B-D+C) [1,000 IDR/year] | | | 145.8 | 296.2 | 1,069.8 | 2,231.5 | 2,990.8 | 3,745.5 | 4,930.0 | 17,492.9 | 57,582.5 | 10,053.9 |
| Production Cost of 1 kg product (D)/(A) [1,000 IDR] | | Seeds | 6.324 | 2.194 | 0.232 | - | 0.077 | 0.031 | 0.358 | - | - | 1.536 |
| | | Fruits | - | - | - | 0.025 | - | - | - | 0.001 | 0.002 | 0.009 |
| Profit of 1 kg product (B-D)/(A) [1,000 IDR] | | Seeds | 9.7 | 14.8 | 23.8 | - | 24.9 | 25.0 | 24.6 | - | - | 20.5 |
| | | Fruits | - | - | - | 3.0 | - | - | - | 3.5 | 4.8 | 3.8 |
| Value-added of 1 kg nutmeg fruits when production is completely self-supplied [1,000 IDR] | | | 1.7 | 2.0 | 3.1 | 3.0 | 3.3 | 3.3 | 3.3 | 3.5 | 4.8 | 4.1 |

Source: Author's calculation based on survey data, 2012

Notes: 1) There was no hired labor reported.
2) Average of annual sales is the sum of annual quantities of each product (seeds and fruits) divided by nine farmers.
3) Average selling price for 2012 and the value-added of 1 kg nutmeg fruit were calculated using weighted average method.
4) Other averages were calculated using arithmetic average method.
5) Added Value = Net profit + Labor Cost + Taxes. One kg nutmeg fruit contains 0.131 kg nutmeg seeds on average.

**Table 3.** Profitability of nutmeg sweets producers (1 USD = 13,324 IDR (Central Bank of Indonesia as of 9 August 2017).

| Items | | | J | K | L | M | Average |
|---|---|---|---|---|---|---|---|
| Laborer | Number of Family Laborers (A) [Persons] | | 2 | 2 | 3 | 3 | 2.5 |
| | Number of Hired Labor [Persons] | | 0 | 5 | 47 | 17 | 17.3 |
| Sales | Fresh Sweets | Annual Sales Quantity (B) [kg] | 6,000.0 | 864.0 | 22,000.0 | 1,200.0 | 7,516.0 |
| | | Year 2012 Selling Price [1,000 IDR/kg] * | 24.0 | 22.0 | 20.0 | 22.0 | 20.9 |
| | Dried Sweets | Annual Sales Quantity (C) [kg] | 0 | 14,400.0 | 50,000.0 | 76,800.0 | 35,300.0 |
| | | Year 2012 Selling Price [1,000 IDR/kg] * | 0 | 21.0 | 20.0 | 25.0 | 22.8 |
| | Total Sales (D) [1,000 IDR/year] | | 144,000.0 | 321,408.0 | 1,440,000.0 | 1,946,400.0 | 962,952.0 |
| Production Cost | Material Self-Supplied Cost (E) [1,000 IDR/year] | | 0 | 2,188.0 | 0 | 0 | 547.0 |
| | Material Purchased Cost | Nutmeg Fruits [1,000 IDR/year] | 27,000.0 | 57,600.0 | 216,000.0 | 216,000.0 | 129,150.0 |
| | | Sugar [1,000 IDR/year] | 86,400.0 | 178,560.0 | 864,000.0 | 1,296,000.0 | 606,240.0 |
| | | Other Material [1,000 IDR/year] | 0 | 0 | 4,800.0 | 0 | 1,200.0 |
| | Family Labor Cost (F) [1,000 IDR/year] | | 864.0 | 3,840.0 | 22,800.0 | 15,120.0 | 10,656.0 |
| | Hired Labor Cost [1,000 IDR/year] | | 0 | 9,600.0 | 128,400.0 | 85,680.0 | 55,920.0 |
| | Packaging Cost [1,000 IDR/year] | | 3,750.0 | 8,352.0 | 45,000.0 | 90,000.0 | 36,775.5 |
| | Transportation Cost [1,000 IDR/year] | | 0 | 0 | 9,600.0 | 0 | 2,400.0 |
| | Tools & Machinery [1,000 IDR/year] | | 311.0 | 418.8 | 21,690.6 | 930.9 | 5,837.9 |
| | Utility Cost [1,000 IDR/year] | | 5,808.0 | 2,460.0 | 20,352.0 | 21,432.0 | 12,513.0 |
| | Service Fee [1,000 IDR/year] | | 0 | 0 | 17.0 | 0 | 4.3 |
| | Taxes [1,000 IDR/year] | | 90.0 | 15.0 | 486.8 | 60.0 | 163.0 |
| | Interest [1,000 IDR/year] | | 0 | 0 | 12,000.0 | 135.0 | 3,033.8 |
| | Total Production Cost (G) [1,000 IDR/year] | | 124,223.0 | 263,033.8 | 1,345,146.4 | 1,725,357.9 | 864,440.3 |
| Net Profit (D-G) [1,000 IDR/year] | | | 19,777.0 | 58,374.2 | 94,853.6 | 221,042.1 | 98,511.7 |
| Family Income (D-G+E+F) [1,000 IDR/year] * | | | 20,641.0 | 64,402.2 | 117,653.6 | 236,162.1 | 109,714.7 |
| Family Income Per Member (D-G+E+F)/(A) [1,000 IDR/year] * | | | 10,320.5 | 32,201.1 | 39,217.9 | 78,720.7 | 43,885.9 |
| Production Cost of 1 kg product (G)/(B+C) [1,000 IDR] * | | | 20.7 | 17.2 | 18.7 | 22.1 | 20.2 |
| Profit of 1 kg product (D-G)/(B+C) [1,000 IDR] * | | | 3.3 | 3.8 | 1.3 | 2.8 | 2.3 |
| Value-added of 1 kg fruit when production is self-supplied [1,000 IDR] * | | | 8.0 | 8.7 | 6.6 | 7.5 | 7.2 |

Source: Author's calculation based on survey data, 2012

Notes: 1) * calculated using weighted average method. Other averages were calculated using arithmetic average method.

2) Added Value=Net profit + Labor Cost + Raw Material Cost (Excluding Seedlings Cost) + Taxes + Interest. Purchased raw materials are assumed to be self-supplied.

**Table 4.** Profitability of nutmeg oil producers (1 USD = 13,324 IDR (Central Bank of Indonesia as of 9 August 2017).

| Items | | | N | O | P | Q | R | Average |
|---|---|---|---|---|---|---|---|---|
| Laborer | Number of Family Laborers (A) [Persons] | | 1 | 4 | 1 | 1 | 7 | 2.8 |
| | Number of Hired Labor [Persons] | | 3 | 0 | 8 | 10 | 6 | 5.4 |
| Sales | Nutmeg Oil | Annual Sales Quantity (B) [kg] | 3,360.0 | 3,600.0 | 3,840.0 | 6,000.0 | 15,600.0 | 6,480.0 |
| | | Year 2012 Selling Price [1,000 IDR/kg] * | 900.0 | 840.0 | 850.0 | 800.0 | 950.0 | 893.0 |
| | Total Sales (C) [1,000 IDR/year] | | 3,024,000.0 | 3,024,000.0 | 3,264,000.0 | 4,800,000.0 | 14,820,000.0 | 5,786,400.0 |
| Production Cost | Material Self-Supplied Cost (D) [1,000 IDR/year] | | 50,000.0 | 26,400.0 | 0 | 0 | 124,800.0 | 40,240.0 |
| | Material Purchased Cost [1,000 IDR/year] | | 1,099,200.0 | 739,200.0 | 800,000.0 | 1,440,000.0 | 1,872,000.0 | 1,190,080.0 |
| | Family Labor Cost (E) [1,000 IDR/year] | | 16,800.0 | 40,320.0 | 8,400.0 | 9,600.0 | 56,840.0 | 26,392.0 |
| | Hired Labor Cost [1,000 IDR/year] | | 50,400.0 | 0 | 67,200.0 | 96,000.0 | 48,720.0 | 52,464.0 |
| | Tools & Machinery [1,000 IDR/year] | | 1,020,000.0 | 146,423.1 | 6,217.5 | 3,570.0 | 49,482.8 | 245,138.7 |
| | Transportation Cost [1,000 IDR/year] | | 0 | 0 | 0 | 0 | 21,600.0 | 4,320.0 |
| | Utility Cost [1,000 IDR/year] | | 4,800.0 | 960.0 | 213,600.0 | 230,400.0 | 480.0 | 90,048.0 |
| | Taxes [1,000 IDR/year] | | 1,700.0 | 62.0 | 400.0 | 1,150.0 | 50.0 | 672.4 |
| | Interest [1,000 IDR/year] | | 18,000.0 | 0 | 0 | 0 | 0 | 3,600.0 |
| | Total Production Cost (F) [1,000 IDR/year] | | 2,260,900.0 | 953,365.1 | 1,095,817.5 | 1,780,720.0 | 2,173,972.8 | 1,652,955.1 |
| Net Profit (C-F) [1,000 IDR/year] | | | 763,100.0 | 2,070,634.9 | 2,168,182.5 | 3,019,280.0 | 12,646,027.2 | 4,133,444.9 |
| Family Income (C-F+D+E) [1,000 IDR/year] | | | 829,900.0 | 2,137,354.9 | 2,176,582.5 | 3,028,880.0 | 12,827,667.2 | 4,200,076.9 |
| Family Income Per Member (C-F+D+E)/(A) [1,000 IDR/year] * | | | 829,900.0 | 534,338.7 | 2,176,582.5 | 3,028,880.0 | 1,832,523.9 | 1,500,027.5 |
| Production Cost of 1 kg product (F/B) [1,000 IDR] * | | | 672.9 | 264.8 | 285.4 | 296.8 | 139.4 | 255.1 |
| Profit of 1 kg product (C-F)/(B) [1,000 IDR] * | | | 227.1 | 575.2 | 564.6 | 503.2 | 810.6 | 637.9 |
| Value-added of 1 kg nutmeg fruits when production is completely self-supplied [1,000 IDR] * | | | 65.5 | 87.9 | 87.2 | 83.7 | 104.0 | 92.5 |

Source: Author's calculation based on survey data, 2012

Notes: 1) * calculated using weighted average method. Other averages were calculated using arithmetic average method.

2) Added Value = Net profit + Labor Cost + Nutmeg Raw Material Cost (Excluding Seedlings Cost) + Taxes + Interest. One kg nutmeg fruit contains 0.11 kg nutmeg oil on average. Purchased nutmeg raw materials are assumed to be self-supplied.

with an average capacity of 200 kg, there is a need to purchase sufficient raw material. Thus, the total cost for processing nutmeg oil, which includes expenditures for materials, tools, and machinery, turn out to be higher than processing sweets (Table 3 and Table 4). From Table 4, it is clear that nutmeg oil producers earned the highest total sales compared to other nutmeg producers. The high price of nutmeg oil, which in 2012 was 892,297 IDR per kilogram on average, played an important role for its high sales. Moreover, the production cost for one kg nutmeg oil for a relatively large-scale producer was lower than a relatively small-scale one. The economies of scale seem to appear in nutmeg oil processing, meaning that by increasing production quantity, the costs decrease, while net profit and family income increase. Therefore, the family income for a large-scale oil producer was higher than that of a small-scale producer per family member, and higher than that of other nutmeg producers. Furthermore, results also showed that the value added per one kg nutmeg fruit processed into oil was about 23 times as much as that of the fruit itself.

## IMPACTS OF QUALITY PROBLEMS ON PROFITABILITY OF NUTMEG PRODUCTS

### Price change of nutmeg products in Bogor Regency

Despite its high profitability, the sustainability of the nutmeg processing industry has been confronted with several challenges, with scarcity of raw materials was the initial problem. Because of the high price and growing demand for nutmeg oil, oil producers were eager to keep on expanding and has reached maximum production until 2013. Meanwhile, with an average capacity of 200 kg for one distillation machine, tons of nutmeg fruit were needed. Although it has not been statistically ascertained, interviewed respondents reported that nutmeg cultivation in Bogor Regency has gradually reached a point of not meeting the high demand for raw material which worsened between 2012 and 2015. The shortage was due to the conversion of cultivation areas into housing land. The lack of proper farm management was a constraint on nutmeg production. Hence, the unbalanced supply and demand of raw materials has brought a significant impact on the price of nutmeg fruit, which rose following an increase in nutmeg oil price from 2010 to 2013 (Table 5). These changes were followed by an increase in the price of nutmeg sweets. Since oil price was high, nutmeg oil producers had a stronger bargaining power to buy more nutmeg fruit than other producers. The competitive situation between oil producers and other nutmeg producers in securing raw material gave farmers the benefit of selling at higher price.

In spite of the increasing price and demand of raw material, this favorable condition for farmers did not last long. Quality issues concerning nutmeg oil occurred in early 2014. Nutmeg seeds and mace contain 12% and 15% oil, respectively. To make good quality nutmeg oil, only nutmeg seeds and mace are used. However, if only seeds and mace are used, then oil producers need to secure more fruits. Thus, many producers resort to using whole fruits, regardless of the quality of their oil components. In addition, it is suspected that some producers might have used nutmeg from eastern Indonesia, where oil processing is considered as unsuitable. The lack of quality awareness has led oil processors to face severe problems in quality and in terms of gaining trust from consumers. Many nutmeg oil producers have received product rejections, because of failed to meet the requirements, especially by the manufacturing and export companies.

The price of nutmeg oil dropped significantly to one third from 2013 to 2015. A price change also occurred in other products. The selling price of nutmeg seeds dropped from 31,353 IDR to 9,135 IDR in a couple of years. Although the gap was smaller, the change in price was also seen in nutmeg sweets. As clarified above, it was confirmed that the price of nutmeg oil influenced the price of nutmeg fruit and sweets (the correlation coefficients between the price of nutmeg oil and other nutmeg products in Table 5 are high). To analyze above mentioned phenomenon in detail, another interview survey was conducted in 2015.

**Table 5.** Price change of nutmeg products
(1 USD = 13,324 IDR (Central Bank of Indonesia as of 9 August 2017)

| Year | Nutmeg Oil Producers | Nutmeg Farmers | | Nutmeg Sweets Producers | | Consumer Price Index (CPI) (*) |
|---|---|---|---|---|---|---|
| | Nutmeg Oil | Seeds | Fruits | Fresh Sweets | Dried Sweets | |
| 2010 | 631,687 | 20,375 | 2,665 | 16,796 | 16,796 | 91.3 |
| 2011 | 715,746 | 22,252 | 3,189 | 17,677 | 18,023 | 96.2 |
| 2012 | 892,297 | 22,000 | 3,767 | 22,000 | 22,000 | 100.0 |
| 2013 | 943,161 | 31,353 | 4,805 | 28,829 | 28,829 | 106.4 |
| 2014 | 518,732 | 18,863 | 3,301 | 23,579 | 23,579 | 113.2 |
| 2015 | 332,171 | 9,135 | 2,491 | 20,761 | 20,761 | 120.4 |
| Average | 669,972 | 20,663 | 3,370 | 21,607 | 21,665 | |
| Correlation coefficient between prices of nutmeg products and raw materials | | **Oil & Seeds** | **Oil & Fruits** | **Sweets & Fruits** | | - |
| | | 0.92 | 0.84 | 0.84 | | |

Sources: Survey data in 2012 and 2015.
* IMF - World Economic Outlook Databases (2016)
Notes: 1) Price unit: IDR/kg
2) All prices are real prices deflated by CPI (Year 2012 = 100.0).

**Profitability change of nutmeg industry in Bogor Regency**

Three nutmeg producers were interviewed in 2012 and 2015, as oil producer P, sweets producer L and X. Sweets producer X was initially omitted from data analysis because he was under a group of non-cultivating producers of nutmeg sweets (Group 2). However, because of his impressive performance between 2012 and 2015, producer X was included in this section to provide a broader perspective on how nutmeg sweets producers respond to the current trend of the nutmeg industry in Bogor Regency. In this section, their financial conditions during the years 2012 and 2015 were compared to analyze how the current price fluctuation affected production.

The profitability of nutmeg oil producer P showed a drop in total sales from 3.3 billion IDR to 698 million IDR, due to a drastic decrease in oil price by 559,350 IDR per kg in three years (Table 6). The other reason was difficulty over securing sufficient good raw material. Although producer P has been cultivating nutmeg, the nutmeg trees were just planted and have not started bearing fruit. Thus, he had to compete with other nutmeg producers in purchasing raw material from local farmers. Dealing with the fall of nutmeg oil price and the scarcity of good raw material, producer P had to reduce production from twice a week to twice a month on a non-regular basis. Total production cost (i.e. materials and labor cost) decreased significantly by 79% in three years. Since nutmeg oil can be preserved longer than food and beverages, producer P intended to sell when the price rose in the market. This strategy helped him secure his income. Thus, producer P was able to have a positive return in 2015.

**Table 6.** Change in profitability of nutmeg oil producer
(1 USD = 13,324 IDR (Central Bank of Indonesia as of 9 August 2017)

| Items | | Nutmeg Oil Producer P | |
|---|---|---|---|
| | | 2012 | 2015 |
| Laborer | Number of Family Labor (A) [Persons] | 1 | 1 |
| | Number of Hired Labor [Persons] | 8 | 1 |
| Sales | Nutmeg Oil Annual Sales Quantity (B) [kg] | 3,840.0 | 2,400.0 |
| | Nutmeg Oil Selling Price [1,000 IDR/kg] | 850.0 | 290.6 |
| | Total Sales (C) [1,000 IDR/year] | 3,264,000.0 | 697,558.5 |
| Production Cost | Material Self-Supplied Cost (D) [1,000 IDR/year] | 0 | 0 |
| | Material Purchased Cost [1,000 IDR/year] | 800,000.0 | 175,386.1 |
| | Family Labor Cost (E) [1,000 IDR/year] | 8,400.0 | 1,993.0 |
| | Hired Labor Cost [1,000 IDR/year] | 67,200.0 | 1,993.0 |
| | Tools & Machinery [1,000 IDR/year] | 6,217.5 | 5,379.1 |
| | Transportation Cost [1,000 IDR/year] | 0 | 0 |
| | Utility Cost [1,000 IDR/year] | 213,600.0 | 45,839.6 |
| | Taxes [1,000 IDR/year] | 400.0 | 332.2 |
| | Interest [1,000 IDR/year] | 0 | 0 |
| | Total Production Cost (F) [1,000 IDR/year] | 1,095,817.5 | 230,923.0 |
| Net Profit (C-F) [1,000 IDR/year] | | 2,168,182.5 | 466,635.5 |
| Family Income (C-F+D+E) [1,000 IDR/year] | | 2,176,582.5 | 468,628.6 |
| Family Income Per Member (C-F+D+E)/(A) [1,000 IDR/year] | | 2,176,582.5 | 468,628.6 |
| Production Cost of 1 kg product (F/B) [1,000 IDR] | | 285.4 | 96.2 |
| Profit of 1 kg product (C-F)/(B) [1,000 IDR] | | 564.6 | 194.4 |
| Value-added of 1 kg nutmeg fruits when production is completely self-supplied [1,000 IDR] | | 87.2 | 29.6 |

Source: Author's calculation based on survey data in 2012 and 2015.
Notes: 1) Added Value = Net profit + Labor Cost + Nutmeg Raw Materials Cost (Excluding Seedlings Cost) + Taxes +Interest. One kg nutmeg fruits contain 0.11 kg nutmeg oil on average. Purchased nutmeg raw materials are assumed to be self-supplied materials.
2) All monetary values are real prices deflated by CPI (Year 2012 = 100.0).

Changes in profitability of producer L and producer X in 2012 and 2015 are shown in Table 7. Total sales of producer L, a relatively large-scale producer of nutmeg sweets in 2012 (Table 3), decreased by 38% in 2015. Production had to be reduced due to the following reasons, (1) the nutmeg trees were still not bearing fruit, and (2) difficulty in purchasing a sufficient supply of good nutmeg fruits. Producer L also decided to reduce costs (especially for materials and labor cost) and raise the selling price of nutmeg sweets in order to maintain profitability in 2015. On the other hand, producer X showed impressive performance in 2015 although he was a middle-scale producer with limited access to the market in 2012. The competitive Bogor Regency market encouraged him to expand target markets to cities outside of Bogor Regency. This strategy worked very well and led to a larger scale in sales and production. The rising demand from several wholesalers in surrounding areas, such as Cianjur and Sukabumi, played an important role in bringing a positive return to their production in 2015.

From the above analysis, it was noted that a decrease in nutmeg oil price and the scarcity of raw material lowered the profitability of nutmeg oil producer P. Nutmeg sweets producer L and X also succeeded in gaining a higher profit by raising their selling price and cutting off costs, or by expanding their market and increasing production. Despite the decrease in profits, results showed that nutmeg oil producer P can still earn a higher family income per member, as well as a higher value added for one kg nutmeg fruit compared to nutmeg sweets producer L and X (Table 6 and 7).

**Table 7.** Change of profitability in nutmeg sweets producers
(1 USD = 13,324 IDR (Central Bank of Indonesia as of 9 August 2017)

| Items | | | Producer L | | Producer X | |
|---|---|---|---|---|---|---|
| | | | 2012 | 2015 | 2012 | 2015 |
| Laborer | Number of Family Labor (A) [Persons] | | 3 | 1 | 3 | 3 |
| | Number of Hired Labor [Persons] | | 47 | 22 | 21 | 9 |
| Sales | Fresh Sweets | Annual Sales Quantity (B) [kg] | 22,000 | 13,000 | 0 | 7,320.0 |
| | | Selling Price [1,000 IDR/kg] | 20.0 | 20.8 | 0 | 16.6 |
| | Dried Sweets | Annual Sales Quantity (C) (kg) | 50,000 | 30,000 | 19,200 | 37,350.0 |
| | | Selling Price [1,000 IDR/kg] | 20.0 | 20.8 | 24.0 | 19.1 |
| | Total Sales (D) [1,000 IDR/year] | | 1,440,000.0 | 892,708.9 | 460,800.0 | 834,952.7 |
| Production Cost | Material Self-Supplied Cost (E) [1,000 IDR/year] | | 0 | 0 | 0 | 0 |
| | Material Purchased Cost [1,000 IDR/year] | | 1,084,800.0 | 526,573.7 | 216,000.0 | 428,528.5 |
| | Family Labor Cost (F) [1,000 IDR/year] | | 22,800.0 | 11,958.1 | 14,400.0 | 58,457.9 |
| | Hired Labor Cost [1,000 IDR/year] | | 128,400.0 | 61,435.0 | 100,800.0 | 67,451.4 |
| | Packaging Cost [1,000 IDR/year] | | 45,000.0 | 22,421.5 | 21,120.0 | 15,709.2 |
| | Transportation Cost [1,000 IDR/year] | | 9,600.0 | 4,783.3 | 31,200.0 | 5,729.9 |
| | Tools & Machinery [1,000 IDR/year] | | 21,690.6 | 18,012.5 | 1,944.4 | 7,583.8 |
| | Utility Cost [1,000 IDR/year] | | 20,352.0 | 14,449.4 | 4,960.0 | 8,167.2 |
| | Service Fee [1,000 IDR/year] | | 17.0 | 14.1 | 0 | 1,993.0 |
| | Taxes [1,000 IDR/year] | | 486.8 | 404.3 | 50.0 | 83.0 |
| | Interest [1,000 IDR/year] | | 12,000.0 | 0 | 0 | 2,882.5 |
| | Total Production Cost (G) [1,000 IDR/year] | | 1,345,146.4 | 660,051.8 | 390,474.4 | 596,586.5 |
| Net Profit (D-G) [1,000 IDR/year] | | | 94,853.6 | 232,657.0 | 70,325.6 | 238,366.1 |
| Family Income (D-G+E+F) [1,000 IDR/year] | | | 117,653.6 | 244,615.2 | 84,725.6 | 296,824.0 |
| Family Income Per Member (D-G+E+F)/(A) [1,000 IDR/year] | | | 39,217.9 | 244,615.2 | 28,241.9 | 98,941.3 |
| Production Cost of 1 kg product (G)/(B+C) [1,000 IDR] | | | 18.7 | 15.4 | 20.3 | 13.4 |
| Profit of 1 kg product (D-G)/(B+C) [1,000 IDR] | | | 1.3 | 5.4 | 3.7 | 5.3 |
| Value-added of 1 kg nutmeg fruits when production is completely self-supplied [1,000 IDR] | | | 6.6 | 6.3 | 10.7 | 13.8 |

Source: Author's calculation based on survey data in 2012 and 2015.
Notes: 1) Added Value = Net profit + Labor Cost + Nutmeg Raw Materials Cost (Excluding Seedlings Cost) + Taxes +Interest. Purchased nutmeg raw materials are assumed to be self-supplied materials.
2) All monetary values are real prices deflated by CPI (Year 2012 = 100.0).

## CONCLUSIONS AND RECOMMENDATIONS

To promote rural development in the nutmeg industry, nutmeg farmers will need more support for skills and technology concerning cultivation to ensure a stable supply of fruits. Producers should also expand the market and realize optimal size of production to enhance their profits and income. To prevent further damage and preserve the sustainability of the industry in Bogor Regency, quality awareness must be emphasized to all concerned stakeholders, from farmers, producers and buyers. Hence, the quality of nutmeg oil as a high price export commodity is extremely important. Government involvement becomes necessary, especially in enacting legal and formal regulation as well as policy enforcement for quality assessment of nutmeg products in Bogor Regency. Lastly, due to the small number of respondents in this study, it is necessary to broaden the scope of this research and conduct further analysis, with consideration of the above conclusions as reference information.

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# SOCIO-ECONOMIC AND ENVIRONMENTAL IMPACTS OF THE CONSERVATION FARMING VILLAGE PROJECT IN UPLAND COMMUNITIES OF LA LIBERTAD, NEGROS ORIENTAL, PHILIPPINES

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## ABSTRACT

Upland agriculture is a major cause of soil erosion in the Philippines. The clearing of forests for agricultural production and improper farming practices combine together to make the uplands highly susceptible to degradation. While a number of soil conservation interventions for upland agriculture have been identified, adoption by farmers has been marginal. The Conservation Farming Village (CFV) approach is a modality that aims to improve the adoption of soil conservation technologies. This approach was implemented in selected barangays (villages) in La Libertad, Negros Oriental, Philippines from October 2008 to December 2011. This paper analyzed the socio-economic and environmental impacts of CFV on upland farms. Specifically, it estimated changes in soil loss, organic matter and nitrogen, phosphorus and potassium content, and farm income. The CFV project has been effective in transforming the traditional monocropping system into a diversified cropping system. While soil erosion was still noted, the rate of soil loss and the total amount of nutrient loss decreased through time. The conservation farming (CF) technologies adopted by farmers were effective in reducing soil erosion. Moreover, the farmers' net returns generally increased after CF adoption due to the increased returns from crop diversification. The CFV project resulted in positive social impact. It improved the farmers' level of living, self-confidence leading to better leadership capability and decision-making as well as increased their social network.

**Key words**: Soil erosion, soil conservation, soil nutrient, impact assessment, technology adoption

## INTRODUCTION

The land resource of the Philippines is deteriorating because of land use change and erosion. Twenty-one percent of the country's agricultural lands and 36 percent of non-agricultural lands are moderately or severely eroded (ADB 2009). Moreover, large portions of our forest are degraded due to shifting agricultural production. There are more than 20 million people living in upland watershed areas, half of whom are dependent on shifting cultivation for their livelihood (Cruz and Zosa-Feranil 1998 as cited by NEDA 2011). With the rapid growth of population and the apparent lack of livelihood opportunities in the lowlands, migration into the uplands will continue. Unfortunately, the upland farmers either do not take soil conservation seriously or are not fully aware of the effects of soil erosion. Hence, the unsustainable land use and improper farming practices make the upland areas highly susceptible to degradation.

In other parts of the world such as in the ASEAN region and in Australia, conservation activities such as conservation agriculture and landcare programs were implemented for natural resource management. The Conservation Agriculture Network for South East Asia (CANSEA) was created in 2009 to develop conservation agriculture (CA) cropping systems in South East Asia in 2009. The network believes that each country can contribute to advancing conservation agriculture (CA) to counteract negative environmental and economic externalities of crop intensification (Panyasiri et al. 2010). In Australia, the Australian Government is investing A$1 Billion through the National Landcare Programme over four years from 2014-15, including support for the Landcare Networks, with the objective of managing landscapes to sustain long-term economic and social benefits from their environment (NRM 2017).

For the Philippines, the Conservation Farming Village (CFV) is a modality for enhancing the transfer of conservation farming technologies and practices anchored on participatory planning, monitoring, and evaluation processes at the community level. The CFV mechanism adopts a multi-level technology promotion mechanism that capacitates local soil conservation extension units of the local government units (LGUs) and change agents (farmer-volunteers). The farmer-volunteers are used as an arm for the promotion of upland farming technologies and approaches. The farmer-to-farmer linkage strengthens the "multiplier effect" of technology diffusion processes at the local level. The major output in each CFV site are the Science &Technology-based model farms, serving as satellite farms with several farmer-adopters around the core model. The criteria used in the selection of CFV sites included the following: (1) located in the uplands, (2) reliable source of water for raising crops, (3) soil erosion is a problem, (4) located within a critical watershed, (5) agricultural production is actively practiced, (6) accessible to motorized transportation, (7) within UPLB's operational coverage, (8) the LGU counterparts are supportive of the technological interventions and are willing to assist in the implementation of the CFV project, and (9) no existing or very few national programs on soil conservation implemented. Under the CFV, the upland farmers are provided with choices of the soil conservation technologies that will suit their farming conditions. These technologies are: (1) conservation agriculture technologies utilizing the three principles of no tillage, permanent soil cover and crop rotation; (2) alley cropping/use of hedge rows; (3) sloping agricultural land technologies (SALT 1-4, including livestock component); (4) contour farming; (5) natural vegetative strips; (6) contour composting/vermi-composting; (7) *Jatropha* as hedgerow vegetation; (8) farming systems suitable for sloping lands (multi-species cropping, conservation tillage, ground cover); (9) pole barriers and other physical barriers (bench terraces, contour rock walls); (10) canals and soil traps; (11)water-saving technologies/water management; (12) nitrogen-fixing trees, silviculture, and improved forage planting; and (13) agro forestry technologies.

This paper analyzed the social, economic and environmental impacts of CFV on the upland communities of La Libertad, Negros Oriental. Specifically, it estimated the changes in soil loss, organic matter and nitrogen, phosphorus and potassium content, as well as net farm income.

## METHODOLOGY

### Selection of Study Sites

The study was conducted in barangays or villages where the Conservation Farming (CF) technologies were introduced: Elecia, Pitogo, Nasunggan and Aya, La Libertad. Farmers from another adjacent village (Talaon) decided to adopt the CF technologies. Talaon and Aya have almost the same topography and crop cover. Large portions of these barangays have slopes of above 18% which are prone to flooding resulting in excessive soil erosion and landslides. The soils in the CFV sites are clay loam to clay and planted with corn, coconut and palay (rainfed and irrigated). These are situated in the upper part of the Pacuan Watershed where conversion of forestlands into agricultural use is rampant, becoming grasslands due to deforestation. Appendix Tables 1 and 2 show the baseline

data on soil fertility taken during project implementation and the condition of the study sites before the CFV project implementation. Baseline data on soil fertility showed that phosphate (K) level was high while organic matter (OM) content was at medium level, however, the nitrogen (N) and potassium (P) levels were low while soil pH was strongly acidic. Excessive soil erosion, flooding in low-lying areas and landslides were reported to be very common in the area. There was lack of access to public services such as roads, potable water, and educational facilities. During that time, the LGU allotted funds for the development of the water system to reach the communities with less access. Also, there was no sustainable livelihood in the upland communities. The LGU decided to include the distribution of livestock (hogs and/or goats) and assorted high-value crop planting materials in the CFV program. The objective was to help improve the low level of household income in the area.

A total of 40 model farms (10 model farms per barangay) were established in the four initially selected barangays (Elecia, Pitogo, Nasunggan and Aya) with a combined area of 80.35 hectares. The CF technologies practiced in these farms included the following: hedgerow intercropping or contour hedgerows (100%), multi-storey planting (100%), contour composting (50%), contour rockwalls (13%) and mulching (5%). Hedgerow intercropping and multi-storey cropping became popular to the farmers because these were simple and easy to establish and effective in preventing soil erosion. Contour farming with rock wall and multi-storey cropping were adopted by farmers whose farms were situated in rocky and forested areas, respectively.

**Selection of respondents**

A total of 40 farmers were interviewed during the survey in the five CF sites in La Libertad. The farmer-respondents were composed of: (1) all of the farmers (n=10) who volunteered the farms that they operate to be used as CFV model/demo farms; and (2) randomly selected farmers (n=30) who adopted the CF technologies during and after the project.

**Data collection and soil analysis**

Farm visits, soil sampling and ground slope determination were conducted in each model farm as well as in other critical areas of the barangays. Present land use, farming systems and occurrences of soil erosion were also observed during field visits and interviews. A total of 23 soil samples were collected from the CFV sites in La Libertad. These soil samples were analyzed by the Department of Soil Science of the University of the Philippines Los Banos for texture, nutrient content (N, P, K), OM and pH.

**Assessment of socio-economic impact**

Social impact was measured by determining the changes in the living condition of the farm households as well as the farm communities as a whole when the CFV project was implemented. Effect on leadership capability and decision making were also measured. Data were generated using personal interview. Change in farm income was used as indicator to measure the economic impact of the CFV. This was determined by comparing net farm income before and after the adoption of CF technologies by the farmers. A t-test of paired two sample means was used to determine if the difference between the farmers' income before and after CFV adoption is statistically significant. Spearman's Correlation Analysis was used to determine whether there is a statistically significant relationship between change in farm income and the social impact indicators.

The over-all economic impact of the adoption of CF technologies was measured using the Benefit-Cost Analysis (BCA). The analysis focused on the annual incremental on-site benefits and costs of the soil conservation measures that were implemented in each of the CFV sites. The cash flow covered the years 2009 to 2015 (a 6-year period). The year 2009 represents the start of implementation of the CFV project while 2015 is the year the impact evaluation was conducted. The Internal Rate of Return (IRR), Net Present Value (NPV), and Benefit-Cost Ratio (BCR) were used to

measure the project worth. The discount rate used was 6% which is the opportunity cost of money representing the minimum attractive rate of return for development projects (PCAARRD 2013).

**Assessment of environmental impact**

Environmental impact was gauged in terms of the changes on the biophysical condition of the soil, particularly level of soil fertility and amount of soil loss. The before-after analytical framework was used. Soil erosion was estimated using the modified Universal Soil Loss Equation developed by David (1976) and David and Collado (1977) as cited in David (1988). Change in soil fertility was measured in terms of the amount of N, P, K that were saved due to the adoption of the CF technologies. The value of the soil nutrients saved was estimated using the Replacement Cost valuation method (Shiferaw et al. 2005). This method estimates the cost of replacing the lost soil nutrients with commercially available fertilizers. The amount of N, P and K was translated into monetary value based on the price per 50kg-bag of urea (PhP1300), Solophos (PhP950) and muriate of potash (PhP2100). The resulting costs per kg were PhP 12 for N, PhP3.80 for P and PhP25.20 for K. The cost of nutrient per kg multiplied by the rate of nutrient loss (converted in kg/ha/year) was used as the value of nutrients saved.

## RESULTS AND DISCUSSION

**Characteristics of the farmer-respondents**

The average age of farmer-volunteers was 46 years and 44 years for farmer-adopters. There was an equal number of male and female respondents among the farmer-volunteers while there were more female farmers (53.33%) in the farmer-adopters group. This finding implies that farming is no longer significantly dominated by male farmers. All of the farmer-respondents have formal schooling and a majority have elementary education. Majority of the farmer-respondents were married, and had 4-6 household members. Majority of the farmer-volunteers and farmer-adopters owned residential land where they built their own houses. Majority of the farmer-volunteers, and farmer-adopters' houses were made of wood or a combination of wood and concrete. All the farmer- respondents sourced their drinking water from a mountain spring, used fuel wood for cooking and with manual-flush toilet facility. Most of the farmer-volunteers (60%) and farmer- adopters (50%) used electricity for lighting. These findings imply that although the CFV farmers were relatively "well-off" than the other farmers in terms of their housing material, they appeared to be less financially able in terms of other housing facilities.

**Technologies adopted by farmer-volunteers farmer-adopters and their perceptions on effectiveness and ease of adoption**

The farmer-volunteers were able to establish demonstration farms and chose the soil conservation technologies based on the physical characteristics of their land. All farmer-volunteers adopted contour hedgerows and composting. Majority adopted boundary planting, organic farming and multi-storey cropping. Likewise, majority of the farmer-adopters practiced contour hedgerows, contour composting, rockwall and boundary planting in order to prevent soil erosion (Table 1). All of the farmer-volunteers tried to convince other farmers to adopt the CF technologies after they found the practices to be effective and easy to adopt. Both male and female farmer-volunteers considered all of the practices to be effective, apart from contour composting which was considered by a few male farmers to be just slightly effective. In terms of ease of adoption, there is a slight difference in the ratings of the males and females. Females rated the following practices as relatively difficult to follow: boundary planting, organic farming, livestock raising, contour composting vermiculture, contour rockwall and pole barriers or contour fences. On the other hand, the males reported that terracing is not easy to implement. Majority of the farmer-adopters practiced contour hedgerows and contour composting in order to prevent soil erosion.

In terms of effectiveness, all CF technologies received higher ratings from farmer-adopters (ranging from 2.78 to 3, with 3 being the highest). These technologies were considered effective in preventing soil erosion and bringing back soil fertility. In terms of ease of adoption, the same technologies were considered as easy to adopt by adopters with a mean rating of 2.48-3. Among the technologies mentioned, the easiest to follow were pole barriers or contour fences and integrated pest management. The relatively difficult practices were establishing terraces and vermiculture.

**Table 1.** Conservation farming technologies adopted by farmer-volunteers and farmer-adopters, La Libertad, Negros Oriental, Philippines, 2015.

| Conservation Farming Technology | Farmer-Volunteers (n=10) | | Farmer-Adopters (n=30) | |
|---|---|---|---|---|
| | Number | Percent | Number | Percent |
| Contour hedgerows | 10 | 100 | 20 | 67 |
| Contour composting | 10 | 100 | 16 | 53 |
| Rockwall | 4 | 40 | 15 | 50 |
| Boundary planting | 9 | 90 | 14 | 47 |
| Organic farming | 7 | 70 | 12 | 40 |
| Livestock raising | 6 | 60 | 12 | 40 |
| Terracing | 6 | 60 | 12 | 40 |
| Multi-storey cropping | 7 | 70 | 10 | 33 |
| Vermiculture | 5 | 50 | 10 | 33 |
| Mulching | 5 | 50 | 10 | 33 |
| Integrated pest management | | | 4 | 13 |
| Pole barriers/Contour fences | | | 4 | 13 |

While there was continuous local government unit (LGU) support to the farmer-adopters, some factors impeded the adoption of the technologies such as lack of planting materials, unavailability of labor to maintain the farm, and non-ownership of the land. Vermiculture had the highest percentage of discontinuance because of the difficulty in rearing the worms (Table 2).

**Table 2.** Discontinuance of CF technologies adopted by 30 farmer-adopters, La Libertad, Negros Oriental, Philippines, 2015.

| CF Technology | Adopted | | Discontinued | |
|---|---|---|---|---|
| | *Frequency* | *Percentage* | *Frequency* | *Percentage* |
| Vermiculture | 13 | 43.33 | 2 | 15.38 |
| Contour hedgerows | 24 | 80.00 | 3 | 12.50 |
| Contour composting | 20 | 66.67 | 2 | 10.00 |
| Mulching | 12 | 40.00 | 1 | 8.33 |
| Multi-storey cropping | 17 | 56.67 | 1 | 5.88 |
| Organic farming | 17 | 56.67 | 1 | 5.88 |
| Contour rockwall | 20 | 66.67 | 1 | 5.00 |

**Socio-Economic Impact**

***Change in farmers' income***

Incremental incomes of the 22 farmer-adopters and 10 farmer-volunteers before and after adoption of CF technologies are shown in Tables 3 and 4, respectively. Of the 30 farmer-adopters, only 22 were included in the impact evaluation because the remaining eight of these farmers were very recent adopters at the time of the impact assessment and the effect of the CF technologies would not be significantly felt at that time. T-test results showed that there is a statistically significant difference between the farmers' income before and after CFV adoption at 95% confidence level. Also,

the percent change in the net income of the farmer-adopters (90%) was found to be higher than the percent change in the net income of the farmer-volunteers (61%). The farmer-adopters' better market access would account for the better performance.

**Table 3**. Net income before and after CFV, 22 farmer-adopters in 2009-2013, La Libertad, Negros Oriental, Philippines.

| Item | Before CFV | After CFV | Difference | % Difference |
|---|---|---|---|---|
| *RETURNS* | | *Pesos per hectare* | | |
| Home consumption | 5,420.81 | 7,173.72 | 1,752.91 | 32% |
| Sales from crops and livestock | 9,096.02 | 19,017.33 | 9,921.31 | 109% |
| Total Returns | 14,516.83 | 26,191.05 | 11,674.22 | 80% |
| *COSTS* | | | | |
| Production Cost | 1,275.00 | 1,081.39 | -193.61 | (15.18) |
| Total Cost | 1,275.00 | 1,081.39 | -193.61 | (15.18) |
| *NET RETURNS* | 13,241.83 | 25,109.66 | 11,867.83 | 90% |

**Table 4.** Net income before and after CFV, 10 farmer-volunteers in 2009-2013, La Libertad, Negros Oriental, Philippines.

| Item | Before CFV | After CFV | Difference | % Difference |
|---|---|---|---|---|
| *RETURNS* | | *Pesos per hectare* | | |
| Home consumption | 8,986.92 | 9,183.85 | 196.92 | 2% |
| Sales from crops and Livestock | 7,365.00 | 16,971.92 | 9,606.92 | 130% |
| Total Returns | 16,351.92 | 26,155.77 | 9,803.85 | 60% |
| *COSTS* | | | | |
| Production Cost | 796.15 | 1,111.92, | 315.77 | 40% |
| Total Cost | 796.15 | 1,111.92, | 315.77 | 40% |
| *NET RETURNS* | 15,555.77 | 25,043.85 | 9,488.08 | 61% |

***Impact at the community level***

At the community level, a little more than half of farmer-adopters (55%) observed that access to market has improved after the CFV program as the LGU provided trucks to transport farm produce at no cost to the farmers every Wednesday and Thursday. The LGU established trading posts and started improving the access roads to the CFV villages by changing the all-weather roads to concrete pavements. Land use and farming systems were changed by the CFV initiative through the introduction of forest and fruit trees, vegetables and hedges. Vegetables and other short duration crops were planted together with the main crop (commonly corn). Diversified farming was also practiced by the farmers. The use of inorganic fertilizers and the practice of burning crop residues were minimized. After the project implementation, an aggregate area of about 520 hectares was under CFV, or about 11% of the total land area of the five barangays. Majority of the farmer-volunteers perceived that the level of living and level of cooperation in the community improved because of CFV. Farmers supported and helped their fellow farmers as well. In contrast, majority (59%) of farmer-adopters did not observe any change.

***Impact at the household and individual levels***

Majority of the farmer-volunteers (90%) mentioned that adoption of CF technologies helped attain security for food and children's education. They were able to buy household items and farm implements because of the additional income from increased production. Majority of the farmer-adopters (82%) became food secure while less than half of the respondents (45%) mentioned that their additional income helped in purchasing household assets, such as television sets and furniture while 41% were also able to buy farm implements like shovel, scythe and plow. In addition, 45% of the farmer-adopters attributed house improvement to CFV adoption. Considering both farmer-volunteers

and farmer-adopters, 82% mentioned that their level of living improved after the CFV project. This is consistent with Newby and Cramb's (2011) findings that adoption of CF practices increased the farmers' productivity which increased income and helped improve their level of living.

Majority of farmer-volunteers and farmer adopters perceived that their leadership ability, self-confidence and decision-making were enhanced. Moreover, their social network (among members of the community and the LGU) expanded and this helped in addressing farm problems. This confirms Cramb's (2005) findings that adoption of CF systems increased the formation of networks and association within the community that could serve as a form of social capital for the farmers. Social network size plays a role in the adoption of natural resource management practices (Wosen et al. 2013). Social capital plays a significant role in the adoption of soil and water conservation practices among 398 farming households in Ethiopia (Husen et al. 2017). Seventy seven percent of the 22 farmer-adopters enhanced their leadership ability as shown by their being officers of the CFV organization established or 'leading' the non-members to follow the CF technologies. This in turn, led to increased self-confidence, better decision-making and an expanded social network.

Results did not show high correlation values with income (INC) that would support the respondents' perception (Table 5). However, the analysis revealed statistical significance between the variables, purchase of farm implements (PFI) and purchase of household items (PHHI) indicating that farmers who were able to buy new farm implements were among those who bought new household items. Furthermore, the correlation among PFI, PHHI, and increased social network (SOCNET) was found to be statistically significant. Leadership also showed statistically significant values with PHHI (a= 5%) and SOCNET (a=1%) indicating that those who claimed that their leadership improved were among the respondents who reported that their social network improved and that they were able to purchase new items for their households. The correlation between household food security (FOODSEC) and INC was not significant even at a 90% confidence level. A number of the respondents who claimed that their household food security improved were those who were also able to increase farm production but did not sell their additional harvests and were used for home consumption. The difference in the number of soil conservation methods that the respondents adopted (SCA) did not make any significant difference. This indicates that the increase in income among the farmers was due to the additional crops that were planted and sold.

**Table 5.** Correlation analysis between change in income and selected socio-economic variables, La Libertad, Negros Oriental, Philippines, 2015.

| Variable | INC | FOODSEC | PFI | PHHI | SOCNET | LEAD | SCA |
|---|---|---|---|---|---|---|---|
| INC | 1.0000 | | | | | | |
| FOODSEC | 0.2666 | 1.0000 | | | | | |
| | 0.1337 | | | | | | |
| PFI | 0.1274 | -0.2840 | 1.0000 | | | | |
| | 0.4799 | 0.1093 | | | | | |
| PHHI | 0.2171 | 0.1304 | 0.4588 | 1.0000 | | | |
| | 0.2248 | 0.4693 | 0.0072 | | | | |
| SOCNET | -0.1458 | -0.2364 | 0.3967 | 0.3758 | 1.0000 | | |
| | 0.4180 | 0.1853 | 0.0223 | 0.0311 | | | |
| LEAD | -0.1185 | -0.0213 | 0.1754 | 0.4287 | 0.5129 | 1.0000 | |
| | 0.5113 | 0.9064 | 0.3289 | 0.0128 | 0.0023 | | |
| SCA | 0.1878 | 0.0832 | 0.0592 | 0.1126 | 0.2938 | 0.1125 | 1.0000 |
| | 0.2952 | 0.6454 | 0.7435 | 0.5329 | 0.0971 | 0.5330 | |

Key: upper level values - rho, lower level values – sig. level

***Farmers' attitude towards conservation***

Majority of the farmers will continue the CF practices due to the benefits derived such as improvement in the level of living and additional income. Some farmers mentioned continuation of the CF practices because of the concern for the future generation. From their perspective it will help in the improvement of the environment because it helps prevent soil erosion. Majority of the farmers said they will convince other farmers to try CF practices so they enjoy the same benefits they are getting from the CF technology. Some farmers mentioned that convincing other farmers will benefit the community more because of the prevention of soil erosion through adoption of CF practices.

**Environmental impact**

The annual rates of soil erosion ranged from 41.1 to 67.6 tons/ha/yr with an average of 54.3 tons/ha/yr (start of the project) and 7.5 to 19.5 tons/ha/yr with an average of 10.2 tons/ha/yr (after the project). In the Philippines, the considered table soil erosion is less than 10/tons/ha/year (PCAARRD 2001). In general, a reduction of 81% in the amount of soil erosion was observed after the project (Table 6).

**Table 6.** Changes in soil erosion rates (in ton/ha/yr) in La Libertad, Negros Oriental, Philippines, 2015.

| Barangay | Average annual erosion rate (ton/ha/yr) | | | | |
|---|---|---|---|---|---|
| | **Before** | **Classification** | **After** | **Classification** | **% decrease** |
| Nasunggan | 67.6 | very high | 11.9 | low | 82 |
| Aya | 41.1 | very high | 8.5 | low | 79 |
| Talaon | no data | - | 19.5 | moderate | - |
| Elecia | no data | - | 12.7 | low | - |
| Pitogo | no data | - | no data | - | - |
| **Average** | **54.3** | **very high** | **10.2** | **low** | **81** |

The level of soil fertility in the CFV sites in La Libertad remained the same for a period of seven years (2008-2015). The levels of P, organic matter and pH remained the same while the level of K decreased based on qualitative description. However, numerical values derived from the soil analyses showed a general downward trend in the soil nutrient levels (Table 7). Several reasons can be cited to explain this situation. First, though the erosion rate was reduced, soil erosion still occurred even in the model farms. Also, the practice of organic farming has not significantly increased among the farmers. Instead, majority of the farmers did not apply any form of fertilizer to recharge the soil nutrients. Thus, with increased cropping intensity, nutrients remaining in the soil were continuously "mined" thereby decreasing soil fertility levels further. Moreover, soil acidity was not consciously addressed by the CFV program.

**Table 7.** Changes in the level of N, P, K, %OM, and pH before and after the CFV project, La Libertad, Philippines.

| Period | N (%) | P | | K | | % OM | | pH | |
|---|---|---|---|---|---|---|---|---|---|
| | | (Ppm) | Level | (Me/100g soil) | Level | Value | Level | Value | Level |
| Before | 0.13 | 5.8 | low | 0.60 | high | 2.7 | medium | 5.5 | strongly acid |
| After | 0.11 | 2.0 | low | 0.33 | medium | 2.1 | medium | 5.1 | strongly acid |
| Change | No change | No change | | Decrease | | No change | | No change | |

Note: P (before & after) - using Bray method

Projected trends in soil erosion rates, N, P, K and organic matter (%OM) are shown in Table 8. As a result of adopting CF technologies, all the parameters mentioned above showed decreasing rates. From a high of 72 tons/ha/yr, projected soil erosion in 2015 reached the tolerable level at 10.2 tons/ha/yr. The reduced rate of N, P and K loss also translates to a reduced rate when these nutrients are translated to kg/ha/yr (Table 8). A large reduction in the amount of soil erosion is a proof that the conservation farming technologies established in La Libertad were effective in reducing soil erosion. This is the reason why, after the CFV project, 28% of farmers did not experience erosion while 55% of the farmers experienced low to moderate erosion. The farmers also observed that the floodwaters became clearer, not muddy as previously described before adoption of the CF technologies.

**Overall Impact of the CFV Project**

An incremental net benefit analysis using a cash flow from 2009 to 2015 was applied to determine the worth of the CFV project. The value of nutrient losses were incorporated in the stream of costs in the cash flow table starting in 2010 until 2012 (Table 9). These losses represent the cost of delayed adoption of the CFV technologies. Note that the values were decreasing over time indicating that more farmers were adopting the CFV practices until 2012 when all target farmers have completely joined the CFV. Thus, by 2013, no nutrient losses appeared as a cost item in the cash flow table.

The incremental increase in income of the 32 farm households who initially adopted the soil conservation measures is presented in the cash flow table starting from the year 2010. For the late adopters, increased income was reflected from 2011 onwards. Prior to this year, only the value of harvests from traditional crops (without the additional production triggered by the LGU assistance) was included in the cash flow to account for the opportunity cost of late adoption. Also appearing in the stream of benefits is the value of nutrients saved due to reduced soil erosion. A decreasing trend in erosion was observed from the period 2011 to 2015. This implies that the soil nutrients being lost from erosion was also decreasing. Conversely, the quantity of nutrients being saved from erosion was increasing over time. In addition, the number of farmer-adopters also increased to 343. Thus, the value of nutrients saved exhibited an increasing trend from 2011 to 2015. Using a 6% discount rate resulted in a positive Net Present Value (NPV) and a BCR equal to 1.14. The Internal Rate of Return turned out to be 10% which is greater than the minimum attractive rate of return (6%) for development projects. The results indicate that the annual net benefits generated by the CFV project at La Libertad from 2009 to 2015 were able to recover the investments for mitigating soil erosion in the area.

## CONCLUSION AND RECOMMENDATIONS

The CFV project was able to increase farm production and conserve land of upland farmers of La Libertad. The conservation farming technologies that were adopted were effective in reducing soil erosion. Crop diversification increased farmers' income. Net returns generally increased after CF adoption. The CFV project resulted in a positive social impact as it improved the farmers' self-worth leading to better leadership capability and decision-making, as well as increased social network. As a whole, the benefits generated by the CFV project outweighed the costs.

The CF technologies should be introduced in other upland areas that are susceptible to soil erosion. The encouraging results of the impact evaluation indicate that the conservation farming technologies would benefit other upland farms around the municipality of La Libertad. In the same vein, soil erosion in upland farms located in other areas of the country can be effectively addressed through the adoption of CF technologies.

**Table 8.** Trends in soil erosion, losses in N, P, K and % OM, La Libertad, Negros Oriental, Philippines, 2008-2015.

| Year | SE[1] tons/ha/yr | N (%) | P (ppm) | K (me/100g soil) | OM (%) | N | | P | | K | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | kg/ha/yr | decrease/ yr | kg/ha/ yr | decrease/ yr | kg/ha/ yr | decrease/ yr |
| 2008 | 72.0 | 0.14 | 7.3 | 0.7 | 3.0 | 103.69 | | 0.53 | | 2.01 | |
| 2009 | 63.2 | 0.14 | 6.5 | 0.7 | 2.8 | 87.46 | 16.23 | 0.41 | 0.11 | 1.67 | 0.34 |
| 2010 | 54.3 | 0.13 | 5.8 | 0.6 | 2.7 | 72.21 | 15.25 | 0.31 | 0.10 | 1.35 | 0.31 |
| 2011 | 45.5 | 0.13 | 5.0 | 0.5 | 2.6 | 57.95 | 14.27 | 0.23 | 0.09 | 1.07 | 0.28 |
| 2012 | 36.7 | 0.12 | 4.3 | 0.5 | 2.5 | 44.66 | 13.28 | 0.16 | 0.07 | 0.82 | 0.25 |
| 2013 | 27.8 | 0.12 | 3.5 | 0.4 | 2.3 | 32.36 | 12.30 | 0.10 | 0.06 | 0.61 | 0.22 |
| 2014 | 19.0 | 0.11 | 2.8 | 0.4 | 2.2 | 21.03 | 11.32 | 0.05 | 0.05 | 0.42 | 0.19 |
| 2015 | 10.2 | 0.11 | 2.0 | 0.3 | 2.1 | 10.69 | 10.34 | 0.02 | 0.03 | 0.27 | 0.16 |

[1] SE values were computed using MUSLE and showed significant decrease from 2008 to 2015

**Table 9.** Cash flow for Benefit-Cost Analysis, 343 CFV farmer-adopters, La Libertad, Negros Oriental, Philippines.

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| Item | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 |
|---|---|---|---|---|---|---|---|
| Total Net Returns from Production | 0 | 358,565 | 2,414,368 | 2,633,134 | 2,633,134 | 7,011,709 | 7,011,709 |
| Nutrients saved | 0 | 12,309 | 25,115 | 28,944 | 95,345 | 88,816 | 82,288 |
| Total benefits | 0 | 370,873 | 2,439,484 | 2,662,079 | 2,728,479 | 7,100,525 | 7,093,997 |
| Cost of mitigating soil erosion | | | | | | | |
| Establishment cost | 115,030 | 20,800 | 18,390 | 0 | 56,255 | 0 | 0 |
| Project cost | 7,200,000 | 0 | 0 | 0 | 0 | 0 | 0 |
| Support of the LGU (tools, planting materials) | 2,000,000 | 2,000,000 | 2,000,000 | 0 | 2,000,000 | 0 | 0 |
| Total cost of mitigation | 9,315,030 | 2,020,800 | 2,018,390 | 0 | 2,056,255 | 0 | 0 |
| Nutrient loss | 0 | 93,328 | 78,775 | 72,929 | 0 | 0 | 0 |
| Total cost | 9,315,030 | 2,114,038 | 2,097,165 | 72,929 | 0 | 0 | 0 |
| NET BENEFIT | -9,315,030 | -1,743,165 | 342,318 | 2, 589,150 | 672,224 | 7,100,525 | 7,093,997 |

**IRR = 10%** **NPV (6%) = 2,224,920** **BCR (6%) = 1.14**

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## APPENDIX TABLES

**Appendix Table 1**. Soil fertility analysis during CFV project implementation, La Libertad, 2010.

| Barangay | N 5% of OM | P | | K | | OM | | pH | |
|---|---|---|---|---|---|---|---|---|---|
| | | Ppm (Bray) | Level | Me/100g soil | Level | Percent | Level | Value | Level |
| Nasunggan | 0.13 | 2.8 | Low | 0.47 | Medium | 2.6 | Medium | 5.6 | Moderately acidic |
| Aya | 0.15 | 2.8 | Low | 0.48 | Medium | 2.9 | Medium | 4.9 | Very strongly acidic |
| Pitogo | 0.12 | 11.8 | Medium | 0.85 | High | 2.7 | Medium | 5.9 | Moderately acidic |
| Average | 0.13 | 5.8 | Low | 0.6 | High | 2.7 | Medium | 5.5 | Strongly acidic |

Source: Silliman University 2011.

**Appendix Table 2**. The condition of the study sites before the CFV project implementation.

| Item | Condition before the project |
|---|---|
| Facilities and services | |
| Roads | Not accessible during rainy season |
| Schools | Primary level |
| Livelihood | Not sustainable |
| Electricity | None |
| Potable water | Limited |
| Landslides | Frequent (during typhoon seasons) |
| Agricultural production | Low level |
| Environmental hazards | |
| Landslides | Frequent (during typhoon seasons) |
| Flooding | Frequent (in low-lying areas) |
| Soil erosion | Excessive |

Source: Survey Data 2015.

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# POPULATION DYNAMICS AND GROWTH PATTERN OF THE BROWN PLANTHOPPER, *Nilaparvata lugens* (Stål) AND ITS NATURAL ENEMIES IN SUSCEPTIBLE AND RESISTANT TROPICAL RICE VARIETIES IN CENTRAL THAILAND

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## ABSTRACT

The brown planthopper (BPH), *Nilaparvata lugens* (Stål), is an important rice insect pest in tropical Asia. This study examined the population dynamics of BPH and its natural enemies in Central Thailand over two cropping seasons in 2015 in rice fields planted with susceptible and resistant varieties to BPH. Our results showed three implications in the behavior of populations of BPH and its natural enemies under non-outbreak conditions: 1. No consistent differences were found in the growth pattern of BPH between rice fields planted with resistant and susceptible rice varieties. 2. Typical seasonal growth pattern of BPH tropical populations, characterized by early density peaks and high initial density was only found in fields planted with susceptible rice in Nakhon Nayok Province in the wet season. 3. The natural enemies *Pardosa pseudoannulata* (Bösenberg & Strand), the wolf spider (WS) and *Cyrtorhynus lividipennis* (Reuter), the mirid bug (MB) responded differently to changes in the density of BPH among rice fields and crop seasons. This study suggests that pest low-density conditions and the crop seasons have an effect on the population dynamics of BPH and its natural enemies and therefore, on the pest natural control by predators and the level of pest suppression in fields planted with BPH-resistant varieties.

**Key words:** community, insect predators, pest, population density, rice cultivars,

## INTRODUCTION

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) is one of the most important rice insect pests in temperate, tropical and sub-tropical regions of Asia (Bottrell and Schoenly 2012, Cook and Perfect 1989, Kuno and Dyck 1985). The development of BPH is incomplete, with life stages from egg to nymph and to dimorphic adults; brachypterous (short-winged) and macropterous (long-winged) (Denno and Roderick 1990). Population growth characteristics of BPH are different in temperate and tropical regions. In temperate areas, the pest growth is abrupt and stable in time. Thus, it is possible to predict potential outbreaks by monitoring density of BPH long-winged adults that migrate into rice fields (Cheng and Holt 1990, Kisimoto 1981). Immigration of BPH in the tropics, is continuous over the crop period and is believed to be basically controlled by natural enemies (Cook and Perfect 1989, Kenmore et al. 1984). Outbreaks of BPH in tropical rice fields have been mainly attributed to the misuses of pesticides that disturbs the natural control of the pest by killing predators and parasitoids (Heinrichs and Mochida 1984), including those related with the spraying of higher concentrations of harmful pesticides than the legally allowed by the authorities and the use of

pesticide cocktails (Arunmit et al. 2012). Abiotic factors such as temperature and rainfall also influence fluctuations of BPH populations (Win et al. 2011) and in-field factors such as rice varietal use and planting methods have been also associated with BPH outbreaks (Cook and Perfect 1989, Magunmder et al. 2013, Tetarwal et al. 2014, Wada and Salleh 1992, Win et al. 2011, Zhu et al. 2004).

Breeding resistant rice varieties to BPH and to other insect pests has been one of the most effective and environmentally friendly approaches for insect pest management in rice crops (Alagar et al. 2007, Brar et al. 2009, Jena et al. 2006). Resistant rice can cause reductions in the pest density by about 100-fold, saving rice farmers the need to use pesticides and its economic cost (Aquino and Heinrichs 1979, Brar et al. 2009, Kenmore et al. 1984). Among the benefits of BPH-resistant rice varieties, lower nymph development, less fecundity of the pest and reduced population build-up have been demonstrated (Alagar et al. 2007, Cohen et al. 1997, Saxena and Pathak 1979). The main mechanisms of resistance are known to come from the manipulation of BPH resistance genes that involve changing the plant phloem chemistry and the loss of important feeding components for the insect pest that affect its behavior and physiology and in some cases producing insecticidal Bt-toxins (Bottrell and Schoenly 2012, Cook and Denno 1994, Fisk 1980, Sogawa 1982, Sogawa 2015, Lundgren et al. 2009). Twenty-one genes for BPH-resistance have been identified from genetic resources such as wild species of *Oryza* and landrace cultivars (Jena and Kim 2010).

Parallel to the benefits for crop pest management, concerns about the advantages of host pest-resistance and its possible risks have been also claimed, especially possible adverse effects of pest-resistant cultivars on the community of natural enemies and the emergence of new BPH biotypes, which are BPH populations that have adapted to a particular resistant rice variety (Bottrell et al. 1998, Jena and Kim 2010, Lundgren et al. 2009, Poppy 2000, Poppy and Sutherland 2004, Wolfenbarger and Phifer 2000). In order to visualize the benefits of pest-resistant rice plants as an alternative for crop protection, risk assessment of potential changes on ecosystem services like the natural pest control provided by natural enemies, needs to be evaluated especially in rice cropping systems, where pest resistant rice is used as the only pest management strategy (Lundgren et al. 2009). Until now, this kind of assessment has been mainly focused on the toxicity of insecticidal chemicals produced by the pest-resistant rice host (Jumin et al. 2000, Li et al. 2007, Lee et al. 2014). However, the pest and its natural enemies interact with the resistant host-plant in ways that are not assessed under the toxicological approach, and an assessment under a community-based approach, is also required (Lundgren et al. 2009).

A community-based assessment of pest-resistant crops is necessary to be conducted under field conditions and involves monitoring the density of the pest population and the populations of natural enemies in fields planted with the pest-resistant and fields planted with non-resistant plants. Although this type of studies are difficult to conduct since they require numerous field sites and years of study, they have relevance for the assessment of some ecological impact of pest-resistant crops (Lundgren et al. 2009, Bernal et al. 2002).

Thailand is an important world rice exporter and outbreaks of BPH seriously affected rice production in the central plain and lower northern regions of the country from 2009 to 2012 (Chaiyawat 2011, Heong and Hardy 2009, Luecha 2010, Rattanakarn et al. 2012, Soitong et al. 2011, Sriratanasak et al. 2011, Thongdeethae 2009, Wattanesk 2010). Previous research on BPH in Central Thailand has been mainly focused on studying the mechanisms for the development of BPH-resistance to pesticides and to BPH-resistant rice cultivars (Arunmit et al. 2012, Chaiwong et al. 2010, Pongprasert and Weerapat 1979, Punyawattoe et al. 2013, Sriratanasak et al. 1996). Meanwhile, studies about the population dynamics of BPH in Central Thailand have been conducted only in resistant rice varieties (den Braber and Meenakanit 1992).

Therefore, this study examined under a community-based approach, the population dynamics and growth pattern of BPH and its natural enemies in two important provinces of rice production in Central Thailand over two cropping seasons in farmers' fields, non-sprayed with insecticide and planted with different BPH-resistant and susceptible rice varieties. This study aimed to contribute to the clarification of the knowledge about the population dynamics of BPH and its natural enemies in tropical rice agroecosystems and to provide insights about the potential ecological impact of BPH-resistant rice varieties for farmers and its implications for the local management of BPH.

## MATERIALS AND METHODS

### Area of study and climate

Population surveys of BPH and its natural enemies were conducted at rice fields in Chai Nat and Nakhon Nayok Provinces in the Central Thailand (Fig. 1) during the dry and wet seasons in 2015.

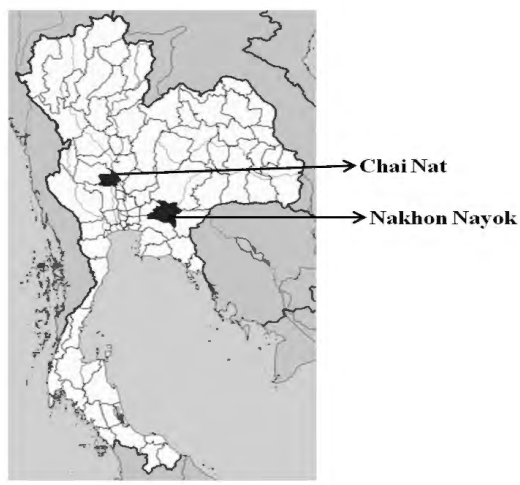


**Fig. 1.** Location of Chai Nat and Nakhon Nayok Provinces in Central Thailand - the survey area for rice fields sampling.

Climate of the central region of Thailand shows characteristics of Köppen's tropical savanna climate with clear dry and wet seasons. There is prevalence of the dry conditions from November until April of the next year and the rainy season occurs from May to October (Ginigaddara and Ranamukhaarachchi 2009). In 2015, the Central Thailand was warmer than an average year (average of 29 years; 1981-2010) (Thai Metereological Department 2015). Annual mean temperature in the Central Thailand was 28°C, 0.8 °C above average (Fig. 2). The central region of Thailand was also drier in 2015; the annual 2015 rainfall was 1202 mm, 73 mm less than the average value (1275 mm) during the same period as shown above (Fig. 2).

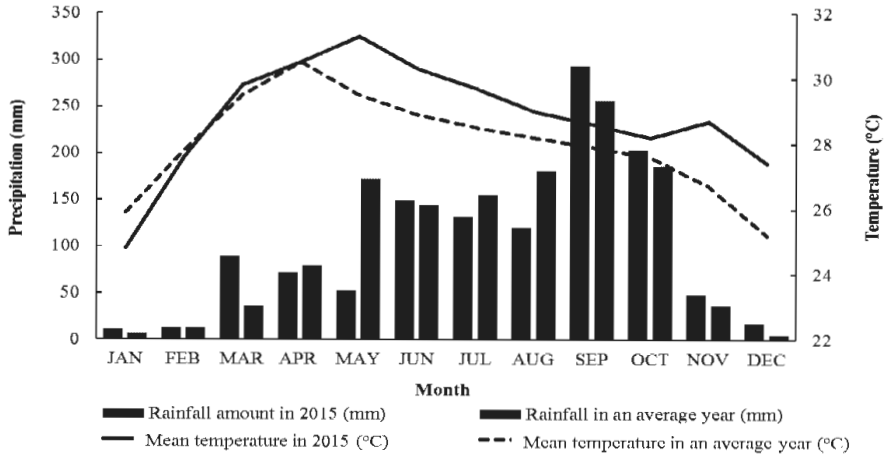


**Fig. 2.** Monthly average temperature and rainfall in Central Thailand in 2015 and in an average year. Source: TMD 2016.

**Rice cultivation in central Thailand**

The central region of Thailand is the most intensively rice cultivated alluvial area in the country. During the rainy season, rice production in Central Thailand represents about 20 percent of the whole rice cultivated area in the country (AEO 2011, IRRI 2016). The system of production is highly mechanized, irrigation is common and the direct-seeded method of cropping is the most common among farmers. Several high yielding rice varieties adapted for the irrigated environment with insect pest resistance have been released in the region, being Chai Nat 1, RD 47 and Pisanulok 2 among some of them (Kupkanchanakul 2000). Details of varietal use, planting methods and transplanting/seeding dates at the sampled fields in this study are shown in Table 1.

Chai Nat and Nakhon Nayok Provinces are considered as two of the most intensive rice producing provinces in the country (AEO 2011). In Chai Nat the current planted rice area ranges from 120,000 to 150,000 ha. In Nakhon Nayok about 60,000 ha of rice fields have been planted during the last six years (AEO 2011). Fallow periods are longer in Nakhon Nayok than in Chai Nat and the main rain-fed rice variety grown in Nakhon Nayok is Thai Jasmine Rice 105 (Table 1).

Fig. 3 shows the details about rice cropping calendar in the two provinces of the study. Rice growing at Chai Nat Province is mainly irrigated, following the cropping system "rice-rice-rice" or “rice-rice” with no alternating crops. Rice is grown once or twice in the dry season and once in the wet season (AEO 2011). In Chai Nat, the first rice crop in the dry season starts from November up to mid March of the next year, the second dry season crop from mid-March to mid-June and the wet season crop from mid-June up to October. When the cropping system “rice-rice” is taken, the wet season crop will happen as described before but the schedule for the dry season crop will mostly depend on the availability of irrigation resources for each rice farmer. At the studied fields in Chai Nat, the wet season crop planting was practiced in June and harvested in September, while the dry season crop planting was from December to late February and harvesting from early March to April (Table 1).

Rice growing in Nakhon Nayok Province is rain-fed or irrigated. In Nakhon Nayok the cropping pattern generally follows the "rice-rice" system with no alternating crop. There are two main rice growing seasons, one in the dry season from November to the end of February and a wet season crop from May until the end of October. At the studied fields in Nakhon Nayok planting during the

wet season was practiced from June until the end of August; harvest was done from September until late October.

Table 1. Crop season, varietal use, planting method, duration of the crop and planting/seeding date of the sampled rice fields at Chai Nat and Nakhon Nayok Provinces in Central Thailand

| Field No. | Province | Season | Resistance to BPH | Variety | Planting method | Crop duration (DAT/S)* | Planting/seeding date |
|---|---|---|---|---|---|---|---|
| 1 | Chainat | Dry | Resistant | Pisanulok 2 | Transplanting | 72 | 19/01/2015 |
| 2 | Chainat | Dry | Resistant | RD41 | Transplanting | 66 | 12/01/2015 |
| 3 | Chainat | Dry | Resistant | RD41 | Transplanting | 60 | 16/02/2015 |
| 4 | Chainat | Dry | Resistant | RD57 | Direct seeding | 50 | 26/02/2015 |
| 5 | Chainat | Dry | Resistant | RD49 | Direct seeding | 69 | 26/12/2015 |
| 6 | Chainat | Dry | Resistant | Pisanulok 2 | Direct seeding | 58 | 06/01/2015 |
| 7 | Chainat | Dry | Resistant | Pisanulok 2 | Direct seeding | 58 | 06/01/2015 |
| 8 | Chainat | Dry | Resistant | RD57 | Direct seeding | 66 | 10/02/2015 |
| 9 | Chainat | Dry | Susceptible | RD31 | Transplanting | 66 | 25/01/2015 |
| 10 | Chainat | Dry | Susceptible | Chainat 1 | Transplanting | 69 | 16/01/2015 |
| 11 | Chainat | Dry | Susceptible | RD31 | Transplanting | 52 | 18/01/2015 |
| 12 | Chainat | Dry | Susceptible | Pathumtani1 | Transplanting | 67 | 18/01/2015 |
| 13 | Chainat | Dry | Susceptible | Pathumtani1 | Direct seeding | 63 | 19/02/2015 |
| 14 | Chainat | Dry | Susceptible | Pathumtani1 | Direct seeding | 63 | 19/02/2015 |
| 15 | Chainat | Dry | Susceptible | Pathumtani1 | Direct seeding | 81 | 27/12/2015 |
| 16 | Chainat | Dry | Susceptible | Pathumtani1 | Direct seeding | 62 | 20/02/2015 |
| 17 | Chainat | Dry | Susceptible | Pathumtani1 | Direct seeding | 63 | 19/02/2015 |
| 18 | Chainat | Wet | Resistant | RD41 | Transplanting | 75 | 19/06/2015 |
| 19 | Chainat | Wet | Resistant | RD41 | Direct seeding | 69 | 25/06/2015 |
| 20 | Chainat | Wet | Resistant | RD41 | Direct seeding | 74 | 20/06/2015 |
| 21 | Chainat | Wet | Resistant | RD47 | Direct seeding | 75 | 20/06/2015 |
| 22 | Chainat | Wet | Resistant | RD49 | Transplanting | 71 | 23/06/2015 |
| 23 | Chainat | Wet | Susceptible | RD31 | Direct seeding | 77 | 17/06/2015 |
| 24 | Chainat | Wet | Susceptible | RD31 | Direct seeding | 77 | 17/06/2015 |
| 25 | Chainat | Wet | Susceptible | RD31 | Direct seeding | 80 | 14/06/2015 |
| 26 | Chainat | Wet | Susceptible | RD31 | Direct seeding | 77 | 17/06/2015 |
| 27 | Chainat | Wet | Susceptible | RD31 | Direct seeding | 72 | 23/06/2015 |
| 28 | Nakhon Nayok | Wet | Susceptible | Thai jasmine rice 105 | Direct seeding | 87 | 22/07/2015 |
| 29 | Nakhon Nayok | Wet | Susceptible | Thai jasmine rice 105 | Direct seeding | 82 | 22/07/2015 |
| 30 | Nakhon Nayok | Wet | Susceptible | Thai jasmine rice 105 | Transplanting | 87 | 22/07/2015 |
| 31 | Nakhon Nayok | Wet | Susceptible | Thai jasmine rice 105 | Direct seeding | 87 | 22/07/2015 |
| 32 | Nakhon Nayok | Wet | Susceptible | Thai jasmine rice 105 | Direct seeding | 93 | 04/08/2015 |
| 33 | Nakhon Nayok | Wet | Susceptible | Thai jasmine rice 105 | Direct seeding | 93 | 04/08/2015 |
| 34 | Nakhon Nayok | Wet | Resistant | RD47 | Direct seeding | 90 | 28/06/2015 |
| 35 | Nakhon Nayok | Wet | Resistant | RD47 | Direct seeding | 77 | 20/06/2015 |
| 36 | Nakhon Nayok | Wet | Resistant | RD47 | Direct seeding | 88 | 23/06/2015 |
| 37 | Nakhon Nayok | Wet | Resistant | RD47 | Direct seeding | 85 | 26/06/2015 |
| 38 | Nakhon Nayok | Wet | Resistant | RD47 | Direct seeding | 93 | 26/06/2015 |

*Duration in days of the crop season after transplanting or seeding in the rice field until harvest; Days after transplanting/seeding (DAT/S)

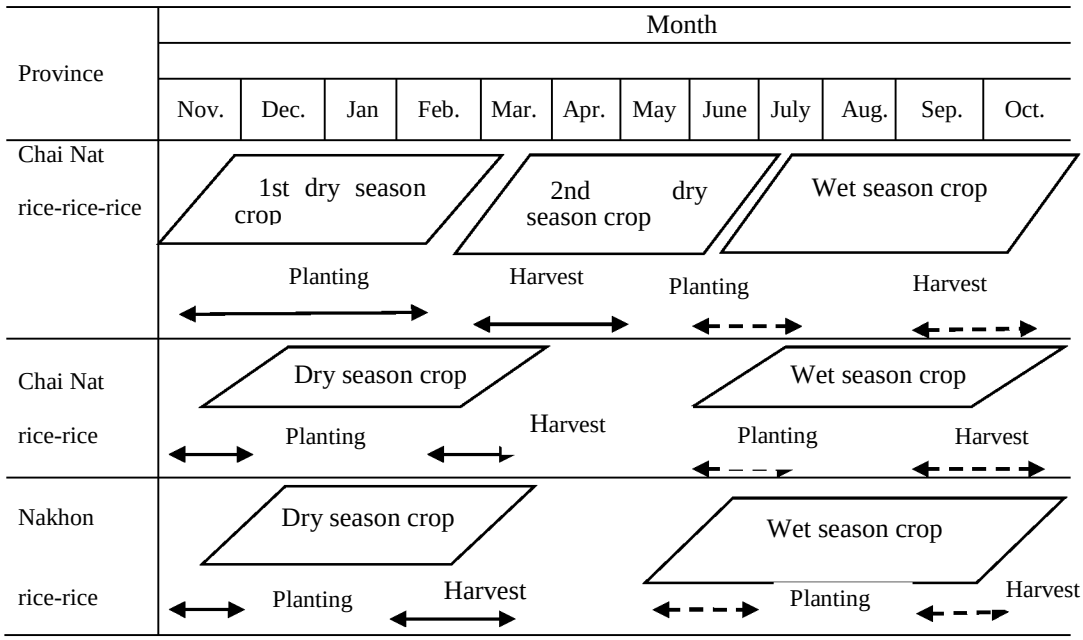


**Fig. 3.** Rice cropping calendar at Chai Nat and Nakhon Nayok.

**Rice samples**

Sampling was carried out in a total of 38 paddy fields during the late dry season (March-May) and wet season (July-September) of 2015. During the dry season, eight fields from resistant rice varieties and nine fields from susceptible rice varieties to BPH were sampled in Chai Nat Province (Table 1) every seven days. In the wet season, five fields from resistant rice varieties and five fields from susceptible varieties to BPH were sampled in Chai Nat every fourteen days. In Nakhon Nayok, five fields from resistant rice varieties and six fields from susceptible varieties to BPH were sampled during the wet season every seven days (Table 1). It was not possible to collect data during the dry season of 2015 in Nakhon Nayok due to limited irrigation resources available for rice farmers in the region and thus, significantly reduced amount of available rice fields to conduct sampling.

Densities of BPH including adults and nymphs and its major natural enemies, were assessed by carrying out sampling in the rice fields from about 13 to 20 days after transplanting/seeding (DAT/S) until harvest (70-90 DAT/S). Susceptible rice fields during the dry season in Chai Nat were sampled earlier after transplanting than in other fields since the presence of BPH was detected at 13 DAT/S. Visual counting from two transects of 10 rice hills in a diagonal way per rice field was conducted on each sampling date. A hill was considered to be the sampling unit. Sweep netting was also carried out in order to collect samples of long-winged BPH macropterous (migrants) adults following the same sampling protocol as mentioned above. Immigration was assessed counting the densities of winged adults present in the rice field up to 45 DAT/S.

Data collected were used to calculate mean densities of BPH (per hill) as well as densities of the major species of BPH natural enemies in Central Thailand (Chaiwong et al. 2010); *Cyrtorhynus lividipennis*, the mirid bug (hereinafter, MB) and *Pardosa pseudoannulatta*, the wolf spider (hereinafter, WS) (Chaiwong et al. 2010). It is important to mention that this study was conducted under two special conditions:

1. Outbreaks of BPH were not reported in Central Thailand in 2014 and 2015 (Rice Department 2015) and sampling occurred under non-outbreak conditions of BPH.

2. Farmers did not spray any pesticides at the sampled rice fields in the two provinces of the study, mainly due to the lack of economical resources that in a normal situation allow them to invest in chemical control and protect the rice harvest (Chaiwong et al. 2012). At present, rice farmers over Thailand face increasing balance due (Pingali 1993, Slayton and Timmer 2008, Sricharoen 2015) and decisions of rice crop management by farmers depend on the profits they get from the crop. If the price of rice is low, farmers try to reduce costs and they stop insecticide spraying (Matteson 2000, Pingali 1993, Sricharoen 2015).

**Data Analysis**

Statistical analysis was performed using the software STATISTICA10 (StatSoft, Inc.). The analysis of variance (ANOVA) was performed to evaluate the effects of three factors, i.e. province, crop season and rice variety. Although three factors are involved, overall ANOVA with three factors was not possible to perform because data for one crop season are missing (Nakhon Nayok, dry season). Thus, we performed ANOVA with two factors (province/season and rice variety). Density peaks of BPH and its growth pattern at each province and season were also compared between BPH-resistant and susceptible rice fields (hereinafter, resistant and susceptible fields) performing Mann-Whitney U-tests. Pearson's correlation analysis was conducted to evaluate the relationship between the mean density of BPH and each of its natural enemies at each study site and season.

## RESULTS

**Incidence of BPH and its natural enemies, MB and WS in resistant and susceptible rice fields**

The analysis of variance with two factors (province/season and rice variety) in the studied rice fields at two Provinces was performed for the density of each one of the life stages of BPH and both natural enemies. As significant or nearly significant interactions between factors were found in most of densities of BPH at different growth stages and natural enemies, data for the average densities of BPH and its natural enemies are shown in Table 2. The average density of BPH and its natural enemies at each season and province were compared at BPH-resistant and susceptible fields as it follows. At fields in Chai Nat during the dry season, the mean density of BPH-long-winged adults and MB were significantly higher at fields with susceptible rice varieties. Densities of BPH nymphs, short-winged adults, and WS were not significantly different between rice varieties (Table 2).

In fields at Chai Nat during the wet season, the mean incidence of BPH nymphs, short-winged adults and long-winged adults as well as the densities of both natural enemies were not significantly different between resistant and susceptible varieties (Table 2). At fields in Nakhon Nayok, the mean density of short-winged adults was significantly higher at susceptible fields. Densities of nymphs, long-winged adults and both natural enemies were not significantly different between resistant and susceptible fields. Although WS did not show significantly different incidence in resistant and susceptible fields, its mean density over the crop season tended to be higher than densities of MB at both study sites and both crop seasons (Table 2).

**Correlations between mean densities of BPH and its natural enemies, throughout the crop season in resistant and susceptible fields.**

Table 3 shows the correlation between the crop season mean densities of BPH and its natural enemies, MB and WS. Densities of BPH long-winged adults were positively correlated with densities of MB and WS in both resistant and susceptible fields at Chai Nat during the dry season. Densities of BPH nymphs and short-winged adults were not significantly correlated with the occurrence of both species of natural enemies at both resistant and susceptible fields at Chai Nat during the dry season. Furthermore, densities of BPH were not correlated with densities of MB and WS neither in resistant

nor in susceptible fields at Chai Nat during the wet season. In resistant rice in Nakhon Nayok during the wet season, densities of BPH nymphs were positively correlated with densities of MB and densities of BPH short-winged adults were negatively correlated with densities of WS (Table 3). Densities of long-winged adults were not correlated with the occurrence of both species of natural enemies. In susceptible fields, densities of BPH nymphs, short-winged adults and long-winged adults were not correlated with densities of both, MB and WS (Table 3).

Table 2. Mean density of BPH and each of its natural enemies in fields planted with resistant and susceptible rice varieties

| Site and Season | Variety | Mean density throughout the crop season ((individuals/hill)±SE**) | | | | |
|---|---|---|---|---|---|---|
| | | BPH | | | Natural enemies | |
| | | Nymphs | Short-winged adults* | Long-winged adults* | *C. lividipennis** (MB) | *P. pseudoannulata* (WS) |
| Chai Nat / Dry Season | Resistant | 0.17±0.07 | 0.04±0.01 | 0.07±0.02 **A** | 0.12±0.04 **A** | 0.43±0.22 |
| | Susceptible | 0.50±0.24 | 0.05±0.02 | 0.25±0.06 **B** | 0.43±0.13 **B** | 0.47±0.16 |
| Chai Nat / Wet Season | Resistant | 0.34±0.08 | 0.08±0.02 | 0.30±0.03 | 0.30±0.14 | 0.62±0.28 |
| | Susceptible | 0.44±0.09 | 0.07±0.02 | 0.38±0.11 | 0.30±0.13 | 0.51±0.23 |
| Nakhon Nayok / Wet Season | Resistant | 0.37±0.06 | 0.05±0.01 **A** | 0.32±0.07 | 0.21±0.09 | 0.44±0.19 |
| | Susceptible | 0.79±0.24 | 0.36±0.11 **B** | 0.48±0.07 | 0.23±0.09 | 0.42±0.17 |

*Means in a column with different letters are significantly different according Fisher's LSD test (*p <0.05).*

**Data are reported as the mean ± standard error

Table 3. Correlation matrix between mean densities of BPH and mean densities of its natural enemies in fields planted with resistant and susceptible rice varieties

| **Site** | **Rice Variery** | | *C. lividipennis* (MB) | *P. pseudoannulatta* (WS) |
|---|---|---|---|---|
| Chai Nat / Dry Season | Resistant | BPH - Nymphs | -0.37 | -0.23 |
| | | BPH - Brachypterous | -0.19 | -0.08 |
| | | BPH - Macropterous | *0.99 | *0.93 |
| | Susceptible | BPH - Nymphs | 0.61 | 0.56 |
| | | BPH - Brachypterous | 0.11 | 0.11 |
| | | BPH - Macropterous | *0.78 | *0.79 |
| Chai Nat / Wet Season | Resistant | BPH - Nymphs | 0.36 | 0.05 |
| | | BPH - Brachypterous | 0.10 | -0.17 |
| | | BPH - Macropterous | -0.12 | -0.09 |
| | Susceptible | BPH - Nymphs | -0.73 | 0.46 |
| | | BPH - Brachypterous | -0.74 | 0.48 |
| | | BPH - Macropterous | 0.78 | -0.54 |
| Nakhon Nayok / Wet Season | Resistant | BPH - Nymphs | *0.99 | -0.82 |
| | | BPH - Brachypterous | 0.85 | *-0.96 |
| | | BPH - Macropterous | 0.28 | -0.07 |
| | Susceptible | BPH - Nymphs | 0.38 | -0.42 |
| | | BPH - Brachypterous | -0.21 | 0.33 |
| | | BPH - Macropterous | 0.49 | 0.28 |

*Pearson's correlation analysis significant at $p < 0.05$

**Growth pattern of BPH and its natural enemies throughout the crop season in resistant and susceptible fields.**

***Chai Nat-Dry season:*** Total BPH population in resistant fields at Chai Nat during the dry season showed lower initial densities followed by higher densities late in the crop season (Fig. 4A). However, the magnitude of total BPH density was low during all the crop period, reaching a peak density of only 0.5 BPH per rice hill at 62 DAT/S. Meanwhile, in susceptible fields, total BPH incidence was higher, with two density peaks of 1 and 1.4 BPH per rice hill at 41 and 70 DAT/S, respectively (Fig. 5A). However, the mean density of total BPH between resistant and susceptible fields was not significant. Contrary to densities in resistant fields, total BPH densities in susceptible fields continued to increase until the end of the crop period (Fig. 5A).

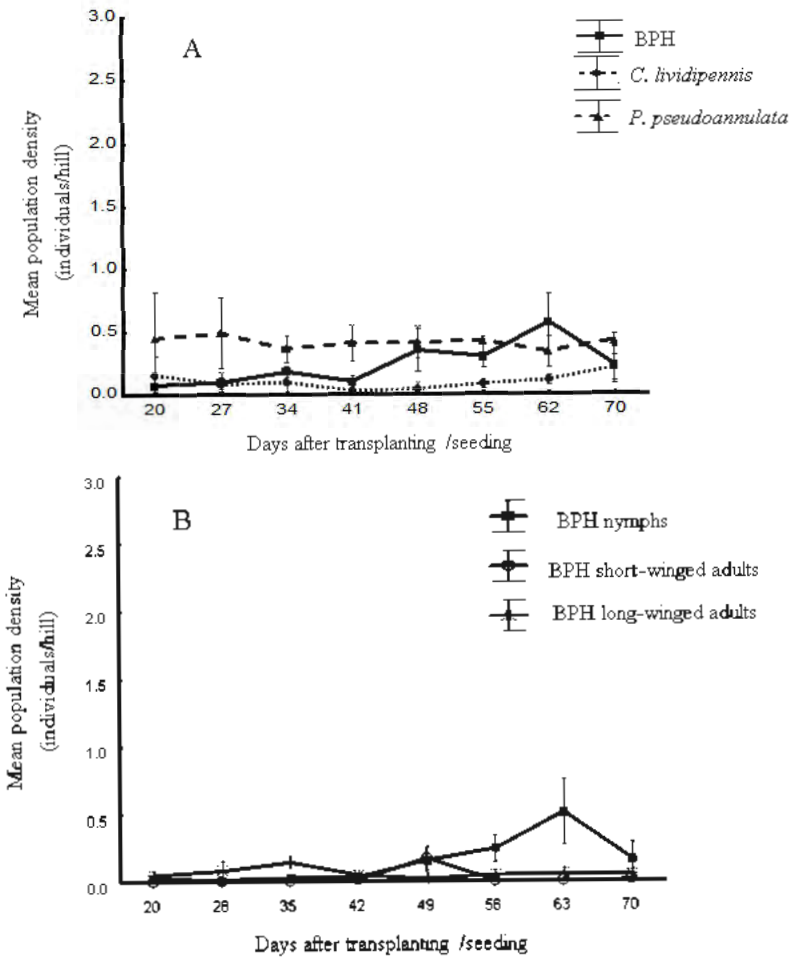


**Fig. 4.** Population growth pattern throughout the rice crop season of *N. lugens* (BPH) and its natural enemies in resistant rice fields at Chai Nat during the dry season. A. Growth pattern of total BPH (nymphs + short winged adults + long-winged adults) and its natural enemies, *C. lividipennis* and *P. pseudoannulata* B. Growth pattern of BPH life stages.

Long-winged adults showed a very low incidence during all the crop period in resistant fields (Fig. 4B) but in susceptible fields, there was an initial peak density of long-winged adults at 27 DAT/S of about 0.75 individuals per hill (Fig. 5B). The magnitude of the peak density of BPH-long-

winged adults was significantly higher in BPH-susceptible fields (*p= 0.048*) as well as the magnitude of the first peak density of nymphs (*p= 0.0087*) than those in resistant fields. BPH nymphs showed a similar growth pattern to the one of total BPH, in both resistant and susceptible fields (Figs. 4 and 5).

Short-winged adults showed very low occurrence during the crop period at both, resistant and susceptible fields (Figs. 4B and 5B) and no significant difference for peak densities between rice varieties was found. The incidence of the natural enemies, MB and WS in resistant fields was low during all the crop period and its growth pattern was not similar to the one of BPH population. Nevertheless, MB showed a slight increasing density after 48 DAT/S until the end of the crop season when BPH-nymphs were also increasing until 63 DAT/S (Fig. 4A and 4B). In susceptible fields, MB showed a similar growth pattern to that of the total BPH (Fig. 5A). Densities of MB continued to increase until the end of the crop period as total BPH and BPH nymph densities did. WS showed a synchronized growth pattern with the one of BPH-long-winged adults, and both, WS and BPH-long-winged adults showed density peaks at 27 DAT/S and 62 DAT/S and decreased in density at the end of the crop period (Figs. 5A and 5B).

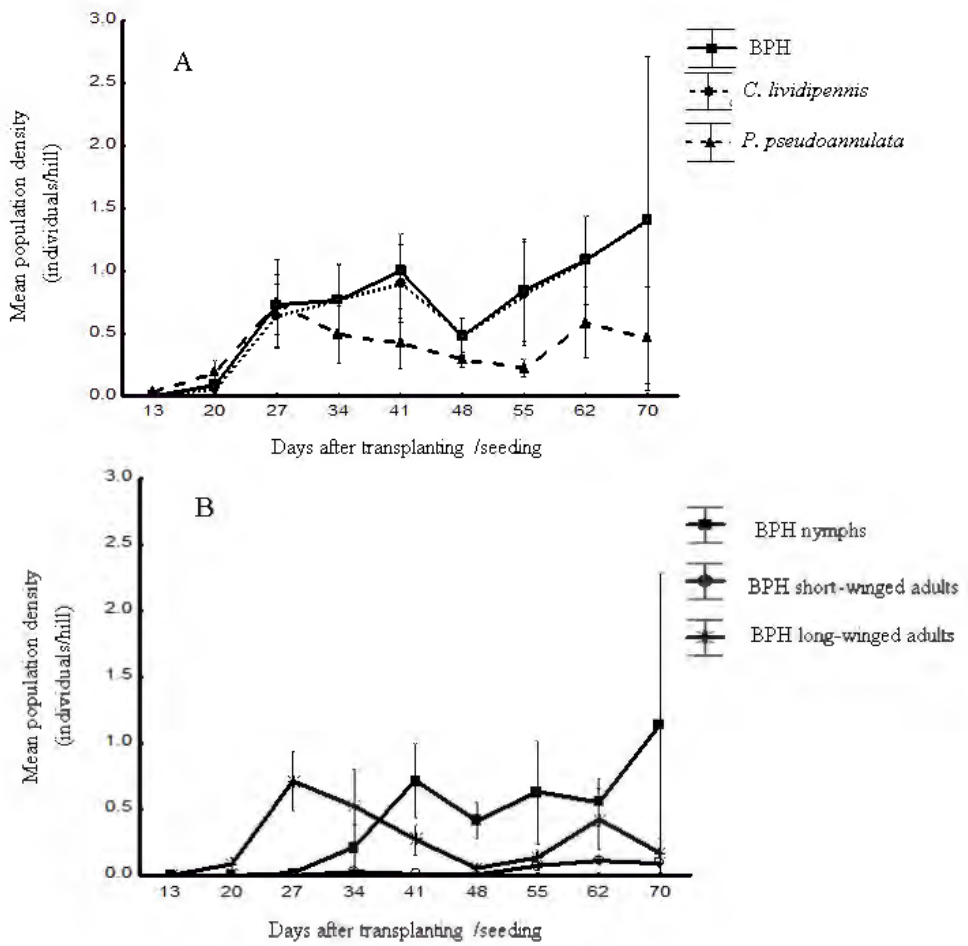


**Fig. 5.** Population growth pattern throughout the rice crop season of *N. lugens* (BPH) and its natural enemies in susceptible rice fields at Chai Nat during the dry season. A. Growth pattern of total BPH (nymphs + short winged adults + long-winged adults) and its natural enemies, *C. lividipennis* and *P. pseudoannulata*, B. Growth pattern of BPH life stage.

***Chai Nat- Wet season:*** Growth pattern of total BPH was not notably different between resistant and susceptible fields in Chai Nat during the wet season (Figs. 6A and 7A) and significant differences were not found for the peak density of nymphs, BPH-long winged adults and BPH-short winged adults. In both cases, densities were lower early in the crop period and higher after 48 DAT/S, reaching a maximum density of about 1 individual per rice hill at 62 DAT/S. However, the magnitude of BPH density in both types of fields was small during the crop season (Figs. 6A and 7A). Peaks of BPH long-winged adults in Chai Nat occurred early (Figs. 4B and 5B) and close to the mid-crop period (Figs. 6B and 7B) during the dry and wet season, respectively in both resistant and in susceptible fields at 48 DAT. In both resistant and susceptible fields, short-winged adults only increased by the end of the crop season in both cases, after 48 DAT/S (Figs. 6B and 7B). MB and WS responded positively to increases in the density of BPH early in the crop period but after 48 DAT/S their growth patterns were not synchronized with that of BPH in resistant fields (Fig. 6A). In susceptible fields, WS showed a similar growth pattern to that of total BPH during all the crop period (Fig. 7A).

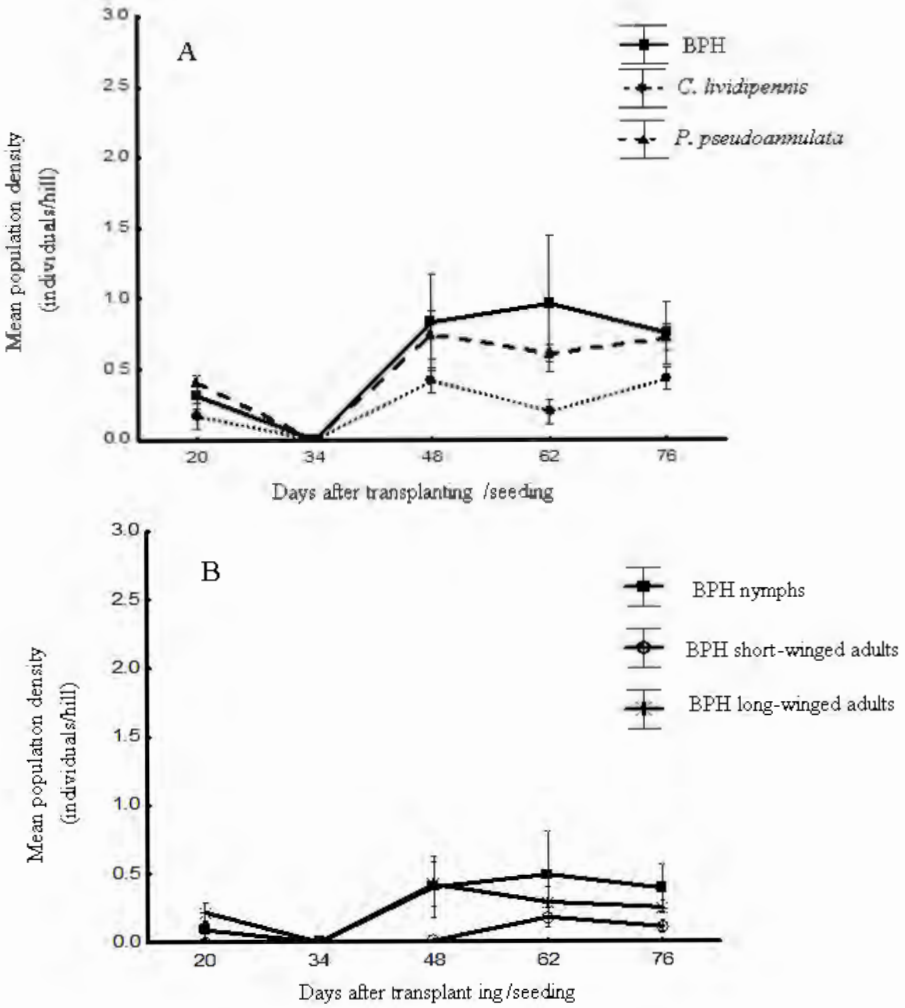


**Fig. 6.** Population growth pattern throughout the rice crop season of *N. lugens* (BPH) and its natural enemies in resistant rice fields at Chai Nat during the wet season. A. Growth pattern of total BPH (nymphs + short winged adults + long-winged adults) and its natural enemies, *C. lividipennis* and *P. pseudoannulata* B. Growth pattern of BPH life stage

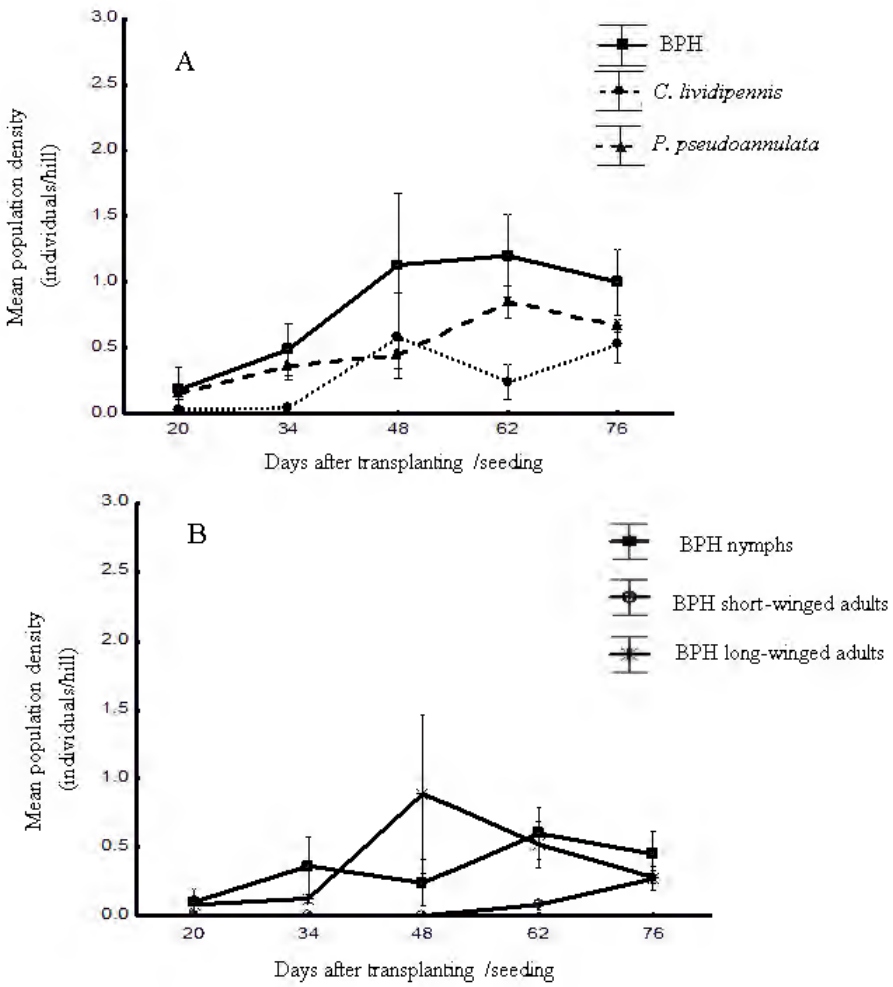


**Fig. 7**. Population growth pattern throughout the rice crop season of *N. lugens* (BPH) and its natural enemies in susceptible rice fields at Chai Nat during the wet season. A. Growth pattern of total BPH (nymphs + short winged adults + long-winged adults) and its natural enemies, *C. lividipennis* and *P. pseudoannulata* B. Growth pattern of BPH life stage

***Nakhon Nayok-Wet season***

Growth pattern of BPH in rice fields at Nakhon Nayok during the wet season was significantly different between susceptible and resistant fields (Figs. 8 and 9). The first nymph peak density was significantly higher in extent and occurred later in the crop season in BPH-resistant fields than in susceptible fields (*p= 0.035; p= 0.012*, extent and time occurrence, respectively). Total BPH population in resistant fields (Fig. 8A) showed higher incidence late in the crop season than at the beginning of the crop period with two density peaks of 2.5 and 2.8 individuals per hill at 62 and 76 DAT/S, respectively. Long-winged adults showed a peak density at 55 DAT/S. Short-winged adults and nymphs increased in density after 55 DAT/S, showing their maximum densities at 62, 76 and 83 DAT /S (Fig. 8B).

On the other hand, total BPH population in susceptible fields showed higher incidence early in the crop season with a density peak of 1.8 BPH per hill at 41 DAT/S. This density peak was followed by smaller densities later in the crop season (Fig. 9A). Nymph growth pattern showed a similar trend to total BPH, initial higher density and smaller occurrence at the end of the crop period (Fig. 9B). Long-winged adults peaked earlier (34 DAT/S) in susceptible than in resistant fields (55 DAT/S) with this time occurrence in the crop period, being significantly different between resistant and susceptible fields ($p= 0.012$). Short-winged adults showed very small incidence over the crop season (Fig. 9B). Generally, densities of BPH in Nakhon Nayok (Figs. 8 and 9) were higher than those in Chai Nat during both the dry (Figs. 4 and 5) and the wet seasons (Figs. 6 and 7).

In resistant fields at Nakhon Nayok, the natural enemies, MB and WS showed very low incidence throughout the crop season with densities being not higher than 0.5 individuals per hill (Fig. 8A). Growth pattern of MB and WS did not show a similar trend to the growth pattern of total BPH neither, to any of the life stages of BPH (Figs. 8A and 8B).

In susceptible fields at Nakhon Nayok, growth pattern of MB (Fig. 9A) was similar to that of long-winged adults and nymphs (Fig. 9B) and it responded more notably to the increases in BPH density early in the crop period than at the end of it. WS (Fig.9A) instead, responded more notably to increases in BPH density after 62 DAT/S, close the end of the crop season (Figs. 9A and 9B).

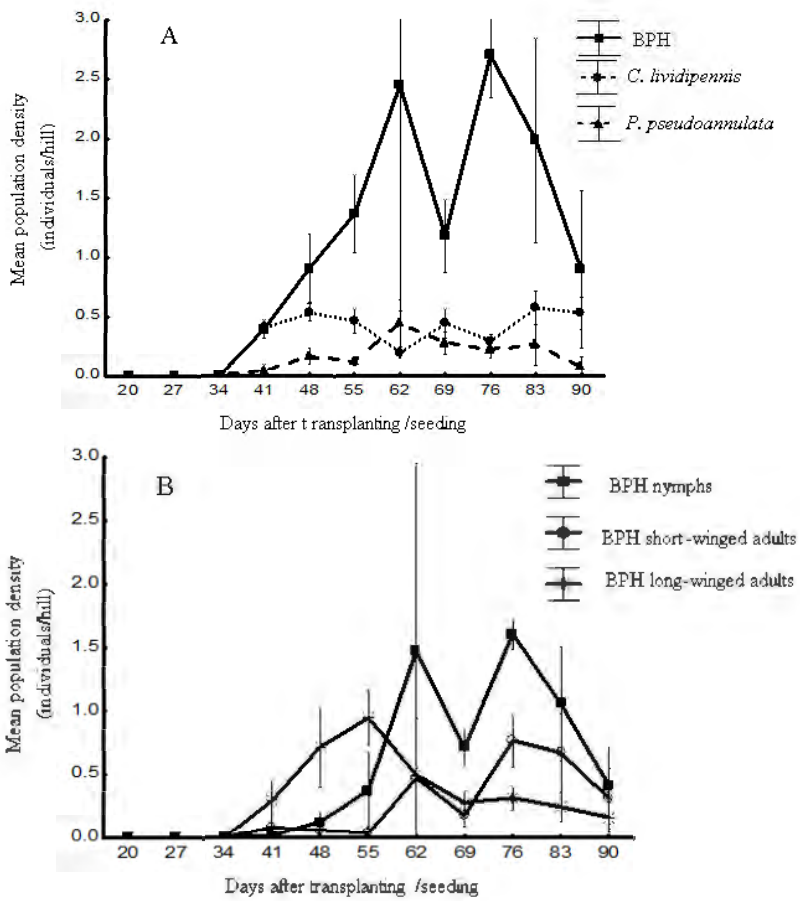


**Fig. 8**. Population growth pattern throughout the rice crop season of *N. lugens* (BPH) and its natural enemies in resistant rice fields at Nakhon Nayok during the wet season. A. Growth pattern of total

BPH (nymphs + short winged adults + long-winged adults) and its natural enemies, *C. lividipennis* and *P. pseudoannulata* B. Growth pattern of each BPH life stage.

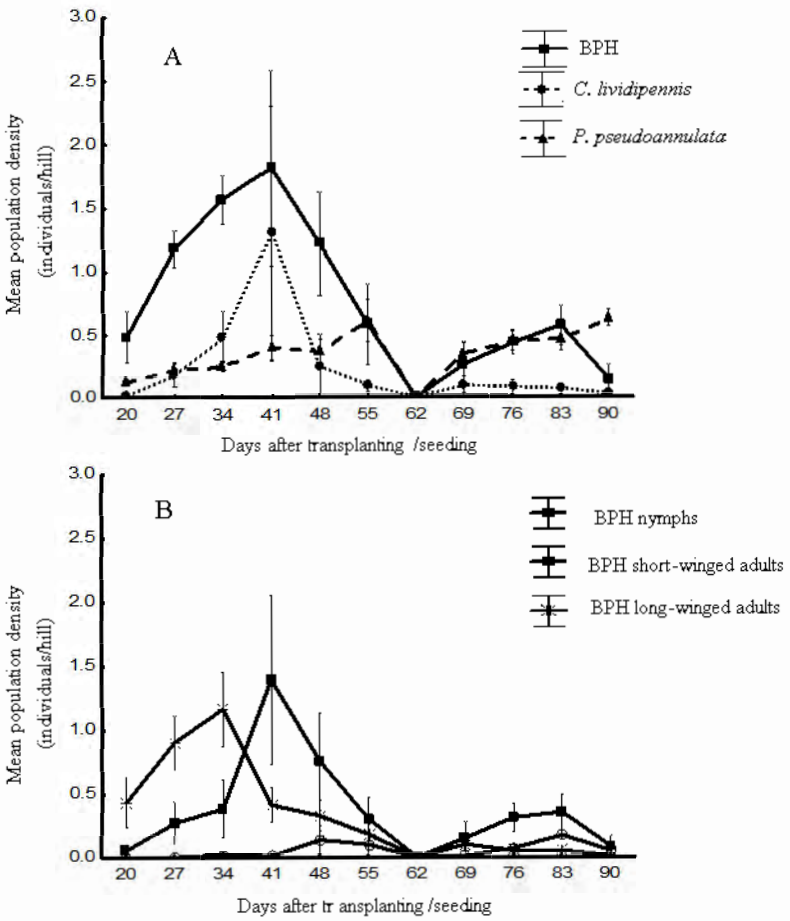


**Fig. 9**. Population growth pattern throughout the rice crop season of *N. lugens* (BPH) and its natural enemies in susceptible rice fields at Nakhon Nayok during the wet season. A. Growth pattern of total BPH (nymphs + short winged adults + long-winged adults) and its natural enemies, *C. lividipennis* and *P. pseudoannulata* B. Growth pattern of each BPH life stage

## DISCUSSION

Significant implications in the population dynamics and growth pattern of BPH and its natural enemies in rice fields planted with BPH-resistant and BPH-susceptible fields were identified in this study:

**1. No consistent differences were found in the population dynamics and the growth pattern of BPH between resistant and susceptible rice fields.**

Resistance allows rice plants to affect pest fertility, development and feeding behavior (Baldwin and Preston 1999). Thus, lower densities of BPH are expected in resistant rice cultivars and less conspicuous density peaks when compared with susceptible rice plants (Alagar et al. 2007, Kartohardjono and Heinrichs 1984). However, the results of this research showed that densities of BPH were not smaller at all the BPH-resistant fields that were studied. Only the average and

maximum density of BPH long-winged adults in resistant fields at Chai Nat during the dry season and the mean density of BPH short-winged adults in resistant fields at Nakhon Nayok during the wet season, were smaller than in BPH-susceptible fields (Table 2). Additionally, mean densities at all BPH life stages were not significantly different between susceptible and resistant fields in Chai Nat during the wet season (Table 2). Furthermore, we only found a different growth pattern between resistant and susceptible fields in Nakhon Nayok during the wet season (Figs. 8 and 9). Nevertheless, resistant fields in Nakhon Nayok during the wet season were planted with the rice variety RD47 and susceptible fields with Thai Jasmine rice. Transplanting dates were clearly different between resistant and susceptible fields (Table 1). We acknowledge that factors related with the difference in the planting dates between resistant and susceptible fields, may also explain our results.

Compared to pest-resistant rice, pest-susceptible rice varieties can be cultivated at a lower cost, due to a less water requirement of susceptible rice plants (Hussain et al. 2008, Third World Network 1990). In case farmers in Central Thailand would require in the future to plant only susceptible rice varieties, the evidence of this study cannot support the idea that the use of such rice varieties could be riskier for pest control than the use of pest-resistant rice varieties. Actually, under natural conditions, a healthy rice plant has a great capacity for damage compensation or insect pest resistance (Sogawa 2015, Way and Heong 1994). It has been demonstrated in tropical rice fields in the Philippines, yield loss did not occur even though susceptible rice had a density of about 30 BPH per plant (IRRI 1990). Actually, some studies have shown that in Thailand some susceptible rice varieties have the potential to develop tolerance to insect pests (Oupkaew et al. 2011, Rerkasem 2015). Other studies in India and Pakistan, revealed susceptible varieties are not as prone to diseases as high-resistant rice varieties are (Hussain et al. 2008, Third World Network 1990). Additionally, evidence demonstrating that improving the diversity of the rice agroecosystem with a mixture of resistant and susceptible varieties in insecticide-untreated fields has reduced the harmful effects by different species of hoppers (Cook and Perfect 1985, Kenmore 1991). This may also represent an alternative for pest management for farmers. However, future research that provides evidence about potential risks of this varietal mixture is necessary.

**2. Typical seasonal growth pattern of BPH tropical populations was only found in fields planted with susceptible rice varieties in Nakhon Nayok during the wet season.**

Although it has been found in several studies that tropical populations of BPH are characterized by low densities late in the crop season that happen after early and high initial pest densities (Hirao 1989, Kisimoto 1981, Kuno and Dyck 1985, Wada and Salleh 1992), in this study, only BPH populations in susceptible fields at Nakhon Nayok during the wet season followed the typical pattern of tropical populations of BPH (Fig.9). BPH populations in resistant and susceptible fields in Chai Nat during the dry (Figs. 4 and 5) and wet (Figs. 6 and 7) seasons and in resistant fields in Nakhon Nayok during the wet season (Fig. 8), showed the opposite pattern; lower initial pest densities followed by higher incidence of BPH later in the crop period.

BPH populations vary significantly in time and space (Denno et al. 1980, Denno and Grissell 1979, Kuno 1977, Strong et al. 1989) and diverse factors like host plant physiology, predation, population density and even planting dates influence the growth and size of the BPH population (Denno and Roderick 1990, Denno et al. 1991). For example, selective pressures related with the finding of mates and habitat conditions seem to affect wing form and dispersal in BPH populations (Denno et al. 1991). When the population density is low, individuals become dispersed in the rice field and potential mates for the reproduction get limited, causing male long-winged adults to be favored over short-winged ones in order to facilitate searching for females (Denno et al. 1991). Habitat stability and changes in the physiology of the rice plant can also favor long-winged

individuals, so that the pest population is able to disperse searching for new rice fields or better plant hosts when conditions are not favorable (Denno et al. 1991, Dyck et al. 1979, Saxena et al. 1981).

Overall incidence of BPH long-winged adults was higher than BPH short-winged adults in the sampled fields in this study (Table 2). With the exception of resistant fields in Chai Nat during the dry season, where incidence of BPH long-winged adults was low over the crop period (Fig. 4B), occurrence of long-winged adults was frequent over the crop season, happening close or after the mid time of the crop period in fields at Chai Nat during the wet season (Figs. 6B and 7B) and in resistant fields at Nakhon Nayok during the wet season (Fig. 8B), or early in the crop season in susceptible fields at Nakhon Nayok (Fig. 9B). Susceptible fields in Chai Nat during the dry season showed the increase in the incidence of long-winged adults early and late in the crop period (Fig. 5B). Although long-winged adults are less fecund than short-winged adults (Cheng and Holt 1990, Kisimoto 1965) they still contribute to build-up the pest population. Immigration of long-winged adults to new rice fields normally explains the typical early high nymph densities of tropical BPH populations (Dyck et al. 1979) as it was observed in susceptible fields in Nakhon Nayok (Figs. 9A and 9B). However, occurrence of long-winged adults close or after the mid time of the crop season may also explain the non-typical higher nymph densities that were observed close to the end of the crop season in fields at Chai Nat during the dry (Figs. 4 and 5) and wet seasons (Figs. 6 and 7) and in resistant fields in Nakhon Nayok (Fig. 8). Actually, only in susceptible fields in Chai Nat during the dry season, BPH densities continued rising close to harvest time where the increase in the incidence of long-winged adults occurred both, early and late in the crop period.

Additionally, the smaller population densities observed in fields in Chai Nat during the dry (Figs. 4 and 5) and wet seasons (Figs. 6 and 7) could have been also caused by the less fertility of BPH long-winged adults. BPH populations can grow rapidly when aggregations of short-winged adults occur (Kisimoto 1965) but when conditions are not favorable, for example, a population low-density condition, a trade-off between fitness and wing form happens (Denno et al. 1991). Predominance of long-winged adults was considered typical of BPH populations at high-density conditions under the effects of crowding that stimulate macroptery (Denno et al. 1991). However, the results of this study support previous research that has shown that population low-density conditions also influence the triggering of macroptery (Denno et al. 1991, Kuno 1979).

On the other hand, although the use of insecticides has decreased dramatically in Central Thailand as a consequence of the termination of subsidies for rice production by the Government, some farmers still use them, especially when their purpose is seed production (non-published data from interviews to farmers in the study area). Sampled fields in this study were not treated with insecticides but the use of chemicals for BPH control in neighboring fields may have occurred. Rice areas in Chai Nat and Nakhon Nayok comprise a matrix of successional planted rice crops. Pests and fauna associated with the rice agroecosystem are connected among this assortment of continuous available rice due to asynchronous planting dates (Way and Heong 1994). It is also possible that the frequent and higher incidence of BPH long-winged adults over short-winged adults could have been the consequence of an "escape" from sprayed surrounding fields.

**3. The natural enemies of BPH seem to respond differently to increasing densities of BPH in different crop seasons and different rice fields.**

The effect of pest resistant crops on the behavior and performance of natural enemies has been widely discussed. Some researchers have suggested that high levels of resistance can be detrimental for natural enemies because resistant cultivars lower the pest to the densities, where food gets limited for natural enemies (Gould et al. 1991, Kenmore et al. 1984). Meanwhile, other studies have proposed that when crop resistance is combined with control by natural enemies, satisfactory pest suppression

occurs (Carriere and Tabashnik 2001, Way and Heong 1994). This study showed results related to multiple scenarios concerning the behavior of natural enemies in resistant rice cultivars.

Firstly, in resistant fields in Chai Nat during the dry season both the pest and natural enemies seemed to be suppressed to some degree. Resistant fields had significantly less number of BPH long-winged adults and less number of MB individuals than in susceptible fields (Table 2 and Figs. 4A and 5A). In these fields, an adverse effect of resistant cultivars on natural enemies as reported in previous studies may have occurred (Gould et al. 1991, Kenmore et al. 1984). BPH could have been suppressed by plant resistance to a population density level, where it may have also caused less available food for natural enemies and thus, causing also lower densities of MB compared to the ones in susceptible fields. This may also explain why although there was a positive response of natural enemies to pest density in resistant and susceptible cultivars (Table 3), only in susceptible fields MB was higher in density and also showed a synchronized growth pattern to the one of BPH (Fig. 5A).

Secondly, in fields at Chai Nat during the wet season there were no consistent differences in the growth of BPH and both natural enemies between resistant and susceptible fields (Figs. 6 and 7) and there was no significant response of natural enemies to increasing densities of the pest in susceptible and resistant fields (Table 3). It is possible that other factors independent of the type of rice variety could have influenced the behavior of the pest and both natural enemies. In this case, density-related effects could have played an important role. When the pest is at a low density condition, BPH individuals get located at the bottom of the rice plant stem (Denno and Roderick 1990), making it more difficult for predators to find BPH. Under this unfavorable condition for the predators MB and WS could have changed their prey preference from BPH to other preys easier to find (Ferry et al. 2006, Mayntz et al. 2005). The lack of correlations between BPH density and both natural enemies in fields at Chai Nat during the wet season may be explained by a niche shift of BPH in the rice plant under low density conditions. In parallel, WS is a predator known for also having nocturnal habits (Maloney et al. 2003). Since sampling in this study occurred in the day-time, an underestimation of the spider response related to BPH is another limitation that cannot be discarded.

Lastly, the growth pattern of BPH in resistant fields at Nakhon Nayok during the wet season showed that BPH was not suppressed late in the crop period, when BPH densities increased and density peaks were clearer (3 BPH per hill) (Fig. 8A). Natural enemies instead, showed very low incidence over the crop period (Fig. 8A). Resistant fields at Nakhon Nayok showed a scenario, in which we observed less degree of BPH suppression and poor incidence of natural enemies, but in this case, not caused by a BPH low-density condition. Not only BPH but also natural enemies are connected to neighboring rice fields (Way and Heong 1994). It is possible that since BPH densities were very low early in the crop season (Fig. 8A), natural enemies could have migrated to other rice fields and their densities only started increasing after BPH also increased after 34 DAT/S. Poor control by natural enemies early in the crop season has been demonstrated to cause high BPH densities later in the crop period (Way and Heong 1994) as it happened in resistant fields in Nakhon Nakhon (Fig. 8A).

A significant amount of research has failed to report consistent differences in the population dynamics and performance of natural enemies between resistant and susceptible crops (Marvier et al. 2007, Rodrigo-Simón et al. 2006, Wolfenbarger et al. 2008). In this sense, the findings of this study support these previous research and report no consistent differences in the population dynamics and growth pattern of natural enemies between resistant and susceptible fields in Central Thailand and rather, suggest that diverse factors may influence the performance of natural enemies and their response to BPH in each rice field, location and crop season. Among these factors, BPH population density-related effects may play a significant role. Other two factors that may be important are the type of resistant variety (Way and Heong 1994) and specific climatic conditions at each cropping

season (Döbel and Denno 1994). In a future study, the discrimination of different types of resistant varieties and a detailed effect of climate on the rice arthropod community may explain in a deeper way than our findings.

## CONCLUSION

The extent of pest suppression on resistant crops has been thought to be location-specific (Gould et al. 1991, Way and Heong, 1994) and therefore, the relevance of assessing the dynamics of BPH populations on a specific region has been widely recalled when applying pest management strategies to a particular area (Kuno and Dyck 1985). Our study is one of the few comprehensive works that has assessed at a community-based level, the population dynamics of BPH and its natural enemies in different resistant and susceptible non-sprayed rice fields in Central Thailand under a non-outbreak condition, contributing with insights about the potential ecological impact of BPH-resistant rice varieties on BPH and its natural enemies and its implications for the local management of the pest. Our findings conclude that the low-density condition of the pest population and the crop season have a strong effect on the dynamics of BPH and its natural enemies and therefore, on the magnitude and effectiveness of natural control by predators and the level of pest suppression in fields planted with BPH-resistant varieties. Although we acknowledge the limitations of our study concerning the difficulty of conducting this type of research in farmers' fields, we do not expect the conclusions and findings of this study to be an absolute evaluation of the extent of pest suppression in BPH-resistant fields but instead, to clarify the knowledge about the dynamics of BPH and its natural enemies in tropical rice fields planted with BPH-resistant and BPH-susceptible varieties where the conditions vary in a way from experimental fields.

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# SEROLOGICAL AND MOLECULAR DETECTION OF DIFFERENTIAL INFECTIONS OF BUNCHY TOP AND MOSAIC CAUSING VIRUSES IN TISSUE CULTURE PLANTLETS OF ABACA (*Musa textilis* Née)

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## ABSTRACT

The abaca planting materials are usually propagated through tissue culture, and its production requires reliable virus indexing protocol to ensure that plants are indeed virus-free. This study aimed to establish a reliable protocol for virus indexing of abaca tissue cultures using serological and molecular methods considering differential infections during the tissue culture process. The objectives were to determine the differential infection of plantlets in tissue culture line and plantlets obtained following subsequent *in vitro* and *ex vitro* cultures. The materials were plantlets of *in vitro* and *ex vitro* culture lines obtained from infected tissue explant. In this study, differential infection of *Banana bunchy top virus* (BBTV), *Banana bract mosaic virus* (BBrMV) and *Sugarcane mosaic virus* (SCMV) were detected by enzyme-linked immunosorbent assay (ELISA) among plantlets obtained from a single tissue culture line, and in plantlets obtained following subsequent *in vitro* (first and second subculture) and *ex vitro* cultures. The differential infection resulted to inconsistency of virus detection by ELISA. Infection of *in vitro* abaca cultures occurred mostly as mixed infection of two or three viruses. The differential infection of BBTV was confirmed by PCR detection. The sensitivity of BBTV detection by PCR was affected by the dilution of template DNA.

**Key Words**: bract mosaic, sugarcane mosaic, polymerase chain reaction, enzyme-linked immunosorbent assay, virus indexing

## INTRODUCTION

Abaca (*Musa textilis* Nee) also known worldwide as the Manila hemp is indigenous in the Philippines, and is one of the most economically important crops in the country. The Philippines dominates abaca fiber production and supplies about 85% of the total abaca fiber worldwide. In 2016, the country produced a total of 496,069 bales in 125.5 kg of abaca fibers, with Bicol Region as the top producer (PhilFIDA 2016). The production of abaca is seriously affected by virus diseases (Thomas et al. 2003) causing significant losses to the industry These diseases include bunchy top, bract mosaic and abaca mosaic which have taken their toll on many farmers and persistently devastated abaca plantations in abaca producing regions in the Philippines (FIDA 2011). Abaca bunchy top is known to be caused by *Banana bunchy top virus* (BBTV) which is transmitted by aphid, *Pentalonia*

*nigronervosa*, in a persistent, circulative, non-propagative manner (Magee 1953). In 2008, a distinct virus species, the *Abaca bunchy top virus* (ABTV) has also been found associated with the disease (Sharman et al. 2008). Bunchy top infected plants are stunted which produces undersized suckers with short, narrow, stiff and upcurled leaves, and chlorotic to necrotic leaf margins (Ocfemia et al. 1930; Raymundo 2000; Bajet and Magnaye 2002). Abaca mosaic is caused by the aphid transmitting abaca strain of *Sugarcane mosaic virus* (SCMV) with symptoms of chlorosis and mosaic on leaves (Eloja et al. 1962; Eloja and Tinsley 1963; Bajet and Magnaye 2002; Gambley et al. 2004). *Banana bract mosaic virus* (BBrMV) also infects abaca (Espino et al. 1990; Magnaye and Espino 1990; Sharman et al. 2000). The virus is transmitted in a non-persistent manner by *P. nigronervosa*, *Aphis gosypii* and *Rhopalosiphum maidis* (Magnaye and Espino 1990, Muñez 1992). The BBrMV causes discontinuous streaks on the bract, spindle-shaped streaks on the petiole, and mottling on the pseudo stem (Rodoni et al. 1997). All of these viruses can also be transmitted through vegetative propagation.

Since 1992, the Philippine government, through the Philippine Fiber Industry Development Authority (PhilFIDA), implemented the abaca rehabilitation program in its effort to effectively manage the virus diseases affecting abaca. The rehabilitation program is part of the production support services by PhilFIDA which include: eradication of infected plants and replanting, development of new abaca areas, facility upgrading, and abaca disease management project. In support of the rehabilitation program, mass propagation of planting materials is needed. The meristem/shoot tip culture technique has been developed for abaca to rapidly propagate disease-free planting materials and produce of cheaper plantlets with high survival rate in the field. This has been adopted by other research laboratories throughout the country, and has long been transferred to the farmer's fields (Aspuria 2003). However, it is crucial to ensure that the source of the explant and the derived plantlets are virus-free.

The BBTV, SCMV and BBrMV have been shown to be transmitted through tissue culture (Diekmann and Putter 1996, Drew et al. 1989 and 1992, Ramos and Zamora 1990, Wu and Su 1991). BBTV is readily transmitted through tissue culture in banana cultivars, and the virus is efficiently detected by ELISA (Thomas et al. 1995). BBrMV is also detectable in *in vitro* cultures (Hwang and Su 1998). For abaca tissue culture, plantlets are obtained using meristematic tissue from a healthy mother plant. The plantlets are separated, subsequently subcultured for further propagation, and then indexed to ensure that they are virus-free. However, a standard protocol for virus indexing of abaca tissue culture needs to be established in the country. Current virus indexing of tissue culture plantlets involves testing the source of the explant and the representative plantlets collected either at the *in vitro* or *ex vitro* stages. However, in case the virus has not been detected in the explant and then used for tissue culture, it is possible that the plantlets obtained from a single explant would be differentially infected. The differential infection is attributed to the irregular distribution and movement of the virus in actively growing meristematic tissues as shown for BBTV (Thomas et al. 1995). The differential infection of plantlets during tissue culture and the subsequent cultures may affect the reliability of virus indexing.

This study sought to establish a reliable protocol using serological and molecular methods for virus indexing of abaca tissue cultures considering the differential virus infection of tissue culture plantlets. The objectives were to determine the differential infection among plantlets of tissue culture lines and among plantlets obtained following subsequent *in vitro* and *ex vitro* cultures.

## MATERIALS AND METHODS

The study was conducted at the Plant Virology Laboratory, IWEP (formerly Crop Protection Cluster) and the Plant Tissue Culture Laboratory, ICropS, formerly Crop Science Cluster (CSC), University of the Philippines Los Baños (UPLB) in 2013-2014.

**Source of *in vitro* abaca cultures**

The source of *in-vitro* abaca cultures cv. Inosa was provided by the Plant Tissue Culture Laboratory, CSC, UPLB. The plantlets were 2-3 year-old established stock cultures acquired from the PhilFIDA in Tacloban, Leyte, and the National Abaca Research Center (NARC) in Baybay City, Leyte. The established cultures were sub-cultured in modified Murashige and Skoog (MS) (1962), a standard multiplication medium supplemented with 5 ppm benzyladenine (BA). There were eight stock cultures from PhilFIDA namely, 1514, H61122, 1541, D21511, L51417, N21478, V1128 and E21516, and these were considered in this study as batch 1 samples. The 10 stock cultures from NARC were ST10, ST11, ST12, ST13, ST14, ST17, ST18, ST19, ST20 and ST22, and these were considered as batch 2 samples. Each stock culture was considered as a tissue culture line, e.g Line ST10, ST11, ST12 etc. Shoot tissue collected from batch 1 and 2 samples was subcultured for three consecutive cycles. After the third subculture, the plantlets were transferred to rooting media, and then maintained as *ex vitro* cultures in the screenhouse. Leaf samples from plantlets of each line (batch 1 and 2) were collected for virus assay at the first and second *in vitro* culture, and then at the *ex vitro* stage. The samples were tested for the presence of BBTV by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). Both methods have been established for detection of abaca viruses (Hwang and Su 1998, Thomas and Dietzgen 1991, Furuya et al. 2006, Mahadev et al. 2013). The presence of BBrMV and SCMV were tested by ELISA.

**Sub-culture of plantlets**

***Multiplication of shoots.*** Plantlets taken from the abaca stock cultures were inoculated to freshly-prepared MS basal medium containing salts (macro and micronutrients) and Nitsch and Nitsch vitamins (1969) supplemented with plant growth regulators (1uM indole-3-acetic acid (IAA) and 10uM benzyadenine purine (BAP), 3 ppm benzyladenine, and 3% sucrose, adjusted to pH 5.8 and solidified using 0.55% plant tissue culture (PTC) agar (Pronadisa). Two to three shoots per plantlet were subcultured for tissue proliferation in a period of three months**.** The first *in vitro* subculture plantlets were generated after one month, and then these were used as the source to produce the second subcultured plantlets one month thereafter. The second subcultures were again used as the source to produce the third subculture plantlets one month thereafter. The subcultured plantlets consisted of the tissue culture lines, with each line corresponds to the stock cultures described above. The plantlets were observed for the presence of virus-like symptoms, such as mosaic and chlorosis.

***Root induction*.** After 3 months of subculture in the multiplication medium, the plantlets were transferred to rooting medium to promote the proliferation of roots. The rooting medium was composed of MS basal salts, Nitsch and Nitsch vitamins, 0.25 ppm indole-3-butyric acid (IBA), 6% sucrose, 0.25% activated carbon solidified with 0.55% PTC agar (Pronadisa) at pH adjusted to 5.8. The plantlets were cultured in the rooting medium for 1 month and then acclimatized for 2 weeks. Acclimatized plantlets were transferred to potting medium (1 sterilized garden soil: 3 coir dust), and these served as the *ex vitro* cultures. The plantlets were pre-treated with fungicide, and were maintained inside an insect-proof net cage to prevent virus transmission by the aphid vector.

**Virus detection**

The plantlets derived from the first and second *in vitro* subculture, and from *ex vitro* culture of each abaca line were assayed for the presence of abaca viruses. For each line, the number of samples subjected for virus indexing depended on the availability of plantlets having adequate leaf tissue for sampling. At the first *in vitro* culture stage, BBTV detection consisted of 23 total samples collected from plantlets of tissue culture lines in batch 2 samples, while BBrMV and SCMV detection consisted of 32 total samples collected from batch 1 and 48 of batch 2 samples.

Analysis of differential infection of BBTV in plantlets following subculture consisted of samples of the first *in vitro* and *ex vitro* cultures. BBTV detection at the *ex vitro* stage consisted of 20 total samples from ST10, ST11, ST12, ST13, ST17, ST18, ST19, ST20 and ST 22 lines. For ST10

line, the *ex vitro* culture samples were collected from plantlets, ST10(4-1), ST10(8-1) and ST10(10-1) which were derived from the subcultured plantlets ST10(4), ST10(8) and ST10(10), respectively. Due to limited samples available for testing, fewer plantlets from the *ex vitro* stage were tested. Analysis of BBrMV differential infection following subculture consisted of samples from the first and second *in vitro* cultures. Detection of BBrMV and SCMV consisted of 17 total samples from ST10, ST11, ST12, ST13, ST14, ST17, ST18, ST19, ST20 and ST 22 lines. For ST10 line, the second *in-vitro* culture sample was collected from plantlet ST10(8-1) derived from the subcultured plantlets ST10(8) of the first *in vitro* culture.

**Direct ELISA.** The Plate Trapped Antigen (PTA) ELISA was employed for BBrMV and SCMV detection using the commercial kit from Agdia, and following the manufacturer's protocol with some modifications. Instead of using the Agdia general extraction buffer, the tissue samples were homogenized at 1:5 dilution in Tris-Na-DIECA buffer (Bajet and Magnaye 2002). The buffer was added at the start of grinding to avoid tissue oxidation. The succeeding steps then followed the Agdia protocol. Samples were considered positive to the virus when the absorbance reading was above the threshold value, which was twice the average of the absorbance of three healthy control samples.

**Indirect ELISA.** Detection of BBTV was conducted by indirect PTA ELISA. Commercial BBTV polyclonal antibody (Agdia) was used following the protocol described by Su (1999) and Bajet and Magnaye (2002).

**Polymerase chain reaction.** Total DNA was extracted following the protocol by Su (1999) with some modifications. The presence of BBTV was also detected by polymerase chain reaction (PCR) while the presence of BrMV and SCMV were not tested in this study. Detection of BBTV by PCR followed the procedure described by Thompson and Dietzgen (1995) using BBTV specific primers, BBT-1 and BBT-2 designed to amplify the DNA-R component of the viral genome with an expected amplicon size of 349 bp. The negative samples were retested using 1:10 diluted DNA template. The reaction mixture was subjected for amplification in a Veriti® 96-well thermal cycler (Applied Biosystems) with the following conditions: initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 20 sec, annealing at 60°C for 1 min, extension at 72° for 1 min, and a final extension at 72°C for 3 min (Thompsom and Dietzgen 1995). The PCR product was subjected to gel electrophoresis to check for the presence of amplified DNA. The DNA was stained using a dye (GelRed™) and then viewed in a gel documentation system (AlphaImager® Mini - Alpha Innotech).

## RESULTS AND DISCUSSION

This study showed the differential infection of BBTV, BBrMV and SCMV among abaca tissue culture plantlets obtained from a single tissue culture line, and among plantlets obtained following subsequent *in vitro* (first and second subculture) and *ex vitro* cultures. Although the plantlets of each line were derived from a single explant, these plantlets were found to be differentially infected. Likewise, the plantlets obtained from a single plantlet following subsequent culture were also differentially infected.

### Virus-like symptoms in abaca tissue cultures

Virus-like symptoms such as deformation, vein chlorosis and swelling were observed on the leaves of some *in vitro* cultures (Figure 1a-b). Vein necrosis which is not a typical virus disease symptom was also observed (Figure 1c-d). However, these symptoms cannot be attributed at all to virus infection since these were observed even on the virus-free plantlets, while not all virus-infected plants had these symptoms. For instance, although some *in vitro* cultures were positive to BBTV, none of these symptoms were observed. The plantlets were also tested for BBrMV and SCMV infection and the presence of symptoms did not correspond to virus infection. The symptoms observed may be morphological and physiological disorders brought about by the process of tissue culture.


**Fig. 1.** Symptoms observed in *in-vitro* cultures of abaca. a) vein swelling; b) leaf deformation; and c-d) vein necrosis.

**Viruses detected in abaca tissue culture lines by enzyme-linked immunosorbent assay**

The BBTV, BBrMV and SCMV were detected by ELISA, but the presence of the virus was differentially detected among plantlets derived from a single tissue culture line (Table 1). The plantlets of tissue culture lines from batch 1 and batch 2 samples with each line derived from a single explant were differentially infected. The eight out of ten ST lines (batch 2 samples) were positive to BBTV, in which all plantlets of five lines ST10, ST11, ST12, ST17, and ST 20 were consistently infected while the plantlets of other lines ST14, ST18, ST19 and ST22 were differentially infected. For instance, one out of two plantlets of line ST14 was positive to BBTV while the other plantlet was negative. The virus was not detected in all plantlets of lines ST13 and ST19.

The BBrMV was consistently detected in all plantlets of the seven lines (1514, H61122, 1541, D21511, L51417, N21478 and E21516) but not V1128 line (Table 1). On the other hand, the plantlets of the ST lines were differentially infected with BBrMV in which all plantlets of five lines (ST10, ST11, ST13, ST19, ST20) were all infected while those of the other lines ST12, ST14, ST17, ST18 and ST22 were differentially infected. Plantlets were also differentially infected with SCMV (Table 1). All plantlets of four (H61122, L51417, N21478, V1128) out of eight lines were consistently positive to SCMV. Plantlets of two ST lines (ST10, ST22) were consistently infected with SCMV while those of the other lines (ST11, ST12, ST13, ST14, ST17, ST18, ST19, ST20) were differentially infected. The BBTV, BBrMV and SCMV were detected mostly as mixed infections.

Infection of *in vitro* abaca cultures occurred mostly as mixture of two or three viruses. Like in banana, mixed virus infections in abaca commonly occur in the field. Abaca plants from different abaca growing areas were found to be mixed infected with BBTV and BBrMV (Sta. Cruz et al. 2016), and with BBrMV and SCMV (Gambley et al. 2004, Sta. Cruz et al. 2016). Thus, it is important to ensure that the explants to be used for tissue culture propagation are tested for infection with multiple viruses including BBTV, BBrMV and SCMV, and with other viruses that may be infecting abaca, e.g. ABTV. In this study, BBTV, BBrMV and SCMV were detectable by ELISA and the method was

reliable enough for virus detection. ELISA proved to be a very reliable method for BBTV detection in micropropagated plants (Thomas et al. 1995). It can detect BBTV even in asymptomatic plantlets (Sta. Cruz et al. 2016).

**Table 1.** Viruses detected by enzyme-linked immunosorbent assay in *in-vitro* abaca tissue cultures.

| Tissue Culture Sample [1] | Tissue Culture Line | Number of Virus Positive Samples/ Samples Tested | | |
|---|---|---|---|---|
| | | **BBTV** | **BBrMV** | **SCMV** |
| Batch 1 | 1514 | nt [2] | 10/10 | 9/10 |
| | H61122 | nt | 3/3 | 3/3 |
| | 1541 | nt | 6/6 | 4/6 |
| | D21511 | nt | 6/6 | 5/6 |
| | L51417 | nt | 2/2 | 2/2 |
| | N21478 | nt | 1/1 | 1/1 |
| | V1128 | nt | 1/3 | 3/3 |
| | E21516 | nt | 1/1 | 0/1 |
| Batch 2 | ST10 | 3/3 | 3/3 | 3/3 |
| | ST11 | 3/3 | 8/8 | 5/8 |
| | ST12 | 1/1 | 3/5 | 4/5 |
| | ST13 | 0/3 | 4/4 | 3/4 |
| | ST14 | 1/2 | 4/5 | 4/5 |
| | ST17 | 3/3 | 1/3 | 2/3 |
| | ST18 | 1/2 | 5/7 | 1/7 |
| | ST19 | 0/1 | 3/3 | 2/3 |
| | ST20 | 2/2 | 4/4 | 2/4 |
| | ST22 | 1/3 | 2/6 | 6/6 |

[1] Batch 1 samples from PhilFIDA while Batch 2 from NARC; Banana bunchy top virus (BBTV); Banana bract mosaic virus (BBrMV); Sugarcane mosaic virus (SCMV);
[2] nt: not tested due to limited samples for testing

**Differential infection of abaca tissue culture plantles following subsequent *in vitro* and *ex vitro* cultures**

Differential infections with BBTV, BBrMV or SCMV of abaca plantlets during subsequent cultures were also observed. Analysis of differential infection was done using ST lines or batch 2 samples.

**BBTV infection.** Detection of BBTV in plantlets obtained from subsequent cultures was not always consistent (Table 2). The presence BBTV was consistently detected in two plantlets, ST10(4) and ST10(8) of line ST10 at the first *in vitro* subculture stage. Although the virus was not tested at the first *in vitro* culture, plantlet ST10 (10-1) which was derived from the same ST10 line was consistently BBTV positive at the *ex vitro* culture stage. The virus was also consistently detected in plantlets ST11(2) and ST11(5) at the first subculture, and plantlet ST11(2-1) derived from ST11(2) was consistently positive at the *ex vitro* culture. BBTV was also consistently detected in plantlets ST17(7), ST17(3), ST17(2). On the other hand, BBTV was not consistently detected in plantlets of lines ST18, ST20 and ST22 (Table 2). For instance, plantlet ST18(10) was BBTV positive at the first subculture. However, the virus was not detectable in ST18(9-1) when tested at the *ex vitro* culture. The plantlet ST20(5) and derived plantlet ST20(5-1) were differentially infected at the first *in vitro* subculture and *ex vitro* culture stages, respectively. Furthermore, plantlet ST12(1) and the derived plantlet ST12(1-1) were also differentially infected at the first subculture and *ex vitro* stages. Plantlets of lines ST13 and ST19 were consistently negative at the first *in vitro* and *ex vitro* culture stages.

**Table 2.** Banana bunchy top virus (BBTV) detected by ELISA in abaca plantlets following *in-vitro* and subsequent *ex-vitro* culture.

| Tissue Culture Line | First *in-vitro* Subculture | | *Ex-vitro* Culture | |
|---|---|---|---|---|
| | Plantlet | Reaction in ELISA | Plantlet | Reaction in ELISA |
| ST10 | ST10(4) | + | 4-1 | nt |
| ST10 | ST10(8) | + | 8-1 | nt |
| ST10 | ST10(10) | nt | 10-1 | + |
| ST11 | ST11(2) | + | 2-1 | + |
| ST11 | ST11(5) | + | 5-1 | nt |
| ST17 | ST17(7) | + | 7-1 | nt |
| ST17 | ST17(3) | + | 3-1 | nt |
| ST17 | ST17(2) | nt | 2-1 | + |
| ST18 | ST18(10) | + | 10-1 | nt |
| ST18 | ST18(9) | nt | 9-1 | - |
| ST20 | ST20(5) | - | 5-1 | + |
| ST20 | ST20(10) | + | 10-1 | nt |
| ST22 | ST22(3) | - | 3-1 | nt |
| ST22 | ST22(5) | + | 5-1 | nt |
| ST22 | ST22(8) | nt | 8-1 | - |
| ST12 | ST12(1) | - | 1-1 | + |
| ST13 | ST13(4) | - | 4-1 | nt |
| ST13 | ST13(1) | - | 1-1 | nt |
| ST13 | ST13(6) | nt | 6-1 | - |
| ST19 | ST19(3) | - | 3-1 | - |

(+) positive to BBTV in enzyme-linked immunosorbent assay; nt-not tested due to limited samples for testing.

**BBrMV infection.** Differential infection of BBrMV was also observed. The BBrMV was consistently detected at the first and second subcultures in four plantlets, ST10(8), ST13(1), ST9(3) and ST20(5). Each of these plantlets was BBrMV positive when tested at the first *in vitro* culture and the plantlets, ST10(8-1), ST13(1-1), ST19(3-1) and ST20(5-1) derived at the from the second subculture were consistently virus positive. However, differential infection of BBrMV was observed in some plantlets during the first subculture and the subsequent culture. Plantlets which were infected at the first subculture generated plantlets which were also BBrMV infected as well as plants negative to the virus. For instance ST14(2) was BBrMV positive at the first subculture but the plantlets derived from second subculture had differential infection with one, ST14(2-1) of three plantlets was infected while the other two, ST14(2-2) and ST14(2-3) were not infected. Likewise, some plantlets ST12(5), ST17(7), ST18(5) had differential infection following subsequent cultures. These plantlets were negative to BBrMV infection at the first subculture but generated plantlets, ST12(5-1), ST17(7-1) and ST18(5-1) which were BBrMV infected at the second subculture stage.

On the other hand, the plantlet ST11(2) that was negative to BBrMV at the first subculture generated plantlets, ST11(2-1) and ST11(2-3) that were also virus negative as well as virus positive plantlet ST11(2-2) at the second subculture. Likewise, plantlet ST22(10) was negative at the first subculture but produced virus positive plantlet, ST22(10-1) at the second subculture. Table 3 not indicated

**Table 3.** Banana bract mosaic virus (BBrMV) detected by ELISA in abaca plantlets following two *in-vitro* subcultures.

| Tissue Culture Line | First *in-vitro* Subculture | | Second *in-vitro* Subculture | |
|---|---|---|---|---|
| | Plantlet | Reaction in ELISA | Plantlet | Reaction in ELISA |
| ST10 | ST10(8) | + | 8-1 | + |
| ST13 | ST13(1) | + | 1-1 | + |
| ST19 | ST19(3) | + | 3-1 | + |
| ST20 | ST20(5) | + | 5-1 | + |
| ST14 | ST14(1) | + | 1-1 | + |
| ST14 | ST14(2) | + | 2-1 | + |
| ST14 | ST14(2) | + | 2-2 | - |
| ST14 | ST14(2) | + | 2-3 | - |
| ST12 | ST12(5) | - | 5-1 | + |
| ST17 | ST17(7) | - | 7-1 | + |
| ST18 | ST18(5) | - | 5-1 | + |
| ST11 | ST11(2) | - | 2-1 | - |
| ST11 | ST11(2) | - | 2-2 | + |
| ST11 | ST11(2) | - | 2-3 | - |
| ST22 | ST22(10) | - | 10-1 | + |
| ST22 | ST22(10) | - | 10-2 | - |
| ST22 | ST22(10) | - | 10-3 | - |

(+) positive to BBrMV in enzyme-linked immunosorbent assay

**SCMV infection.** Detection of SCMV was not consistent following subsequent culture (Table 4). Although consistent SCMV infection was observed for some plantlets the other plantlets had differential infection. The presence of SCMV in plantlets of four lines ST10, ST12, ST13 and ST17 was consistent when tested at the first and second subcultures. For instance, plantlet ST10(8) at the first subculture stage and the plantlet ST10(8-1) derived from it were both virus positive. Likewise, plantlets ST12(5-1), ST13(1-1) and ST17(7-1) were virus positive. However, SCMV positive plantlet ST14(2) at the first subculture stage generated plantlets at the second subculture which had inconsistent reaction wherein ST14(2-1) was virus positive while ST14(2-2) and ST14(2-3) were negative. Plantlet ST19(3) was positive at the first subculture but produced plantlet ST19(3-1) that was virus negative at the second subculture. On the other hand, ST18(5) and ST20(5) were negative to the virus at the first subculture stage but produced plantlets, ST18(5-1) and ST20(5-1) which were virus positive at the second subculture. Although ST22(10) was virus negative at the first subculture, one of the plantlets ST22(10-1) produced at the second subculture was virus positive and the two plantlets ST22(10-2), ST22(10-3) were negative. Plantlet ST11(2) was consistently negative at the first and second subcultures.

The differential infection observed in this study can be attributed to the irregular distribution and movement of the virus in actively growing meristematic tissues as shown for BBTV (Thomas et al. 1995). Inconsistent transmission of BBTV in micropropagated banana was reported by Thomas and co-workers (1995). In this way, subculturing can result in the appearance of BBTV-free plantlets which were derived from virus infected plant. They found that nine-month extended subculturing can result in the appearance of BBTV-free plantlets which were derived from virus infected plant. This inconsistent transmission also means that the plants are differentially infected during *in vitro* culture. The differential infection of plantlets during tissue culture and the subsequent subcultures would

affect the reliability of virus indexing. The critical time of virus indexing then would be testing the tissue explants before they are subjected to the tissue culture process, and the tissue must be ensured to be virus-free using sensitive method like ELISA or PCR. Monitoring of virus infection during the tissue culture process is also necessary to ensure that the plantlets would be virus-free, particularly before they are used for the subsequent culture. Finally, virus indexing is needed at the *ex vitro* stage before the plantlets are finally taken out for field planting. Random sampling of about 1-10% of the plantlets which is usually practiced for virus indexing may not ensure that the plantlets that will be released would be virus-free. Thus, testing of more number of plantlets would be necessary, however an efficient system for mass indexing needs to be developed.

**Table 4.** Sugarcane mosaic virus (SCMV) detected by ELISA in abaca plantlets following two *in-vitro* subcultures.

| Tissue Culture Line | First *in-vitro* Subculture | | Second *in-vitro* Subculture | |
|---|---|---|---|---|
| | Plantlet | Reaction in ELISA | Plantlet | Reaction in ELISA |
| ST10 | ST10(8) | + | 8-1 | + |
| ST12 | ST12(5) | + | 5-1 | + |
| ST13 | ST13(1) | + | 1-1 | + |
| ST17 | ST17(7) | + | 7-1 | + |
| ST14 | ST14(1) | + | 1-1 | + |
| ST14 | ST14(2) | + | 2-1 | + |
| ST14 | ST14(2) | + | 2-2 | - |
| ST14 | ST14(2) | + | 2-3 | - |
| ST19 | ST19(3) | + | 3-1 | - |
| ST18 | ST18(5) | - | 5-1 | + |
| ST20 | ST20(5) | - | 5-2 | + |
| ST22 | ST22(10) | - | 10-1 | + |
| ST22 | ST22(10) | - | 10-2 | - |
| ST22 | ST22(10) | - | 10-3 | - |
| ST11 | ST11(2) | - | 2-1 | - |
| ST11 | ST11(2) | - | 2-2 | - |
| ST11 | ST11(2) | - | 2-3 | - |

(+) positive to SCMV in enzyme-linked immunosorbent assay

**BBTV infection as detected by polymerase chain reaction (PCR) in *in vitro* abaca cultures**

The presence of BBTV in *in vitro* and *ex vitro* cultures was confirmed by PCR using the BBT1/BBT2 primers which amplified a region of the DNA R component of the BBTV genome (Table 5). The expected PCR amplification product of 349 bp was obtained in the positive samples. BBTV was detected in all the lines tested. The virus was consistently detected in all plantlets of lines ST12(1,4,5), ST13(2,3,5) and ST18(2,4,5) of the undiluted DNA template. BBTV was also consistently detected in ST17, ST10 and ST11. However, the virus was detected when the template DNA was diluted at 1:10, except in ST17(4). On the other hand, the virus was not consistently detected in ST19 and ST14. For instance, BBTV was detected in ST19(1) and ST19(5) while ST19(2) was negative even in the undiluted template DNA.

BBTV infection of *in vitro* and *ex vitro* cultures was confirmed by PCR detection. The sensitivity of BBTV detection by PCR was affected by the dilution of template DNA. Detection of BBTV by PCR using DNA extracted from leaves of mature plant was found to be more efficient using

diluted (1:10 or 1:20 dilution) than undiluted samples (Sta. Cruz et al. 2016). This is because dilution of template DNA may have reduced the concentration some inhibitors allowing more efficient PCR detection. In this study, although the efficiency of PCR detection increased with template DNA dilution (1:10), most of the *in vitro* cultures were PCR positive even with undiluted DNA. However, this results need to be confirmed using more number of samples in the test.

**Table 5**. Banana bunchy top virus infection detected by PCR in *in-vitro* abaca cultures.

| Tissue Culture Plantlet | Undiluted DNA template | 1:10 template DNA dilution |
|---|---|---|
| ST12 (1) | + | nt |
| ST12 (4) | + | nt |
| ST12 (5) | + | nt |
| ST13 (2) | + | nt |
| ST13 (3) | + | nt |
| ST13 (5) | + | nt |
| ST18 (2) | + | nt |
| ST18 (4) | + | nt |
| ST18 (5) | + | nt |
| ST17 (4) | + | nt |
| ST17 (1) | - | + |
| ST17 (5) | - | + |
| ST10 (1) | - | + |
| ST10 (2) | - | + |
| ST11 (2) | - | + |
| ST19 (1) | + | nt |
| ST19 (5) | + | nt |
| ST19 (2) | - | - |
| ST14 (4) | + | nt |
| ST14 (2) | - | + |
| ST14 (5) | - | - |

(+) positive to BBTV in polymerase chain reaction
nt-not tested due to limited samples for testing.

This study generated information useful for the establishment of reliable virus indexing of abaca tissue cultures. Since, multiple viruses are transmitted in *in vitro* cultures, it is suggested that the source of the explants must be tested for multiple infections to ensure that the explants are clean to prevent the multiplication of the virus during propagation through tissue culture. The explants can be tested by ELISA and confirmed by PCR for the presence of the virus. Further study to compare the sensitivity of ELISA and PCR is needed to determine which method is more efficient for virus indexing of tissue cultured abaca.

## CONCLUSION

In this study, differential infections of BBTV causing bunchy top, as well as BBrMV and SCMV causing mosaic diseases were detected by enzyme-linked immunosorbent assay. The virus was

detected in plantlets obtained from a single tissue culture line, and in plantlets obtained following subsequent *in vitro* (first and second subculture) and *ex vitro* cultures. Infection occurred as mixtures of BBTV, BrMV and SCMV. Although the plantlets of each line were derived from a single explant, these plantlets were found to be differentially infected. Likewise, the plantlets obtained from a single plantlet following subsequent culture were also differentially infected. The differential infection resulted to inconsistency of virus detection by ELISA. Differential infection of BBTV was also detected by PCR. The sensitivity of BBTV detection by PCR increased with the dilution of template DNA. The information generated from this study will be useful for the establishment of a reliable method for virus indexing considering the differential virus infection of tissue culture plantlets.

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# SURVEY OF INSECT PESTS AND DISEASES OF GABING SAN FERNANDO, *Xanthosoma sagittifolium* (L.) SCHOTT and MELET IN SELECTED AREAS OF LUZON AND ZAMBOANGA CITY, PHILIPPINES

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## ABSTRACT

Different insect pests were observed on Gabing San Fernando (GSF) (*Xanthosoma sagittifolium* (L) Schott and Melet) in GSF growing areas in Luzon and Zamboanga City, Philippines through monitoring conducted from August, 2015 to October, 2016. The most prevalent were the leaf feeders, 'harabas' or cutworm (*Spodoptera litura* Fabricius) and the wooly bear caterpillar (*Olepa ricini* Fabricius). The striped albatross caterpillar (*Appias olferna peducaea* Fruhstorfer), noted in Kasibu, Nueva Vizcaya is a novel host that will be of interest to butterfly breeders and has potential for livelihood. Tussock moth caterpillar (*Orgyia spp*.) attacked GSF occasionally at the Central Experiment Station of University of the Philippines Los Baños (UPLB), and the National Crop Protection Center (NCPC) experimental plots. The lepidopterous pests' damage only affects 2-3 leaves and 1-2 plants. Banana aphids (*Pentalonia nigronervosa* Cocquerel) were also found in the inner stalk at the NCPC area. The aphids were suspected vectors of the virus, Dasheen Mosaic (DsMV). Majority of the surveyed areas had 2 or 3 plants with DsMV symptoms but remained healthy. It was observed that the GSF populations in most areas were resistant to DsMV. Notable among the destructive insects found attacking GSF was the grub of the coconut rhinoceros beetle (*Oryctes rhinoceros* Linnaeus), covered with decaying debris and found feeding in the corm. The infested GSF had dried leaves. Other insects were observed on GSF plants but they were occasional dwellers, transient visitors, and many were beneficials. The insect pests, disease and beneficials are new records for GSF in the Philippines.

**Key words**: leaf feeders, new records

## INTRODUCTION

Gabing San Fernando (GSF) or *Xanthosoma sagittifolium (*L.) Schott and Melet belongs to the Araceae family or Aroids. Almost similar to Taro (*Colocasia esculenta* [L.]), GSF, also known as the arrow leaf elephant ear, is an important tropical and subtropical tuber crop grown in several countries in the world. Its corms and cormels are consumed as staple or subsistence food in developing countries (Lokesh et al. 2014). The *Xanthosoma* species are plants of the tropical rain forest and, although in their natural habitat they grow under the forest canopy. Under cultivation, they are usually sown with full exposure to sunlight. They require well-drained soils and do not tolerate the permanent presence of water. The mean temperature for their optimum growth must exceed 20°C (Bermejo and De Leon 1994). In reality, aroids are fairly robust plants with leathery leaves, which are difficult for most insects to chew. As a group, these are often left to grow without pesticides and still manage to produce significant yields. There are, however, several pests and disease which deserve attention, especially in intensive, commercial production (Lebot 2009). Compared to *Colocasia* and other root and tuber crops, GSF is

supposedly resistant to pests and disease. However, there were reports of pest infestation of the plant from farmers. In a study by Pillai et al. (1993), however, yield losses of up to 29% was noted due to infestation of *C. esculenta*, *X. sagittifolium* and *Amorphophallus sp*. by *Spodoptera litura*, *Aphis gossypii* Glover and spider mites [*Tetranychidae*].

Mealybugs, which are injurious on tuber crops, also infest *Xanthosoma sagittifolium*, although mainly cassava, taro, yam, sweet potato, elephant foot yam, and yam bean are much more affected. (Mani et al. 2016). On the other hand, Coleson and Miller (2005) found out that *X. sagittifolium* consistently exhibited strong aphid resistance (antixenosis), specifically on *Aphis gosyphii* than in taro (*Colocasia esculenta*).

*Xanthosoma* does not suffer from any severe pests or disease in the Pacific islands (Weightman and Moros 1982 as cited by Manner 2011). However, in the Caribbean, its pests include nematodes, a hairy caterpillar, mealybug, cotton lace bug, woolly aphids, scale insects, and red spider mites. Other pests include wireworms, white grubs, and a smooth, black or dark brown boring caterpillar (Morton 1972). Dasheen mosaic virus (DsMV) is the most important viral pathogen of cultivated aroids world-wide (Chen et al. 2001). Viruses, however, do not appear to be a serious problem for *X. sagittifolium* (Kay 1987), although Castro (2006) mentioned that DsMV is the most important virus problem in Nicaragua. Other infrequent pests include *Aphis gossypii* in the Antilles and Surinam, *Euetheola bidentata* in Surinam, *Graphocephala propior*, *Quinta cannae*, and *Cacographis ortholatis* in Venezuela, *Aspidiotus destructor* in the Antilles and Polynesia, and *Pentalonia nigronervosa*, *Tetraleurodes ursorum*, and *Corythucha gossypii* in the Antilles (Manner 2011; Reddy 2015).

This paper presents some insects observed to attack GSF through a survey and monitoring conducted in different areas of Luzon and Mindanao, Philippines. The survey also includes the beneficial arthropods that have been observed in GSF plants and relates the pest management practices on some of the prevalent pests that attacked GSF.

## METHODOLOGY

The monitoring, identification, and assessment of insect pests, diseases and beneficials on GSF plants were done in four ways. First, there was the establishment of GSF plots in the NCPC experimental area, and weekly monitoring of the plants within their growing period until harvest. Second, there was weekly monitoring of the three GSF experiments and a GSF production area established at the Central Experiment Station of UPLB-College of Agriculture. Third, there was periodic monitoring of GSF areas in farmers' fields in the neighboring municipalities/city of San Pablo City, Bay, and Los Baños in Laguna province, and in Dolores, Lucena, Lucban, and Pagbilao of Quezon province. Fourth, there was a one-time observation in some GSF areas in the country, like in Mindanao, during the extensive germplasm collections. The monitoring was made from August 2015 to October 2016.

Insect pests and diseases were collected, identified, damage characterized, and documented through photos. The assessment of the pest and disease infestation was based on their occurrence, succession, frequency, and seasonality. The incidence of viruses in the GSF plants was also assessed with monitoring of insect vectors that may carry the virus.

## RESULTS AND DISCUSSION

A total of 14 insect pests, one disease (Table 1), and 8 beneficial arthropods (Table 2) were observed to attack GSF during the monitoring period.

Notable among the insect pests that have brought considerable damage to one or two plants/leaves were the 'harabas' or common cut worm (*Spodoptera litura* Fabricius), banana aphid (*Pentalonia nigronervosa* Cocquerel) (Fig. 1), the wooly bear caterpillar (*Olepa ricini* Fabricius) a new pest of crops and weeds in the Philippines (Cayabyab et al. 2015), and the Tussock moth caterpillar (*Orgyia spp*. Ochsenheimer) (Fig. 2).

There were, however, other insect pests on GSF but were few in numbers on the leaves and stalks of the plant, namely, the grey mealy bug (*Ferrisia virgata* Cockerell), pineapple mealybug (*Dysmicoccus brevipes* Cockerell), and cottony cushion scale (*Planococcus lilacinus* Cockerell) (Fig. 3). Other insects which were occasional pests of GSF were the spiralling white fly (*Aleurodicus dispersus* Russell), long horn grasshopper (*Phaneroptera furcifera* Stål), bag worm (Psychidae), citrus grasshopper (*Melecodes tenebrosa* Walker), taro grasshopper (*Gesonula mundata zonocera*) (Navás 1904), and rice grasshopper (*Oxya hyla* Serville). The striped albatross caterpillar (*Appias olferna peducaea* Fruhstorfer) noted in Kasibu, Nueva Vizcaya, is a novel host for GSF that will be of interest to butterfly breeders and has potential for livelihood (Fig. 4).

(a) (b)

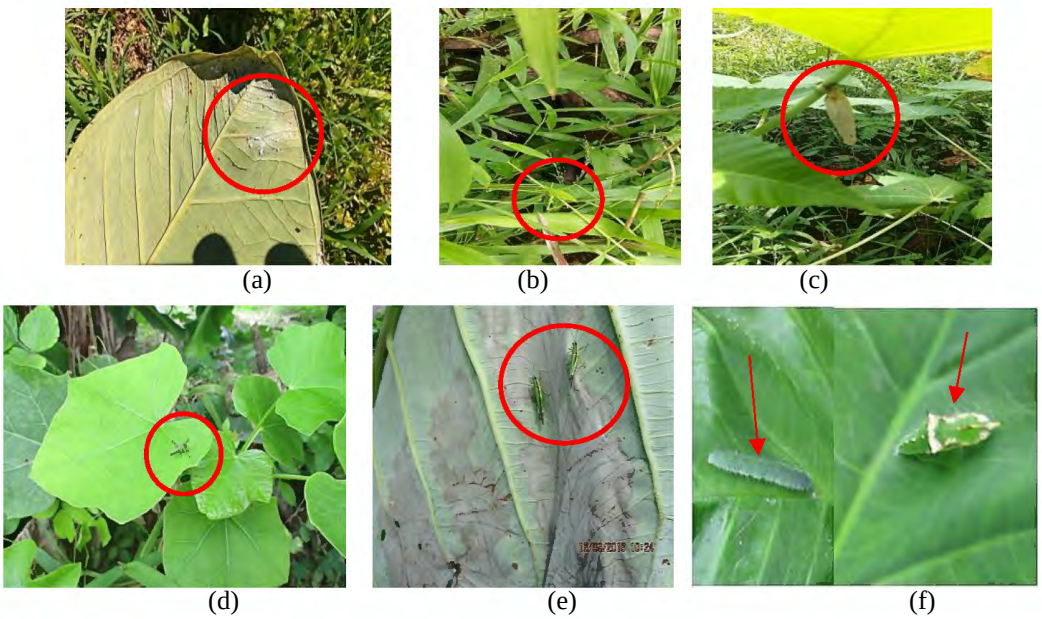
**Fig. 1**. Harabas or common cut worm on leaves (*Spodoptera litura* Fabricius) (a) and aphids (*Pentalonia nigronervosa Cocquerel)* (b) on inner stalk of GSF.

(a) (b)

**Fig. 2.** Wooly bear caterpillar (*Olepa ricini* Fabricius) (a), and Tussock moth caterpillar (*Orgyia spp*. Ochsenheimer) (b).

(a) (b) (c)

**Fig. 3**. Grey mealybug (*Ferrisia virgata* Cockerell) (a), Pineapple mealybug (*Dysmicoccus brevipes* Cockerell) (b), and Cottony cushion scale (*Planococcus lilacinus* Cockerell) (c).



(a) (b) (c)

(d) (e) (f)

**Fig. 4.** Spiraling white fly (*Aleurodicus dispersus* Russell) (a), Long horn grasshopper (*Phaneroptera furcifera* Stål) (b), Bag worm (*Psychidae*) (c), Citrus grasshopper (*Melecodes tenebrosa* Walker) (d), Rice grasshopper (*Oxya hyla* Serville) (e), and the Striped albatross butterfly caterpillar (*Appias olferna peducaea* Fruhstorfer) (f).

Another insect pest that was observed in GSF was the grub of the coconut rhinoceros beetle (*Oryctes rhinoceros* (Linnaeus). It was found feeding on the corm of the GSF at Dolores, Quezon. The usual host of the rhinoceros beetle are old decaying trunks of fallen coconut and its living trees. *Oryctes rhinoceros* (L.) is one of the most damaging insects to palms in Asia and the Pacific Islands (Giblin-Davis 2001). Adults are a major pest of *Cocos nucifera* (L.) and *Elaeis guineensis* Jacq. (Giblin-Davis 2001), but so are minor pests of many other palms and other plant species. By feeding on healthy leaves, the *Oryctes rhinoceros* causes physical damage, which can stunt growth and lead to secondary infections from bacteria or fungi (Hinckley 1973; Castro 2006). As was observed, GSF affected plants due to the feeding damage, and plants turned yellow, senesced and died (Fig. 5). This is a new finding for this insect pest which feeds on GSF as an alternate host.

(a) (b)

**Fig. 5.** Rhinoceros beetle (*Oryctes rhinoceros* (L.) damage (a) and grub (b) on GSF.

Most of the GSF plants observed in different parts of Luzon and Zamboanga City, Mindanao, where the survey was made, showed the presence of the Dasheen Mosaic virus (DsMV) (Fig. 6). DsMV incidence, however, did not show heavy infection and did not indicate a risk or grave threat. There were GSF plants in a population that do not show the symptoms and if infected, only 1 to 3 plants were affected and did not succumb to death. This is consistent with the findings that GSF is resistant to diseases and insect attack (Crop Protection Compendium (Open Source); Lebot 2009). However, depending on the host-virus strain combination and the location, DsMV can severely impact yields of the edible aroids *Colocasia* and *Xanthosoma* in the Pacific (Nelson 2008).

**Fig. 6**. Dasheen Mosaic Virus (DsMV) symptoms observed in GSF leaves.

DsMV is a typical member of the genus Potyvirus, and the most important viral pathogen of cultivated aroid plants worldwide, including the genera Aglaonema, Alocasia, Amorphophallus, Anthurium, Arisaema, Caladium, Colocasia, Cryptocoryne, Dieffenbachia, Monstera, Philodendron, Richardia, Spathiphyllum, Xanthosoma and Zantedeschia (Chen et al. 2001).

All of the above results related to GSF insect pest, disease and beneficials are new records in the Philippines and serve as benchmark data. It is also imperative to conduct a regular inventory of pests and diseases of GSF to check for threats on this alternative crop used as animal feed and food for human consumption.

**Table 1.** Insect pests on GSF observed at different locations in Luzon.

| Location | Insect/Disease Pest Observed | Date(s) Observed | Severity | | |
|---|---|---|---|---|---|
| | | | Frequency | Number / Appearance | Prevalence |
| CES, UPLB | Cut worm | September 2015 November 2015 | Occasional | 1 - 2 | seen in 2 GSF plant |
| | Virus | February–November 2015 | Occasional | As mosaic | 3-5 GSF plant affected |
| NCPC, UPLB | Banana aphids | November 2015 | Occasional | >20 as colony | Only 1 GSF affected |
| | Wooly bear caterpillar | July 11, 2016 | Occasional | 4-5 | 1 GSF attacked |
| | Tussock moth caterpillar | December 3, 2015 | Occasional | 3-4 | 1 GSF attacked |
| | Virus | December 3, 2015 | Occasional | As mosaic | 3-5 GSF plant affected |
| | Grey mealy bug | Sept – Nov., 2015 | Occasional | >10 | 3-5 GSF plant affected |
| | Pineapple mealy bug | Sept – Nov., 2015 | Occasional | >10 | 4-5 GSF plant affected |
| Dolores, Quezon | Cut worm | September 16, 2016 | Occasional | 2-3 | 1 GSF attacked |
| | Rhinoceros beetle grubs | September 16, 2016 | Occasional | 1 | 1 GSF plant attacked |
| | Virus | September 16, 2016 | Occasional | As mosaic | 4-5 plant affected |
| Lucena, Quezon | Long horn grasshopper (Katydid) | October 21, 2016 | Occasional | 1 | 1-2 found in plant |
| | Bag worm (Psychidae) | May 25, 2016 | Occasional | 1 | Found in 1 GSF |
| Pagbilao, Quezon | Long horn grasshopper (Katydid) | October 21, 2016 | Occasional | 1 | Found in 1 GSF |
| | Cottony cushion scale | October 21, 2016 | Occasional | >10 | Colony found in 3 GSF |
| Kasibu, Nueva Vizcaya | Striped albatross caterpillar | February 12, 2016 | Occasional | 1-3 | 2-3 found in >10 GSF plants |
| Lucena City | Citrus grasshopper | May 25, 2016 | Occasional | 1 | Found in 1 GSF |
| | Virus | September 16, 2016 | Occasional | As mosaic | 3-5 GSF plant affected |
| Aurora, Quezon | Spiraling white fly | April 22, 2016 | Occasional | 5-10 | 3-4 GSF with colonies |
| | Rice grasshopper | April 22, 2016 | Occasional | 2 | Found in 1 GSF |
| | Virus | April 22, 2016 | Occasional | As mosaic | 4-6 GSF plant affected |
| Zamboanga City | Virus | October 06, 2016 | Occasional | As mosaic | 4-6 gsf affected |

**Beneficial**

A number of beneficial insects were also observed during the survey (Table 2). The orb weaver spider (*Argiope spp*.), crab spider (*Misumena sp*.), and huntsman spider (*Heteropoda venatoria* Linnaeus) helped in the reduction or elimination of insect pests that attack GSF. Other predators, such as the yellow crazy ants (*Anoplolepis gracilipes* Smith) and praying mantis (*Mantis religiosa* Linnaeus), were observed. Natural enemies are often encountered in the growing of crops. Palaniswami and Pillai (1980) found *Aphis gossypii* to be parasitized by the aphelinid *Aphelinus mali* (Hald.), a species of *Coccophagus* (*C. cowperi* Gir.) and by the encrytid *Aphidencyrtus aphiivorus* (Mayr).

The passerine birds, Maya, (*Passer montanus (*Linnaeus) were frequently observed in the GSF surroundings, and as such, their contribution in reducing pest population cannot be discounted for.

**Table 2.** Beneficial insects/natural enemies.

| Location | Beneficial Insect Observed | Date(s) Observed | Severity | |
|---|---|---|---|---|
| | | | Frequency | Prevalence |
| CES, UPLB | House spider | November, 2015 | Occasional | 1 |
| | Orb weaver | October 21, 2016 | Occasional | 1 |
| | Crab spider | October 21, 2016 | Occasional | 1 |
| NCPC, UPLB | House spider | November, 2015 | Occasional | 1 |
| | Orb weaver | November, 2015 | Occasional | 1 |
| | Crab spider | October, 2015 | Occasional | 1 |
| Lucban, Quezon | House spider | October 21, 2016 | Occasional | 1 |
| | Orb weaver | October 21, 2016 | Occasional | 1 |
| | Crab spider | October 21, 2016 | Occasional | 1 |
| Lucena, Quezon | Yellow crazy ants | October 21, 2016 | Occasional | 3-5 |
| | Lady Bug (Coccinelid Beetle) | October 21, 2016 | Occasional | 1 |
| | Yellow coccinelid beetle | October 21, 2016 | Occasional | 1 |
| | Black coccinelid beetle | October 21, 2016 | Occasional | 1 |
| Pagbilao, Quezon | Black ant | October 21, 2016 | Occasional | 3-5 |
| Lucena City | Crab spider | October 21, 2016 | Occasional | 1 |
| Aurora, Quezon | Praying mantis | May 25, 2016 | Occasional | 1 |

**Pest management on Gabing San Fernando**

Cut worms, as mentioned earlier, were observed on two occasions infesting GSF planted in the experimental area of Central Experiment Station (C8), UPLB, while banana aphids (*Pentalonia nigronervosa* Cocquerel) were seen at the lower stalk of GSF planted at NCPC, UPLB experimental plots. The presence of these two insect pests, however, did not pose a major threat and the extent of damage was not large enough to be considered a problem. But to ensure a rational management approach, the former was controlled by spraying a biological control agent, nucleopolyhedrosis virus (NPV), from NCPC, while the banana aphid was controlled by spraying a combination of cypermethrin 5 EC and thiocarbamate (cartap hydrochloride ES) at rate of 1.125L a.i/ha and 1.25kg a.i./ha, respectively (Cayabyab et al. 2015).

The infestations of wooly bear caterpillar (*Olepa ricini Fabricius*) and Tussock moth caterpillar (*Orgyia spp*) were only sporadic and very limited. Hence, their larvae were only mechanically crushed. No further presence or incidence of these insects was observed thereafter.

## CONCLUSION

Insect pests namely, cutworm/'harabas' (*Spodoptera litura* Fabricius), Tussock moth (*Orgyia spp*.), and wooly bear caterpillar (*Olepa ricini* Fabricius) attacked GSF in certain occasions but their infrequent presence do not pose a major threat to the growing of GSF. This suggests that these insects attacked the GSF in cases when their most preferred hosts are not present. Occasional occurrence of the said pest was observed in the months of September to February, but their population was not sufficient to cause alarm. A nucleopolyhedrosis virus (NPV) from NCPC was used to control cutworms.

The presence of banana aphids (*Pentalonia nigronervosa* Cocquerel) in the GSF stalks may indicate the possibility for the insect to be carriers of DsMV, since the typical symptoms were noted. Its incidence, however, in most GSF planting sites pose minor problem to the growth of GSF as it did not severely affect the plants. The observed estimate of affected plants was only 1 – 3% in a GSF planting area, while banana aphids were controlled by spraying a combination of cypermethrin and thiocarbamate. Regular monitoring of pest and diseases of GSF is recommended for a pre-emptive management strategy to avoid or lessen damage.

There is a need to seriously consider the emerging threat of rhinoceros beetle to the growth of GSF in an area where coconuts and other palms are abundant because the grubs can cause severe damage that can affect the quality of the corms and the cormels produced.

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# CHEMICAL BASIS FOR REPELLENCY OF *Sargassum cinctum* J. AGARDH (Sargaceae) AGAINST ASIAN CORN BORER, *Ostrinia furnacalis* (Guenee) (Lepidoptera:Crambidae)

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## ABSTRACT

Anecdotal claims reveal that Sargassum seaweed has long been used by farmers to reduce insect pest populations in cacao and various vegetables. A study was conducted at the National Crop Protection Center, College of Agriculture and Food Science, University of the Philippines Los Baños from 2015-2017 to validate farmer knowledge on reduced Asian corn borer populations when brown seaweed, *Sargassum cinctum* is placed in plot borders. Prominent volatile organic chemicals (VOCs) emitted by seaweed under sunlight and room conditions were identified by GCMS and differences in emission patterns were observed. In general, the fresh seaweed extract showed a higher percent repellency as compared to the dried seaweed extract. We identified for the first time the nature of the compounds emitted by *S. cinctum* and repelling the Asian corn borer. Some VOCs presented in combination did not produced synergistic effect. The number of eggmasses and number of eggs laid by female moths was reduced when brown seaweed was applied on corn under field cage conditions. Sargassum seaweed can be used together with other effective IPM strategies, especially in coastal areas, to reduce corn borer population. Brown seaweed can be an effective pest management strategy tool for low input and/or organic green corn production

**Key words:** insect behavior, semiochemicals, allomone, pest management

## INTRODUCTION

Anecdotal claims indicate that brown seaweed, *Sargassum cinctum* has long been used by farmers to reduce insect pest populations in cacao and various vegetables. Preliminary trials in corn, conducted in barangay Poblacion, San Andres, Quezon in Bondoc Peninsula from June to September 2013 by Felipe Duaso, a farmer-scientist trained under the Farmer Scientist Training Program (FSTP) of the University of the Philippines, demonstrated higher yields in the plots with seaweed at the edges of the plot, compared to control plots. He claims this is farmer-knowledge gained from the experiences of his father and other farmers in their farming community. This observed phenomenon is similar to the larvicidal activity of certain seaweeds and algae against mosquitoes (Hira et al. 2010; Thangam and Kathiresan 1996).

The production of volatile organic compounds (VOC) by plants is essential for chemical ecology interactions. Headspace collection of volatiles have demonstrated the presence of major volatile compounds found in seaweeds: hydrocarbons, terpenes, phenols, alcohols, aldehydes, ketones, esters, fatty acids and halogen or sulfur-containing compounds (Gresslera et al. 2009). Non-host plant

volatiles interfere with orientation to the host plant and affect the olfactory, feeding and oviposition behavior of major insect pests and even natural enemies (Brevault and Quilici 2010; Bruce and Picket 2011; Finch and Collier 2000; Forbes and Feder 2006; Koschier et al. 2000; van Toll and Visser 2002). Biologically active plant volatiles may be used in the development of integrated pest management strategies (Koschier et al. 2000; Calumpang et al. 2013 and 2014).

Filipino farmer knowledge on insect pest reduction when tagbak (*Alpinia elegans* (C. Presl) K. Schum) stalks are placed in rice fields also served to reduce insecticide use in rice production in Infanta, Quezon. Volatile organic chemicals emitted by tagbak such as α-terpinene, α-pinene, and cymene demonstrated 67 to 70% repellency against the green leaf hopper, a vector of tungro in rice. Therefore, the use of tagbak for insect pest management in rice production can be promoted in areas where these abound, to reduce dependence on synthetic insecticides (Calumpang et al. 2012; Calumpang et al. 2013).

Kakawate (*Glyricidia sepium*) (Jacq.) Walp., is also believed to repel insect pests of rice, while leaf bunches are placed close to lighted bulbs in night fairs and markets to repel insects. Volatile chemical components of kakawate were demonstrated to repel green leaf hopper, *Nephottetix virescens* in olfactometric bioassays. Volatile chemicals emitted by kakawate were identified by GC/MS (Calumpang et al. 2013). Both plants are used by organic rice farmers in the Philippines (Pantoja et al. 2016).

Asian corn borer is a major pest of corn, damage has been found to result in tremendous yield reduction ranging from 20 to 80% (Sanchez 1971). It comes as a result of infestation from two generations during a cropping season. The main point of establishment and survival of the 1st and 2nd instars of the first generation are on the whorled leaves while the 3rd and 4th generation prefer the leaf sheaths (Magsino 1995). The Asian corn borer is repelled by a plant volatile, 1-methylethyl propyl disulfide, which is emitted by both corn and a weed closely associated with corn, *Ipomoea triloba*. The emission is significantly increased when corn is entwined by the weed (Calumpang et al. 2000). This study provided the chemical basis for the population reduction of corn borer in corn fields with *I. triloba*.

Other cultural management practices should therefore be tested to make available additional alternatives to chemical pesticides. The increase in yield would redound to less inputs and lower risk for farmer applicators, consumers and the environment. The potential impact of this research, includes among others: reduced insecticide use in corn production, additional pest management options for organic agriculture and less environmental and human health concerns regarding pesticide use in corn production.

In the present paper, our objective was to validate farmer knowledge on reduced insect pest populations in corn with fresh brown seaweed.

## MATERIALS AND METHODS

### Establishment of stock culture

The parental stock for the mass rearing of the Asian corn borer (ACB) was collected from Los Baños, Laguna, Philippines. The larvae were reared at the National Crop Protection Center (NCPC), College of Agriculture, UPLB. An artificial diet was used to feed the larvae which were 1-2 weeks old and were placed in plastic rearing cups. On the third week, the larvae were placed in a large container until they pupate and were fed with young corn and its stalks. The pupae were collected daily and transferred to a separate container. The newly-emerged adults were fed with a sugar solution-soaked cotton hung at the top of the container. Also, egg masses collected in the cage bioassays served as an additional source ACB for rearing/stock culture.

**Corn planting**

Six corn seeds were sown in pots and were thinned to 3 plants per pot after 2 weeks. The corn plants were then fertilized with complete fertilizer (14-14-14) at the rate of 15g per 4 li water, every 2 weeks and watered daily. Corn planting was done weekly in order to ensure staggered growth of the plants.

**Seaweed**

The seaweed samples used in various experiments were collected from Catanauan, Quezon Province, Philippines in 2015 and from Calatagan, Batangas in 2016. These were used as fresh material or air dried. The seaweed was identified as brown seaweed, *Sargassum cinctum* J. Agardh, (Fig. 1) by Dr. Garmino C. Trono, Professor Emeritus, Institute of Marine Science, University of the Philippines Diliman, Quezon City, Philippines.

**Fig. 1.** Immature and mature brown seaweed, *Sargassum cinctum.*

**Extraction of headspace volatiles emitted by Sargassum seaweeds**

The volatile organic chemicals (VOCs) emitted by *Sargassum* seaweed samples were collected by enclosing the sample in a Tygon bag and subjecting it to Tenax adsorption, followed by Soxhlet extraction (42 °C) in *n*-pentane and concentrated in a micro Kuderna Danish set-up (34 °C). The final extracts were diluted in *n*-hexane and were kept frozen until analysis by GCMS.

The volatile components were analyzed using a Shimadzu 2010 GCMS at Natural Science Research Institute (NSRI), University of the Philippines Diliman. The temperature settings of the capillary gas chromatography-mass spectrometer was maintained at 60 °C for 3 min, and programmed at 5°C /min to 250 °C and held for 5 min. Injector temperature was maintained at 250 °C. Compound identification was based on mass spectra analysis completed with the mass spectral database NIST107.Lib.

**Olfactometric bioassays**

Seaweed extracts (0.5 mL) were pipetted to a filter paper and were allowed to dry. Treated and control (hexane-treated) filter papers were inserted at opposite ends of the Y-tube olfactometer. Adult female Asian corn borers were placed at the open end of the olfactometer and vacuum was applied. It was then covered with carbon paper and was observed for 10-15 minutes. One ACB per replicate was done, and a total of 30 replicates were conducted.

Individual reference materials of volatile organic compounds (Sigma, USA), identified by GC-MS, were assayed for repellency against corn borer females.

**Effect of *S. cinctum* on oviposition of ACB in field cage experiments**

One hundred twenty (120) corn plants per set-up were used (6 rows with 20 corn plants each) for the field plot bioassay. One set-up contained the corn plants with seaweeds, while the other set-up contained corn plants only. A two meter distance between the two treatments was provided. The set-up were enclosed in a muslin cloth cage, properly fastened on the sides to ensure that no insect can escape. Twenty five (25) pairs of newly emerged Asian corn borers were released at the center of the cage to allow ovipositing females to select oviposition sites.

**Statistical analysis**

The data obtained in the field cage experiments were analyzed statistically and significant differences of means were determined using Independent Sample T-test (SAS Institute 2001).

## RESULTS AND DISCUSSION

**Identification of headspace volatiles emitted by *Sargassum cinctum***

The compounds present as volatile organic chemicals (VOC) emitted by seaweed were elucidated through GC-MS analysis. Tentative identification showed the prominent chemical components are: 2-ethyl-1-hexanol, 2,2,4-trimethylheptane, 1,3,5-undecatriene, 6-[(Z)-1-butenyl]-4-cycloheptadiene, 2,4-di-tert-butylphenol and 1-butoxy-2-ethylhexane. The minor components are 3-eicosene, 9-eicosene, 3-butyl-4-vinylcyclopentene, 3-(1-butenyl)-4-vinyl-cyclopentene, 6-butylcyclo-1,4-heptadiene, and dipropyl disulphide..

Prominent VOCs of mature *S. cinctum* were: 1,3,5-undecatriene and 6-(1-butenyl)cyclohepta-1,4-diene (Fig. 2). These were consistently emitted by Sargassum samples for 3 successive days.

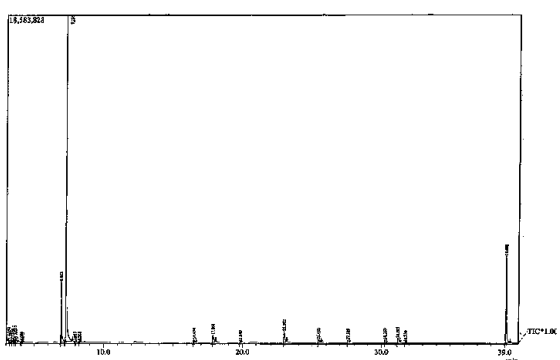

**Fig. 2**. Representative gas chromatography-mass spectrometry trace of headspace volatiles released from mature *Sargassum cinctum*

Volatile organic chemicals emitted by immature *S. cinctum*were slightly from the emission profile of mature seaweed. Prominent VOCs in immature *S. cinctum* were: 2-ethyl-1-hexanol, 6-[(Z)-1-butenyl]-1,4-cycloheptadiene and 1,3,5-undecatriene (Fig. 3).

6-[(Z)-1-butenyl]-1,4-cycloheptadiene is reported to be present in brown alga *Dictyopteris membranacea,* (Hattab et al. 2007) and (*Ectocarpus siliculosus*) as algal sexual pheromone (Tringali 1997) while 1,3,5-undecatriene was part of the volatile fractions of brown alga, *D. membranacea,* along with 3-butyl-4-vinylcyclopentene and 6-butylcyclo-1,4-heptadiene (Hattab et al. 2007). Minor components include: 5-methyl-3-heptanone, 3,5,5-trimethyl-1-hexene, 2-methyl-4-heptanone, 12-methyltetradecanoic acid methyl ester which is reported to be present in green alga (*Spirogyra longata*), possibly derived from fatty acids (Abdel-Aal et al. 2015).

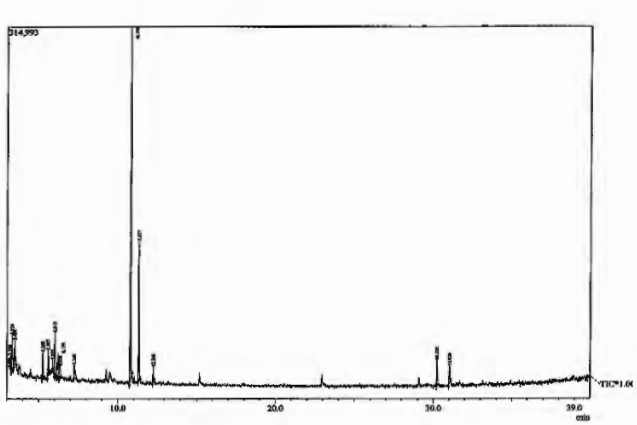

**Fig. 3.** Representative gas chromatography-mass spectrometry trace of headspace volatiles released from immature *S. cinctum*.

The production of VOC is closely related to the physiology of the species (Gresslera et al. 2009). Different growth conditions of the same species are implied by differences in VOC profiles (Kajiwara et al. 1989).

Several volatile compounds emitted by *S. cinctum* have been determined from other brown algae and *Sargassum* species. The 3-(1-butenyl)-4-vinyl-cyclopentene, 6-(1-butenyl)cyclohepta-1,4-diene and 1,3,5-undecatriene are known algal sexual pheromones of brown marine alga (Tringali 1997; Maarse 1991; Boland et al. 1983; Hattab et al. 2007 ). 2-Ethyl-1-hexanol has also been identified in *Sargassum subrepandum* (Forsk) (Abou-El-Wafa et al. 2011).

Full sunlight conditions however, resulted in an entirely different emission pattern. VOC emissions under full sunlight showed a different pattern from the emissions made under room conditions (24$^{o}$C, ambient room light). Prominent emissions at higher temperatures (30 – 35$^{o}$C) under full sunlight were 2-ethylhexylglycidyl ether and 2,4-di-tert-butylphenol (Fig. 4). These were not detected in samples collected under room temperature conditions. Minor compounds include: 2,3,8 trimethyldecane, eicosene, and 4-heptadecenal. The emission of volatile alcohols was noted, namely, 1-dodecanol, and heptanol.

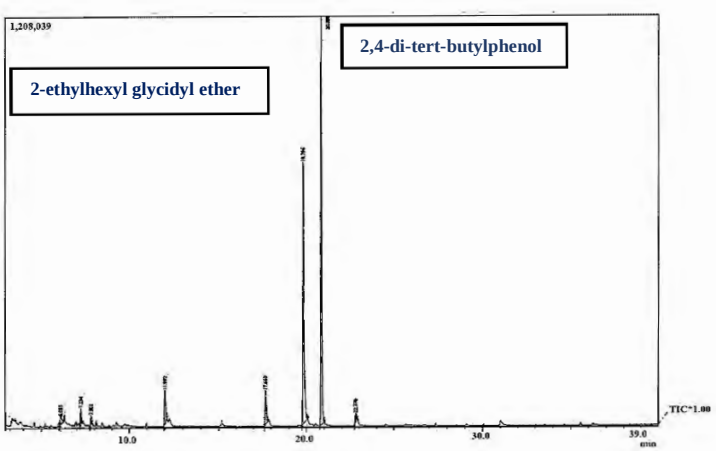


**Fig. 4.** Representative gas chromatography-mass spectrometry trace of headspace volatile profile released from *Sargassum cinctum* under full sunlight (30 – 35$^{o}$C).

Eicosene has been reported in *Sargassum wightii* (Pandiyan et al. 2014). The presence of 2,4-di-tert-butylphenol has likewise been reported in four red algae: *Gracilaria bursa-pastoris, Phyllophora crispa, Laurencia obtusa* var. pyramidata and *Jania rubens* (Guven et al. 2014; Karabay-Yavasoglu et al. 2007) as well as in steam distillate of *Dictyopteris membranaceae and Cystoseira barbata* (Ozdemir et al. 2006).

Volatile emissions under full sunlight is expected to be different from volatile emissions under room temperature. It has been established that temperature has a strong effect on the quantity of volatiles released in the headspace. Emission at 10°C was significantly lower than at 15°C and 20°C. This difference can be attributed to a temperature effect on the secretion of volatiles rather than on the evaporation rate of volatiles. Light influenced fragrance emission significantly, the most intense emission being noted at high irradiances (Jakobsen and Olsen 1994).

Water stress is also another factor that affects release of VOCs and explains the difference in repellency produced by fresh and dried Sargassum.

In maize, headspace volatile compounds emitted by watered and water-deprived maize plants had different profiles. The most abundant compounds from watered plants are limonene, linalool, benzoic acid, indole, β-caryophyllene and acetophenone, whereas, in water-deprived plants, limonene, acetophenone, hexanoic acid, benzoic acid and indole are dominant. In addition, (E)-4,8-dimethyl-1,3,7-nonatriene, 6-methyl-5-hepten-2-one, anisole and 1-carvone are undetected in the water-deprived plants (Sole et al. 2010).

The compound, dipropyl disulfide present in the Sargassum extract has a similar structure as dimethyl disulfide, a known volatile compound from marine algae (Moore 1976).

**Olfactometric bioassays**

Olfactometric bioassays demonstrated the repellency of some *S. cinctum* volatile emissions against the ACB. The Y-tube used in the assays was initially established to be effective in assessing repellency using phenylacetaldehyde, a known attractant of ACB (Cantelo and Jacobson 1979). Phenylacetaldehyde produced 30% repellency in ACB or 70% attractancy (Table 1).

In general, the fresh seaweeds extract showed a higher percent repellency (76.7%) compared to the dried seaweeds extract (61.3%). A lower repellency of the dried seaweeds extract may be due to losses as the chemicals are volatile in nature. A lower concentration of the repellent chemicals renders a lower percent repellency. Thus, fresh brown seaweeds were used in the field trials, as a validation trial of Filipino farmer practice.

Among the VOCs tested, 2-ethyl-1-hexanol, 1-eicosene and 2-ethylhexyl glycidyl ether were the most repellent of the seaweed volatile organic chemicals at concentration levels that approximated the natural ratio. Although 2-4-ditertbutyl phenol is a prominent volatile chemical emitted under sunlight, it did not demonstrate repellency against corn borer moths (Table 1). A mixture of 2-ethyl-1-hexanol and 1-eicosene, both highly repellent (>70% repellency), however did not produce synergistic effect. 2-Ethyl-1-hexanol and dodecanol were reported to be found in *Sargassum subrepandum.* (Forsk) (Abou-El-Wafa et al. 2011) and red alga, *Corrallina elongata* (Dembitsky and Srebnik 2002), respectively. There are no reports of these seaweed species being used for pest management.

Volatile organic chemical emissions play a crucial role in plant-insect interaction. Experiments in which insects have been exposed to plant volatiles alone and in combination have revealed that stronger behavioral responses are obtained with appropriate blends or combinations of volatiles than with single compounds (Bruce and Pickett 2011). 2-Ethyl-1-hexanol was demonstrated to be a part of a six-component blend that was attractive to orange wheat blossom midge, *Sitodiplosis mosellana*

(Diptera: Cecidomyiidae) in an olfactometer bioassay but individual compounds were not attractive at the same dose (Birkett et al. 2004). It was also detected from aeration samples of tea shoots using GC-MS (Mu et al. 2012).

**Table 1.** Olfactory response of Asian corn borer female moths to volatile organic chemicals reference standards identified in brown seaweed, *S. cinctum.*

| Volatile Organic Chemical | Concentration (ug/ml) | % Repellency |
|---|---|---|
| 2-ethyl-1-hexanol* | 30 | 70 |
| 1-dodecanol | 0.77 | 47 |
| 1-tetradecanol | 0.77 | 57 |
| 1-undecanol | 5 | 57 |
| 1-octadecene | 5 | 50 |
| 1-eicosene | 15 | 77 |
| 2-ethylhexyl glycidyl ether** | 5 | 67 |
| 5-methyl-3-heptanone | 99.75 | 37 |
| 2-4-ditertbutyl phenol** | 60 | 33 |
| 2-ethyl-1-hexanol + 1-eicosene | 30 + 15 | 70 |
| Wet *S. cinctum* | - | 77 |
| Dry *S. cinctum* | - | 61 |
| Phenylacetaldehyde | 15 | 31 |

Major peaks: * Room temperature ** Full sunlight

Damaged maize leaves attacked by Asian corn borer larvae release herbivore-induced volatiles that affect orientation behaviors and oviposition of the females. Nineteen volatile chemicals, with terpenes being the major components, were identified. Females responded to (E)-2-hexenal, nonanal, (Z)-3-hexen-1-ol and three unknown compounds while the male only responded to (E)-2-hexenal, nonanal and one unknown compound (Huang et al. 2009).

On a tri-trophic level, studies have demonstrated that herbivore injured plants produce specific blends of odors which can attract certain insect predators and parasitoids (Dicke 1994; Turlings et al. 1995). Herbivore cues are not very detectable to carnivores at a distance, herbivore-induced plant volatiles increase herbivore detectability enormously. Among the terpenoids, the two homoterpenes 4,8-dimethyl-1,3(E),7-nonatriene and 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene are the most often reported herbivore-induced plant volatiles. These can be synthesized by plants of many species from the terpene alcohols nerolidol and geranyllinalool without any mediation of herbivory (Dicke 1994).

Females of the braconid larval parasitoid, *Cotesia marginiventris*, are strongly attracted to volatiles emitted by the caterpillar damaged plants. Volatiles emitted by corn seedlings just after caterpillars start feeding on them, include: (Z)-3-hexenal; (E)-2-hexenal, (Z)-3-hexenol, (Z)-3-hexen-l-yl acetate, linalool, (3E)-4,8-dimethyl-1,3,7-nonatriene, indole, a-trans-bergamotene, (E)-,3-farnesene, (E)-nerolidol and (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Turlings et al. 1995). None of these chemicals were detected in the brown seaweed VOCs. Future work should cover the effects of brown seaweed on the natural enemies and beneficial arthropods in a corn agro-ecosystem.

**Effect of *S. cinctum* on oviposition and hatchability of eggs of ACB laid in field cage**

There are fewer number of egg masses or eggs laid by adult female ACBs in corn with brown seaweed at 45 days after planting (DAP), both in 2016 and 2017 cropping periods (Fig. 5A and B).

Likewise, hatchability of eggs was greater in corn without seaweed. These indicate that the female corn borer was less attracted for oviposition to corn with brown seaweed and eggs exposed to its volatiles may have weakened the developing embryo resulting to lower hatchability. Air, particularly oxygen is vital for metabolic processes within the egg and the chorion of the egg is permeable to gases. A continuous film of air is held between vertical columns in the inner part of the chorion and the air in the trachea can be kept saturated (Hinton 1960; Hinton 1969). Since VOCs mix with ordinary air, these could penetrate the chorion and possibly exert deleterious effect to the developing embryo. The exact mechanism is still unclear. These results confirmed our earlier laboratory results that fecundity of female ACB and hatchability of eggs was affected by volatiles emitted by the brown seaweed both in rearing pan and Petri plate bioassays (Navasero et al. 2016).

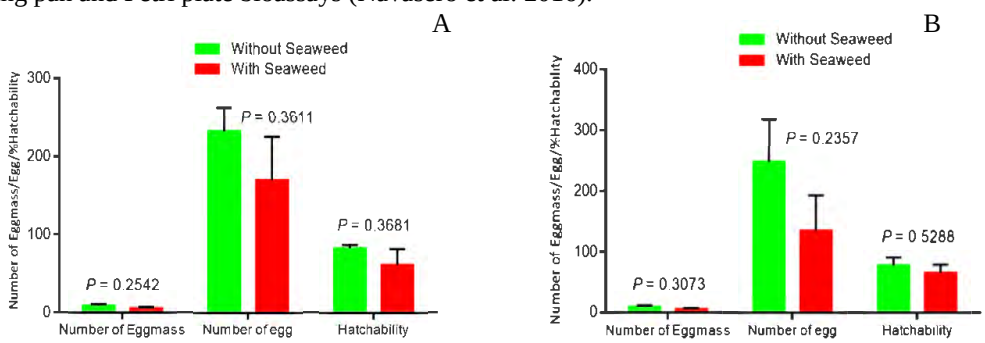


**Fig. 5**. Mean (±SE) number of egg mass , total number of eggs and percent hatchability of eggs in *O. furnacalis* released in field cage with 45 DAP corn with and without seaweed, *S. cinctum* during 2016 (A) and 2017 (B) cropping period.(t-test; p-value<0.05 was considered significant).

## CONCLUSIONS

Filipino farmer innovation in using brown seaweed for corn pest management has scientific basis. Female corn borer moths were less attracted for oviposition on corn with brown seaweed. A reduction in number of egg masses and number of eggs was observed when seaweed was placed on the corn plant. Olfactometric assays demonstrated the presence of volatile organic compounds emitted by brown seaweed, *S. cinctum* that repel female corn borer moths.

Sargassum seaweed can be used together with other effective IPM strategies, especially in coastal areas, to reduce corn borer population. Brown seaweed can be an effective pest management strategy tool for low input and/or organic green corn production.

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# EFFECT OF ORGANIC AMENDMENTS AND MICROBIAL INOCULANT ON NITROGEN, PHOSPHORUS AND POTASSIUM USE EFFICIENCY OF SUGARCANE UNDER ACID TYPIC HAPLUDAND

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## ABSTRACT

A field experiment was established in an acid Typic Hapludand in Isabela, Negros Occidental, Philippines from January to December 2016 to optimize the use of mudpress, bagasse ash and microbial inoculant in sugarcane production using 'Phil 2004-1011' sugarcane variety. Twelve treatments were imposed including no fertilization, with full fertilization using inorganic fertilizer, and with full fertilization + lime. The recommended N rate ($RR_N$) was reduced to 75, 50 and 25% with subsequent application of mud press to satisfy the full $RR_N$. Bagasse ash at 10 t $ha^{-1}$ and microbial inoculant were likewise used to supplement the nutrient sources. Standard cultural practices for sugarcane production were followed. Cane yield and NPK use efficiency indices were determined and analyzed to come up with a judicious fertilization program. Integrated nutrient management practices including mudpress, bagasse ash and microbial inoculant improved cane yield, partial factor productivity and agronomic efficiency of applied N, and physiological efficiency of applied P. Application of lime resulted to better partial factor productivity and apparent recovery efficiency of applied P. Combined use of inorganic fertilizer and mudpress enhanced partial factor productivity of applied K, while addition of bagasse ash and microbial inoculant increased physiological efficiency of applied K.

**Key words**: amendments, microbial inoculant, nutrient use efficiency

## INTRODUCTION

Application of fertilizers is an indispensable practice in sugarcane production as it augments indigenous soil nutrient supply needed for cane growth and sugar production. As in most of the sugarcane growing countries, efficient fertilization practices have contributed much to the productivity of sugarcane. It is still undeniable that nitrogen (N) is the most influential plant nutrient in global sugarcane production, as too little can impact negatively on sucrose production and too much can cause lodging, reduced cane quality and increased risk of pest and disease infestation (Meyer et al. 2007). Among the sources of N, inorganic fertilizer has been widely utilized because of its immediate effect, availability and easy handling. However, the negative environmental impact and escalating cost of inorganic fertilizer have become a public concern. This prompted farmers to recycle farm and sugar mill wastes as organic fertilizers. Mudpress or filter cake, a waste by-product from sugar factories, has a great potential to supply nutrients in addition to its favorable effects on physico-chemical and biological properties of soil (Shankaraiah and Murthy 2005). Another waste from sugar manufacturing,

boiler or bagasse ash is rich in $K_2O$ which can be used as fertilizer (Cosico 1985) and liming material due to its high pH (Vance, 1996). Despite the fertilizer and soil amendment value of mudpress and bagasse ash, their application is not widely practiced because of their relatively low nutrient content, indirect nutrient availability and difficulty in handling. These often accumulate at mill sites and generally considered a waste disposal and air pollution problem.

For sustainable sugarcane production, neither chemical fertilizers nor organic manures alone, but their integrated use is highly beneficial (Shankaraiah and Murthy 2005). Complete substitution of inorganic fertilizers by organic fertilizers is not possible to fulfil the large crop nutrient demand. This provides an impetus to develop strategy for a sound combination of different nutrient sources, which will not only improve the efficiency of both the sources but will also minimize the negative effect of over use of chemicals (Chatterjee et al. 2014).

The proper application of fertilizers concerns not only their quality, but also their quantity, as too little of the right fertilizer does not give the most economic return, while too much adversely affects the crop (Chaudhry and Corpuz 1984 as cited by Abayomi 1987). Unbalanced fertilization is also a cause of low nutrient use efficiency (NUE) (Krauss 2001). Indices of NUE include partial factor productivity, agronomic efficiency, apparent recovery efficiency and physiological efficiency (Mosier et al. 2004). Estimates of overall efficiency of applied fertilizer have been reported to be about or lower than 50% for N, less than 10% for P, and about 40% for K (Baligar et al. 2001). Previous studies suggested that commercial sugarcane varieties differ in their nutrient use efficiency particularly nitrogen (Gascho et al. 1986, Stevenson et al. 1992, Robinson et al. 2007), thus indicating a need for variety-specific N recommendations (Meyer et al. 2007). There is likewise an increasing interest in improving nutrient use efficiency of cultivated crops (Schumann et al. 1998) and this is driven by a growing public belief that crop nutrients are excessive in the environment and farmer concerns about rising fertilizer prices and stagnant crop prices (Roberts 2008).

The present study aimed to assess the cane yield and nutrient use efficiency of applied nitrogen, phosphorus and potassium in sugarcane, in terms of partial factor productivity, agronomic efficiency, apparent recovery efficiency and physiological efficiency.

## MATERIALS AND METHODS

### Treatments and Field Experiment

A field experiment was conducted in an acid Guimbalaon sandy clay loam classified as Typic Hapludand (Carating et al. 2014) in Isabela, Negros Occidental ($10^0$ 10' N, $122^0$ 59' E) situated 41.86 meters above sea level. Treatments were laid out in Randomized Complete Block Design with three replications. The recommended N rate ($RR_N$) applied through inorganic fertilizer was reduced by 25%, 50% and 75%. This reduction in the amount of inorganic fertilizer was substituted by the application of mudpress. The amount of mudpress used to satisfy the remaining recommended N rate was computed based on its total N content. From these three combinations of inorganic fertilizer and mudpress, another two sets of treatments were made – those which were supplemented with bagasse ash at 10 t $ha^{-1}$, and those which were added with bagasse ash at 10 t $ha^{-1}$ and microbial inoculant (BioGroe™) which contains plant growth promoting bacteria (PGPB). Except for the control, all treatments received the same amount of inorganic fertilizer P (105 kg $P_2O_5$ $ha^{-1}$) and K (520 kg $K_2O$ $ha^{-1}$). The detailed amount of inorganic fertilizers and amendments used for each treatment is presented in Table 1. Nutrient contents of the organic amendments in terms of % N (Kjeldahl digestion), % $P_2O_5$ (extraction and spectrophotometry) and % $K_2O$ (extraction and AAS) were determined and the values are shown in Table 2.

**Table 1**. Amount and kind of amendments and fertilizers applied per treatment.

| Treatments | Inorganic Fertilizers (kg ha$^{-1}$) | | | Lime (kg ha$^{-1}$) | Mud-press (kg ha$^{-1}$) | Bagasse ash (kg ha$^{-1}$) | Microbial inoculant (PGPB) |
|---|---|---|---|---|---|---|---|
| | 46-0-0 | 0-18-0 | 0-0-60 | | | | |
| T1 - Control (no fertilizer application) | | | | | | | |
| T2 - $RR_N$ IF | 304.35 | 583.33 | 866.67 | | | | |
| T3 - $RR_N$ IF + lime | 304.35 | 583.33 | 866.67 | 5,000 | | | |
| T4 - 75% $RR_N$ IF: 25% $RR_N$ MP | 228.26 | 583.33 | 866.67 | | 6,363.64 | | |
| T5 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA | 228.26 | 583.33 | 866.67 | | 6,363.64 | 10,000 | |
| T6 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA + MI | 228.26 | 583.33 | 866.67 | | 6,363.64 | 10,000 | √ |
| T7 - 50% $RR_N$ IF: 50% $RR_N$ MP | 152.17 | 583.33 | 866.67 | | 12,727.27 | | |
| T8 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA | 152.17 | 583.33 | 866.67 | | 12,727.27 | 10,000 | |
| T9 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA + MI | 152.17 | 583.33 | 866.67 | | 12,727.27 | 10,000 | √ |
| T10 - 25% $RR_N$ IF: 75% $RR_N$ MP | 76.09 | 583.33 | 866.67 | | 19,090.91 | | |
| T11 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA | 76.09 | 583.33 | 866.67 | | 19,090.91 | 10,000 | |
| T12 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI | 76.09 | 583.33 | 866.67 | | 19,090.91 | 10,000 | √ |

Thirty-six plots were prepared each having a dimension of 160m$^2$. Cultural and management procedures provided in the Sugar Regulatory Administration Sugarcane Production Manual (SRA-OPSI 2004) were followed. Quality cane points having 3-4 active buds were planted horizontally with buds at both sides. A population density of 40,000 stools ha$^{-1}$ was derived by planting 4 cane points per linear meter. Ridge busting and alternate off-barring and hilling-up were practiced using animal-drawn cultivator. Water, fertilizer and pest management were practiced according to the standard agronomic methodologies of the locality. Sugarcanes were harvested 10 months after planting (February to December) upon attaining physiological maturity, when yellowing of the leaves was uniform, cane stalks turned dark purple and internodes at the terminal portion of the stalks shortened.

**Table 2**. Nutrient analysis of sugar mill wastes used as organic amendments.

| Amendment | % N | % P2O5 | % K2O |
|---|---|---|---|
| Mudpress | 0.55 | 0.22 | 0.22 |
| Bagasse ash | 0.02 | 0.22 | 1.43 |

**Data Collection and Analysis**

Cane yield was obtained from the harvestable area of 108 m$^2$ consisting of the inner 18 furrows and excluding 4 stools on both sides of the furrow. Total soluble solid ($^o$ Brix) determination using a hand refractometer was made to monitor the maturity of cane in the field.

The following indices were computed to measure nutrient use efficiency of applied fertilizer (Mosier et al. 2004):

$$\text{Partial factor productivity (PFP)} = \frac{\text{kg crop yield}}{\text{kg nutrient applied}}$$

$$\text{Agronomic efficiency (AE)} = \frac{\text{kg crop yield increase}}{\text{kg nutrient applied}}$$

$$\text{Apparent recovery efficiency (RE)} = \frac{\text{kg nutrient taken up}}{\text{kg nutrient applied}}$$

$$\text{Physiological efficiency (PE)} = \frac{\text{kg yield increase}}{\text{kg nutrient taken up}}$$

Statistical analysis was performed by the analysis of variance (ANOVA) for randomized complete block design (RCBD) using the Statistical Tools for Agricultural Research (STAR Nebula 2013 version). The significant difference between treatment means was tested by Tukey's Duncan Multiple Range Test (DMRT) at $p \leq 0.05$ when the F-test indicated effects on the significance level of $p \leq 0.05$.

## RESULTS AND DISCUSSION

**Cane yield**

Application of inorganic fertilizer (IF), mudpress (MP), bagasse ash (BA) and microbial inoculant (MI) significantly influenced cane yield (Table 3). The highest cane yield of 120.24 TC ha$^{-1}$ was obtained from plots fertilized with 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI. Comparable cane yields were also recorded from the following treatments in descending order: 50% $RR_N$ IF: 50% $RR_N$ MP + BA + MI, 25% $RR_N$ IF: 75% $RR_N$ MP + BA, 75% $RR_N$ IF: 25% $RR_N$ MP + BA + MI, 25% $RR_N$ IF: 75% $RR_N$ MP, 50% $RR_N$ IF: 50% $RR_N$ MP + BA, 50% $RR_N$ IF: 50% $RR_N$ MP. These treatments with organic amendments and microbial inoculant can be characterized as high tonnage since yields were ≥ 100 TC ha$^{-1}$ (SRA 2014).

**Table 3.** Cane yield of Phil 2004-1011 sugarcane variety applied with inorganic fertilizer, organic amendments and microbial inoculant under acid Typic Hapludand.

| Treatments | Cane Yield (TC $ha^{-1}$) ** |
|---|---|
| T1 - Control (no fertilizer application) | 69.73e |
| T2 - $RR_N$ IF | 93.27d |
| T3 - $RR_N$ IF + lime | 99.39cd |
| T4 - 75% $RR_N$ IF: 25% $RR_N$ MP | 105.08bcd |
| T5 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA | 105.39bcd |
| T6 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA + MI | 114.39ab |
| T7 - 50% $RR_N$ IF: 50% $RR_N$ MP | 107.76abc |
| T8 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA | 110.04abc |
| T9 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA + MI | 117.09ab |
| T10 - 25% $RR_N$ IF: 75% $RR_N$ MP | 110.95abc |
| T11 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA | 116.85ab |
| T12 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI | 120.24a |

***=highly significant; CV=6.61; means having same letter are not significantly different at the 5% level by DMRT*

Similar to the findings of Chatterjee et al. (2014), yield attributing characters were significantly influenced by combined application of inorganic, organic and biological sources of nutrients. Addition of biofertilizer under reduced IFs and higher organic sources showed significant positive results over uninoculated treatments. They attributed this to the production of humic acid and humic substances from organic sources which might have enhanced the soil physical condition. Also, fulvic acids released from organic sources are good source of energy for beneficial soil organisms which will have positive impact on nitrogen mineralization and mobilization. Fulvic acids likewise helped in solubilizing the reserved mineral substances making them available for plant uptake throughout the growth period. Also, organic amendments have likely increased balanced availability of essential nutrients, improved soil physical condition, and with microbial inoculant, have improved soil biological fertility status. In sugarcane production, increasing mudpress application resulted to increased cane yield (Bangar et al. 2000, Tiwari and Nema 1999, Quilloy 1983 as reported in SRA-OPSI 2004).

**Nitrogen use efficiency**

Among the indices of nitrogen use efficiency, treatments significantly affected partial factor productivity (PFP) and agronomic efficiency, but not the apparent recovery efficiency and physiological efficiency (Table 4). The highest crop yield obtained per unit N applied (846.74) was obtained from plots applied 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI**.** This PFP value was found comparable with those recorded from plots amended with MI, plots applied with IF and MP alone, those with 50% $RR_N$ IF: 50% $RR_N$ MP + BA and 25% $RR_N$ IF: 75% $RR_N$ MP + BA. Increasing amount of MP also resulted in improving PFP values, and the trend generally followed the order observed in cane yield data.

Partial factor productivity of applied N increased gradually with the application of higher amount of organic materials (Chatterjee et al. 2014). This indicated that application of higher amount of organic amendment can efficiently transform the applied nitrogen to economic yield. They likewise found that inoculation of biofertilizer showed marked effect on PFP.

**Table 4.** Efficiencies of applied fertilizer nitrogen in sugarcane production under acid Typic Hapludand.

| Treatments | Partial Factor Productivity ($PFP_N$) * | Agronomic Efficiency ($AE_N$) ** | Apparent Recovery Efficiency ($ARE_N$) ns | Physiological Efficiency ($PFP_N$) ns |
|---|---|---|---|---|
| T1 - Control (no fertilizer application) | - | - | - | - |
| T2 - $RR_N$ IF | 666.19d | 168.14d | 8.86 | 18.77 |
| T3 - $RR_N$ IF + lime | 709.93cd | 211.88cd | 9.59 | 22.52 |
| T4 - 75% $RR_N$ IF: 25% $RR_N$ MP | 750.60abcd | 252.55bcd | 9.55 | 26.52 |
| T5 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA | 742.21bcd | 251.17bcd | 9.90 | 27.52 |
| T6 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA + MI | 805.54abc | 314.51ab | 9.05 | 36.01 |
| T7 - 50% $RR_N$ IF: 50% $RR_N$ MP | 769.74abc | 271.69abc | 9.22 | 30.13 |
| T8 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA | 774.93abc | 283.90abc | 10.76 | 27.14 |
| T9 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA + MI | 824.58ab | 333.54ab | 9.61 | 36.92 |
| T10 - 25% $RR_N$ IF: 75% $RR_N$ MP | 792.52abc | 294.48abc | 9.04 | 33.15 |
| T11 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA | 822.91ab | 331.88ab | 11.20 | 29.32 |
| T12 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI | 846.74a | 355.70a | 10.29 | 37.25 |
| *Mean* | **773.26** | **279.04** | **9.73** | **29.57** |

** = significant; CV =6.57; means with the same letter suffix are not significantly different at the 5% level by DMRT*

The highest increase in crop yield obtained per unit of N applied, i.e. agronomic efficiency (AE) of 355.70 was likewise obtained from plots applied with 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI. Similarly higher AE was obtained from plots applied with MI and those plots receiving 50%:50% and 25%:75% combinations of IF and MP, with and without the addition of BA (Table 4). Agronomic efficiency varied remarkably with the source of nutrients and showed an increasing trend with increased level of organic materials (Chatterjee et al. 2014). Improvement in agronomic efficiency using maximum amount of organic material with biofertilizer inoculation could be due to optimum availability of N as per crop demand and reduced N loss leading to efficient uptake and utilization of applied N (Singh et al. 2008).

Apparent recovery efficiency of applied N ($ARE_N$), which is the ratio of nitrogen uptake with nitrogen applied, was not significantly different among treatments. $ARE_N$ values ranged from 8.86 obtained from plots applied with $RR_N$ IF to 11.20 from plots applied with 25% $RR_N$ IF: 75% $RR_N$ MP + BA. Response to applied N is higher for the ratoon crop than for planted cane, with a mean $ARE_N$ of 30 percent on farms and 40 percent for research plots (Basanta et al. 2003 as cited by Balasubramanian et al., 2004). They further conveyed an $ARE_N$ for planted cane to vary from 0 to 40 percent. Apparent recovery followed an increasing trend with increased level of N application through organic materials (Chatterjee et al. 2014). They found that apparent recovery is the expression of N uptake by the fertilized plants rather than the amount of N applied. Since not all of the amount of N applied is taken up by crops due to different venues of nutrient loss, the apparent recovery thus reflects the fraction that was actually taken-up and utilized by the fertilized plants.

In a similar manner, physiological efficiency (PE) values was comparable among treatments, which implies that differences in N uptake among treatments did not cause significant variation in increasing crop yield. PE values ranged from 18.77 ($RR_N$ IF) to 37.25 (25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI). In contrast to this finding, superior value of PE under higher organic material combination could be the result of higher yield and better conversion of source to sink (Chatterjee et al. 2014).

**Phosphorus use efficiency**

Treatment effects were noted on the partial factor productivity, apparent recovery efficiency and physiological efficiency of applied P, but not on the agronomic efficiency (Table 5). The highest partial factor productivity (2151.30) of applied P was derived from plots with $RR_N$ IF + lime. This is attributed to the ability of lime in neutralizing soil acidity which in turn improved P availability (Baligar et al. 2001). This PFP value was found comparable with those obtained from plots applied with $RR_N$ IF and plots fertilized with 75% $RR_N$ IF: 25% $RR_N$ MP. On the other hand, agronomic efficiency values ranged from 509.52 to 719.86 which shows that the magnitude of increase in crop yield per unit of P applied did not differ markedly. The quantity of P taken up per unit of P applied, i.e. ARE, varied with treatments where the application of $RR_N$ IF + lime recorded the highest ARE of 2.92. This was followed by the following treatments in descending order of ARE: $RR_N$ IF (2.65), 75% $RR_N$ IF: 25% $RR_N$ MP (2.60), 75% $RR_N$ IF: 25% $RR_N$ MP + BA + MI (2.06).

In terms of physiological efficiency of applied P, the highest magnitude of crop yield increase per unit of P taken up was obtained from plots applied with 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI which is 391.94.. Likewise, treatments comprising of IF + MP + BA + MI, IF + MP + BA, and IF + MP (except the 75%:25% combination) had comparably higher PE. These treatments were able to efficiently convert the nutrient taken up into biological and economic yield. The recovery of applied fertilizer P ranges from less than 10 % to as high as 30 % in the initial year of application (Ghosh et al. 2015). However, because fertilizer P is considered immobile in the soil and reaction (fixation and/or precipitation) with other soil minerals is relatively slow, long-term recovery of P by subsequent crops can be much higher.

**Table 5.** Efficiencies of applied fertilizer phosphorus in sugarcane production under acid Typic Hapludand.

| TREATMENTS | Partial factor productivity ($PFP_P$) ** | Agronomic efficiency ($AE_P$) ns | Apparent recovery efficiency ($ARE_P$) * | Physiological efficiency ($PFP_P$) * |
|---|---|---|---|---|
| T1 - Control (no fertilizer application) | - | - | - | - |
| T2 - $RR_N$ IF | 2018.76ab | 509.52 | 2.65ab | 189.59d |
| T3 - $RR_N$ IF + lime | 2151.30a | 642.06 | 2.92a | 218.32cd |
| T4 - 75% $RR_N$ IF: 25% $RR_N$ MP | 2006.94ab | 675.26 | 2.60ab | 257.21bcd |
| T5 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA | 1698.80cd | 574.90 | 2.06bc | 279.11abcd |
| T6 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA + MI | 1843.76bc | 719.86 | 2.06bc | 353.63ab |
| T7 - 50% $RR_N$ IF: 50% $RR_N$ MP | 1841.48bc | 649.98 | 2.17bc | 300.87abcd |
| T8 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA | 1613.49d | 591.10 | 2.16bc | 282.26abcd |
| T9 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA + MI | 1716.86cd | 694.48 | 1.92bc | 375.95ab |
| T10 - 25% $RR_N$ IF: 75% $RR_N$ MP | 1715.42cd | 637.39 | 1.99bc | 321.71abc |
| T11 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA | 1571.45d | 633.76 | 2.34abc | 278.47abcd |
| T12 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI | 1616.95d | 679.26 | 1.77c | 391.94a |
| *Mean* | **1799.56** | **637.05** | **2.24** | **295.37** |

** = significant; CV =6.57; means with the same letter suffix are not significantly different at the 5% level by DMRT*

**Potassium use efficiency**

Partial factor productivity and physiological efficiency of applied K were affected by treatments, while comparable results were manifested in agronomic efficiency and apparent recovery efficiency (Table 6). The most efficient treatments in terms of producing crop yield per unit of K applied were those involving IF and MP regardless of proportion. The PFP values obtained from plots applied with $RR_N$ IF and $RR_N$ IF + lime also had higher PFP values. Increase in crop yield per unit of K applied, i.e. agronomic efficiency was not affected by treatments. On the average, a kilogram of K applied will cause a yield increase by 76.16 kg. In terms of apparent recovery efficiency, values ranged from 1.72 to 2.53 which indicates higher K recovered in plant tissue than what was applied through fertilizers and amendments. The soil had considerable level of indigenous K which were taken up by the plant in conjunction with K from external inputs. There was a significant increase in crop yield per unit of K taken up which is revealed in varying PE values. The different treatments affected the rate at which sugarcane converted the absorbed K into dry matter and cane yield. The highest PE value of 50.37 was obtained from plants fertilized with 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI. This would mean that a kilogram of K taken up would cause a kilogram increase in crop yield by 50 times. Statistically similar PE values were obtained from plants amended with MP, MP + BA, and MP + BA + MI. First-year recovery of applied K can range from 20 to 60 % (Ghosh et al. 2015). Potassium is generally considered to have a higher use efficiency than N and P because it is immobile in most soils and is not subject to the gaseous losses that N is or the fixation reactions that affect P.

**Table 6.** Efficiencies of potassium fertilizer in sugarcane production under acid Typic Hapludand.

| Treatments | Partial Factor Productivity ($PFP_K$) ** | Agronomic Efficiency ($AE_K$) *ns* | Apparent Recovery Efficiency ($ARE_K$) *ns* | Physiological Efficiency ($PFP_K$) * |
|---|---|---|---|---|
| T1 - Control (no fertilizer application) | - | - | - | - |
| T2 - $RR_N$ IF | 216.10abc | 54.54 | 2.18 | 24.69c |
| T3 - $RR_N$ IF + lime | 230.28ab | 68.73 | 2.53 | 26.86bc |
| T4 - 75% $RR_N$ IF: 25% $RR_N$ MP | 237.09a | 79.77 | 2.23 | 35.93abc |
| T5 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA | 187.56d | 63.47 | 1.84 | 34.76abc |
| T6 - 75% $RR_N$ IF: 25% $RR_N$ MP + BA + MI | 203.57cd | 79.48 | 1.89 | 42.22ab |
| T7 - 50% $RR_N$ IF: 50% $RR_N$ MP | 236.93a | 83.63 | 2.16 | 38.77abc |
| T8 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA | 191.86cd | 70.29 | 2.02 | 36.26abc |
| T9 - 50% $RR_N$ IF: 50% $RR_N$ MP + BA + MI | 204.16cd | 82.58 | 1.75 | 48.23a |
| T10 - 25% $RR_N$ IF: 75% $RR_N$ MP | 237.86a | 88.38 | 2.13 | 43.64a |
| T11 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA | 199.70cd | 80.54 | 2.07 | 39.79abc |
| T12 - 25% $RR_N$ IF: 75% $RR_N$ MP + BA + MI | 205.48bcd | 86.32 | 1.72 | 50.37a |
| *Mean* | **213.69** | **76.16** | **2.05** | **38.32** |

** = significant; CV =6.57; means with the same letter suffix are not significantly different at the 5% level by DMRT*

## CONCLUSION

The findings demonstrated the potential of combined inorganic and organic sources in improving the N and K use efficiency of sugarcane. Integrated nutrient management where organic and

inorganic fertilizers are used simultaneously as a source of diversifying N recorded improved nitrogen use efficiency. Application of lime resulted to better partial factor productivity and apparent recovery efficiency of applied P. Combined use of inorganic fertilizer and mudpress enhanced partial factor productivity of applied K, while addition of bagasse ash and microbial inoculant increased physiological efficiency of applied K. Integrated nutrient management through the combined use of inorganic, organic and biofertilizers increased cane yield and improved N and K use efficiency. Thus, this practice is recommended for sustainable and efficient sugarcane production.

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# SEGREGATION PATTERN OF RESISTANCE TO SOYBEAN MOSAIC VIRUS ON TANGGAMUS X TAICHUNG CROSSED POPULATIONAT $F_{2:3}$ GENERATION

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## ABSTRACT

Segregation is one of the genetic parameters used to determine the proportion of progenies of a particular phenotype The research aimed to determine the distribution pattern of gene frequency of resistance to a disease caused by *Soybean Mosaic Virus* on the $F_{2.3}$generation of Tanggamus X Taichung crossed progenies. In addition to the segregation of characters resistant to the disease, the research also generated patterns of segregation for other characters namely, plant height, pod number per plant , 100 seed weight per plant and total seed weight per plant. The research was conducted at the Integrated Field Laboratory and the Laboratory of Seeds and Plant Breeding, University of Lampung, Bandar Lampung, Indonesia from June to September 2014. Conformity to a normal distribution and analysis of segregation pattern were tested using a Chi-squared test at $\alpha_{0.01}$. The results showed that resistance to Soybean mosaic virus did not spread following a normal distribution, indicating that the characters were semi-dominant.

**Key words**: soybean breeding, soybean mosaic virus, segregation patterns

## INTRODUCTION

Low productivity in soybean (*Glycine max* [L.] Merrill) is caused by several factors including disease, such as mosaic due to *Soybean Mosaic Virus* (SMV). The disease was considered extremely important in many countries (Hill 1999), with SMV infection during vegetative phase resulting in yield loss of up to 25 %, whereas infection during the beginning of growth resulting in a yield loss reaching 90 % (Prayogo 2012). An effective way of preventing yield loss due to oybean mosaic disease is by using resistant varieties. To develop soybean varieties resistant to SMV, while maintaining high yield is by crossing two varieties having those complementary desirable characters. According Sa'diyah et al. (2016), the $F_1$ generation of a cross between Yellow Bean X Tanggamus, Tanggamus X Orba, and Tanggamus X Taichung were resistant to SMV. When the $F_2$ generation of the Tanggamus X Taichung cross was retested for SMV resistance, the progenies continued to show resistance to SMV, and the weight of seeds per plant exceeded that of the two parents (Aslichah et al. 2014, Wanda et al. 2015).

SMV resistance was controlled by a single dominant gene (Buss et al. 1985, Shi et al. 2008), or a single intermediate dominant gene (Shi et al. 2008). According to Shi et al. (2008) resistance properties were controlled by a single dominant gene for the $G_1$SMV, while a single intermediate

dominant gene controlled for $G_7$ SMV. Therefore, it was necessary to study the segregation pattern of resistance to soybean mosaic disease in the $F_{2:3}$ generation of Tanggamus X Taichung.

The research sought to determine the distribution pattern of gene frequency of SMV resistance at the $F_{2:3}$ generation of Tanggamus X Taichung and, evaluate the segregation on plant height, pod number per plant, 100 seed weight per plant and seed weight per plant.

## MATERIALS AND METHODS

The research was conducted at the Integrated Field Laboratory and the Laboratory of Seeds and Plant Breeding at the University of Lampung, Bandar Lampung, Indonesia from June to September 2014 using a non-repetitive experimental design. Soybean $F_{2.3}$ seeds were developed through a non-reciprocal diallele mating, which crossed 10 parental lines (Barmawi et al. 2012). The $F_1$ generation was tested against SMV in 2013 (Sa'diyah et al. 2016) and the $F_2$ generation in 2015 (Wanda et al. 2014). The inoculum of SMV was isolated frm a natural source. The inoculum was augmented by infecting it on Tanggamus susceptible to SMV. The inoculum was prepared by crushing 5 mg of naturally infected leaves in 50 ml phosphate buffer solution at pH 7. Phosphate buffer was made of two solutions, Solutions A and B. Solution A consisted of 1.36 g $KH_2PO_4$ and solution B was 1.78 g $Na_2HPO_4$. $2H_2O$ dissolved in 1 L distilled water. Phosphate buffer was made by mixing 510 ml of solution A with 490 ml solution B.

The inoculum (sap extracted from the infected leaves) was used to infect sample plants. Virus inoculation was performed on soybean plants that already had fully open leaves at 7 – 10 days after planting (DAP). The leaves were sprinkled with zeolite to inflict abrasion and were sprayed with SMV suspension. After inoculation, the leaves were washed with distilled water.

Data was analysed through chi-square test for their normal distribution and segregation to test for conformation between observed and expected values (Gomez and Gomez 1995). Observations were made on each soybean plant involving variables: (1) disease severity was calculated at 42 DAP on 10 leaves of the test plants following the protocol of Campbell and Madden (Mulia 2008); (2) plant height; (3) pod number per plant ; (4) 100 seed weight per plant ; and (5) total seed weight plant.

The severity of the disease (%) was evaluated as

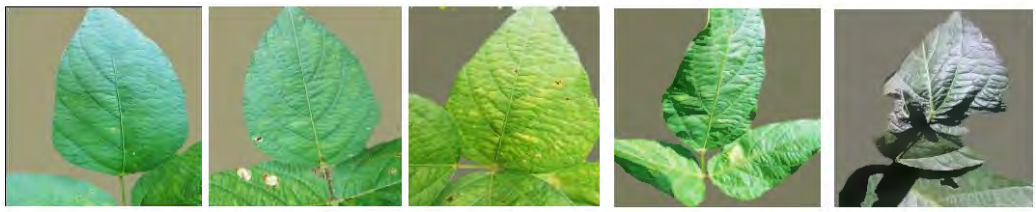
$$DS = \frac{\sum(nxv)}{NxZ} \text{ x } 100\%$$

where DS = Disease severity
N = Number of sample plants
Z = Highest score
n = Number of sample plants per infection category
V = Score for infection category

Figure 1 presented pictures of infected leaf and scoring method in categorising the severity of the disease (Akin and Barmawi 2005).

Gene inheritance, which controlled the characters having a fitted ratio between the observed and the expected values, was considered as the number of genes which controlled the characters. When the controlling genes were simple, the $F_{2:3}$ population would match some ratios in the form of graphs as follows: (1) if with two peaks, the likelihood ratios of phenotypic segregation would be 3: 1, 9: 7, 13: 3, and 15: 1; (2) if with three peaks, the likelihood ratios would be 1: 2: 1, 9: 3: 4, 9: 6: 1, and 12: 3: 1; (3) if with more than three peaks, the likelihood ratios would be either 9: 3: 3: 1 or 6: 3: 3: 4; and (4) if with a unimodal shape, segregation would be polygenic (Snyder and David 1957).

Segregation ratios evaluated in the research was the ratio of the $F_2$ population since the plants came from a number of genotypes of the $F_2$ population. It was assumed that the selected genotypes were segregating heterozygous. Individuals which heterozygous would segregate following the pattern of $F_2$ segregation



(A) (B) (C) (D) (E)

**Fig. 1.** Symptoms on infected leaves and their disease scoring
(A) No symptom= 0; (B) Chlorosis and decoloration on midrib= 1; (C) Mosaic with chlorosis on midrib and leaf surface= 2; (D) Heavy mosaic and chlorosis, leaf bended upward or downward= 3; (E) Leaf malformation= 4.

Resistance category based on Disease Severity (%):(Akin and Barmawi, 2005).
0 – 15 = Highly Tolerant
16 – 25 = Partially Tolerant
26 – 35 = Partially Susceptible
36 – 55 = Susceptible
56 – 100 = Highly Susceptible

## RESULTS AND DISCUSSION

The severity of disease caused by SMV as measured on plant height, 100 seed weight per plant characters did not follow the normal distribution (Table 1; Figure 2-6) indicating that these characters were controlled by one or two major genes. The pattern of segregation followed the Mendelian ratio or its modification (Fehr 1987). The result corroborated with that of Wanda et al. (2014) who indicated that severity of SMV infection was in accordance to the Mendelian ratio or its modification.

**Table 1.** Chi-square test for the fit to the normal distribution.

| No. | Character | $\chi^2$count | $\chi^2$table | The Frequency Distribution |
|---|---|---|---|---|
| 1. | Severity of disease | 54.15 | 12.59 | Abnormal |
| 2. | Plant height | 116.99 | 18.47 | Abnormal |
| 3. | Pod number per plant | 5.11** | 18.47 | Normal |
| 4. | 100 seed weight per plant | 22.22 | 13.28 | Abnormal |
| 5. | Seed weight per plant | 8.31** | 18.47 | Normal |

Note: * = different at $\alpha_{0.01}$

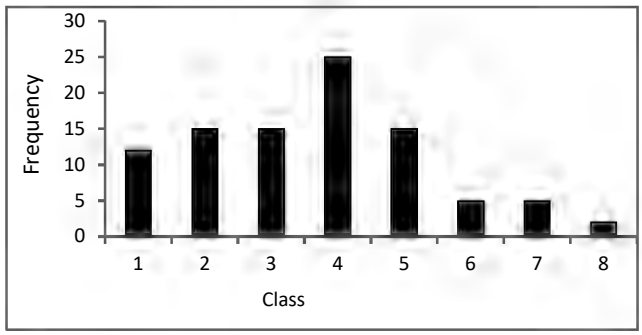



**Fig. 2.** The normal distribution for pod number per plant on Tanggamus X Taichungcrossed population at $F_{2:3}$ generation.

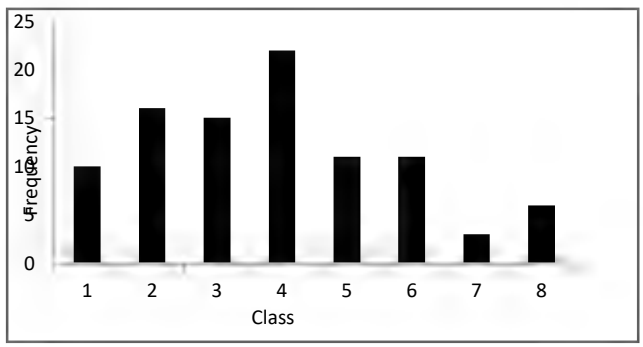



**Fig. 3**. The normal distribution for grain weight per plant on Tanggamus X Taichung crossed population at $F_{2:3}$ generation.

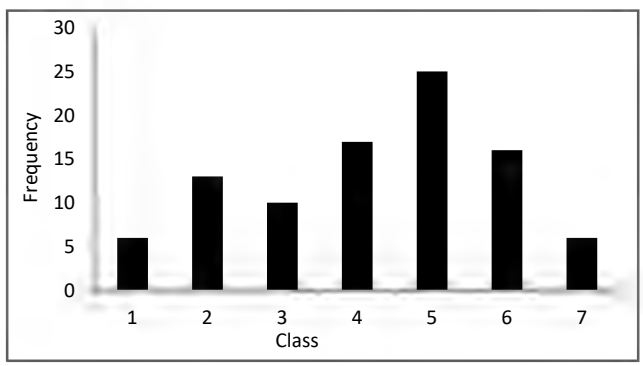



**Fig. 4**. The abnormal distribution with three peaks for severity of disease on Tanggamus X Taichung crossed population at $F_{2:3}$ generation.

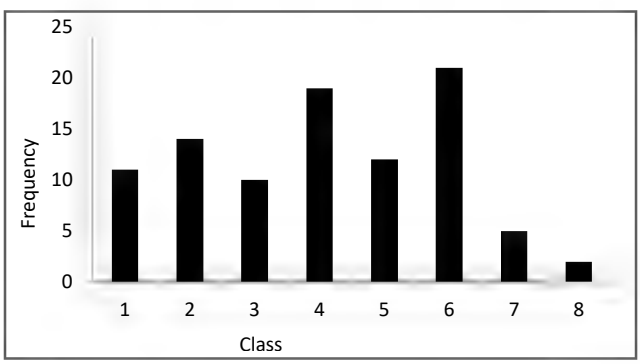



**Fig. 5.** The abnormal distribution with two peaks for plant height on Tanggamus X Taichung crossed population at $F_{2:3}$ generation.

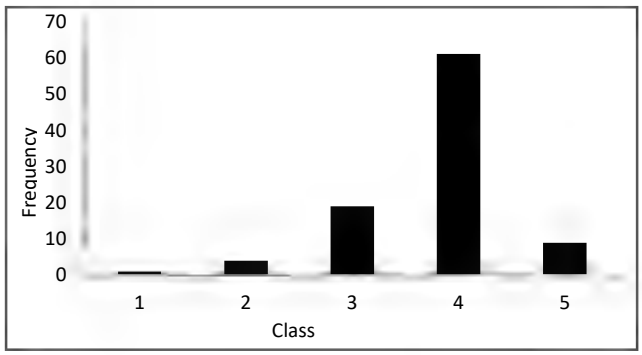



**Fig. 6.** The abnormal distribution with two peaks for 100 seed weight per plant on Tanggamus X Taichung crossed population at $F_{2:3}$ generation.

The segregation ratio of the severity of the SMV infection on the $F_{2:3}$ generation of Tanggamus X Taichung cross was 1: 2: 1 with of 50-75 % of augmentation suggested that the character was controlled by one dominant gene (Synder and David 1957; Table 2). The differences in the disease severity on each plant were observed in the field. Low infection rate observed on the resistant plants might be due to their ability to inhibit viral replication, contain the virus in the infected cells and prohibit the virus to infect other cells or plant tissues (Akin 2006). Agrios (1996), indicated that the infection rate showed by a high severity of the disease might be caused by substances in the cell fluid suitable for the growth and development of the SMV. The infection worsened when the virus replication was supported by environmental conditions which reduced the host capability to impede the growth of the virus.

**Table 2.** Chi-squared segregation ratios for the severity of disease caused by SMV infection on the Tanggamus X Taichung crossed population at $F_{2:3}$ generation.

| **Ratio** | **Observation** | **Expectation** | **$\chi^2$count** | **$\chi^2\alpha_{0.01}$** | **P $\chi^2>\alpha_{0.01}$** |
|---|---|---|---|---|---|
| Two Classes | | | | | |
| 3:1 | 33 : 61 | 70.5 : 23.5 | 78.74** | | <0.005 |
| 9:7 | 33 : 61 | 52.88 : 41.13 | 16.98** | 6.64 | <0.005 |
| 13:3 | 33 : 61 | 76.38 : 17.63 | 129.50** | | <0.005 |
| 15:1 | 33 : 61 | 88.13 : 5.88 | 543.01** | | <0.005 |
| Three Classes | | | | | |
| 1:2:1 | 20:48:26 | 23.5 : 47 : 23.5 | 0.81 | | 0.50-0.75 |
| 9:3:4 | 20:48:26 | 52.88 : 17.63 : 23.5 | 73.05** | 9.21 | <0.005 |
| 9:6:1 | 20:48:26 | 52.88 : 35.25 : 5.88 | 93.99** | | <0.005 |
| 12:3:1 | 20:48:26 | 70.5 : 17.63 : 5.88 | 68.94** | | <0.005 |
| Four Classes | | | | | |
| 9:3:3:1 | 16 : 17 : 42 : 19 | 52.88 : 17.63 : 17.63 : 5.88 | 88.77** | 11.35 | <0.005 |
| 6:3:3:4 | 16 : 17 : 42 : 19 | 35.25 : 17.63 : 17.63 : 23.5 | 45.11** | | <0.005 |

Note: **= different at $\alpha_{0.01}$

There were three selected genotypes that expressed 25% of disease severity and therefore considered as resistant. The third genotype was also selected due to high pod number and seed weight per plant and resistance to SMV infection. The severity of infection was measured from the leaf

damage because the infection decreased the biochemical processes of damaged chloroplasts as well as decreased other photosynthetic pigments such as carotene and xanthophyll (Akin 2006).

In a segregating generation, if the frequency of gene which controlled a particular phenotype segregated to a normal distribution, the phenotype would be a quantitative character. On the other hand, if the frequency of the controlling gene did not segregate to a normal distribution, the phenotype would be a qualitative character (Allard 2005). The result of segregation analysis on the Tanggamus X Taichung cross at $F_{2:3}$ generation progeny fitted the frequency of normal distribution for pod number per plant and seed weight per plant thus, these characters were quantitatively controlled by many minor genes having small influence of each (Crowder 1997). The expression and extent of a quantitative character were dependent on environmental factors (Baihaki 2000). The result was in accordance with the results of Sriwidarti (2010) and Wulandari (2013) which concluded that pod number and seed weight per plant on soybean and peanut followed a normal distribution with a peak and therefore were controlled by many genes.

The segregation ratio of plant height was 3: 1 indicating that the character was controlled by a dominant gene having 25 – 50 % of augmentation (Table 3). The segregation ratio of 100-seed weight per plant was 13: 3 indicating for a dominant gene with only 10 – 25 % of augmentation (Table 4). Therefore, the 100 seed weight per plant character was regulated by two genes that interacted as dominant-recessive epistasis; dominant gene at one locus and the recessive gene on another locus effecting the appearance of the same phenotype (Crowder 1997).

**Table 3.** Chi-squared segregation ratios for plant height on the Tanggamus X Taichung crossed population at $F_{2:3}$ generation

| **Ratio** | **Observation** | **Expectation** | **$\chi^2$count** | **$\chi^2\alpha_{0.01}$** | **P $\chi^2>\alpha_{0.01}$** |
|---|---|---|---|---|---|
| Two Classes | | | | | |
| 3:1 | 67 : 27 | 70.5 : 23.5 | 0.60 | | 0.25-0.50 |
| 9:7 | 67 : 27 | 52.88 : 41.12 | 8.71** | 6.64 | <0.005 |
| 13:3 | 67 : 27 | 76.38 : 17.63 | 5.75 | | 0.01-0.05 |
| 15:1 | 67 : 27 | 88.12 : 5.88 | 77.71** | | <0.005 |
| Three Classes | | | | | |
| 1:2:1 | 58 : 34 : 2 | 23.5 : 47 : 23.5 | 73.91** | | <0.005 |
| 9:3:4 | 58 : 34 : 2 | 52.88 : 17.62 : 23.5 | 35.38** | 9.21 | <0.005 |
| 9:6:1 | 58 : 34 : 2 | 52.88 : 35.25 : 5.88 | 3.10 | | 0.10-0.25 |
| 12:3:1 | 58 : 34 : 2 | 70.5 : 17.63 : 5.88 | 19.99** | | <0.005 |
| Four Classes | | | | | |
| 9 : 3 : 3 : 1 | 58 : 30 : 5 : 1 | 5.88 : 17.63 : 17.63 : 52.88 | 22.27** | 11.35 | <0.005 |
| 6 : 3 : 3 : 4 | 58 : 30 : 5 : 1 | 35.25 : 17.63 : 17.63 : 23.5 | 53.96** | | <0.005 |

Note: **= different at $\alpha_{0.01}$

Based on the results of the study the selection for the pod number and seed weight per plant could not be done in early generations. The characters were controlled quantitatively by many minor genes, or polygenic, where each gene contributes small to the appearance or expression of a certain quantitative character in an additive fashion (Baihaki 2000). Similarly, 100 seed per plant character was not effective to be used on early generation selection since the character was controlled by a dominant-recessive gene epistasis. The results also showed that the gene action in controlling disease severity included a dominant gene action over positive and negative, as well as the most dominant positive and negative (Sa'diyah et al. 2016). Therefore, the selection for resistant characters would only be effective when done on further generations.

**Table 4.** Chi-squared segregation ratios for the 100 seed weight per plant on the Tanggamus X Taichung crossed population at the $F_{2:3}$ generation

| Ratio | Observation | Expectation | $\chi^2$count | $\chi^2\alpha_{0.01}$ | P $\chi^2>\alpha_{0.01}$ |
|---|---|---|---|---|---|
| Two Classes | | | | | |
| 3:1 | 81 : 31 | 70.5 : 23.5 | 6.56 | | 0.05-0.01 |
| 9:7 | 81 : 31 | 52.87 : 41.12 | 34.36** | 6.64 | <0.005 |
| 13:3 | 81 : 31 | 76.38 : 17.63 | 1.71 | | 0.25-0.10 |
| 15:1 | 81 : 31 | 88.12 : 5.87 | 8.13** | | <0.005 |
| Three Classes | | | | | |
| 1:2:1 | 58 : 34 : 2 | 23.5 : 47 : 23.5 | 73.91** | | <0.005 |
| 9:3:4 | 58 : 34 : 2 | 52.87 : 17.63 : 23.5 | 35.38** | 9.21 | <0.005 |
| 9:6:1 | 58 : 34 : 2 | 52.87 : 35.25 : 5.8 | 3.10 | | 0.25-0.10 |
| 12:3:1 | 58 : 34 : 2 | 70.5 : 17.63 : 5.87 | 19.99** | | <0.005 |
| Four Classes | | | | | |
| 9 : 3 : 3 : 1 | 58 : 05 : 30 : 1 | 52.87 : 17.63 :17.63 : 5.87 | 531.10** | 11.35 | <0.005 |
| 6 : 3 : 3 : 4 | 58 : 05 : 30 : 1 | 35.25 : 17.63 : 17.63 : 23.5 | 53.96** | | <0.005 |

Note: **= different at $\alpha_{0.01}$

The segregation of characters resistant to SMV evaluated on the $F_{2:3}$ generation of Tanggamus X Taichung progenies was expected to facilitate for the selection method in the next generation. The results of the study indicated that the selection would not be effective in early generation $F_{2:3}$ because the heterozygosity level was high. Selection would be more effective when done in later generations when the level of heterozygosity was greatly reduced to increase homozygosity to a higher level. The study showed three $F_{2:3}$ genotypes which retained the resistance to the SMV infection as well as yielded greater than both parents, Tanggamus and Taichung (Table 5).

**Table 5**. The genotype selection based on of pod number per plant, seed weight plant plant and disease severity characters as expected from Tanggamus X Taichung crossed population at $F_{2:3}$ generation

| No. | Genotype No. | Pod Number per plant | Seed Weight per plant (g) | Disease Severity (%) | Class Criteria of Disease Severity |
|---|---|---|---|---|---|
| 1 | 6.6.22 | 126 | 21.28 | 25.00 | Tolerant |
| 2 | 6.6.24 | 136 | 31.03 | 25.00 | Tolerant |
| 3 | 6.6.65 | 86 | 16.05 | 25.00 | Tolerant |
| Average $F_{2:3}$ | | 77 | 13.40 | 32.81 | Partial Tolerant |
| Average $F_{2:3}$ Selected | | 116 | 22.79 | 25.00 | Tolerant |
| Average Tanggamus | | 42.17 | 5.55 | 28.27 | Partial Tolerant |
| Average Taichung | | 69.67 | 13.76 | 33.61 | Partial Tolerant |

Note: Class criteria of Disease Severity were Highly Tolerant (0 – 15 %); Tolerant (16 – 25 %); Partial Tolerant (26 – 35 %); Partial Susceptible (36 – 55%); Susceptible (56 – 75 %); and Highly Susceptible (76 – 100 %), respectively.

## CONCLUSIONS

The segregation patterns of soybean resistance to SMV infections of the progenies of Tanggamus X Taichung $F_2$:$_3$ generation were in accordance to the Mendelian ratio or its modification of 1: 2: 1, plant height of 3: 1, and 100 seed weight per plant of 13: 3. There were three genotypes resistant to SMV infection and yielded greater than the both parent, Tanggamus and Taichung. The $F_{2:3}$ generation of Tanggamus X Taichung crossed progenies showed pod number per plant and seed weight per plant characters distributed normally. The resistance to SMV infections segregated following Mendelian modification of 1: 2: 1 indicating that the resistance was controlled by a partially dominant single gene. Therefore, it would not be complicated to develop soybean lines resistant to SMV.

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# COMBINED EFFECTS OF SALINITY, RICE VARIETY AND RICE GROWTH STAGE ON THE DIVERSITY OF BACTERIAL COMMUNITIES ASSOCIATED WITH RICE (*Oryza sativa L.*)

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## ABSTRACT

The combined effects of salinity, rice variety, and rice growth stage on the diversity of bacterial communities associated with rice were analyzed using a molecular approach. Samples were taken from a field experiment that was set-up from January to May 2011 at the International Rice Research Institute (IRRI). Culture-independent isolation of total rhizosphere DNA was performed using a commercially available soil DNA extraction kit while the modified CTAB method was used to isolate the cultivable microbial community DNA from culture enrichments from bulk soil, rhizosphere, and sterilized root samples. The V6 to V8 region of the bacterial 16s rRNA gene was amplified through Polymerase Chain Reaction (PCR) and the amplicons were separated using Denaturing Gradient Gel Electrophoresis (DGGE). Rhizosphere bacterial diversity, measured by the Shannon index, was significantly higher under saline conditions compared to normal conditions. Diversity at the reproductive stage was also significantly higher than the vegetative stage. Combined DGGE profile and sequence analysis of the cultivable bacterial community revealed that salinity has a strong influence on the bacterial diversity in the bulk soil. The influence of crop growth stage on the bacterial community was more evident in the rhizosphere while the effect of salinity on specific microbe-plant interactions were observed in surface-sterilized roots. Salt-tolerant varieties were able to maintain association with bacteria that are reported to have plant-growth promoting properties even under saline conditions.

Key words: DGGE, bacterial diversity, salt tolerant variety

## INTRODUCTION

Soil salinity is the second most widespread soil problem in rice growing countries after drought and is considered as a serious constraint to increased rice production worldwide (Gregorio et al. 1997). Millions of hectares in the humid regions of South and Southeast Asia are technically suited for rice production but are left uncultivated or are grown with very low yields because of salinity and abiotic stress (Gregorio et al. 2002). Saline-prone areas in the Philippines are small compared with other countries in the South and Southeast Asia, but are still a potential and important resource base for the production of rice and other related staple food. Although other crops are more tolerant to salinity, for most farmers, there is often no alternative to growing rice in these regions because it is the only crop that can tolerate flooding during the wet season (Yeo et al. 1990; Flores, 2004).

To combat problems of salinity, plant breeders in the International Rice Research Institute (IRRI) developed rice varieties that can tolerate salinity. Aside from this, studies were also performed to reveal the underlying mechanisms of salt-tolerance. One view point that has not been looked into closely is the fact that plants have the ability to interact with soil microorganisms, some of which may have the ability to provide the plant with beneficial substances that enhance growth and tolerance to stresses. The relationship between plants and soil microbial community is very complex and occurs in many ways, resulting in beneficial effects such as stimulation of plant hormones synthesis and enhancement of biological nitrogen fixation and phosphate solubilization, or in negative effects like occurrence of diseases (Ferreira et al. 2008). The rice rhizosphere represents the soil area under the direct influence of roots and serves as a favorable aerobic-anaerobic interface suitable for diverse groups of microorganisms. Plants are capable of increasing soil microbial population through root exudates, which are used as nutrient source for their growth (Sung et al. 2006). Plant rhizodeposition in the rhizosphere results in increased microbial population size and community structures distinct from that in bulk soil (Bais et al. 2006). There is a need to understand the microbial community that is associated with the rice root and its response to salinity. Salinity is the major environmental determinant of microbial community composition rather than extremes of temperature, pH, or other physical and chemical factors (Lozupone and Knight 2007). Unfortunately, very few researches worked on the combined effects of salinity, plant type or growth stage on the microbial community associated with the rhizosphere of salt-tolerant and susceptible rice varieties. Molecular biology analysis of complex microbial communities can be done through genetic fingerprinting. The most common technique is the use of denaturing gradient gel electrophoresis (DGGE) which separates PCR-generated DNA products based on sequence differences (Nakatsu 2007). Changes in the rice rhizosphere microbial communities can be traced using PCR-DGGE (Organo et al. 2013) making it a very useful tool in comparing samples from different environments.

This paper analyzes the effects of salinity, rice variety, and growth stages on the diversity of soil bacterial communities associated with rice (*Oryza sativa L.*) using the PCR-DGGE fingerprinting technique as well as determines which factor has the major contribution in shaping the bacterial community in the rhizosphere. This information will be useful in determining if soil microorganisms can definitely contribute to the resistance and productivity of crops grown on salt-affected soils. This can also serve as a guide in the selection and utilization of beneficial soil microorganisms that can be applied in such conditions.

## MATERIALS AND METHODS

### Field experiment

A field experiment using four rice varieties (IR29, PSB Rc 82, FL478, and Salinas1) with various responses to salinity was set-up for this study. IR29 is an improved *indica* cultivar that is used as a salt-sensitive standard while PSB Rc 82, a popular variety among farmers, is categorized as moderately tolerant to salt stress. FL478, a recombinant inbred line derived from a population developed for salinity tolerance studies, has high tolerance to salinity stress, particularly at the vegetative stage of growth (Gregorio et al. 1997). Salinas1 is a salt-tolerant rice variety released by IRRI in the Philippines in 2010. The rice plants were transplanted in concrete plots that are specifically designed for experiments on salinity tolerance. All plots were initially flooded with normal irrigation water prior to transplanting. Three plots were salinized using sea water with three plots remaining as control at two weeks after transplanting. Electrical conductivity (EC) was initially maintained at 6 to 7 dS $m^{-1}$ during the vegetative stage and was raised to 9 dS $m^{-1}$ during the reproductive stage. The experiment was laid-out in Split Plot Arrangement and Randomized Complete Block Design (RCBD) with salinity level as the main plot and rice variety as the subplot. Thirty-six rice seedlings were transplanted for each treatment.

**Sample collection**

Three replicated bulk soil and rice plant samples (with intact roots) were randomly collected for each variety on each plot during the vegetative (30 days after transplanting, DAT) and reproductive stage (50 DAT) of rice. Sterile metal core samplers, measuring approximately 10 cm in height, were used to collect bulk soil samples from the plots. Rhizosphere and plant root samples, on the other hand, were gathered from randomly selected rice plants. The plants were uprooted using a spade to ensure that the root system was still intact. Bulk soil and plant samples were placed in sterile polypropylene bags and sealed in a container with ice while being transported to the laboratory.

***Bulk soil samples*.** The contents of the core samplers were transferred to a sterile polypropylene bag and mixed thoroughly. Once mixed, a five-gram portion was placed in 45 mL sterile saline solution (NaCl 0.85%) to create a $10^{-1}$ dilution.

***Plant samples*.** Separation of the rhizosphere from the plant roots was performed as described previously (Organo et al. 2013). The obtained root samples were gently washed with sterile distilled water to remove the remaining clay particles. It was then cut to approximately 1.5 inches, enough to fit a 2 mL collection tube. Four to five root slices were placed in 2 mL microcentrifuge tubes. Root-surface sterilization was performed by immersing the roots in 70% ethanol for 3 min, washing with fresh sodium hypochlorite solution (2.5% available $Cl^{-}$) for 5 min, rinsing with 70% ethanol for 30 seconds, and finally, washing five times with sterile distilled water. To confirm root surface sterility, 100 µL of the sterile distilled water used in the last rinse was set on Tryptic Soy Agar (TSA) plates and the plates were incubated at room temperature for 2 days. Rice root samples that were not contaminated were used as source for culture enrichments for endophytic microorganisms. The rhizosphere sample was transferred to sterile 50 mL tubes and served as the source of samples for direct DNA extraction and culture enrichments from the rhizosphere.

**Preparation for direct soil DNA extraction and culture enrichments**

To obtain samples for direct rhizosphere DNA extraction, 2 mL of the rhizosphere suspension was transferred to a collection tube and centrifuged at 10,000 x g for 1 minute. The supernatant was removed, and the soil pellet was washed with TE Buffer then stored at -20 °C prior to DNA Extraction.

To prepare culture enrichments from the bulk soil and rhizosphere suspensions, serial dilutions of up to $10^{-6}$ were prepared using sterile water as diluent. One hundred µL samples from dilutions $10^{-4}$ to $10^{-6}$ were inoculated in 10 mL vials of Nutrient Broth (NB) and Tryptic Soy Broth (TSB). To prepare endophytic culture enrichments, two tubes containing surface-sterilized roots were used. The roots were macerated while inside the tubes using a flame-sterilized rod. One mL of sterile distilled water was mixed to the macerated samples and 100 µL of the suspension were inoculated in 10 mL vials of NB and TSB. All the inoculated vials were placed in a rotary shaker for 5 days at ambient temperature. After 5 days, 2 mL of cultures were transferred to a collection tube and centrifuged at 10,000 x g for 1 minute. The supernatant was removed, and the cell pellet was washed with TE buffer then stored at -20 °C prior to DNA extraction.

**DNA extraction**

Total rhizosphere DNA was extracted as previously described (Organo et al. 2013) using a DNA Isolation Kit (MoBio Ultraclean™) following the manufacturer's instructions with some modifications. The modified CTAB method was used to extract DNA from the pellets obtained from culture enrichments (William et al. 2004). The quality and quantity of extracted DNA was assessed by both agarose gel electrophoresis and spectrophotometric analysis using Nanodrop ®. Samples with poor quality DNA extracts were not used as template for PCR.

**PCR Amplification**

A 17-mer forward primer, designated 968f (5'AA CGC GAA GAA CCT TAC 3'), to which a 40-mer GC clamp (5'-CGCCCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G 3') was attached at the 5' end, was paired with 1378r (5'GCG TGT GTA CAA GGC CCG GGA ACG 3') to amplify the bacterial 16S rRNA gene fragments (Brons and van Elsas 2008). PCR reaction was performed as previously reported (Organo et al. 2013).

**Denaturing Gradient Gel Electrophoresis**

DGGE was performed using DCode™ (Bio-Rad, Hercules, Calif., USA) universal mutation detection system using 8% polyacrylamide gels with a gradient of 30% to 60% denaturing conditions. Electrophoresis was initially started at 100V for 10 minutes and was then lowered to 60V and allowed to run for 15 hours. The gel was stained with ethidium bromide for 5 minutes then destained with deionized water for 20 minutes. The gel was viewed and photographed using QuantityOne™ 1-D Gel Analysis Software (Bio-Rad).

**DGGE Profile Analysis**.

The indices of diversity and dominance of bacterial populations were calculated using the images of DGGE profiles. To determine the diversity and evenness of the bacterial communities, the Shannon index of diversity (H') and Simpson Index of Dominance (D) was calculated for each of the gel lane using the trace quantities generated by Quantity One™ 1-D Gel Analysis Software.

H' is defined as:

$$H' = -\sum_{i=1}^{S}(p_i \ln p_i)$$

where S is the species richness, P*i* is the importance probability of the bands in the lane. P*i* was calculated using the formula P*i* =n*i*/N, where n*i* is the trace quantity (peak intensity x width of the band) of the *i*th band and N is the total trace quantities of all the detected bands in the lane (Shannon and Weaver 1963).

The obtained Shannon index of diversity (H') for each of the gel lane were subjected to ANOVA and treatment means were compared using Duncan's Multiple Range Test at 5% level of significance. Statistical analysis was performed using the Statistical Analysis Software System®.

**16S rDNA sequence analysis**

Selected bands in the DGGE gel were excised using sterile plastic forceps and were carefully transferred to labelled collection tubes containing 50 µL HPLC water. The excised gel was macerated using a sterile yellow tip, centrifuged for 30 seconds at 5,000 x g, then incubated at 37 °C for 30 minutes. The resulting DNA was stored at -20 °C prior to re-amplification using primers without GC-clamp. The amplified DGGE bacterial DNA fragments were submitted to Macrogen, Inc., Korea for further purification and sequencing using the 1378r primer. The quality of the sequences obtained was assessed using Finch TV Version 1.4.0 (Geospiza Inc.) Chimera check with Decipher was the program used to check for chimeric sequences (http://decipher.cee.wisc.edu/index.html). The obtained sequences were processed and compared to those available in GenBank using the BLASTn tool for the 16s ribosomal DNA database for bacteria and archaea. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura et al. 2011). Multiple sequence alignment was carried out using the ClustalW alignment function of the MEGA software. The phylogenetic tree was constructed based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using Maximum Composite Likelihood as nucleotide substitution model. *Methanobacterium oryzae* was used as the out-group and the tree topology was evaluated by 1,000 replications of bootstrap analysis.

## RESULTS AND DISCUSSION

### Soil chemical analysis

The $EC_{1:5}$ values were measured and were converted to the ECe based on the recommendation of Watling (2007). In addition, sodium adsorption ratio (SAR) was used to assess soil salinity. SAR indicates the extent to which sodium contributes to the total salinity. Soils can be categorized into non-saline, saline, sodic and saline-sodic based on ECe, SAR and pH (Table 1). Soil chemical analysis confirmed that salinity has been maintained in the saline plots (Table 2). The $EC_e$ levels in the saline plots were greater than 4 dS/m. In addition, very high concentrations of $Na^+$ and $Cl^-$ ions in the saline plots were measured. Although high amounts of Na were measured, the saline plots will not be considered as saline-sodic because the SAR values are still below the limit of 13. All the other parameters were not significantly different between the normal and saline plots.

A few days after salinization, symptoms of salt stress were already seen on IR29 and PSB Rc82 plants in the salinized plots. The plants are smaller compared to those planted in normal plots and the tips of the leaves initially turned white, and this later on progressed as tip burns. While the first two varieties showed serious negative response to salinity at the vegetative stage, FL478 and Salinas 1 remained green and healthy just like the plants under normal conditions. During the reproductive stage, all plants showed symptoms of salt stress such as reduced tillering and spikelet sterility, but it was noted that Salinas 1 performed better than the other varieties during the reproductive stage.

**Table 1.** Classification of salt-affected soils and their distinguishing properties.

| Class | ECe | SAR | pH |
|---|---|---|---|
| **Non-saline** | <4 | <13 | <8.5 |
| **Saline** | >4 | <13 | <8.5 |
| **Sodic** | <4 | >13 | <8.5 |
| **Saline-Sodic** | >4 | >13 | <8.5 |

ECe: Electrical Conductivity (dS/m) of extract of saturated soil paste
SAR: Sodium Adsorption Ratio
pH: pH of saturated soil paste

### Analysis of the DGGE profiles of cultivable bacterial communities

Figure 1 shows the representative DGGE profiles of cultivable bacterial communities from bulk soil, rhizosphere and surface-sterilized roots. Plants are capable of increasing soil microbial population through root exudates, which are used by microorganisms as nutrient source for their growth (Sung et al. 2006). The resulting DGGE profiles of the culture enrichments from this study is in accordance with this statement, as the DGGE profiles from bulk soil clearly differs from that of the rhizosphere. The effect of salinity on the bacterial community is very evident on the banding patterns of bulk soil samples at both vegetative and reproductive stages. Some bands were very intense at normal condition, but were no longer present at saline condition such as the lane represented by bands N10 and N12. On the other hand, some bands are more prominent in samples under saline conditions. Band N7, for example, is very intense during saline condition, but although still visible at normal salinity levels, it is very faint and difficult to recognize.

It can be observed that salinity also plays a role in the bacterial community in the rice rhizosphere as indicated by band N16, which is present in all varieties during normal EC levels, but is no longer observed under saline conditions. N20, a band found under Variety B (PSB Rc82), is very intense during normal EC level, but is very faint under saline condition. Interestingly, some bands are specific to a rice variety and salinity level during the reproductive stage, regardless of the enrichment media. These bands are: N26 and T20, which are all observed only in Variety C (FL478) under saline condition.

The DGGE profiles from the culture enrichment of surface-sterilized roots showed specific bands for each rice variety, especially during saline conditions. These include: N38 and T29 for IR 29, N32 and T30 for PSB Rc82, and N35 and T32 for Salinas 1. This is an indication that each variety may have interaction with particular groups of endophytic bacteria that may have helped illicit their response to salinity. These bands need to be identified to determine if the corresponding bacteria were those that have PGPR properties. Based on the DGGE profiles alone, it can be concluded that salinity plays a great role in the bacterial community in the bulk soil and rice rhizosphere. On the other hand, the bacterial profiles inside the plant roots tend to be highly dependent on the rice variety.

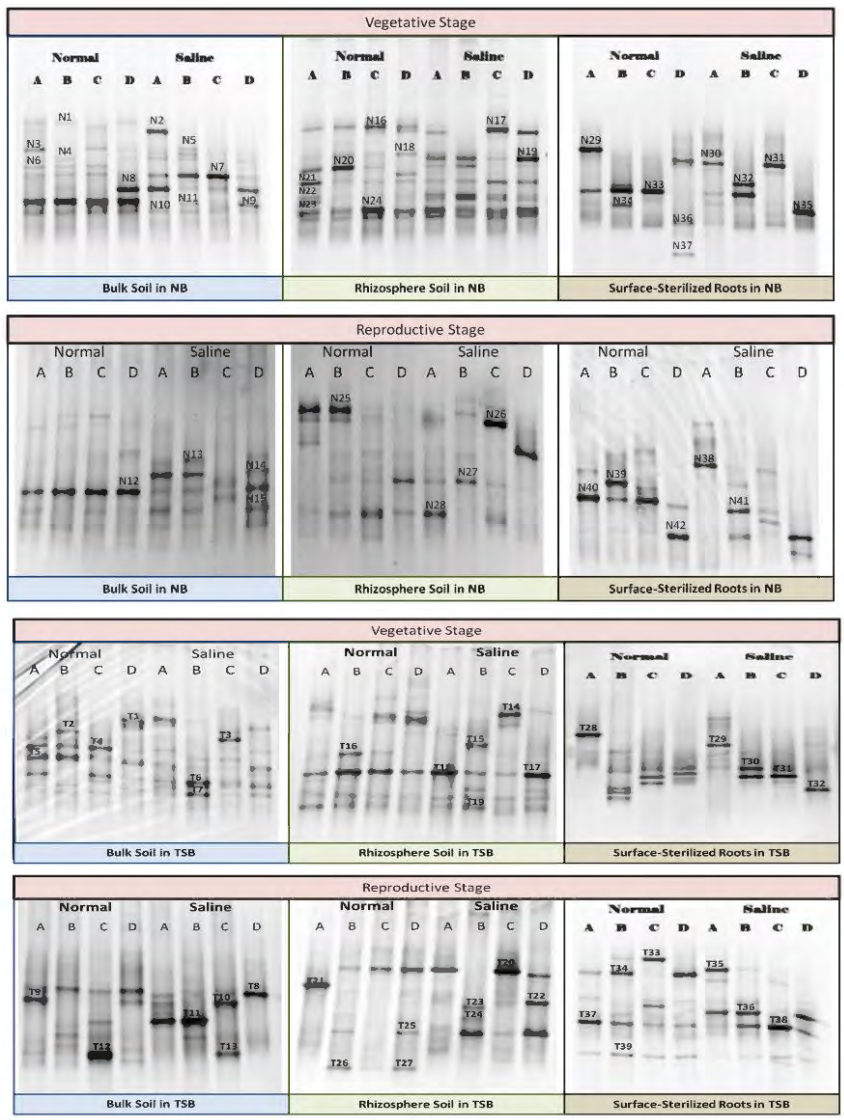


**Fig. 1.** DGGE profiles generated from culture enrichments using the primer pair 968fGC-1401r. (A) IR29; (B) PSB Rc 82; (C) FL478; (D) Salinas 1.

**Table 2.** Soil chemical properties of the soil samples from the IRRI B14 demo plot at the end of the experiment.

| Result of Analysis | Normal 1 | Normal 2 | Normal 3 | Saline 1 | Saline 2 | Saline 3 |
|---|---|---|---|---|---|---|
| **pH** | 6.10 | 6.20 | 6.20 | 6.10 | 6.00 | 6.00 |
| **OM%** | 2.45 | 2.36 | 2.54 | 2.60 | 2.59 | 2.51 |
| **N%** | 0.11 | 0.06 | 0.11 | 0.11 | 0.13 | 0.11 |
| **P (ppm, Olsen)** | 75 | 67 | 79 | 82 | 89 | 89 |
| **K (me/100g soil)** | 0.94 | 2.40 | 1.03 | 1.07 | 0.87 | 0.84 |
| **Na (me/100g soil)** | 4.24 | 8.81 | 6.67 | 15.01 | 13.47 | 13.27 |
| **Ca (me/100g soil)** | 12.28 | 12.96 | 12.01 | 10.66 | 11.88 | 10.53 |
| **Mg (me/100g soil)** | 7.83 | 7.96 | 8.09 | 7.19 | 6.81 | 8.60 |
| **Cl (ppm)** | 324.00 | 324.00 | 417.00 | 1390.00 | 1205.00 | 1298.00 |
| **Fe (ppm)** | 463.00 | 362.00 | 386.00 | 448.00 | 411.00 | 368.00 |
| **Zn (ppm)** | 5.00 | 3.00 | 4.00 | 4.00 | 4.00 | 3.00 |
| **$EC_{1:5}$ (µS/cm)** | 150.50 | 145.70 | 225.45 | 981.50 | 772.50 | 936.50 |
| **Ece (dS/m)** | 1.35 | 1.31 | 2.03 | 8.83 | 6.95 | 8.43 |
| **SAR** | 1.34 | 2.72 | 2.10 | 5.02 | 4.41 | 4.29 |

**Bacterial diversity analysis**

Statistical analysis revealed that the Shannon index of diversity in the rhizosphere is significantly affected by salinity and rice growth stages (Table 3). The bacterial diversity in the rhizosphere is higher under saline conditions compared to normal conditions, especially in the reproductive stage. For both rice growth stages, the increase in bacterial diversity were higher for the salt-tolerant varieties, FL478 and Salinas 1. However, the effect of rice variety on the rhizosphere bacterial diversity was not significant. These observations are consistent with findings demonstrating the dynamic nature of microbial communities in the rhizosphere which varies during the life cycle and with the seasonal response of plants (Hussain et al. 2012).

**Table 3.** Statistical analysis of the Shannon diversity (H') values of the four rice varieties grown at normal and salinized conditions during vegetative and reproductive growth stages.

| **Salinity Level** | **Mean Diversity Index*** |
|---|---|
| Normal | 2.26193 b |
| Saline | 2.43282 a |
| **Rice Growth Stage** | **Mean Diversity Index** |
| Vegetative | 2.27883 b |
| Reproductive | 2.42262 a |
| **Variety** | **Mean Diversity Index** |
| IR 29 | 2.33932 a |
| PSB Rc82 | 2.28611 a |
| FL 478 | 2.36668 a |
| Salinas1 | 2.40972 a |
| **Variety** | **Mean Difference Between Saline And Normal Levels** |
| IR 29 | 0.0615 a |
| PSB Rc82 | 0.1053 a |
| FL 478 | 0.2460 a |
| Salinas1 | 0.2656 a |

* Means followed by similar letters within the same variable are not significantly different at 6% level DMRT.

**Comprehensive analysis of the DGGE profile and band identity**

A total of forty-two bands were excised for the DGGE profiles from NB culture enrichments, thirty-nine were selected from the TSB culture enrichment, and seven bands were excised from the DGGE profile of the total rhizosphere DNA. Nine out of the 88 excised bands did not meet the required 97% level of similarity through BLASTn. Majority of the sequence matches from enriched samples have high level of similarity to the well-studied members of the Gammaproteobacteria.

Aside from the overall microbial diversity, it is important to fully understand the combined effects of salinity, rice growth stage, and rice variety on specific bacterial populations. The matrix combining the band identities and the DGGE banding patterns indicates that salinity has a strong effect on the bacterial diversity in the bulk soil (Fig. 2). Bands identified to belong to genus *Providencia* was found only in normal plots. The genus *Aeromonas*, *Pseudomonas, Serratia, Shewanella* and *Vibrio*, on the other hand, represent those that are present in both normal and saline conditions.

While results from the bulk soil profiles revealed the impact of soil salinity on the soil bacterial community, DGGE profiles from rhizosphere enrichments showed the "rhizosphere effect" on the bacterial community. The genus *Providencia,* which was found only in normal EC levels of the bulk soil, was observed in the rhizosphere of all rice varieties under both normal and saline conditions. Members of the genus *Providencia* have been reported to promote rice growth in wheat and rice. On

the other hand, the genus *Serratia* and *Shewanella* which were both observed at both normal and saline in the bulk soil, were observed only under normal conditions in the rhizosphere. It appears that salinity, somehow, has an influence on the ability of the plant to interact with microorganisms.

| Genus | Bulk Soil | | | | | | | | Rhizosphere | | | | | | | | Roots | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | | | | Saline | | | | Normal | | | | Saline | | | | Normal | | | | Saline | | | |
| | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| *Aeromonas sp.* | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| *Pseudomonas sp.* | B | B | B | B | B | B | B | B | | | | | | | | | | | | | | | | B |
| *Serratia sp.* | V | V | V | V | V | V | V | V | R | R | | | | | | | R | R | R | R | | | | |
| *Shewanella sp.* | B | B | B | B | B | B | B | B | V | V | V | V | | | | | | | | | | | | |
| *Vibrio fluvialis* | B | B | B | B | B | B | B | B | | | | | | | | | | | | | R | R | | |
| *Providencia sp.* | B | B | B | B | | | | | V | V | V | V | V | V | B | V | | | | | | | | |
| *Bacillus sp.* | | | | | | | | | | V | | | | | | | V | V | V | V | B | V | V | V |
| *Cedecea sp.* | | V | | | | | | | | | | | | | | | | | | | | | | |
| *Azomonas macrocytogenes* | | | | | | | | | | | | | | | | | | | | | | V | | |
| *Brevibacterium halotolerans* | | | | | | | | | | | | | | | | | | | V | | | | V | |
| *Enterobacter asburiae* | | | | | | | V | | | | | | | | | | V | | | | | | | |
| *Klebsiella variicola* | | | | | | | | | | | | | | | | | | | R | | | | | |
| *Lysinibacillus boronitolerans* | | | | | | | | | | | | | | | | | | | | | V | | V | |
| *Methylobacterium populi* | | | | | | | | | | | | | | | | | | | | V | | | | |
| *Morganella morganii* | | | | | | | | | R | | | | | | | | | | | | | | | |

Legend:

| | |
|---|---|
| V | Bacteria was observed during the vegetative stage |
| R | Bacteria was observed during the reproductive stage |
| B | Bacteria was observed on both stages |

**Fig. 2.** Consolidated 16s rDNA analysis and DGGE profile observation.

The effect of salinity was visible on the bacterial community in surface-sterilized rice roots. Several root-bacterial interactions were observed only under normal conditions. These were *Enterobacter* for IR29, *Klebsiella* for FL 478 and *Methylobacterium* for Salinas 1. In addition, *Azomonas macrocytogenes* was detected only in the roots of PSB Rc82 under saline condition during the vegetative stage. *Vibrio* sp was observed for both IR 29 and PSB Rc82 under saline condition. Interestingly, members of the genus *Aeromonas* were found in almost all regions and conditions. An iron reducing bacterial strain was identified as a member of the *Aeromonas* group by 16S rRNA gene sequence analysis with a very wide range of tolerance to salinity (Wang et al. 2009).

*Serratia* sp. was observed in both normal and saline conditions in the bulk soil, but only during the vegetative stage. During the reproductive stage, *Serratia* sp. were found in the rhizosphere of IR29 and PSB Rc82 under normal conditions, and inside the roots of all varieties under normal conditions. These results suggest the possible role of *Serratia* sp. during the reproductive stage of rice. This interaction, however, was not observed during saline conditions. This is an indication that salinity has an influence on several plant-microbe associations. A strain of *Serratia marcecens*, IRBG500, was reported to significantly increase the root length and root dry weight, but not the total N content of rice variety IR72. Its initial entry was at the points of lateral root emergence and root tip (Gyaneshwar et al. 2001).

Although the effect of salinity was very evident, several plant-specific interactions were still observed. FL478, a salt- tolerant variety, was observed to be associated with *Klebsiella sp.* and *Brevibacterium halotolerans. Klebsiella* is a genus widely known to have the ability to fix nitrogen

(Mano and Morisaki 2008) while *Brevibacterium* species are known to exist in a number of different habitats, especially in those having a high salt concentration. Most members of this family grow well in the presence of 8% NaCl, and many strains also grow in 15% NaCl (Collins 2006). *B. halotolerans* has been isolated from the halophyte, *Prosopis strombulifera*, which was grown under extreme salinity. It was found to have ACC-deaminase activity, nitrogen fixation and even possible biocontrol activity (Sgroy et al. 2009). In this study, FL478 was the only variety that was shown to interact with *B. halotolerans*. This plant-microbe interaction may have contributed to FL478's tolerance at the vegetative stage.

In this study, the presence of *Pseudomonas* species was observed in the DGGE profiles of enriched bulk soil samples at both salinity levels and reproductive stage. No *Pseudomonas* species were found in the rhizosphere. The only other *Pseudomonas* present was found inside the roots of Salinas 1, which was a released tolerant variety. It is highly possible that the rice plant is in a mutualistic interaction with a Pseudomonas species, which could have already been present during the seedling stage, and could have established itself inside the roots, even prior to salinization. Under saline conditions, *Pseudomonas sp.* was only observed inside the roots of Salinas 1 at both stages. This interaction was not observed in other rice varieties. Members of the genus *Pseudomonas* is widely known to promote plant growth via different mechanisms, and is reported to promote salinity tolerance in corn (Mano and Morisaki 2008). Several pseudomonads are used as plant growth promoting bacteria. Specifically, fluorescent pseudomonads were found to have catabolic versatility, excellent root colonizing ability and their capacity to produce a wide range of enzymes and metabolites that enable the plant to withstand varied biotic and abiotic stress conditions (Mayak et al. 2004). A *Pseudomonas fluorescens* strain possessing ACC deaminase activity was reported to enhance the saline resistance in groundnut plants (Saravanakumar and Samiyappan 2007) and a P-solubilizing *Pseudomonas* strain was also found to have a positive influence on plant nutrition under salt-stressed conditions when co-inoculated with *Rhizobium* sp (Bano and Fatima 2009).

Some endophytic bacteria have beneficial effects on the host plant, such as plant growth promotion, the induction of increased resistance to pathogens, as well as the supply of fixed nitrogen to the host plant (Mano and Morisaki 2008). In this study, several known plant growth promoting bacteria were found, not only in rice rhizosphere, but inside the roots as well. The combined result of DGGE profile analysis and band identification revealed that salt-tolerant varieties FL478 and Salinas1 interact with bacteria that have PGPR-like properties inside their roots. This indicates possible interaction of rice roots with endophytic PGPR. Further analysis should be done to determine these possibilities. Isolation of DNA directly from rice roots and analysis through PCR-DGGE is strongly recommended to confirm these findings and to check for the presence other microorganisms with plant-growth promoting properties.

## CONCLUSION AND RECOMMENDATIONS

The results of this study suggest the interplay of salinity level and rice variety as factors affecting the characteristics of the microbial community in the rhizosphere. A detailed physiological analysis of the four rice varieties is highly recommended. An analysis of the exudates that the rice varieties release under salt stress can provide a great insight into how the microbial community in the rhizosphere is affected by salinity. Based on the results of this study, it is possible that under salt stress, the plants may have released substances which tend to enhance the bacterial diversity. Salt-tolerant varieties may also have greater capability to produce more variety of root exudates that can promote the growth of a more diverse and, possibly, beneficial microorganisms.

The dominance of several Deltaproteobacterial bacteria in the rhizosphere is also interesting. Since salinity is associated with increased amounts of solutes and ions in the rhizosphere, it appears that *Geobacter* species play an important role in saline environments. It is therefore necessary to further

analyze the community, focusing on Deltaproteobacterial species as well as the archaeal community in the rhizosphere. The presence of several plant-microbe interactions in the rhizosphere and in surface-sterilized roots of salt-tolerant varieties is noteworthy. Further research needs to be done to determine if these specific organisms can contribute to the crop's ability to tolerate salinity.

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# ULTRAVIOLET–B INDUCED FLAVONOID PRODUCTION IN *IN VITRO* CULTURES OF SHALLOT (*Allium cepa* var. *Aggregatum* G. Don cv Batanes)

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## ABSTRACT

Onions are a rich source of health-enhancing flavonols, however research in enhancing the levels of these compounds, especially in shallots, is limited. This study was undertaken to investigate the relationship between flavonol production and UV-B exposure in shallots in an *in vitro* system and at the greenhouse. The experiment was conducted from January 2006 to April 2007 at the Plant Tissue Culture Laboratory, Institute of Crop Science, College of Agriculture and Food Science, UPLB. UV-B was used as an elicitor of flavonol production at two different stages of shallot tissue culture. *In vitro* established plantlets of shallots (cv *Batanes*) were subjected to UV-B (~290 nm) at 0, 3, and 6 h per day for 7 and 14 days at shoot induction and bulbing stages. Bulb diameter and total flavonol content was measured and the profiles of the three predominant flavonoids: quercetin, myricetin, and kaempferol, were described using paper chromatography. These were grown *in vivo* under greenhouse conditions. UV-B exposure did not affect bulb diameter of *in vitro*-grown plantlets but had increased total flavonols. Cultures at the bulbing stage had higher total flavonol than in the shoot induction stage. Flavonoid accumulation tends to increase with prolonged exposure to UV-B but could not be generalized for the different growth stages. There were no differences in flavonol accumulation regardless of the duration or exposure to UV-B. Percent survival was highest among plants that did not receive UV-B treatment. Bulb weight, diameter, plant height, and flavonol accumulation were not affected by UV-B treatments. Quercetin was found to be the most abundant flavonol followed by myricetin and kaempferol in *in vitro* cultures and among plants grown in the greenhouse.

**Key words**: flavonol, quercetin, myrcetin, kaempferol

## INTRODUCTION

Flavonoids are ubiquitous in higher plants and is a diverse family of aromatic molecules which are subdivided into six major subgroups: the chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins, and a seventh group, the aurones, which are widespread but not ubiquitous (Winkel-Shirley 2001). Flavonoids are potent antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals (Miller 1996). The flavonoids are UV-absorbing compounds and this characteristic implies a direct role of the compound in UV photoprotection. Arguably, the flavonols are the most important flavonoids that participate in stress responses such as that of UV radiation. Flavonol compounds have been shown to be specifically induced by UV light across a wide range of species (Falcone Ferreyra et al. 2012).

Shallots as part of the genus *Allium* are naturally rich in flavonoids. Quercetin, a flavonol, is the most abundant flavonoid in onion that had attracted special interest (Price and Rhodes 1996) in

regard to human nutrition. Quercetin has been reported to reduce the risk of cardiovascular diseases and certain cancers (Patil and Pike 1995, Patil et al 1995b). It is believed to be mostly found or compartmentalized in cell vacuoles. In intact onion plants, total quercetin is concentrated on the drying skin or scale (Patil and Pike 1995, Takahama and Hirota 2000) and may be due in part to mobilization of the molecule towards the drying skin (Gubb and MacTavish 2002).

*In vitro* or tissue culture model systems are widely accepted as an indispensable tool to investigate biosynthesis and physiology of secondary metabolites. It provides a clearer view that could be used as an alternative to the whole plant (Luckner 1972, Thorpe 1981, Kyte 1987). Plant tissue culture systems provide easier manipulation when elicitors are applied since tissue culture-derived plant materials have simpler organization, grows in a controlled environment, has shorter growth cycles, and minimizes the complexity of the whole intact plants (Staba 1980). Thus, possibilities to obtain fundamental knowledge about relationships between primary and secondary metabolism could be obtained. Secondary metabolite profiles particularly flavonoids in shallot onion cultivar of the Philippines have not been strongly established specifically for in *in vitro* grown cultures. Since the production of flavonoids is highly regulated by UV activating the biosynthetic pathway of flavonoids, using UV as an abiotic elicitor is a strategy to increase total flavonoid content in plants. Thus, this study focused on the flavonoids that may be induced by ultraviolet-B (UV-B) irradiation in *in vitro* cultures of shallot, which could offer substantial basis for further biotechnological manipulation of the crop.

## MATERIALS AND METHODS

### Plant material

The shallot *Batanes* cultivar (*Allium cepa* var. *Aggregatum* G. Don cv Batanes) was used in this study (Fig. 1A). The bulbs are red to light purple in color and produce small oblate to round bulbs held in a common basal plate in clusters of two to six (Dahilig 1992).The shallots were purchased from Central Luzon State University (CLSU) in Muñoz, Nueva Ecija to assure purity of the cultivars. Dried leaves, scales and roots were trimmed and the good quality bulbs with the favorable sizes were selected. .

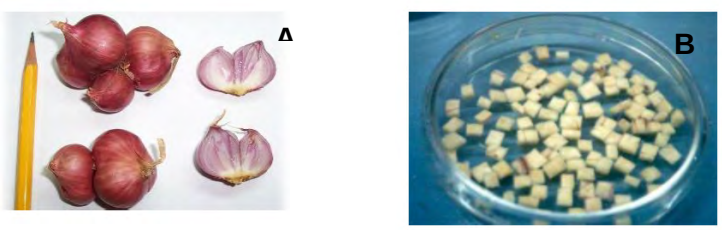


**Fig. 1.** Shallot used as source of explants are the small to medium sized bulbs of approximately1-2 cm diameter (A); and excised basal plates (B).

### Culture media

The modified Murashige and Skoog (MS; 1962) nutrient medium was used as basal medium. Two different culture media were prepared: Shallot Induction Medium (SIM), for shoot multiplication; and Shallot Bulbing Medium (SBM), for bulb formation (Pateña et al. 1998). The pH of the culture media was adjusted to 5.8 using 1N KOH or 1N HCl before dispensing into 15 ml aliquots culture vessels. The culture media were autoclaved at 15 psi for 20 minutes.

### Preparation of explants and establishment of sterile cultures

Bulblets with the outer scales removed were placed in a container, drenched with fungicide (benomyl) powder for at least 12 h and finally washed with running water. The bulblets were subsequently soaked in liquid detergent for at least 15 minutes and rinsed with water. Sterilization was done inside the inoculating chamber following the double sterilization regime (15 + 15 min). The

bulbs were initially sterilized with 10% sodium hypochlorite solution, followed by 5.25% hypochlorite commercial bleach (Zonrox®) for another 15 mins. In between and after sterilization procedures, the bulbs were rinsed thrice with sterile distilled water before dissection. The basal plates at approximately 3-4 $mm^2$ x 1-2 mm thick were excised (Fig. 1B) and sterilized with 1% sodium hypochlorite for 5 min, and rinsed with sterile distilled water three times. These were inoculated onto Shallot Induction Medium (SIM) for the establishment of sterile cultures and shoot multiplication. Adequate size of shoots was obtained three weeks after inoculation. These served as the initial cultures.

**Culture maintenance and induction of flavonol production by UV-B radiation**

The established cultures of shallots (cv *Batanes*) were either incubated unto SIM for shoot induction and SBM for bulbing. All cultures were incubated in culture room equipped with white cool fluorescent lamps at 8.86 µmol/$m^2$/s PAR at 24 ± 4ºC room temperature under 16-h photoperiod for shoot induction and 10-h photoperiod for bulb formation. Shallot cultures at shoot and bulb stages were exposed to UV-B radiation (≈290nm) at different exposure durations similar to the method described by Lavola (1998), Olsson et al. (1998) and Wilson et al. (1998). The treatments were as follows: $T_1$ – 0 UV-B for 7 days; $T_2$ – 3 h UV-B for 7 days; $T_3$ – 6 h UV-B for 7 days; $T_4$ – 0 h UV-B for 14 days; $T_5$ – 3 h UV-B for 14 days; $T_6$ – 6 h UV-B for 14 days

UV-B (280-320nm) lamps (Kandolite, India) were installed in separate culture cabinets enclosed with black cloth. Supplementary UV-B was provided with UV tubes (Kandolite FL 30W). Other wavelengths were filtered using 0.13 mm thick cellulose acetate which were replaced every other day. Sample tissues for the determination of flavonoids were gathered 24 h after the last day of exposure to UV-B. Plant tissue extracts were collected at the end of each treatment duration. The extracts were kept in the freezer (-20ºC) until further analysis.

**Establishment of plant materials, maintenance and sampling**

UV-B irradiated plantlets were acclimatized in the greenhouse for a week. After acclimation, the plantlets were washed, cleaned of agar and dipped in 0.1% fungicide (benomyl) solution for one minute. The plantlets were sown in individual pots containing (1:1:1) sterilized garden soil, compost, and carbonized rice hull (CRH) mixture. Bulbs from the non-irradiated cultures served as control. Four plants per pot were maintained for flavonoid analysis. Proper cultural management practices for the plants were employed simulating field conditions. Random destructive sampling was employed, washed with water, and blot dried. Composite samples of plants from each treatment were collected 50 days after transplanting for flavonoid analysis. Bulb diameter, weight, plant height and number of bulbs per cluster of the sample plants were measured.

**Flavonol extraction**

Extraction of flavonols was carried out using the method of Thompson et al (2005). Approximately 2 grams of tissue sample from each treatment were homogenized with 10 ml 80% ethanol. The homogenized samples were centrifuged at 5000 rpm for 15 minutes. The supernatant was collected and filtered using Whatman #5 filter paper. The filtrates were stored in screw-capped vials at –20ºC for further analysis.

**Total flavonol content**

The extracts from each treatment in three trials, were warmed to room temperature prior to spectrophotometric analysis, and a 0.5-mL sample was diluted with 4.5 mL of 80% ethanol. The absorbance was determined at 362 nm (Shimadzu, UV-1800). Two technical replicates were performed on each sample and the average readings were taken. Quercetin dihydrate (Sigma) was used as a standard and different standard concentrations ranging from 0.00625 to 0.100 mg/mL were used to create a standard curve (absorbance vs concentration) using linear regression. Total flavonol

content was quantified on a wet matter basis using the equation for the best fitting line based on the standard curve.

**Flavonol profile**

Samples were taken from the composite of extracts from the different treatments in three trials. Paper chromatography was performed using the protocol of Markham (1982). Chromatographic paper strips (Whatman 3MM) were washed with acetone-water mixture (1:1 v/v) and air-dried before loading the samples. Three flavonol standards, quercetin, kaempferol, and myricetin, and the sample extract were loaded on each strip. The loaded strips were equilibrated for at least 12 h and developed by ascending chromatography using butanol: acetic acid: water (BAW; 4:1:5) and visualized by ammonia vapors. Each visible spot was eluted with ethanol and absorbance was determined at 362 nm.

**Statistical design and analysis**

Both tissue culture and greenhouse experiments were set-up in a 2 x 3 x 2 factorial in CRD. The factors were growth stage, time (h) exposure to UV-B, and duration (days) of UV-B treatment. The *in vitro* induction experiment had 20 replicates per treatment while the greenhouse study had five replicates. The Statistical Analysis Software (SAS) System (SAS Institute, Cary, NC, USA) was used to analyze all the data gathered following the general linear model (Proc GLM). Significant results of the ANOVA were further tested for difference among treatments using LSD at $\alpha = 0.05$

## RESULTS AND DISCUSSION

**Establishment of initial shoot cultures**

Normal shoots that formed from basal plates are shown in Fig. 2A. The basal plates visibly enlarged 24 h after inoculation, while shoots started to protrude three days thereafter. An average of 92% of the total explants developed shoots with 4.4 shoots per explant. After 21 days of inoculation, 83.28% of the total number of cultures remained contamination-free. Contaminated cultures were immediately discarded and replaced to maintain the number of replicates in each treatment. Developed shoots were held at a common base. Leaf sheaths were white and gradually changed to green towards the leaf region. Explants that did not produce shoots and vitrified (Fig. 2B), formed flattened shoots but remained green (Fig. 2C) and those that assumed 'callus-like' appearance (Fig. 2D) were considered dead. Selected shoots were maintained and used as experimental materials in subsequent experiments.

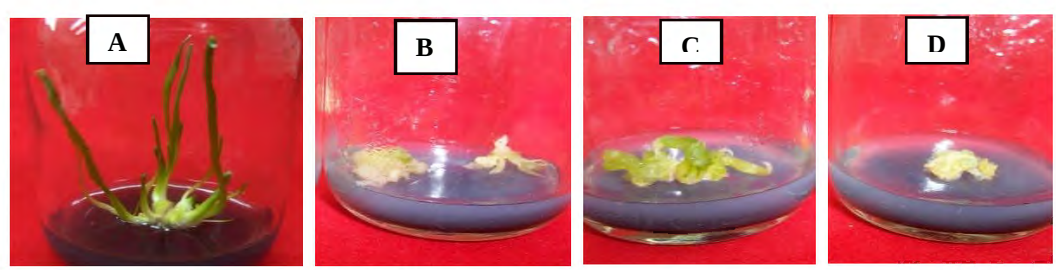


**Fig. 2.** Normal shoots formed from basal plates (A); dead cultures of shallot (cv *Batanes*) exhibiting vitrified and no shoot formation (B); flattened shoots (C); and 'callus-like' growth (D).

**Growth stage and UV-B radiation exposure**

UV-B exposure at the shoot and bulb stages did not affect bulb diameter in shallot (cv *Batanes*) cultures. In contrast, total flavonol accumulation was higher in cultures incubated in SBM (27.59 µg/g sample) than in SIM (18.08 µg/g sample) (Table 1). Induction of flavonol production in bulblets exposed to UV-B at bulbing stage is further evidenced by the presence of anthocyanin (Fig.

3B), also a flavonoid. Bulbs at shoot induction stage did not show anthocyanin pigmentation (Fig. 3A).

**Table 1.** Bulb diameter and total flavonol of *in vitro*-grown shallot (cv *Batanes*) as affected by the number of days and h per day of UV-B radiation exposure in different growth stages.

| Exposure Time (Hrs) | [1/]Bulb Diameter (mm) | | | [2/]Total Flavonol (µg/g sample) | | |
|---|---|---|---|---|---|---|
| | 7 | 14 | Mean | 7 | 14 | Mean |
| **Shoot Induction Stage** | | | | | | |
| 0 | 6.3a | 6.6a | 6.4a | 20.13a | 26.40a | 23.27a |
| 3 | 6.3a | 6.3a | 6.3a | 13.23a | 16.00a | 14.62a |
| 6 | 6.5a | 6.4a | 6.5a | 16.03a | 16.70a | 16.37a |
| Mean | 6.4a | 6.4a | **6.4a** | 16.47a | 19.70a | **18.08**b** |
| **Shallot Bulbing Stage** | | | | | | |
| 0 | 6.4a | 6.2a | 6.3a | 18.66a | 17.40a | 18.03a |
| 3 | 6.4a | 6.2a | 6.3a | 23.90a | 35.37a | 29.63a |
| 6 | 6.0a | 6.2a | 6.1a | 28.57a | 41.67a | 35.12a |
| Mean | 6.3a | 6.2a | **6.2a** | 23.71a | 31.48a | **27.59**a** |
| Standard Deviation | | | 0.028 | | | 0.006 |
| CV (%) | | | 4.503 | | | 25.115 |

[1/]-After acclimatization for 13 weeks in culture. Bulbing started at 9 weeks after inoculation.
[2/]-Total flavonol was determined 24 h after the last days of exposure to UV-B
Means under each column and row heading having the same letters are not significant using LSD at α=0.05
** - Significant using LSD at α=0.01 level

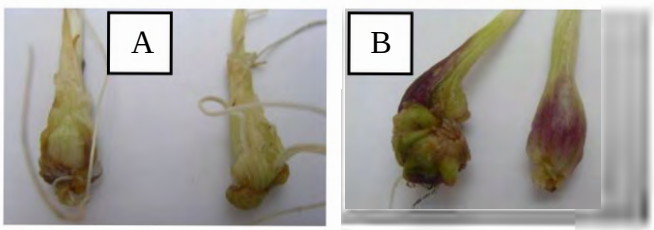


**Fig. 3.** Bulblets of plants exposed to UV-B at shoot induction stage (A); and at bulbing stage (B) which show anthocyanin pigmentation.

Shallot is a bulb forming species of onion and the onset of bulb formation is affected by environmental factors such as photoperiod and temperature (Lancaster et al. 1996). In the absence of physical cues, specifically formulated tissue culture medium such as SBM may promote bulb formation (Pateña et al. 1998). One of the general responses of plants to UV-B radiation is reduced growth characteristics (Teramura 1983) and differs widely between genera, species and among cultivars (Teramura and Sullivan 1991, Jansen et al. 2001). UV, a minor component of sunlight, affects the accumulation of flavonoids. Several studies have demonstrated the change in flavonoid composition because of excess light or UV-radiation (Lois 1994, Olsson et al. 1998). Flavonoids and sinapate esters are UV-B screening pigments which were produced in response to elevated levels of UV-B in *Arabidopsis* (Li et al. 1993) and *Brassica napus* (Wilson et al. 2001).Enzymes and other precursors in the flavonoid biosynthetic pathway are limiting during the immature stage or specific developmental stage (Verhoeyen et al. 2002).In hydroponically and potting soil grown spring onions, total flavonol increased throughout the growing period (Thompson et al. 2005).Differences of nutrient components and total flavonol of onions were affected by age (Thompson et al. 2004) and nitrogen stress (Patil et al. 1995a),but ambient and elevated levels of carbon dioxide had no effect.

**Flavonol profile**

In this study, the distribution of three flavonols in shallot extracts as influenced by exposure to UV-B radiation was determined. Qualitative analysis by paper chromatography identified seven distinct spots in the extracts. Quercetin was found to be the most abundant flavonol in *in vitro* cultures in both shoot induction and bulbing stages and across UV-B treatments (Fig 4). This finding is consistent with the estimates of Lombard (2000) that quercetin conjugates contribute 90 percentage to the total flavonol of bulb onions. Myricetin was the second most abundant flavonol followed by kaempferol. Quercetin was higher in cultures at the bulb stage than at the shoot induction stage, indicating that quercetin contributed most to the total flavonol accumulation (Fig. 4) and that UV-B exposure at relatively mature stage could have further enhanced its synthesis.

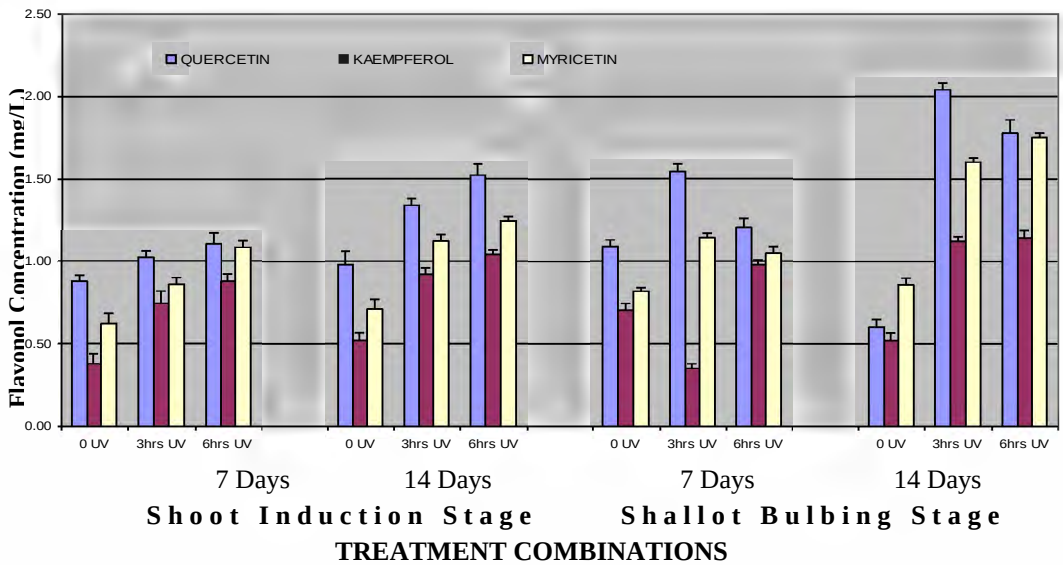


**Fig. 4.** Flavonoids concentration of *in vitro*-grown shallot (cv *Batanes*) as affected by the number of days and h per day of UV-B radiation exposure in different growth stages determined by paper chromatography and quantified through UV-Vis spectrophotometry. Error bars represent the standard error of the mean.

Quercetin, kaempferol, and myricetin are abundant in plants (Sellapan and Akoh 2002, Miean and Mohamed 2001, Hertog et al. 1992) and are commonly found in vegetables (Herrmann 1976). Flavonols, in general, are implicated in plant cells as UV protectant. However, these are also involved other in plant physiological activities. Quercetin and kaempferol (Vogt et al. 1995) are essential for pollen germination and tube growth in petunia (*Petunia hybrida*) and maize (Mo et al. 1992). Myricetin is suspected to play a role in co-pigmentation as its level increased with accumulation of anthocyanins in bilberries (Jaakola 2006). A number of studies showed that multiple forms of precursor enzymes exist in plants and that their expression patterns vary depending on the growth conditions as in the case of chalcone synthase (CHS), the key enzyme for flavonoid pathway. In barley leaves, CHS with a preference for ferrulic acid and caffeic acids instead of *p*-coumaric acid is induced by UV-B radiation (Christensen et al. 1998), which leads to quercetin synthesis over kaempferol biosynthesis (Ryan et al. 1998, Olsson et al. 1998, Wilson et al. 2001).

**Detection and quantification of flavonols in i*n vitro*-derived plantlets grown *in vivo*.**

Shallot cultures at both stages, that did not receive any UV-B treatment had the highest survival at the greenhouse. The lowest percentage survival was observed at 6 h UV-B exposure

(Table 2) regardless of the number of days of exposure. It is evident that there is a critical length of exposure period where UV-B damage is irreversible. Significant differences were observed in percentage survival between the control (43.13 %) and at 6 h per day of exposure to UV-B (35.65 %) but not with 3 h exposure. Increasing the UV-B exposure time resulted in a decrease in percentage survival of plantlets grown *in vivo*.

**Table 2.** Percentage survival of *in vitro*-derived plantlets of shallots (cv *Batanes*) grown *in vivo*.

| UV Exposure Time (Hrs/ day) | Percentage Survival (%) | | | | | | Overall % survival (Mean) |
|---|---|---|---|---|---|---|---|
| | Shoot Induction Stage | | | Shallot Bulbing Stage | | | |
| | 7 | 14 | Mean | 7 | 14 | Mean | |
| 0 | 44.44a | 46.30a | 45.37a | 39.81a | 37.95a | 38.89a | 43.13a* |
| 3 | 37.96a | 37.96a | 37.96a | 36.11a | 37.96a | 37.04a | 37.50ab |
| 6 | 34.26a | 37.96a | 36.11a | 35.19a | 35.19a | 35.19a | 35.65b* |
| Mean | 38.89a | 40.74a | 39.81a | 37.04a | 37.04a | 37.04a | **38.46** |
| S.D. | | | | | | | 5.801 |
| CV (%) | | | | | | | 15.096 |

Means under each column and row heading having the same letters are not significant at 0.05 level (LSD test)
* Significant using LSD at α=0.05 level

Control plants tended to be more vigorous with greener and larger leaves (Fig. 5A) than plants exposed to UV-B in both growth stages at 30 days after potting out (Fig. 5). Necrotic lesions on the leaves were evident on plants exposed to UV-B radiation for 7 days at shoot induction stage (Fig. 5B) and bulbing stage (Fig. 5D), but more pronounced among plants exposed for 14 days to UV-B at both growth stage (Fig 6C and 6E). However, while these abnormalities were observed among UV-B treated plants, their overall growth were comparable with control plants. There were no significant differences on bulb diameter, bulb weight, plant height, and the number of bulbs per cluster, although there is a decreasing trend in their values as UV-B exposure is prolonged (Table 3).


**Fig. 5.** Growth of *in vitro*-derived shallots (cv *Batanes*) under greenhouse conditions after 30 days of transfer: control (A); exposed to 3 h UV-B radiation at shoot induction stage for 7 days (B); and for 14 days (C); exposed to 3 h UV-B radiation at bulbing stage for 7 days (D) and for 14 days (E).

As in the *in vitro* stage, quercetin was the most abundant flavonol in shallots (Fig. 6). There is an increasing trend in the levels of quercetin, myricetin, and kaempferol in both culture stages previously subjected to UV-B treatment. It seemed that age of the tissue and the accumulation of flavonols as a response to UV-B are directly related.

**Flavonol profile using paper chromatography**

The *in vitro*-derived plantlets of shallot were grown *in vivo* to determine the effect of UV-B radiation on the accumulation of flavonols in onion. Among the three flavonols determined through paper chromatography, quercetin was consistently the highest, followed by myricetin and kaempferol in plant previously exposed to UV-B radiation in both growth stages (data not shown). These were consistent with the results obtained in the *in vitro* cultures.

**Table 3.** Bulb diameter, bulb weight, plant height, and number of bulb per cluster of *in vitro*-derived plantlets of shallots (cv *Batanes*) grown *in vivo*.

| UV-B Exposure Time (Hrs per day) | Bulb Diameter (mm) | | | Bulb Weight (g) | | | Plant Height (cm) | | | Number of Bulbs per Cluster | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 14 | | 7 | 14 | | 7 | 14 | | 7 | 14 | |
| **Shoot Induction Stage** | | | | | | | | | | | | |
| 0 | 83.0a | 83.6a | 83.3a | 0.580a | 0.533a | 0.557a | 12.68a | 12.60a | 12.64a | 1.23a | 1.23a | 1.23a |
| 3 | 82.7a | 79.7a | 81.2a | 0.523a | 0.513a | 0.518a | 12.14a | 12.12a | 12.13a | 1.13a | 1.17a | 1.15a |
| 6 | 76.7a | 80.3a | 78.5a | 0.523a | 0.557a | 0.540a | 11.64a | 11.66a | 11.65a | 1.20a | 1.13a | 1.17a |
| Mean | 80.8a | 81.2a | 81.0a | 0.542a | 0.534a | 0.538a | 12.15a | 12.13a | 12.14a | 1.19a | 1.18a | 1.19a |
| **Shallot Bulbing Stage** | | | | | | | | | | | | |
| 0 | 82.2a | 85.7a | 83.9a | 0.550a | 0.543a | 0.547a | 12.60a | 12.55a | 12.58a | 1.23a | 1.23a | 1.23a |
| 3 | 82.5a | 79.7a | 81.1a | 0.537a | 0.537a | 0.537a | 11.95a | 11.49a | 11.72a | 1.13a | 1.13a | 1.13a |
| 6 | 82.1a | 80.0a | 81.0a | 0.513a | 0.520a | 0.517a | 11.63a | 11.54a | 11.58a | 1.13a | 1.10a | 1.12a |
| Mean | 82.3a | 81.8a | 82.0a | 0.533a | 0.533a | 0.533a | 12.06a | 11.86a | 11.96a | 1.17a | 1.16a | 1.17a |
| Standard Deviation | | | 0.069 | | | 0.040 | | | 0.682 | | | 0.097 |
| CV (%) | | | 0.815 | | | 7.484 | | | 5.662 | | | 8.290 |

Means under each column and row heading having the same letters are not significant (LSD test).

In the shoot induction stage, quercetin accumulation increased with prolonged UV-B exposure time and days of exposure. In contrast, the level of kaempferol and myricetin varied with different lengths of exposure time and days of exposure to UV-B. In the bulbing stage, the level of quercetin peaked at three h exposure and declined with prolonged exposure time and days of exposure to UV-B. A similar trend was also observed on kaempferol accumulation, however, much lower than quercetin. In contrast, myricetin accumulation was directly proportional to the length of exposure time at 14 days of exposure but not at seven days of exposure. It was also noted that accumulation of myricetin was relatively higher at 14 days than at seven days of exposure to UV-B. These results conformed with the findings of Lavola et al. (1998) on birch tree exposed to UV radiation.

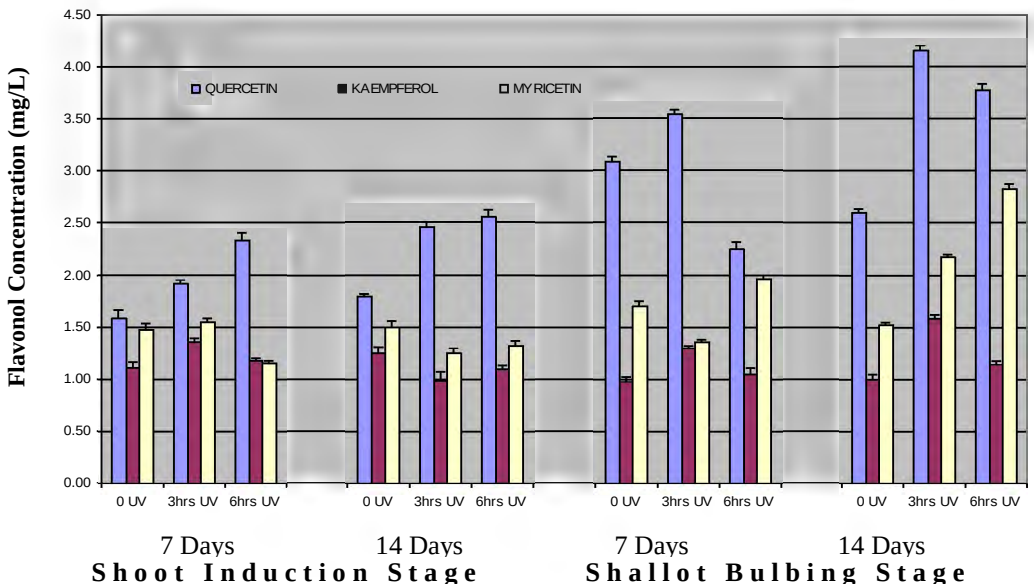


**Fig. 6.** Flavonol concentration of *in vitro*-derived plantlets of shallot (cv *Batanes*) grown *in vivo* determined by paper chromatography and quantified through UV-Vis spectrophotometry. Error bars represent the standard error of the mean.

## CONCLUSION

Collectively, this study demonstrated that accumulation of flavonols (quercetin, myricetin and kaempferol) in *in vitro* conditions was regulated by UV-B. Specifically, UV-B enhanced the accumulation of quercetin while it had minimal effect on the accumulation of myricetin and kaempferol. This preliminary result established the flavonoid profile of shallot (cv. *Batanes*) specifically for the *in vitro* grown cultures. While UV-B affected quercetin accumulation, it did not affect growth characteristics such as bulb diameter, bulb weight, plant height and number of bulbs per cluster. Important considerations for future experiments may include the age of culture and number of days of UV-B exposure for *in vitro*-grown shallots (cv *Batanes*).

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# POLITICAL ECONOMIC ANALYSIS OF INDONESIAN RICE MARKET

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## ABSTRACT

Many studies on rice self-sufficiency in Indonesia have been extensively conducted. Most of the studies analyzed the magnitude and trend of harvested area, production, productivity, consumption, and trade of rice. There have been no studies found related to political economic perspective for rice self-sufficiency yet. Such study is considerably important as rice self-sufficiency is not only a national economic issue, but also a politically related one as well. The strategic role of rice goes beyond economical reason for the country to conduct rice self-sufficiency program. Therefore, it is thought necessary to find out the political preference of the government in rice market along with rice self-sufficiency program to answer the question of who benefit most from such a policy. A political econometrical model was then developed to estimate the political weight of vested interest groups in rice market of Indonesia. This study was carried out using the secondary aggregate data at the national level from 2001 to 2014. This study used a political preferential function model to represent the objective of the government of Indonesia to maximize social welfare of rice producer, consumer, and the government. The rice policy in Indonesia is biased to the government as evidenced by the measured highest political weight for the government, compared to those of the others. Both political weight of the government and producer could affect the achievement level of rice self-sufficiency. Hence, the government is strongly recommended to reformulate rice policy to improve the role of vested interest group in the rice market.

**Key words*:*** self-sufficiency, vested interest group, political weight

## INTRODUCTION

Self-sufficiency policy is theoretically inefficient (Monke and Pearson 1989). Nevertheless, it has been being an important agenda and goal of the agricultural development since the establishment of the Government of Indonesia according to the strategic role of rice in the economy (Baharsjah et al. 2014). The government aimed to achieve four main targets of the sustainable self-sufficiency, i.e. the increasing rice production, the stable price and reserve stocks, and zero import, hence the welfare of rice producer and consumer could be improved. In order to realize that condition, the government intervened the market by implementing four policy instruments, i.e. production policy, price policy, distribution policy, and import policy. The government allocated fund for implementing the policies and the allocation of fund tended to increase. Besides, the government provided subsidies from on farm to off farm activities (such as post-harvest, marketing).

Besides the production policy, the three policy instruments (price, distribution, and importation) were implemented by a state owned-parastatal of Indonesia, namely Badan Urusan Logistik (Bulog). Many studies revealed that the involvement of parastatals could cause market failure in accordance with the rent-seeking activities, thus generating high social cost. The consideration of the government to involve Bulog along with the attainment of self-sufficiency targets were not only based on economic reason, but also political background. Researches on self-sufficiency and state owned-parastatal conducted by many researchers and academicians however were either purely economics or politics. These did not cover political economic perspective about rice self-sufficiency by including the involvement of Bulog with its privileges on rice policy instruments.

During the analysis period, the allocation of self-sufficiency fund tended to increase from around IDR 10 trillion in 2002 to more than IDR 67 trillion in 2014; however, did not attain sustainable rice self-sufficiency. The level of self-sufficiency fluctuated between 95-100%. For this reason, research questions arose accordingly, i.e. Whose interest actually counts among the main vested interest groups in the rice market in Indonesia in accordance with the implementation of rice policies as very responsible for state-owned agency executing the policies? The answer to such question can either provide quantitative data for supporting aforementioned criticisms or disprove them. As there is no quantitative data supporting the criticisms, a political econometric model of rice self-sufficiency is then developed to estimate the political preference of the government to measure political weight of producers, consumers, and the government in the rice market. For that reason, this study aims to analyze the political weight of vested interest groups in the rice market where the government implements the rice policy instrument to achieve the rice self-sufficiency in Indonesia.

The strategic role of rice in Indonesia compels the government to achieve self-sufficiency (Sawit 2001, Timmer 2010). Increasing production, affordable rice prices, stable and enough reserve stock, and zero imported rice are set as the targets of rice self-sufficiency program in Indonesia. The government has provided input and output subsidies for rice commodity (Timmer 2004) and involved National Logistics Agency (Bulog) to stabilize rice prices, to manage reserve stock, and to import rice (Sawit 2001, Timmer 2004 and 2010) since the New Order Government Era (Amang and Sawit 2001, Timmer 2004). However, there have been some criticisms concerning the Bulog's involvement. Although it has been criticized that rice self-sufficiency program is not economically efficient (Barker and Hayami 1976) and is a high-cost policy (Timmer 2004), it is commonly executed by most developing countries to avoid vulnerability from the world's rice price and supply (Barker and Hayami 1976). Consequently, the budget allocations could become rather costly due to input subsidy such as fertilizer, seed, and machineries; and for price support during rice procurement and distribution that are conducted by Bulog. This price support policy is supposed to maintain the domestic rice price higher than the world rice price (Barker and Hayami 1976, Timmer 2004). Therefore, the self-sufficiency program results in decreasing national income (Monke and Pearson 1989).

Apart from Indonesia, strategic role of a state-owned parastatal, in Indonesian case is Bulog, is also found in African and other Asian developing countries (Rashid et al. 2005). In Indonesia, with the fact that it uses budget allocation from the government in its operational activities, there could be corruption found to exist in Bulog especially after the early 1990s (McCulloch and Timmer 2008). That is just found similarly taking place in some African countries where such state-owned parastatals are considered as subsidy predators (Neube and Maunganidze 2014), leading to government failure in budget allocation for food policy (Wambua et al. 2005, Zvavahera and Ndoda 2014).

The Indonesian government gives its mandate to Bulog to stabilize the domestic rice prices using the governmental procurement price and the rice sale price during rice procurement and distribution. Bulog maintains the reserve stock and imports rice to fulfill the annual rice procurement

(Amang and Sawit 2001). In addition, Timmer (2004) suggested that such policy is the final policy option. By that, Bulog, the only organization that receives such a privilege and an authority, represents the government to manage the rice market in Indonesia, as those are not given to the others. During 2001-2014 the distribution of rice for the poor through Bulog increased tremendously from around 1.48 million ton to 2.77 million ton which affected the domestic rice supply. This period will then be used to investigate the impact of pro poor regime under the Susilo Bambang Yudoyono (SBY) administration. Such privilege received by Bulog is concerned with market distortion (Lee and Kennedy 2007). Though in a certain commodity market, government plays a significant role as regulator, subsidy provider, and tax collector (Ortiz 1999). In order to regulate the market, government implements policy instruments in the given market structure of commodity where interest groups exist in the market (Barret 1999). For that reason, and also to cope with market failure, the government of Indonesia intervenes in the domestic rice market to achieve self-sufficiency by giving the mandate to a state-owned parastatal, i.e. Bulog.

## METHODOLOGY

### Data

This study was carried out using the secondary aggregate data at the national level from 2001 to 2014. The macroeconomic indicators consisted of the real national income per capita, the exchange rate of rupiah to US dollar, the import tariff of rice from the Central Agency of Statistics (BPS), the Central Bank of Indonesia (BI), the Ministry of Agriculture, and the Ministry of Finance.

As most of the rice produced, traded, and consumed are in medium quality, this study used the retail price of Indonesian Rice III (IR III) as the consumer price calculating the inflation of the food sector. Moreover, Bulog mostly imports Vietnamese 15 per cent broken that is equivalent to the medium rice and IR III. As a consequence, the nominal producer rice price came from the addition between dried unhusked-rice price and the milling cost of medium rice. For this reason, this study excluded steamed rice, aromatic rice, and premium rice (Basmati, Thai hom malli[1], Japonica) in the analysis. In order to calculate the political weight, the share of procurement to the national rice production and the share of distribution to the national consumption are determined as percentage. Two presidential eras or government periods, i.e. Megawati (2001-2003) and SBY (2004-2014) were transformed into dummy variable to distinguish both.

### Analysis method

This study used a political preferential function model to represent the objective of the government of Indonesia to maximize social welfare of rice producer, consumer, and the government. Prior to further analysis using a political preferential function, there were three steps to be done firstly. The first step was to test the variables integration order using the Augmented Dickey-Fuller's unit root test. The only stationary and free from unit root were then used in the following step.

The second step was to estimate the rice demand and supply relation by using an ordinary least square method. The actual rice market structure in Indonesia tends to be a competitive market, which was indicated by free entry to and exit from market, many market players, and homogenous products. Since the analysis focused on the involvement of Bulog in "trading-likewise activity", thus the other traders were then unified into one part as non-Bulog. Therefore, the rice market structure was assumed as an oligopolic market. Hence, a dynamic-oligopolic model (Bresnahan 1982, Lau 1982) was then used to estimate the rice demand and supply relation. The third step was to determine the demand elasticity and the rice supply elasticity. Upon calculation of the demand and supply elasticities, the political weight of vested interest groups in the Indonesian rice market was computed. The function of the demand and supply relation are as follows:

[1] Premium rice quality from Thailand

*The rice demand function*

By adopting the dynamic-oligopolic model, an error correction model (ECM) with short-run parameters represented by one-lagged autoregressive distributed lag (ADL) model was specified below:

$$\Delta Qd_t = \alpha_0 + \alpha_{Pd}\Delta Pd_t + \alpha_Y \Delta Y_t + \alpha_Z \Delta Z_t + \alpha_{PY}\Delta PY_t + \alpha_{PZ}\Delta PZ_t + \alpha_{Qd}\Delta Qd_{t-1} + \gamma[Qd_{t-1} - \Theta_{Pd}Pd_{t-1} - \Theta_Y Y_{t-1} - \Theta_Z Z_{t-1} - \Theta_{PY}PY_{t-1} - \Theta_{PZ}PZ_{t-1}] + \alpha_D D_t + U_t \quad (1)$$

Where Qd is rice demand (kg), Pd is consumer price (IDR/kg), Y is income (IDR/cap), Z is consumer price of rice substitute (IDR/kg), PY is multiplication between Pd and Y being the shifting variable of demand, and PZ is multiplication between Pd and Z being the rotation variable of demand, D is dummy variable of government period, D = zero for 2001-2003 government period; and D = 1 for 2004-2014. The intercept is $\alpha_0$, the estimated coefficients of the long-run parameters are $\alpha_{Pd}$, $\alpha_Y$, $\alpha_Z$, $\alpha_{PY}$, $\alpha_{PZ}$, $\alpha_{Qd}$, $\alpha_D$, and the error term is $U_t$. In the short-run parameters, the estimated coefficients of the variables are $\Theta_{Pd}$, $\Theta_Y$, $\Theta_Z$, $\Theta_{PY}$, and $\Theta_{PZ}$. Hence, prior to the estimation of dynamic demand function, firstly was to compute the adjustment parameter ($\gamma$) from the ADL model to estimate the static demand function.

*The supply relation function*

In terms of the estimated elasticities of supply, the dynamic demand function provides reasonable econometric predictions, because in the supply relation there is a variable that computed using long-run parameters in the dynamic demand function, i.e. Q* and the formula is $Q_t^* = \frac{Qs_t}{\theta_{Pd}+\theta_{PY}Y_t+\theta_{PZ}Z_t}$. By using the same method, firstly computed the adjustment parameter () from short-run to long-run showing by the ADL model in the dynamic supply relation function as follows:

$$\Delta Ps_t = \beta_0 + \beta_{Qs}\Delta Qs_t + \beta_W \Delta W_t + \lambda \Delta Q_t^* + \beta_{Ps}\Delta Ps_{t-1} + \Psi\left[Ps_{t-1} - \xi_{Qs}Qs_{t-1} - \xi_W W_{t-1} - \Lambda Q_{t-1}^*\right] + \beta_D D_t + V_t \quad (2)$$

Where $Ps$ is producer price (IDR/kg), $Qs$ is rice supply (kg), W is rice milling cost (IDR/kg), D is dummy variable represents the governmental period, and $V_t$ is error term. The intercept is $\beta_0$, the estimated coefficients of the long-run parameters are $\beta_{Qs}$, $\beta_W$, $\lambda$, $\beta_{Ps}$, and $\beta_D$. In the short-run paremeters, the estimated coefficients of the variables are $\xi_{Qs}$, $\xi_W$, and $\Lambda$. The adjusted parameter from short-run to long-run is . There are two parameters of market power, i.e. $\lambda$ is long-run market power, while $\Lambda$ is short-run market power. Similar to the estimation of demand, so prior to the estimation of supply relation, was to estimate the adjustment parameter ($\Psi$) from the ADL model.

*The elasticity of demand*

The computation of demand elasticity was conducted, adopting the same formula of Steen and Salvanes (1999). The formula was used previosuly by modifying the dynamic-oligopolic model that was developed by Bresnahan (1982) and Lau (1982).

Short-run elasticity of demand:

$$\left(\eta_{SR}\right) = [\alpha_P + \alpha_{PY}\bar{Y} + \alpha_{PZ}\bar{Z}].\left[\frac{\bar{P_d}}{\bar{Q_d}}\right] \quad (3)$$

Long-run elasticity of demand:

$$\left(\eta_{LR}\right) = [\Theta_P + \Theta_{PY}\bar{Y} + \Theta_{PZ}\bar{Z}].\left[\frac{\bar{P_d}}{\bar{Q_d}}\right] \quad (4)$$

*The elasticity of supply*

The computation of supply elasticity was relatively complicated as it included *Q** in the supply relation function. Zaini (2011) used the same model with Steen and Salvaned (1999) and derived the elasticity formula from supply relation function and found that:

$$(\varepsilon_{SR}) = \left[\frac{\alpha_{Pd}+\alpha_{PY}\bar{Y}+\alpha_{PZ}\bar{Z}}{\beta_{Qs}(\alpha_{Pd}+\alpha_{PY}\bar{Y}+\alpha_{PZ}\bar{Z})+\lambda}\right].\left[\frac{\overline{PS}}{\overline{Qs}}\right] \quad (5)$$

The long run supply elasticity:

$$(\varepsilon_{LR}) = \left[\frac{\Theta_{Pd}+\Theta_{PY}\bar{Y}+\alpha_{PZ}\bar{Z}}{\xi_{Q}(\Theta_{Pd}+\Theta_{PY}\bar{Y}+\Theta_{PZ}\bar{Z})+\Lambda}\right].\left[\frac{\overline{PS}}{\overline{Qs}}\right] \quad (6)$$

Once the elasticities are obtained, the computation of the political weights in rice market was then conducted using the political preferential function model. Firstly, it was to compute the optimum price range as percentage of producer price (A, %), optimum price range as percentage of consumer price (B, %), CIF price of imported rice (*Pw*, IDR/Kg), share of Bulog's procurement to national rice production ($B_{RP}$, %), and share of Bulog's distribution to national rice consumption ($B_{RD}$, %). The quantitative parameters including demand and supply elasticity indicate the potential role of political weight on each implemented price intervention policy (Lee and Kennedy 2007). Through price normalisation, the political weights computed using formula as follows:

$$W_G = \frac{3}{1-B_{RP}\varepsilon_{SR}A+B_{RD}\eta_{SR}B} \quad (7)$$

$$W_P = \frac{-3B_{RP}\varepsilon_{SR}A}{1-B_{RP}\varepsilon_{SR}A+B_{RD}\eta_{SR}B} \quad (8)$$

$$W_C = \frac{3B_{RD}\eta_{SR}}{1-B_{RP}\varepsilon_{SR}A+B_{RD}\eta_{SR}B} \quad (9)$$

$$W_G = 3 - W_P - W_C \quad (10)$$

$$A = \left\{\frac{P_S-P_W}{P_S} + \left(1-\frac{1}{B_{RP}}\right)\frac{P_W}{P_S} + \frac{1}{\varepsilon_{SR}}\right\} \quad (11)$$

$$B = \left\{\frac{P_D-P_W}{P_D} + \left(1-\frac{1}{B_{RD}}\right)\frac{P_W}{P_D} + \frac{1}{\eta_{SR}}\right\} \quad (12)$$

Where *Pw* is CIF price (Rp/Kg), $W_G$ is political weight of the government, $W_P$ is political weight of producer, and $W_C$ is political weight of consumer.

Once the political weights of the three vested interest groups were obtained, estimation of political econometric model to analyze rice self-sufficiency ratio from political economic perspective as follows:

$$SSR = \delta_0 + \delta_P W_P + \delta_C W_C + \delta_G W_G + \delta_A A_R + \delta_Y Y + \mu_t \quad (13)$$

$$SSR = \frac{Q_S}{Q_S+M-X} \quad (14)$$

where SSR is rice self-sufficiency ratio, $A_R$ is national rice harvested area (ha), M is import of rice (kg), X is export of rice (kg), $\delta_0$ is intercept, $\delta_P, \delta_C, \delta_G, \delta_A,$ and $\delta_Y$ respectively are estimated coefficients of the corresponding variable, and $\mu_t$ is error term.

## RESULTS AND DISCUSSION

### Demand and supply relation estimates

Based on the results of the variables integration order test, it was found that the variables were non-stationary at the levels, but stationary in the first differences at reasonable level of significance. Hence, those stationary ones, the first different, were then used in the estimation of the rice demand and supply relation to obtain its elasticities. On the demand side, the current consumer price ($\Delta Pd_t$), the real national income per capita ($\Delta Y_t$), the current substitute price ($\Delta Z_t$), the current rotating factor ($\Delta PY_t$), the current shifting factor ($\Delta PZ_t$), the lagged demand ($Qd_{t-1}$), and the government period ($D^2$) affect rice demand significantly at 90-95 per cent confidence level. The inelastic price elasticity of demand ($\eta_{SR}$ = -0.11; $\eta_{LR}$ = -0.14) emphasizes that rice is the main staple food in Indonesia. While the estimate coefficient of substitute price shows that maize is not a substitute, but, is a complement since the rice demand decreases when the maize price increases (Table 2). It may indicate that the consumers do not solely change to maize's price decreases. This finding may imply that diversification from rice to non-rice staple food does not work well. The other fact of demand is the distribution of rice for the poor family (Raskin) during 2004-2014 caused the decrease in rice demand.

**Table 2.** OLS Estimates of the dynamic demand function.

| Variable | Parameter | Coefficient | St. Dev. | t-Statistic | Prob. |
|---|---|---|---|---|---|
| C | $\alpha_0$ | 1.36E+10 | 2.78E+09 | 4.8957* | 0.0163 |
| $\Delta Pd_t$ | $\alpha_{Pd}$ | -6.10E+08 | 85245803 | -7.1530** | 0.0056 |
| $\Delta Y_t$ | $\alpha_Y$ | 722.0440 | 151.5221 | 4.7653* | 0.0176 |
| $\Delta Z_t$ | $\alpha_Z$ | -9.46E+08 | 1.96E+08 | -4.8225* | 0.0170 |
| $\Delta PY_t$ | $\alpha_{PY}$ | -20.7517 | 3.218325 | -6.4480** | 0.0076 |
| $\Delta PZ_t$ | $\alpha_{PZ}$ | 26162996 | 5172820. | 5.0578* | 0.0149 |
| $\Delta Qd_{t-1}$ | $\alpha_{Qd}$ | -1.7735 | 0.300563 | -5.9006** | 0.0097 |
| *D* | $\alpha_D$ | -2.30E+09 | 4.32E+08 | -5.3178* | 0.0130 |
| $U_{t-1}$ | $\gamma$ | -0.322197 | 0.094080 | -3.4247* | 0.0417 |
| *R-squared* | | 0.9569 | | | |
| Long-run parameter | | | | | |
| $Pd_{t-1}$ | $\Theta_{Pd}$ | 7.14E+08 | | | |
| $Y_{t-1}$ | $\Theta_Y$ | 246.1786 | | | |
| $Z_{t-1}$ | $\Theta_Z$ | 8.18E+08 | | | |
| $PY_{t-1}$ | $\Theta_{PY}$ | -1.7776 | | | |
| $PZ_{t-1}$ | $\Theta_{PZ}$ | -20584571 | | | |
| Elasticity | | | | | |
| Short-run | $\eta_{SR}$ | -0.1122 | | | |
| Long-run | $\eta_{LR}$ | -0.1415 | | | |

Source: Secondary data analysis, 2016.
Notes: * Prob. < 0.90%; ** Prob. < 0.95%.

The estimates of the supply relation model shows that the variables of current rice supply were obviously influencing the current rice price. Regardless to the low significance level of the estimates, the short run own price supply elasticity ($\varepsilon_{SR}$) was 1.43 (Table 3). This implies that price was the only factor affecting farmers to cultivate their land. Phiri (2013) conducted political economic analysis of the maize market in Malawi and revealed that the price was the most important concern of the producers and affected the formulation of the political policy. The implemented public policies in agriculture may impart negatively to producers and even taxed through the policy that transfer income

[2] *D* = 0 government period 2001-2003, D =1 government period 2004-2014.

from the producers to the consumers through the price mechanism. This occurred because the non economic motives gave more influence to the most influential policy maker.

**Table 3.** OLS Estimates of the supply relation function.

| Variable | Parameter | Coefficient | St. Dev. | t-Statistic | Prob. |
|---|---|---|---|---|---|
| $\Delta Qs_t$ | $\beta_{Qs}$ | 9.42E-10 | 1.39E-09 | 0.6753 | 0.5247 |
| $\Delta W_t$ | $\beta_W$ | -0.9351 | 0.9033 | -1.0352 | 0.3405 |
| $\Delta Q^*_t$ | $\lambda$ | -0.0003 | 0.0003 | -0.9855 | 0.3624 |
| $\Delta Ps_{t-1}$ | $\beta_{Ps\ t-1}$ | 0.0657 | 0.3800 | 0.1729 | 0.8684 |
| D | $\beta_D$ | 17.4824 | 10.9106 | 1.6023* | 0.1602 |
| $V_{t-1}$ | $\Psi$ | -0.3040 | 0.1816 | -1.6742* | 0.1451 |
| *R-squared* | | 0.4245 | | | |
| Long-run parameter | | | | | |
| $Qs_{t-1}$ | $\xi_Q$ | 1.82E-09 | | | |
| $W_{t-1}$ | $\xi_W$ | -0.5341 | | | |
| $Q^*_{t-1}$ | $\Lambda$ | -0.0003 | | | |
| Elasticity | | | | | |
| Short-run | $\varepsilon_{SR}$ | 1.4310 | | | |
| Long-run | $\varepsilon_{LR}$ | 0.7406 | | | |

Source: Secondary data analysis, 2016.
Note: * Prob. < 90%.

**Political weight estimates**

The estimation of political weight used the short-run price elasticities of rice demand and supply in the adopted formula of Johnson (1995). The estimation showed that the biggest political weight is achieved by the government, followed by the producer and lastly the rice consumer (Table 4). As political weight indicates political preference of government and lobbying power of vested interest groups, it implies that the implemented rice policy instruments is biased to the government itself, rather than to the rice producers and the consumers. It proves that Bulog received the highest political preference, representing the government interest.

**Table 4.** The average[1)] political weight of vested interest groups in rice market of Indonesia.

| Vested Interest Group | Political Weight |
|---|---|
| Producer ($W_P$) | 0.5375(17.67%) |
| Consumer ($W_C$) | 0.2324(7.67%) |
| Government ($W_G$) | 2.2401(74.67%) |

Source: Secondary data analysis, 2016.
Note: 1) 2001-2014.

**Political econometric estimates**

Due to the colinearity problem, the estimation of the political econometric function model excluded the constant coefficient of the regression. The estimation result of the political econometric function model of rice self-sufficiency (Equation 13) shows that the magnitude of political weight of the producer and the government directly affect the achievement level of the rice self-sufficiency in Indonesia (Table 5). This indicates that the greater political weight of producer and government will lead to the higher achievement level of rice self-sufficiency. On the other hand, the magnitude of political weight of consumer affects the decreasing level of rice self-sufficiency. It implies that the

achievement of self-sufficiency only could be achieved by involving the producer and government groups and minimizing the involvement of the consumer group.

As political weight is the approach of power or coefficient of lobby (Becker 1983, Zusman 1994), therefore the degree of government intervention in the rice market should be shifted from regulation to facilitation of the producer groups. The active participation of the producer under a good facilitation from the government can result in more productive rice farming. Hence, rice production is increased. As a result, sustainable rice self-sufficiency can be achieved, with resultant improvement of both producer and consumer welfare.

**Table 5.** OLS Estimates of the political econometric function model.

| Variable | Parameter | Coefficient | St. Dev. | t-Statistic | | Prob. |
|---|---|---|---|---|---|---|
| $W_P$ | 0.246652 | 0.098804 | 2.496365 | 0.246652 | * | 0.0341 |
| $W_C$ | -0.21119 | 0.260554 | -0.81056 | -0.21119 | | 0.4385 |
| $W_G$ | 0.266694 | 0.097933 | 2.723224 | 0.266694 | * | 0.0235 |
| $A_R$ | 2.81E-11 | 2.75E-11 | 1.021846 | 2.81E-11 | | 0.3335 |
| Y | -2.01E-09 | 2.08E-09 | -0.96578 | -2.01E-09 | | 0.3594 |
| R-squared | 0.339926 | | | | | |

Source: Secondary data analysis, 2016.
Note: * p < 0.90%.

The real example from the policy choices are, the price subsidy may be reallocated from import expenditure to agricultural infrastructure subsidy, such as agricultural roads, public irrigations, extensions, and post-harvest technology as the strategies that increased rice production successfully during the New Order Government Era (Amang and Sawit 2001, Timmer 2010). The involvement of Bulog in stabilizing rice prices and reserve stock is still needed. Yet the involvement of Bulog in stabilizing rice prices and reserve stock is important. Therefore, Bulog's procurement from domestic production must be endorsed and encouraged more than importation of rice. However, the existing procurement system using the governmental procurement price will obviously squeeze profit margin of private rice traders, i.e. non-Bulog (Timmer 1986). Consequently, by implementing price policy through Bulog, it shows that the government has more preference to Bulog than to non-Bulog. This emphasizes why the political weight of the government is the highest among the three vested interest groups in the rice market of Indonesia. This implies that this political economic study can simplify the actual rice market in Indonesia from a perfect competition market structure to an oligopolistic market structure.

Comparing to the other political economic studies on agricultural commodity, in producing country such as sugar in the Philippines (Lopez 1994) and rice in USA, Republic of Korea, and Japan (Lee and Kennedy 2007), the policies were biased to producer. In 13 developing countries, wheat policies were biased to consumer, while in 12 developed countries, wheat policies were biased to producer (Sarker et al. 1993). Those prove that commodity policy does not depend on the development level and function of commodity in certain countries, but depends on the political preference of the government.

Government intervention is a common nature in agricultural sector. Hence, typical intervention depends on political preference of the government. In case of rice in Indonesia, the subsidy is the dominant one. This choice of such policy is in line with the target to increase production that is mostly conducted in developing countries. It is confirmed with the budget allocation of the food security program during 2002-2014, that was dominated by the subsidy, followed by transfer and government expenditure (MOF 2016). During the analysis period, the budget of the food security programs increased from around IDR 10.26 trillion in 2002 to more than IDR 67.77 trillion in

2014, or increased by 5.77 per cent of growth rate. These findings are consistent with that of Amang and Sawit (2001) where the subsidy is provided starting from on farm and off farm activities to marketing activities, from regional level to national level, from individual life support to collective or public investment.

By design, the intervention in terms of subsidies is to support rice producer. Among the given subsidies, seed and subsidy are given to support and encourage rice producer to apply the recommended dosage of fertilizers, especially urea, the most influencing fertilizer on rice yield (Osorio et al. 2011). Other types of input subsidies, the government provides general food subsidy in term of output subsidy that is given to targeted consumer, i.e. rice for the poor family (Raskin). However, based on the analysis, the aggregate rice policies shows that those policies are biased to the government. The future rice policies are suggested to be designed to be neutral among the three vested interest groups of the rice market in Indonesia accordingly.

The distinguished magnitude of political weight between the government and the producer may help in elaborating next budget allocation related to typical subsidies of rice. The future rice policies must not substitute each other, but complement one another. The existing policies may be sustained by combining with intensive agricultural investment such as irrigations, agricultural roads, extensions, and postharvest management. Such policies have been conducted by the current presidential era, where irrigation is one substantial program among others in *Upaya Khusus Padi Jagung Kedelai* Program (Special Effort on Rice Maize and Soyabean). By then, the domestic production would increase, self-sufficiency could be achieved, the price could be stable, and lower risk of the world's price and supply vulnerability. Hence, the welfare of the producers and the consumers would be improved systematically.

## CONCLUSION

Rice policies in Indonesia are biased to government wherein the highest political preference of the government is received by the government itself, followed by the rice producers and the rice consumers. Sustainable rice self-sufficiency could only be achieved by involving the producer and the government groups, as both vested-interest groups contribute to the achievement level of rice self-sufficiency. The shifting of the rice market structure from a perfect competition to an oligopolistic competition, can provide proof that giving privilege to Bulog to stabilize rice prices and to import rice result in the highest political weight of government in the rice market. The responsiveness of the producers to the rice prices, the price-stabilization policy instrument gives more incentive to the producers rather than save government expenditure. In order to improve the rice self-sufficiency performance, the government needs to increase the active participation of the producer groups under support and facilitation from the government groups in order to achieve the self-sufficiency target.
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# ANALYSIS OF POSTHARVEST HANDLING OF TABLE EGGS PRODUCED IN SAN JOSE, BATANGAS, PHILIPPINES

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## ABSTRACT

The study analyzed the postharvest handling practices done on table eggs by the market participants in San Jose, Batangas, Philippines through a survey conducted from February to May 2016. Data obtained from 60 farmer-respondents and 31 market intermediary-respondents were subjected to cost and returns analysis, partial budgeting, and combination of price margin and ladder pricing analysis along with descriptive analysis. Forward tracing was performed to identify the channels where table eggs passed before reaching final consumers. Farmers, assembler-wholesalers, wholesalers and retailers were involved in the marketing of table eggs produced in San Jose, Batangas. Practices performed can be a combination of the following: assembly, cleaning, sorting and grading, storing, packaging and transporting. Assembly and transporting are usually done by the assembler-wholesalers. Farmers store the eggs at their houses while market intermediaries have their own storage houses. Farmers used polystyrene trays whereas market intermediaries used the cheaper egg carton trays. Results revealed that small- to medium-scale farmers and traders manually sorted and graded the eggs while only a few large-scale farmers used mechanized grading facilities. Both did not conform to the Philippine National Standards. Re-grading was practiced along the marketing chain resulting to re-grading profit. It was concluded that farmers and market-intermediaries who adopt postharvest handling practices generated more profit. Promoting awareness on the proper performance of cleaning as a postharvest handling practice to minimize losses was recommended. The use of uniform standards by the BAFPS-PNS was likewise suggested to be properly implemented in cooperation with the local government units (LGU) serving as the front-liner.

**Key words:** ladder pricing, re-grading, egg quality standards, profitability

## INTRODUCTION

The poultry and chicken industry is the most progressive enterprise and the world's fastest producer of meat (PCAARRD 2010). The country's total chicken population is shared by broilers, native chicken and layers. In 2014, the United States Department of Agriculture (USDA) International Egg and Poultry section reported that the total chicken population of the Philippines was 172.41 million heads broken down into 64.70 million heads of broilers, 78.48 million heads of native chicken and 29.23 million heads of layers. According to the Philippine Statistics Authority (PSA 2017) the inventory of broilers, native chicken, and layers dropped compared with the previous year by 3.16%, 0.04% and 7.40%, respectively. As of January 2017, total chicken inventory of the Philippines was estimated at 175.33 million birds, 1.93% lower than that of 2016. However, layer chicken inventory increased by 6.97%. In 2017, this inventory accounted for 20% of the total chicken inventory in the country, 36.59% of which is reported to be in Region IV-A. Production-wise, in 2016, chicken egg was estimated to be 461,719 metric tons with 23.01% contributed by Batangas (PSA 2017).

The average farm gate price of eggs in San Jose, Batangas increased by 2.42% from PhP4.04 to PhP4.29 per piece in 2014 (PSA 2017). Likewise, wholesale and retail prices increased by 2.28% and 2.4%, respectively. By 2016, average annual farm gate price was 5.12% higher than its 2015 level (PSA 2017). The question of what could have caused such price increases is a relevant and important one considering that table eggs are one of the cheapest sources of protein.

Table eggs are prone to breakage leading to postharvest losses suggesting the need to employ postharvest handling practices. The flow of activities from harvest to the point of consumption is referred to as postharvest handling (Nagpala as cited by Lantican 2015) which aids in strategically selling the eggs to maximize the market participants' profit. It is usually done to minimize the losses incurred along a marketing chain and to facilitate distribution. In the Philippines, postharvest handling practices for table eggs involve the assembly, cleaning, sorting and grading, storing, packaging and transporting. While it is expected to lead to efficiency in the egg marketing system, such needs to be verified as it also entails cost that could undermine the possible net gain from any practice.

Chicken eggs produced also vary in size and quality and prices fluctuate accordingly. Grading is done "to eliminate inedible and defective eggs; separate eggs into high and lower acceptable categories; and establish uniform weight classifications" (FAO 2003). It makes possible the orderly marketing of eggs by avoiding confusion and uncertainty with respect to quality values. Uniform standards in grading also promote efficiency in the distribution system (USDA 2000). Quality of eggs is based on interior factors such as condition of the white and yolk and size of the air cell as well as cleanliness and soundness of the shell. In the Philippines, more frequently, eggs are classified according to weight (or size) expressed in grams per egg. Although eggs are not sold according to exact weight, they are grouped within relatively narrow weight ranges or weight classes.

Standardization is the implementation of rules or any specifications for a repeated use to attain consistency that should help the farmers to obtain optimum profit (Lakhotia 2015). It is advantageous to standardize the grades as it results to high quality eggs that consumers are willing to pay establishing trust between the buyers and sellers (FAO 2003). Despite being the "Egg Basket of the Philippines," grading and standardization in eggs is not regularly and properly practiced in San Jose, Batangas (Calora 2015). Initially, the local government unit posted a billboard listing the standard weights that should be followed in sorting and grading of eggs. However, such posting is no longer existent in San Jose. As a consequence, during grading, it is common practice to modify the grade set for eggs as these are moved along the marketing chain in order to take advantage of the price premium for "large" eggs. Sometimes, there is re-grading being done violating the PNS for eggs and literally exploiting not only the farmers but consumers as well.

This study determined the profitability of performing egg postharvest practices, verified and documented instances of re-grading or non-standardization, and quantified the undue advantage in terms of additional profit being charged to consumers or have been lost to the farmers. Given the importance of eggs in poor man's diet, it is imperative that egg prices are at its most efficient. An important egg industry player such as San Jose Batangas, should be at the helm when it comes to promoting fair trade practice. The findings of the study could therefore help local chief executives to craft measures that will further improve not only the efficiency of pricing but the whole gamut of processes involved in bringing the eggs to the consumer's table. Along with the improvement of the industry should be the protection of the consumers in terms of reasonable prices for table eggs.

The general objective of the study was to analyze the postharvest handling practices done for table eggs produced in San Jose, Batangas. The study also: identified the market participants involved in the marketing chain of table eggs; described the postharvest practices performed by the market participants along the chain; assessed the profitability of performing postharvest practices; and determined the price differences in table eggs along the marketing chain. The study was anchored on

the hypotheses that performing postharvest practices adds profit and market participants are able to enjoy undue economic advantage when standardization in egg grades is not implemented.

## RESEARCH METHODOLOGY

The study was conducted in the municipality of San Jose, Batangas because of its large number of small- to medium-scale egg producers. As discussed earlier, San Jose, Batangas is well-known as the "Egg Basket of the Philippines" because it provides a large portion of the country's supply of table eggs. Farmers were randomly chosen from the list of the registered table egg farmers per barangay. Other participants in the chain were traced through the interviewed farmers and traders. Primary data were collected through personal interviews using pre-tested interview schedule. Data collected included cost of performing postharvest practices, volume bought and sold, sorting and grading procedure, grades, and prices of eggs produced and marketed which were subjected to descriptive analysis using means, frequencies, and percentages. Those performing similar postharvest practices were identified and grouped for the conduct of cost and returns analysis. Results of this analysis were used in partial budgeting which compared the groups with similar practices except for the one whose profitability is being tested. In addition, a combination of profit margin and ladder pricing analysis was performed to assess the differences in table egg prices along the identified marketing chain. Price ladder allowed a clearer understanding of how market participants within a particular market level (price ladder) set their price in order to increase their profit. This is very important in the case of table eggs because of the greater tendency of the egg grade to be changed as it moved from one level to another within the same marketing chain. Analyzing the marketing margin within the ladder allows one to distinguish the amount of price change due to regular margin and the price change due to re-grading. Re-grading is a violation of the standards set by the PNS.

## RESULTS AND DISCUSSION

### Market participants and postharvest practices performed

A total of 91 respondents comprised of farmers (60); assembler-wholesalers (5); wholesalers (15); and retailers (11) were personally interviewed. Assembler-wholesalers also known as *viajeros* are the type of middlemen who only deal in large quantities. They usually go from one farm to another to pick up the eggs and assemble for bulk distribution to either wholesalers or retailers. Wholesalers also pick up and buy eggs in bulk for selling to retailers or fellow wholesalers. On the other hand, retailers buy the eggs from wholesaler or directly from the farmers. Retailers are the final link in the marketing system and usually offer the highest price in small quantities. Two marketing channels were identified in the study: farmer→assembler-wholesaler→wholesaler→retailer and farmer→wholesaler→retailer. It is expected that the longer the channel the higher the price consumers had to pay because of additional marketing margin per added intermediary or level. In other commodities, a longer channel and/or wider marketing margin does not automatically imply marketing inefficiency because other necessary services that entail cost might have been performed in order to satisfy the need of consumers. However, in the case of eggs, additional channel participant might only result to price padding without necessarily resulting to added value because nothing is usually done to the product except, in some cases, transport them.

The postharvest practices for table eggs include a combination of the following: assembly; cleaning/washing; sorting and grading; storage; packaging; and transporting (Table 1).

#### *Assembly*

Assembly is the practice of collecting eggs from different farms and storing them at a warehouse or storage house. This is the main function of the assembler-wholesalers who go from one farm to another to buy eggs in bulk and lump them for distribution to wholesalers. Some retailers who

are neighbors of the farmers also bought from several farms but they no longer distribute them to big buyers unlike the assembler-wholesalers.

**Table 1**. Distribution by postharvest handling practice done on table eggs, 60 farmer-respondents and 31 market intermediary-respondents, San Jose, Batangas, Philippines, 2016

| Postharvest Practice | Farmers (n=60) | % | Intermediaries (n=31) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A-W (n=5) | | W (n=15) | | R (n=11) | |
| | | | Number | % | Number | % | Number | % |
| Assembling | 0 | 0 | 5 | 100 | 0 | 0 | 0 | 0 |
| Cleaning | 34 | 57 | 5 | 100 | 11 | 73 | 3 | 27 |
| Sorting and Grading | 29 | 48 | 5 | 100 | 15 | 100 | 11 | 100 |
| Storing | 60 | 100 | 5 | 100 | 15 | 100 | 11 | 100 |
| Packaging | 60 | 100 | 5 | 100 | 7 | 46 | 7 | 64 |
| Transporting | 29 | 48 | 4 | 80 | 15 | 100 | 0 | 0 |

A-W = assembler-wholesaler; W=wholesaler; R = retailer

***Cleaning/washing***

Even during egg collection, primary sorting is being performed to separate the "*luno*" or those with soft shells and are "dirty" (contaminated with blood or fecal matter). Cleaning of dirty eggs is usually done to ensure their good quality because food safety is important. Farmer-respondents however, are of differing opinions regarding washing of eggs with some believing that washing them will cause spoilage. As such, only 57% of them clean dirty eggs by wiping them out with a piece of cloth in the case of small-scale farmers and using tap water for large-scale farmers. In the case of intermediary-respondents, those who picked-up from the farms were the ones who cleaned dirty eggs. In countries where poultry farms are at their cleanest possible state, washing of eggs is prohibited as it is believed to cause the incidence of salmonella infection. However, in the Philippines where clean production areas cannot be assured, especially for backyard raisers, cleaning of dirty eggs is regarded as good practice to promote food safety. Technically, spoilage of washed eggs can be prevented if the cleaning water is set at 10 to 20 degrees warmer than the eggs. This also facilitates easy removal of the dirt (VSU 2009).

***Sorting and grading***

Sorting and grading is vital as it can improve price setting. The practice of grading and sorting is simultaneously done and it can either be manual or mechanized. Manual sorting and grading is the easiest and an inexpensive way of classifying eggs. However, such practice leads to inconsistencies in grading because it is performed based only on size and exterior quality. Egg size is determined using "eyeball" estimation or by "feeling" egg weight on the sorter's hand and assigning it to particular grade therefore, time consuming. On the other hand, mechanical egg sorter is costly but it does not only give accurate grades – it is also able to check the interior quality of the eggs in a shorter period of time (USDA 2000). Aside from the expected improvement in labor efficiency, exact selection of the eggs will save good eggs from being wasted due to erroneous grading. Despite this advantage, only 48% of the farmers interviewed performed sorting and grading and among them were only a few large-scale ones who had the financial capability to acquire mechanical egg sorter. It would have been better for the study to compare the profitability of using the two methods of egg sorting and grading but the lack of respondents performing mechanized egg sorting prevented the inclusion of this analysis.

On top of the required weights for each class of eggs, the PNS has the following minimum requirements: must be fresh; must be clean and free from visible cracks; must be practically normal in shape; and must be free from foreign odors. Nothing has been mentioned that would require eggs to

be graded according to interior quality. This has some implications in terms of harmonizing standards with trading partners if ever eggs produced in the Philippines will be traded internationally. For instance, Thailand has the following egg interior quality requirements: air cell shall be small with height of not more than 0.8 cm and it does not move when the egg is twirled; for the broken-out egg, the yolk shall not attach to the inner shell; it shall be firm and surrounded by the thick egg white; the egg shall not be spoiled and must be free from abnormal odor; the yolk shall have normal and consistent color; the egg white shall not be cloudy; and free of visible mold (TAS 2010). Almost the same are being required by the USDA for traded eggs in the USA (USDA 2000). The grades used by the market participants and those set by the BAFPS-PNS are shown in Table 2. There are wide discrepancies in the assigned weights per grade by the different market participants and all of them are lower than the PNS. The large-scale farmers had the highest weights assigned per grade but still such were much lower than the PNS set weights suggestive of non-standardization in the egg industry.

**Table 2**. Grades set by 29 farmer-respondents and 31 trader-respondents versus Philippine National Standards, San Jose, Batangas, Philippines, 2016.

| Grades | Small-Scale to Medium-Scale Farmers | Large-Scale Farmers | Traders | Philippine National Standards |
|---|---|---|---|---|
| | | Weight in Grams | | |
| Small | <40 | 38-44 | <35 | 50-55 |
| Medium | 41-50 | 45-54 | 36-40 | 56-60 |
| Large | 51-55 | 55-60 | 41-50 | 60-65 |
| Extra Large | 56-60 | >61 | 46-50 | 65-70 |
| Jumbo | 61 | >63 | >51 | 70 |

Generally, the traders had the least weight assignment per grade among all the respondents. This is because they practice re-grading specifically when there is high demand and yet low supply of eggs. On the average, 61% of them practiced re-grading. Retailers have the highest proportion (64%) of those who re-graded (Table 3). Re-grading essentially implies non-standardization.

**Table 3**. Distribution of 31 market participants by practice of re-grading, San Jose, Batangas, Philippines 2016.

| Market Participant | Re-Grading | | Not Re-Grading | |
|---|---|---|---|---|
| | Number | % | Number | % |
| Assembler-Wholesaler | 3 | 60 | 2 | 40 |
| Wholesaler | 9 | 60 | 6 | 40 |
| Retailer | 7 | 64 | 4 | 36 |
| Total/Average | 19 | 61 | 12 | 39 |

***Storage***

Another postharvest handling practice done on eggs is storing. The main objective of storage is to prolong the shelf life of the eggs and its performance is heavily dependent on the speed eggs are disposed. Eggs not disposed within the day are immediately transferred to the storage area. In the tropics, eggs can deteriorate very quickly unless they are stored at low temperatures. The ideal temperature for storage in such climate is 13°C or lower, usually between 10° and 13°C. The relative humidity should be between 80 and 85% to minimize the loss of egg moisture (FAO 2003). In the Philippines, small- to medium-scale farmers usually store eggs in their house or in a simple cemented building. This is in contrast to well-ventilated storage houses used by large-scale farmers. All the farmers who store eggs do so mostly during summer time at the maximum of only three days.

***Packaging***

Proper packaging is very important during transportation because eggs are frangible, thus all farmers are performing this activity. There are two common types of primary packaging for eggs and the most popular and the cheapest are carton trays made from wood pulp. The more durable ones are plastic or polystyrene trays. During delivery, trayed eggs are placed in carton boxes. Each carton box contains 30 trays for a total of 900 eggs per box. All the farmers interviewed used polystyrene instead of carton trays. Traders provide their own egg carton trays during pick-up from the farms. The usual practice is while being transferred to their own trays eggs are already being sorted and graded at the same time. This maximizes the use of man-hours among the hired laborers. All of the traders used carton egg trays for the primary packaging of the eggs. They are mostly selling the carton trays along with the eggs.

***Transporting***

Transporting is vital to the marketing of eggs as it makes them available where they are demanded by the consumers. Only 29 (48%) of the farmers transported their eggs since the majority opted for them to be picked up (Table 1). These are usually the small- to medium-scale farmers who do not have the vehicle necessary to deliver the eggs directly to their buyers. This implies that most of the delivery of the eggs was done by the large-scale farmers. Assembler-wholesalers and wholesalers do the transporting of eggs for those farmers who do not deliver.

**Geographic and product flows**

The geographic flow of eggs from 60 farmer-respondents is shown in Figure 1. It can be seen that the major market for eggs produced in San Jose, Batangas is Metro Manila since it captured the largest proportion (35%) amounting to 570 boxes and 25 trays. The least proportion with 6 boxes (0.36%), were sold to Bicol traders due to long distance traveled. Laguna is another major market capturing 32% and this is mainly due to accessibility and good markets. Within Laguna, Sta. Rosa and Calamba were the noted main destinations since the two municipalities are densely populated with workers employed by their in-house industrial and science parks. Around 17% were also distributed within Batangas while the nearby Cavite had 12% share.

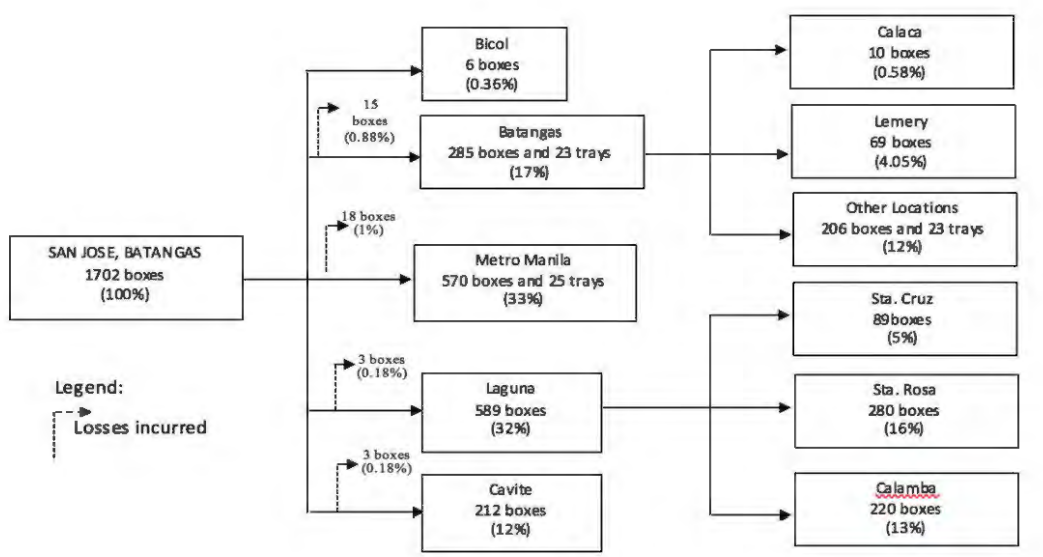


**Fig. 1.** Geographic flow of table eggs produced per week, 60 farmer-respondents, San Jose, Batangas, 2016.

Considering product flows, the marketing channel for eggs was found to be relatively short with only four participants or levels as the longest. The most number of table eggs sold by the farmers were to the wholesalers (932 boxes/week) while the least were sold to individual consumers (228 boxes/week), equivalent to almost 55 and 13%, respectively (Fig. 2). Individual consumers who bought from the farmers are those who live near the farms. In San Jose, Batangas, layers are literally raised in the backyard of many farmers that is why it is easier for the consumers to buy from the farms.

During transport from the farm to the destination markets, losses were incurred and as expected, Metro Manila deliveries which were the second farthest (against Bicol) sustained the highest with around 1% equivalent to 18 boxes per week. Overall, a total of 39 boxes of eggs were lost to farmer due to breakage, "*luno*," and those that were given away. Eggs that were given away are usually to relatives and friends and those that are small and have thin and dark colored shells.

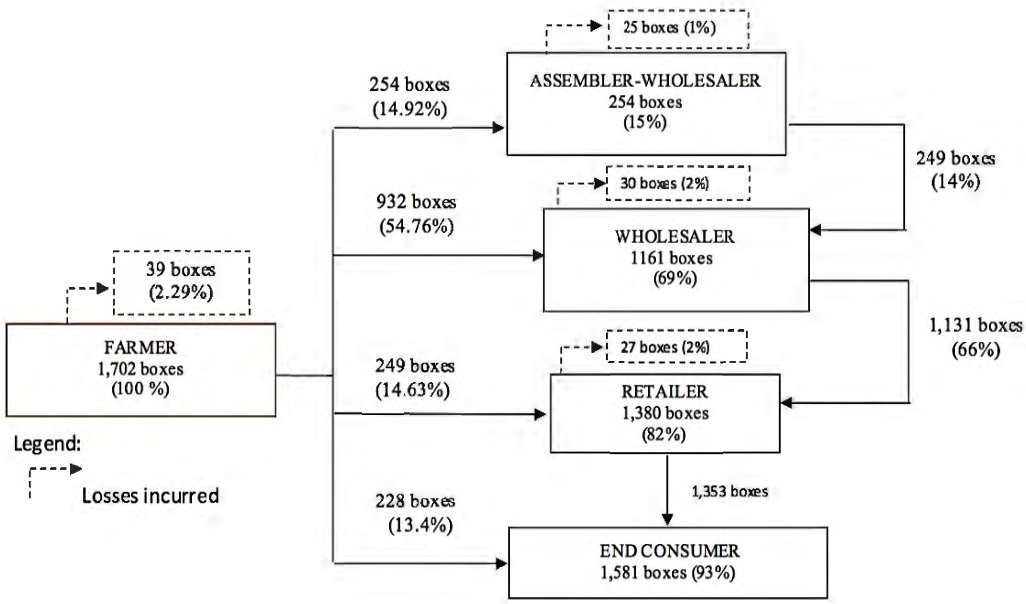


**Fig. 2.** Weekly product flow of table eggs, 60 farmer-respondents, San Jose, Batangas, Philippines

**Table egg prices**

Results of the study further revealed that prices paid for eggs are dependent on the type of intermediaries handling the eggs, mode of sale, and size. All the farmers sold on pick-up basis. Assembler-wholesalers who picked up eggs from farmers paid PhP3,454 per box of large eggs and only PhP2,890 per box of small eggs. For those assorted, they paid an average of PhP3,364 per box (Table 4). The market intermediary-respondents preferred buying the eggs assorted and do the sorting and grading themselves because of the advantage of being able to classify them based on their own set standard. While they claimed that they usually follow the grades set at the farmer's level, they also admitted that they are re-grading the eggs when there is low supply but high demand in the market.

Among those who picked-up the eggs, wholesalers paid an average of PhP3,674 per box to assembler-wholesalers, which is much lower than the average price paid by the retailers (PhP4,298/box). On the other hand, the retailers paid a higher price (PhP4,416/box) when they bought from the wholesalers. The consumers who bought from the wholesalers enjoyed a lower price of PhP4,433 per box as opposed to PhP4,975 per box that they paid to the retailers (Table 4). These

suggest that indeed buying in bulk is advantageous and that buying direct cuts the prices down. Delivered eggs are priced higher than those that were picked up due to transfer cost.

**Table 4**. Average prices paid for eggs (PhP/Box**)** by market outlet to different sources and by grade, 31 market intermediary-respondents, San Jose, Batangas, 2016.

| Market Outlet/Egg Grade | Farmer | Assembler-Wholesaler | | Wholesaler | | Retailer |
|---|---|---|---|---|---|---|
| | Picked-Up | Picked-Up | Delivered | Picked-Up | Delivered | Picked-Up |
| **Assembler-Wholesaler** | | | | | | |
| Small | 2,890 | - | - | - | - | - |
| Medium | 3,267 | - | - | - | - | - |
| Large | 3,454 | - | - | - | - | - |
| Assorted | 3,364 | - | - | - | - | - |
| **Wholesaler** | | | | | | |
| Small | | 3,467 | - | - | - | - |
| Medium | | 3,613 | 3,645 | - | - | - |
| Large | | 3,845 | - | - | - | - |
| Assorted | | 3,770 | - | - | - | - |
| **Average** | | 3,674 | - | - | - | - |
| **Retailer** | | | | | | |
| Small | | 4,374 | - | 4,367 | 4,745 | - |
| Medium | | 4,428 | 4,543 | 4,390 | 4,974 | - |
| Large | | 4,773 | - | 4,680 | - | - |
| Extra Large | | - | 4,689 | 4,875 | - | - |
| Jumbo | | - | - | 4,880 | - | - |
| Assorted | | 4,095 | - | 3,976 | - | - |
| **Average** | | 4,416 | 4,616 | 4,298 | 4,860 | - |
| **Consumer** | | | | | | |
| Small | | - | - | 4,320 | - | 4,350 |
| Medium | | - | - | 4,456 | - | 4,650 |
| Large | | - | - | 4,650 | - | 5,100 |
| Extra Large | | - | - | 4,680 | - | 5,400 |
| Jumbo | | - | - | 4,727 | - | 5,450 |
| Assorted | | - | - | 4,743 | - | 4,900 |
| **Average** | | - | - | 4,433 | - | 4,975 |

**Profitability of performing postharvest handling practices**

Performance of postharvest practices proved profitable as shown by the highest net income (PhP4,456.26) generated by those (Group 4) who did five practices and the least (PhP4,246.48) by those (Group 1) who had two practices only (Table 5). Similarly, for intermediary-respondents, Group 5 with the most number of practices gained the highest net income (PhP5,795.31/box) while Group 1 had the lowest at PhP5,673.81 per box (Table 6).

Using partial budget analysis, net income from each postharvest practice was determined by comparing the groups with similar practices except for one the profitability of which was measured. Under this approach only cleaning and sorting and grading were isolated for the farmer-respondents and transporting and cleaning for intermediary-respondents. Table 7 revealed that sorting and grading generated a net income of PhP347.30 for the farmers while for cleaning they were able to gain an additional net income of PhP186.96 per box. For the intermediary-respondents, additional net incomes of PhP23.60 and PhP19.33 per box for transporting and cleaning, respectively, were acquired. Farmers and intermediaries were able to charge a premium price for cleaned and sorted eggs hence the additional income.

**Table 5**. Net income (PhP/box) by grouping on postharvest handling practices adopted, 60 farmer-respondents, San Jose, Batangas, Philippines, 2016

| Item | Group 1[a] n=17 | Group 2[b] n=14 | Group 3[c] n=9 | Group 4[d] n=20 |
|---|---|---|---|---|
| REVENUE | | | | |
| Quantity sold (per box) | 1.00 | 1.00 | 1.00 | 1.00 |
| Selling price (PhP/box) | 4,858.02 | 4,917.90 | 5,036.10 | 5,332.20 |
| TOTAL REVENUE (PhP) | 4,858.02 | 4,917.90 | 5,036.10 | 5,332.20 |
| COSTS | | | | |
| Cost of production | 356.95 | 340.64 | 358.05 | 360.70 |
| Depreciation | 0.27 | 0.34 | 6.14 | 9.43 |
| Labor | 40.00 | 31.00 | 53.00 | 130.00 |
| Electricity | 167.00 | 98.00 | 155.00 | 205.00 |
| Water/cleaning | 31.25 | 27.00 | 40.00 | 72.14 |
| Transportation | - | - | 38.00 | 44.00 |
| Packaging materials | 16.07 | 12.78 | 21.34 | 54.67 |
| TOTAL COSTS | 611.54 | 509.76 | 671.53 | 875.94 |
| NET INCOME | 4,246.48 | 4,408.14 | 4,364.57 | 4,456.26 |

[a]Group 1 (storing-packaging); [b]Group 2 (cleaning-storing-packaging); [c]Group 3 (sorting and grading-storing-packaging-transporting); [d]Group 4 (cleaning-sorting and grading-storing-packaging-transporting)

**Table 6**. Net income (PhP/box) by grouping on postharvest handling practices adopted, 31 market intermediary-respondents, San Jose, Batangas, Philippines, 2016

| Item | Group 1[a] n=3 | Group 2[b] n=4 | Group 3[c] n=7 | Group 4[d] n=5 | Group 5[e] n=12 |
|---|---|---|---|---|---|
| REVENUE | | | | | |
| Quantity sold (per box) | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Selling price (PhP/box) | 5,690.90 | 5,725.08 | 5,760.32 | 5,860.50 | 5,895.67 |
| TOTAL REVENUE (PhP) | 5,690.90 | 5,725.08 | 5,760.32 | 5,860.50 | 5,895.67 |
| COSTS | | | | | |
| Depreciation | 0.13 | 0.03 | 0.06 | 5.88 | 9.77 |
| Labor | 13.08 | 23.75 | 27.50 | 32.92 | 27.25 |
| Electricity | - | - | - | 24.83 | 19.50 |
| Transportation | - | 24.02 | 38.75 | 30.25 | 32.42 |
| Packaging materials | 3.88 | - | - | 5.33 | 5.75 |
| Cleaning materials | - | - | 7.22 | - | 5.68 |
| TOTAL COSTS | 17.09 | 47.80 | 73.53 | 99.22 | 100.36 |
| NET INCOME | 5,673.81 | 5,677.28 | 5,686.79 | 5,761.28 | 5,795.31 |

[a]Group 1 (sorting and grading-storing-packaging); [b]Group 2 (sorting and grading-storing); [c]Group 3 (assembly-cleaning-sorting and grading-storing); [d]Group 4 (sorting and grading-storing-packaging-transporting); [e]Group 5 (cleaning-sorting and grading-storing-packaging-transporting)

**Price differences at different market outlets**

In order to assess the effect of re-grading or non-standardization on the prices of table eggs, a combined ladder pricing and margin analysis was performed for the following three scenarios based on the identified marketing chains and the extent of re-grading done: farmer→assembler-wholesaler →wholesaler→retailer and farmer→wholesaler→retailer. The latter had two distinct re-grading standards - one for medium-sized eggs bought from the farm but sold at the retail level as jumbo and the other one was large-sized eggs at the farm level that reached the consumers as jumbo-sized.

**Table 7**. Summary of partial budget per box of table eggs, 60 farmer-respondents and 31 market intermediary-respondents, San Jose, Batangas, Philippines, 2016.

| Impacts | Postharvest Handling Practice | | | |
|---|---|---|---|---|
| | Farmers | | Market Intermediaries | |
| | Cleaning | Sorting and Grading | Transporting | Cleaning |
| Positive | | | | |
| Added return | 296.10 | 414.30 | 69.42 | 35.17 |
| Reduced cost | 0 | 0 | 0 | 0 |
| Total positive impact | 296.10 | 414.30 | 69.42 | 35.17 |
| Negative | | | | |
| Added cost | 109.14 | 67.00 | 50.09 | 0 |
| Reduced return | 0 | 0 | 0 | 0 |
| Total negative impact | 109.14 | 67.00 | 50.09 | 0 |
| Net impact (income) | 186.96 | 347.30 | 19.33 | 23.60 |

Results show that differences in prices were triggered by undue margin enjoyed from re-grading. Without re-grading, that is, small egg bought at the farm will be sold as small also, the assembler-wholesaler would have gained only PhP2.80/tray or PhP84.00 per box as profit. However, as practiced, small-sized eggs bought by the assembler-wholesaler at PhP116.60 per tray were re-graded as medium-sized and sold at PhP124.40 per tray. This resulted to an additional profit of PhP5.00 or a total gain of PhP4,335 for 867 trays of eggs re-graded per week by the assembler-wholesalers when they sold to the wholesalers. In addition, the same eggs were re-graded as large by the wholesalers and were sold at PhP131.00 per tray enjoying a gain in profit of PhP3.60 per tray for a total of PhP874.80 additional re-grading profit from 243 re-graded trays (Fig.3).

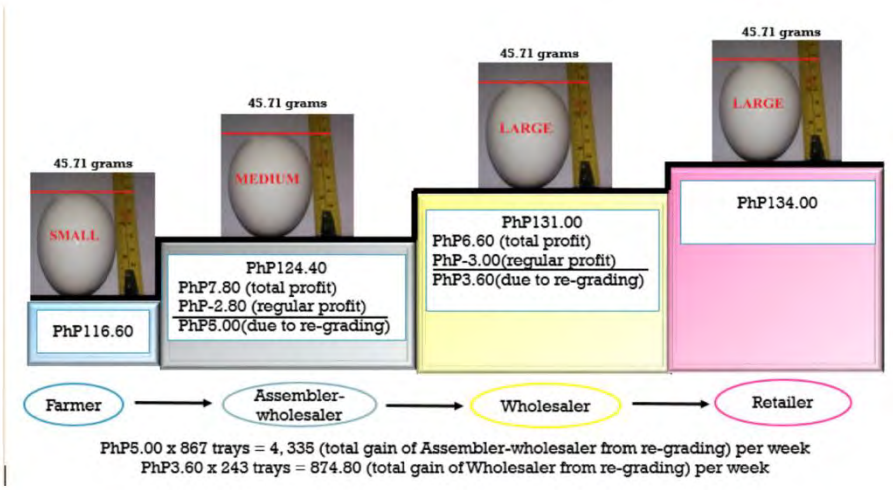


**Fig. 3**. Gain from re-grading small eggs at the farmer level to large at the retail level along F-AW-W-R channel.

On the average, 269 trays of medium sized eggs were re-graded as large sized by the wholesalers with a total profit of PhP13.50 per tray whereas if not for re-grading they would have gained only PhP5.50/tray. As these eggs were transferred to retailers, eggs were again re-graded as "jumbo" giving a total profit gain of Php18.00/tray (regular profit was only PhP14.50/tray). This

resulted to a re-grading profit of PhP2,152 by wholesalers from re-grading and PhP1,263.50 per week by the retailers (Fig. 4).

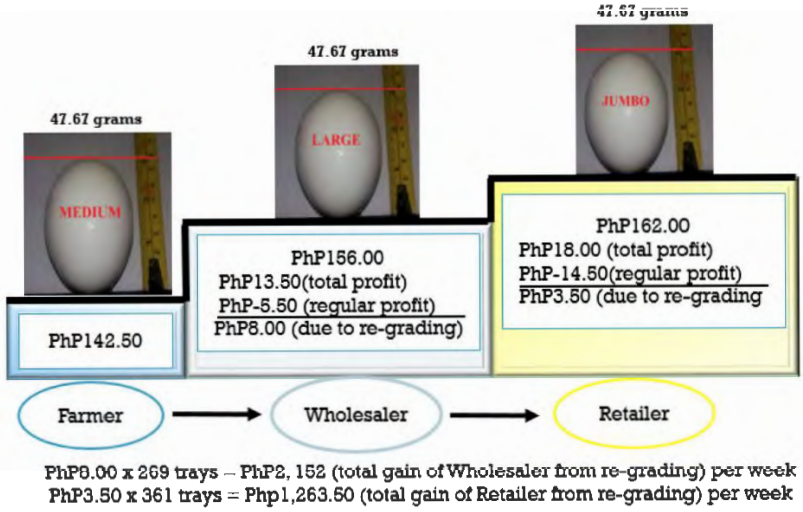


**Fig. 4.** Gain from re-grading medium eggs at the farmer level to jumbo at the retail level along F-W-R channel.

On the other hand, a total of 386 trays of eggs, on the average, were graded as "large" by the farmers and sold to the wholesale-retailers for PhP144 per tray. These were however re-graded by wholesalers as "extra-large" with an added gain of PhP10.50 per tray. The same was re-graded by retailers and sold to consumers as "jumbo" for PhP184 per tray reflecting re-grading gain of PhP9.50 per tray. Overall, wholesalers were able to enjoy undue profit of PhP1,407 while the retailers had PhP2,394 per week (Fig. 5).

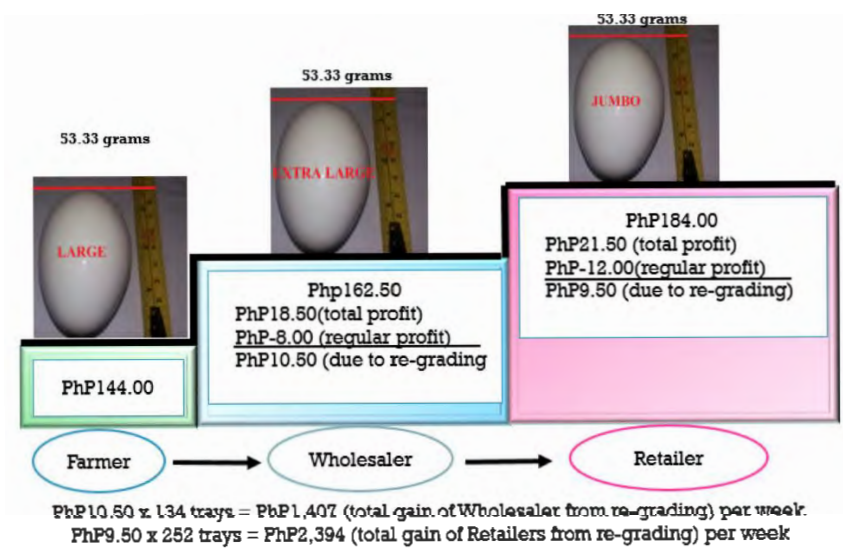


**Fig. 5.** Gain from re-grading large eggs at the farmer level to jumbo at the retail level along F-W-R channel.

## CONCLUSIONS AND RECOMMENDATIONS

Farmers and market intermediary-respondents who adopted postharvest handling practices generated more profit than those who did not adopt such practices. In particular, sorting and grading will give farmers additional net income. Likewise, market intermediary-respondents can increase their net income if they will adopt the additional practice of cleaning. It is therefore recommended that farmers and market intermediaries be encouraged to adopt these practices. Information dissemination on how this should be done properly must be made a priority by the local government unit (LGU) in cooperation with experts from nearby universities and related government agencies. This is particularly true for cleaning or washing where proper cleaning procedure is a requirement for ensured food safety.

The non-compliance to PNS for eggs in San Jose, Batangas had a negative effect on the egg prices at the farmer level and positive effect to the egg prices paid by consumers. There is thus, a need for BAFPS-PNS to ensure that all the participants involved in the marketing of table eggs are adopting uniform standards. It is also recommended that the LGU, from the municipal down to the barangay level, should serve as the implementing arm since they are in the best position to do so being always in close contact with the farmers and the traders. On the spot or random checking on the farms and weighing stations/checkpoints along the road can facilitate monitoring. The *Sangguniang Bayan* should give police powers to the assigned barangay officials to apprehend and penalize violators. In relation to this, proper labeling as indicators of assigned sizes should also be promoted together with corresponding standard sizes of trays for different grades to expedite random checking of lots bought from the area and to be sold elsewhere. And since, eggs from San Jose, Batangas find their way in many areas around the region and in other areas it might be of national interest to implement the same nationwide.

There is also a need for BAFPS-PNS to take a closer look at the issue of interior quality as a grading/standard requirement. Being tagged as "The egg basket of the Philippines" is a pride in itself and such should be given importance by the LGU. The need to maintain uniform standard is crucial in marketing with buying and selling online increasingly becoming the norm. Under this scheme of marketing, all participants are always on the assumption that the products being referring to are of standard quality.

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# PARTIAL CLONING AND EXPRESSION OF *ScBADH* AND *ScMIPS* GENE IN WILD AND CULTIVATED SUGARCANE UNDER MIMICKING SALINE SOIL CONDITIONS

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## ABSTRACT

Partial sequences of *ScBADH* and *ScMIPS* were cloned and sequenced. Partial cDNA sequences of *ScBADH* and *ScMIPS* encoded for 138 and 116 amino acid residue proteins, respectively. The deducted amino acid sequence of ScBADH showed high similarly to BADH of many plant species. When the seedlings of wild and cultivated sugarcane, KPS 94-13, were subjected to nutrient solutions containing 1% NaCl for 0, 12, 24, 48 and 72 h, the expression of *ScBADH* in leaves was highest at 72 h, and after 12 h in roots. The accumulation of *ScMIPS* transcripts in the leaves of both sugarcane genotypes was highest at 48 h. Expression of *ScMIPS* gene in roots was highest at 24 h after receiving salt stress, and then decreased slowly. Results indicated that NaCl-stress could upregulate the expression of *ScMIPS* and *ScBADH*. Wild and cultivated sugarcane showed the same pattern of *ScMIP* and *ScBADH* transcript accumulation. However, wild sugarcane showed a higher transcript accumulation than the cultivated variety.

**Key words:** salinity**,** nutrient solution**,** qRT-PCR, phylogenetic tree, osmoprotectant

## INTRODUCTION

Currently, areas used for growing plants are affected by abiotic stress factors, such as drought, salinity, flooding, and extreme temperatures that have long been known as major limitating factors for crop productivity (Boyor 1982). Salinity has been increasing annually. The increase in soil salinity is due to improper irrigation and the excessive use of fertilizers. Salinity appears to affect growth due to either the toxic effects of $Na^+$ or $Cl^-$ accumulation or the low osmotic potential of the soil or solution (Pitman 1984), which can adversely crop plant productivity. Salt-tolerant plants are able to maintain an acceptably low concentration of NaCl in the cytosol by exclusion or sequestration within the cellular compartment, such as the vacuole and accumulation of $K^+$ and organic osmotica in the cytosol (Kramer 1984). Salt stress distorts homeostasis at both the cellular and whole plant, involving developmental, morphological, physiological, biochemical, and molecular mechanisms (Parida and Das 2005). Some of the most severe effects caused by salinity include cell membrane disruption, generation of reactive oxygen species, reduction in enzymatic and photosynthetic activities, and decreased nutrient acquisition (Hanumantha et al. 2016).

Accumulation of osmoprotectants is a common metabolic adaptation for salt-tolerant plants (Rathinasabapathi 2000, Suleiman et al. 2013). Many osmoprotectants, such as glycine betaine and *myo*-inositol, enhance the protection of plants from such stress. Betaine aldehyde dehydrogenase (BADH), which is encoded by *Betaine aldehyde dehydrogenase* (*BADH*) gene, is the key enzyme for betaine synthesis, favoring salinity tolerance (Tabuchi et al. 2005) and catalyzes the final step in the

synthesis of the osmoprotectant glycine betaine from choline. Choline is converted by choline monooxygenase (CMO) to betaine aldehyde, which is then converted to glycine betaine by betaine aldehyde dehydrogenase. *BADH*s genes have been cloned and characterized from many plant species, such as spinach (Rathinasabapathi 2000), sweet potato (Chen et al. 2014), and *Suaeda corniculata* (Wang et al. 2016). The *BADH* gene was cloned from *S. corniculata* and results suggested that *BADH* might be a positive regulator in plants during NaCl-stress response (Wang et al. 2016).

*Myo*-inositol-1-phosphate synthase (MIPS) catalyzes the conversion of D-glucose-6-P to D-*myo*-inositol-1-phosphate, followed by its specific dephosphorylation to free *myo*-inositol by the $Mg^{++}$ dependent *L-Myo-inositol 1-phosphate phosphatase* (IMP) (Loewus and Murthy 2000). The *MIPS* gene has been isolated from several plant species such as *Arabidopsis thaliana* (Johnson 1994), *Nicotiana tabacum* (Hara et al. 2000), *Oryza sativa* (Yoshida et al. 1999), *Zea mays* (Larson and Raboy 1999), and *Ipomoea batatas* (Zhai and Liu 2009). Abreu and Aragão (2007) isolated the *MIPS* gene from yellow passion *PeMIPS* transcripts were expressed in the ovule, pollen grand, and leaves. The *MIPS* gene has also been shown to improve tolerance to abiotic stress in several plant species (Zhai et al. 2015).

Sugarcane is a naturally salt-sensitive plant. Saline soil is the primary problem causing low yield for sugarcane. A sugarcane breeding program aiming to improve salt tolerance is difficult to achieve due to trait complications. Understanding the response of sugarcane under salt stress at a molecular level is an important step in accelerating the realization of this program. This study sought to clone and identify the partial of *ScBADH* and *ScMIPS* sequences from wild (*Saccharum spontaneum* L.) and cultivated sugarcane (*S. officinarum* L.cv. KPS 94-13), and to investigate the expression of the genes under mimic saline soil condition.

## MATERIALS AND METHODS

### Plant samples and RNA extraction

Two-month old seedlings of wild sugarcane (*Saccharum spontaneum*) and cultivated sugarcane (*S. officinarum* cv. KPS 94-13) were grown in 1/10 Hoagland's nutrient solution containing 200 mM NaCl. Wild sugarcane is diploid with 80 chromosomes while the cultivated sugarcane is polyploid with 128 chromosomes. Leaf and root samples were collected at 0, 12, 24, 48 and 72 h after salt stress for RNA extraction. Total RNA was extracted from 0.1 g of leaves or root by the method described previously by Laksana and Chanprame (2015). RNA quality was determined through PCR using actin specific primers, while RNA quantity was determined using a nanodrop-spectrophotometer.

For first strand cDNA synthesis, the 12.5 uL reaction mixture consisted of 1 µg total RNA sample, 2 µM Oligo $(dT)_{18}$ primer, 0.8 mM dNTP, and RNase-free water were added and mixed gently. The reaction was incubated at 65 ºC for 5 min and then cooled at 4 ºC for a minimum of 2 min. After which, 1x reaction buffer, 0.5 U RiboRock RNAse inhibitor (Fermentas, Canada), 1 mM dNTP, and 1 µL Revert Aid M-MuLVRT (Fermentas) was added to the reaction tube, mixed gently and incubated at 42 ºC for 1 h. The reaction was thermally inactivated at 70 ºC for 10 min, then cooled at 4 ºC for at least 2 min. One-fourth U/ µL RNAseH was added for the degradation of any remaining total RNA.

### Amplification of *ScBADH* and *ScMIPS* gene partial length from wild and cultivated sugarcane

The primers used in this study are listed in Table 1. The degenerated primers were designed based on the highly conserved region of the *BADH* and *MIPS* gene sequences of numerous plant species from the NCBI database, such as *Elaeis guineensis* (XP_010913436.1), *Oryza sativa* (ABI84118.1), *Hordeum vulgare* (ABO93605.1), *Triticum aestivum* (AAL05264.1), *Sorghum bicolor* (XP_002444357.1), and *Zea mays* (NP_001105781.1). PCR reaction was carried out in a total

volume of 20 µL containing 100 ng of the first strand cDNA template, 50 µM dNTPs, 1U of *Taq* polymerase (Fermentas), 5 mM $MgCl_2$, 1x buffer (Fermentas), 0.125 µM forward primer, and 0.125 µM reverse primer. The amplification was performed under the following conditions: initial denaturation at 95 °C for 3 min; then 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 1 min; then a final extension at 72 °C for 5 min. The amplification products were resolved on a 0.7% (w/v) agarose gel electrophoresis at 100 V for 40 min.

**Table 1.** Degenerate primers for amplifying partial of *BADH* and *MIPS* gene sequences.

| Genes | Primers |
|---|---|
| *BADH* | Forward: 5'- ATGGGACATGGAYGATGT -3'<br>Reverse: 5'- TTTTKCCACCAAGTTCCA -3' |
| *MIPS* | Forward: 5'- TGTTCATCGAGAGCTTCCG -3'<br>Reverse: 5'- ATGGGMAGGAGGCTCTTGAA -3' |

**Note:** Y = C/T, K= G/T, M= A/C

**Sequencing and phylogenetic tree construction**

The PCR products were eluted from the 0.7% (w/v) agarose gel using a PCR clean up and gel extraction kit (NucleoSpin® Extract II) following the manufacturer's protocol and were sequenced at First Base Laboratory (Malaysia). The sequences were compared with GenBank databases (Nucleotide BLAST) (www.ncbi.nlm.nih.gov/BLAST/) using default parameters, and then translated into amino acid sequences using Genetyx 5.0 (http://genetyx.software.informer.com/). The derived BADH and MIPS amino acid sequences were aligned to BADH and MIPS amino acid sequences of other plant species by using the Clustal X (http://www.clustal.org/clustal2/) and Genedoc 2.7 programs (http://genedoc.software.informer.com/2.7/). Clustal X was used for alignment. After which, alignment results were imported to GeneDoc 2.7 to generate a picture. A phylogenetic tree was constructed based on BADH and MIPS family members using the Neighbor joining method with 1,000 bootstrap replication using the ClustalW2 program (http://www.ebi.ac.uk/tools/msa/clustalw2).

**Analysis of *ScBADH* and *ScMIPS* gene expression under mimic saline soil via quantitative PCR**

The expression of *ScBADH* and *ScMIPS* in wild and cultivated sugarcane were investigated by real-time quantitative PCR. The accuracy of quantification was confirmed through normalization of S*cBADH* and *ScMIPS* expression to a reference transcript encoding for glyceraldehydes-3-phosphate dehydrogenase: *GAPDH* (GenBank accession no. CA254672). *GADPH* is identified as suitable reference gene for the normalization of gene expression under salinity/drought-treatment in sugarcane (Guo et al. 2014). Specific primers of the two genes for real-time PCR (Table 2) were designed from partial length *ScBADH* and *ScMIPS* cDNAs using Primer3 ver 0.4.0 (http://simgene.com/Primer3).

**Table 2.** Specific primers used for real-time quantitative PCR.

| Genes | Primers |
|---|---|
| *GADPH* | Forward 5' CACGGCCACTGGAAGCA 3' |
| | Reverse 5' TCCTCAGGGTTCC TGATGCC 3' |
| *BADH* | Forward 5' TTGAACATTGTGACAGGATTAGG 3' |
| | Reverse 5'AGTTCCAGCGTAACAGGCTT 3' |
| *MIPS* | Forward 5' GCACAACACCTGTGAGGACT-3 |
| | Reverse 5' TGAGGTAGCTCAGGATGGTG 3' |

PCR reactions were performed in a total volume of 20 µL containing 500 ng of first strand cDNA template, 1x SensiFAST SYBR No-ROX mix buffer (Bioline Reagent Ltd.), 0.4 µM forward primer, and 0.4 µM reverse primer. The amplification was performed under the following conditions: initial denaturation at 95 °C for 30 sec; then 45 cycles of denaturation at 94 °C for 5 sec, annealing at 58 °C for 15 sec, and extension at 72 °C for 10 sec in a Mastercycler® ep realplex4 from Eppendorf®.

The expression of these genes was compared to control conditions (0 day) and the reference gene was *GADPH* gene for sugarcane. For each sample, the reactions were carried out in three biological replicates with three technical replicates each.

## RESULTS AND DISCUSSION

The wild sugarcane (*Saccharum spontaneum*) is naturally more tolerant to abiotic stress compared to cultivated sugarcane. Cultivated sugarcane, KPS 94-13, is one of the most popular cultivars planted in the western region of Thailand and is more sensitive to abiotic stress than wild sugarcane. We expect that they may have some differences in the nucleotide sequences of their genes responsive to stress conditions. To prove this hypothesis we cloned the partial *BADH* and *MIPS* gene from both sugarcane genotypes and investigated the expression of the genes under salt stress conditions.

### Cloning of partial *BADH* and *MIPS* gene

Saline soil is one of the major factors causing low yield in plants (Senger et al. 2013), affecting plant growth and reducing agricultural productivity worldwide. Sugarcane is one plant that shows high sensitvity to salinity. In this study, partial *BADH* and *MIPS* genes were first cloned from cDNAs from both wild and cultivated sugarcane cv. KPS 94-13. The PCR products were DNA fragments had a size of approximately 450 bp for *BADH* and 350 bp for *MIPS*, both corresponding to the predicted product size based on primer design results. The *BADH* amplicon had a 96% similarity with betaine-aldehyde 2, partial sequence from *Sorghum bicolor* (AGZ15752.1), while the *MIPS* amplicon had 99% similarity with myo-inositol-1-phosphate synthase sequence from *Saccharum spontaneum* (ALO50704.1). The sequences of these DNA fragments were translated to amino acid sequences of 138 and 116 amino acid residues for *BADH* and *MIPS*, respectively (Fig. 1).


**Fig. 1.** The deduced amino acid sequences of partial *BADH* (A) and *MIPS* (B) cloned from wild and cultivated sugarcane c.v. KPS 94-13.

The deduced amino acid sequences of BADH from both wild and cultivated sugarcane showed 99.3% identity, while sequences from both MIPS proteins showed 100% identity. These amino acid sequences were compared with sequences of other plant species from the GenBank database. Deduced amino acid sequence from both *BADH* cDNAs of wild and cultivated sugarcane cv. KPS 94-13 showed high similarity to *Sorghum bicolor* (96%, AGZ15752.1), *Zea mays* (96%, AQK52373.1), *Zoysia tenuifolia* (88%, BAD34956.1), *Oryza sativa japonica* (89%, BAT05491.1), and *Hordeum vulgare* (83%, BAB62846.1). We named it as *ScBADH*. Meanwhile, the amino acid sequence deduced from *MIPS* cDNA showed high similarity to *Zea mays* (99%, ACG33827.1) *Zoysia*

*matrellam* (97%, AIN39843.1) *Sorghum bicolor* (97%, KXG39974.1), *Triticum aestivum* (96%, EU371115.1), *Oryza sativa japonica* (96%, BAA25729.1), *Triticum aestivum* (96%, AGK06903.1), and *Nicotiana tabacum* (91%, NP_001311846.1). The start codon of the gene is included in this nucleotide sequence, we named it as *ScMIPS*. Partial of *ScBADH* and *ScMIPS* will be used for cloning the full length of *ScBADH* and *ScMIPS* in the future.

The *BADH* and *MIPS* gene in many plant species have been cloned and the gene expression level determined. Wang et al. (2016) cloned *BADH* gene from *Suaeda corniculata* and analyzed the expression profile of this gene. Meanwhile, *MIPS* gene of several plants have been cloned and studied, including rice (Yoshida et al. 1999), corn (Larson and Raboy 1999), and sweet potato (Zhai and Liu 2009). Abreu and Aragão (2007) isolated *PeMIPS* gene from *Passiflora edulis* and the expression of the gene was analyzed. The gene was expressed in the ovules, pollen grains, and leaves during stress conditions, and suggested that it is important for environmental stress response.

The amino acid sequences deduced from partial *ScBADH* and *ScMIPS* were used for phylogenetic tree construction. This revealed that *ScBADH* and *ScMIPS* from both wild and cultivated sugarcane are closely related to sorghum and corn (Fig. 2 and 3). There was segregation of these genes in monocotyledonous plants, especially sorghum, since sorghum and sugarcane are close relatives, and this evolutionary divergence is estimated as occurring as early as 5 million years ago (Dillon et al. 2007).

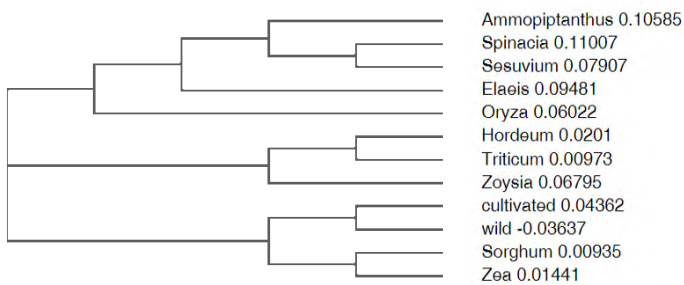


**Fig. 2.** Phylogenetic tree of *ScBADH* among different species was constructed based on deduced amino acid sequences by Neighbour-joining method with 1,000 bootstrap replication using ClustalW2 program .GenBank accession numbers of amino acid sequences used: *Ammopiptanthus nanus* (AIG52060.1), *Spinacia oleracea* (AAN52929.1), *Sesuvium portulacastrum* (AEK98521.1), *Elaeis guineensis* (XP_010913436.1), *O. sativa* (ABI84118.1), *Hordeum vulgare* (ABO93605.1), *T. aestivum* (AAL05264.1), *Zoysia tenuifolia* BAD34947.1), *S. bicolor* (XP_002444357.1) and *Z. mays* (NP_001105781.1).

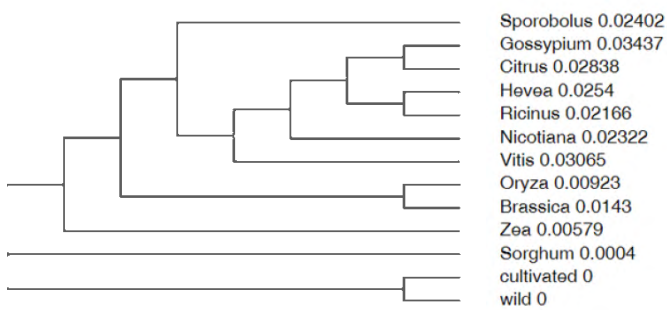


**Fig. 3.** Phylogenetic tree of *ScMIPS* among different species was constructed based on deduced amino acid sequences by Neighbour-joining method with 1,000 bootstrap replication using ClustalW2 program .GenBank accession numbers of amino acid sequences used : *Sporobolus*

*alterniflorus* (ADC33414.1), *Gossypium hirsutum* (ACJ11714.1), *Citrus sinensis* (XP_006464258.1), *Hevea brasiliensis* (AFD61599.1), *Ricinus communis* (NP_001310667.1), *Nicotiana tabacum* (NP_001311846.1),*Vitis vinifera* (XP_010652823.1), *Oryza brachyantha* (XP_006651124), *Brassica juncea* (ABY74556.1), *Z. mays* (ACG33827.1) and *S. bicolor* (KXG39974.1)

The *BADH* and *MIPS* genes regulate the production of osmoprotectants which plants use for protection from the harmful effects caused by abiotic stress, such as salinity (Santos et al. 2010). These molecules accumulate in cells and balance the osmotic difference between the cell's surroundings and the cytosol. In the present study, expression patterns of *ScBADH* and *ScMIPS* genes in different tissues (leaves and roots) exposed to salt stress (200 mM NaCl) at different lengths of time (0, 12, 24, 48 and 72 h) were analyzed through quantitative real-time PCR (qRT-PCR). Roots, in particular, were selected since this is the first organ affected by salt stress.

Results showed that the expression of *ScBADH* and *ScMIPS* genes in leaves and roots were up-regulated compared to the control (0 h). Accumulation of the *ScMIPS* transcript in leaves for both sugarcane species was highest after 48 h (Fig. 4A) whereas, the accumulation of *ScMIPS* transcript in roots of each sugarcane species after 24 and 48 h exhibited highest expression, with no-significant difference between the two time periods (Fig. 4B). Expression in leaves was higher than that in the roots, except after 24 h, where expression in the roots of wild sugarcane was a little bit higher than in leaves. This finding is in accordance with An-jun et al. (2016) who analyzed the expression of *MIPS* gene in pumpkin and suggested that the expression of this gene exhibited tissue specificity, with highest expression level in leaves when subjected to salt, abscisic acid (ABA), and drought stress. It is important to recognize that different tissues and cells in a plant are adapted for specific and often very different purposes. It is therefore not surprising that the expression levels of genes will differ from tissue to tissue and from cell to cell, depending on the tissue/cell's function (Roy et al. 2014). The accumulation of *ScBADH* transcript in leaves was highest after 72 h (Fig. 4C), whereas in roots, the expression was highest after 12 h (Fig. 4D).

The comparison of relative expression of *ScBADH* between roots and leaves revealed that its expression in roots was higher than in leaves. This result is similar to the expression of *IbBADH* in *Ipoea batatas*, which is strongly expressed in roots (Chen et al. 2014). These results indicate that the expression of both *ScMIPS* and *ScBADH* were induced by NaCl-stress. This agrees with Wang et al. (2016) who reported that *BADH* gene is a positive regulator in plants during its response to NaCl-stress. Wild and cultivated sugarcane showed the same pattern of expression for both genes. However, the relative expression of both genes in wild sugarcane was higher than in cultivated sugarcane. This phenomenon may correlate with observations that wild plants species are generally more tolerant to abiotic stress than cultivated species (Bolger et al. 2014, Li et al. 2014). Both genes regulate the production of osmoprotectants. The higher the gene expression, the more osmoprotectants produced and may result in higher salt-stress tolerance. Comparison between the relative expression of *ScBADH* and *ScMIPS* genes showed that *ScBADH* expression was lower than *ScMIPS* expression. This may be because MIPS enzyme is the only known enzyme to catalyze the conversion of glucose 6-phosphate to inositol phosphate (Lackey et al. 2003), which in turn is a precursor for many inositol-containing compounds that are implicated in various physiological and biochemical processes, including growth regulation, cell membrane biogenesis, hormonal regulation, stress signaling, and plant immunity (Kaur et al. 2013). However, BADH enzyme catalyzes the final step in the synthesis of the osmoprotectant glycine betaine from choline (Chen and Murata 2002). The results indicate that the expression pattern for both genes were similar when expression was up-regulated to the highest level, a short time after receiving salt stress, and then gradually decreased.

The higher expression of *ScMIPS* in sugarcane compared with *ScBADH* expression might indicate a better osmotic adjustment of inositol to salt stress than glycine betaine. The expression level

of both genes, especially *ScMIPS*, may be useful as a parameter to assist in the selection for salt tolerance in sugarcane breeding.

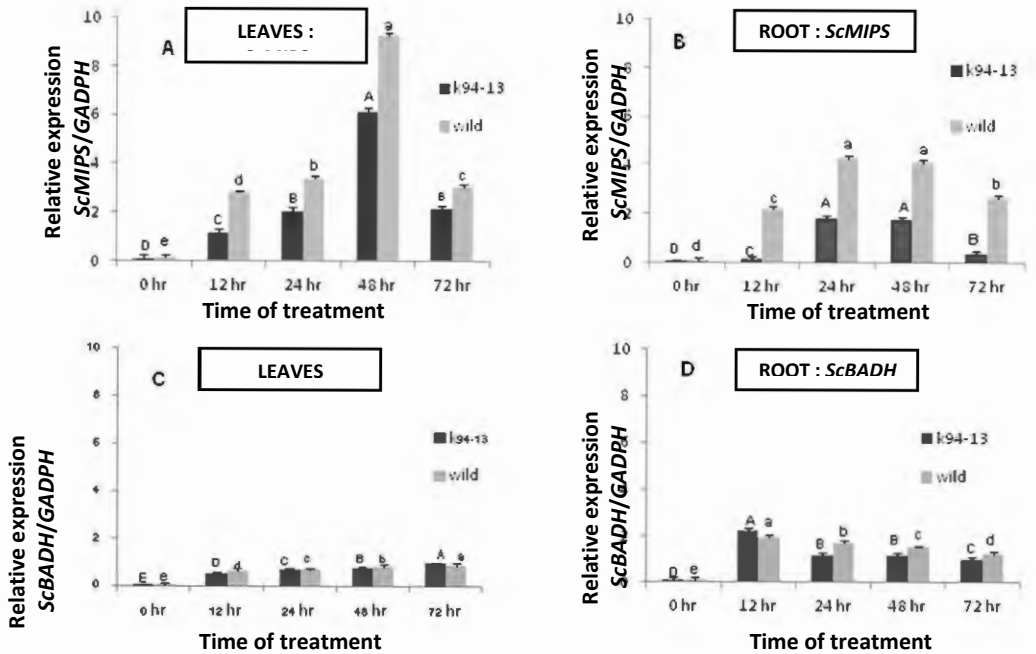


**Fig. 4.** The expression of *ScMIPS* and *ScBADH* gene in leaves (A: *ScMIPS*, C: *ScBADH*) and root (B: *ScMIPS*, D: *ScBADH*) of wild and cultivated sugarcane subjected to 200 mM NaCl for 0, 12, 24, 48 and 72 h. The different capital letters or small letters on the boxes indicate significant differences with $p<0.05$ while the error bars represent standard error. Each treatment composed of three biological and three technical replicates.

## CONCLUSION

Partial cDNA sequences of *ScBADH* and *ScMIPS* were cloned from wild and cultivated sugarcane (KPS 94-13). The deduced amino acid sequence from *ScBADH* showed a high similarly to the BADH of other plant species, such as *Sorghum bicolor* (96%), *Zea mays* (96%) *Oryza sativa japonica* (89%), while ScMIPS deduced protein showed a high similarly to MIPS from *Zea mays* (99%), *Zoysia matrellam* (97%), *Sorghum bicolor* (97%), *Triticum aestivum* (96%), *Oryza sativa japonica* (96%), and *Nicotiana tabacum* (91%). Wild and cultivated sugarcane showed the same pattern of *ScMIP* and *ScBADH* transcript accumulation. Salt stress could up-regulate the expression of both genes, wherein the expression of *ScBADH* from the roots was higher than in leaves, while the expression of *ScMIPS* dominated in leaves. Investigations for gene expression correlation with physiological parameters responding to salt stress in sugarcane may be used as an index for salt tolerance selection in sugarcane breeding.

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# EFFECTS OF AERENCHYMA FORMATION ON THE ROOT POROSITY, LATERAL ROOT DEVELOPMENT, TOTAL WATER UPTAKE AND SHOOT BIOMASS ACCUMULATION OF GLOBAL MAGIC RICE UNDER SEEDLING-STAGE DROUGHT STRESS

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## ABSTRACT

Drought stress negatively affects rice productivity due to the lack of water necessary for growth and development. Previous studies have shown that the formation of root cortical aerenchyma (RCA) can influence the development of lateral roots, which aid in the uptake of water and accumulation of biomass during drought. The present study evaluated aerenchyma formation in several genotypes of global Multi-Parent Advanced Generation Intercross (MAGIC) population in rice, and its relationship with lateral root development, root porosity, total water uptake, and shoot biomass accumulation during seedling-stage drought stress. The following methods were used in the study: aerenchymatous regions were measured in pixels using GIMP v.2.8.14 (GNU Image Manipulation Program); percent lateral roots were determined using a root scanner coupled by Winrhizo v. 2005, and root porosity was estimated using the buoyancy method. Half of the genotypes studied exhibited 2-35% RCA development under drought-stress (DS), compared with 7-25% under the well-watered (WW) treatment. Mean percent RCA was also higher in DS (14.03%) than in WW (12.98%), but was not significantly different at p=0.053. In addition, high RCA genotypes, like MG-6865, did not show highest or lowest shoot biomass nor total water uptake for both treatments. Thus, RCA formation in the global MAGIC rice genotypes did not influence total water uptake, shoot biomass accumulation, root porosity, and the production of lateral roots. Under seedling-stage during severe drought stress, formation of aerenchyma was observed to be a mere response to the absence of water through cell lysis. Therefore, root traits other than RCA might be more correlated with drought-tolerance in the studied genotypes.

**Key words:** global MAGIC rice, biomass, water uptake

## INTRODUCTION

Drought is a major factor limiting crop growth and development and negatively affects the agricultural sector. It is the primary reason for major production losses around the world. The Asia-Pacific Region produces and consumes about 90% of the total rice in the world (Serraj et al. 2005, FAO 2000), unfortunately, approximately 20% of Asia's rice fields are severely affected by drought (Pandey and Bhandari 2008). In 2016 alone, the Philippines lost 81 million pesos worth of agricultural

produce due to the onslaught of El Niño (International Federation of Red Cross and Red Crescent Societies 2016). The occurrence of drought events is expected to increase, based on current climate projections (IPCC 2012) and competition among agricultural, urban and industrial sectors (Haro von Mogel 2013). International interest on drought studies has increased through the years, focusing mainly on root biology (Comas et al. 2013; Gowda et al. 2011). As the primary organ for water and nutrient uptake, root morphology, topology, distribution and its entire architecture limit shoot functioning (Lynch 1995, Nardini et al. 2002, Sperry et al. 2002). Thus, expanding our knowledge and understanding on how the root system interacts with different soil-water regimes, especially during drought, would aid in further improving drought-tolerant rice varieties and ultimately, global food security. A variety of root adaptations of rice under drought stress have been identified, such as the development of well-branched roots (Yoshida and Hesagawa 1982) and increased maximum root depth via vertical elongation (Singh et al. 2010, Gowda et al. 2011, Henry et al. 2011, Lynch 2013, Wasson et al. 2012).

Rice roots are typically characterized by the presence of cortical aerenchyma which disintegrates the root cortex to provide space for the diffusion of oxygen during flooding and anaerobic conditions (Evans 2004). Aerenchyma tissue also lowers the metabolic costs of respiration to maximize growth (Zhu et al. 2010), and nutrient and water uptake during drought (Colmer et al. 2005). Rice under transient drought-to-waterlogged conditions was found to induce the production of lateral roots through the development of aerenchyma tissues, with positive correlations between root porosity and lateral root production (Niones et al. 2013). Although changing water regimes affects the development of aerenchyma in rice roots, its actual function in water uptake during drought is poorly understood (Henry et al. 2012).

The present study investigated aerenchyma formation under drought stress; determined whether biomass accumulation and water uptake are affected by aerenchyma formation; and determined if genotypes with a high percentage of root cortical aerenchyma (RCA) have greater root porosity and lateral roots. We used genotypes from the global Multi-parent Advanced Generation Intercross (MAGIC) rice population, characterized by its diverse genetic background and contrasting shoot biomass under drought. The global MAGIC population was developed by crossing eight parental lines from an *indica* MAGIC population and eight parental lines from a*japonica* MAGIC population making the global MAGIC population a representation of 16 parents from a total of 150 multiple crosses. The population was called as "global MAGIC" because it "encompasses the *indica* and *japonica* rice ecotypes that are known globally"; also, it has undergone multiple crosses that boosted genetic diversity, which is said to be higher than the two parental lines (Bandillo et al. 2013). The study was conducted at the Drought Physiology greenhouse and laboratory of the International Rice Research Institute from May-September 2014.

## MATERIALS and METHODS

### Plant Materials

Five hundred forty genotypes from the global MAGIC population were grown in the field under drought conditions. Ten genotypes with high shoot biomass and ten with low shoot biomass were selected from the previous screening. The genotypes are as follows: (High shoot biomass) MG-6157, MG-6638, MG-6160, MG-6781, MG-6148, MG-6810, MG-6863, MG-6865, MG-6806 and MG-6164; (Low shoot biomass) MG-6415, MG-6490, MG-6411, MG-6419, MG-6261, MG-6353, MG-6420, MG-6462, MG-6485 and MG-6256.

### Experimental Design

The greenhouse experiment was a randomized complete block design with a 2 x 20 factorial arrangement of treatments. Each genotype was replicated five times and was exposed to two treatments: drought-stress (DS) and well-watered (WW).

**Growth Conditions**

Seeds were germinated Petri dishes with filter paper (Whatman, No.41) moistened with tap water, with each dish having about 50 seeds. Each Petri dish was labeled according to genotype and placed inside an incubator at 37˚C for 3 days. Water was continually added until the radicle and small portion of the epicotyl emerged. Soil was collected, sieved, sterilized in the oven for 6-7 hours, and sun-dried until moisture was completely removed. Two hundred Mylar tubes were filled with 950 g of soil each, with its height in the tube adjusted to 40 cm. Plastic cylinders were placed inside the tubes to hold the soil. Cheese cloth was placed at the bottom of each plastic cylinder to allow water to seep through. These Mylar tubes were kept in boxes assisted with wire racks on top of a table at the IRRI greenhouse. The tubes were saturated with water for 2 hours and were left overnight to reach the field capacity. Both drought-stressed and well-watered treatments were observed starting at field capacity. Each Mylar tube was then planted with three pre-germinated seedlings and were thinned out to one after complete emergence of the epicotyl. The plants were allowed to grow under both treatments starting at field capacity for 21 days.

**Total Water Uptake**

Prior to planting, tubes were soaked with water for 2 h and were drained overnight to reach field capacity. The tubes were then weighed in a top loading balance to serve as basis for the measurement of water uptake by weight. The measurement of water uptake for both DS and WW conditions started at field capacity; however, for the DS, the soil was left to dry until the duration of the experiment whereas for WW, lost water was brought back with the field capacity weight of the tube as the basis. Hence, if the field capacity weight of the tube is 1600 g and weighed 1500g after two days, then 100g of water should be added back to the tube so as to maintain the WW in field capacity. The tubes were weighed three times a week (MWF) for 21 days. Total water uptake was computed using the formula below.

**Total Water Uptake (TWU in g)** = (Tube weight at Field Capacity**, g**)- (Tube weight at harvest**, g**)

**Percent Lateral Roots**

Root systems were separated from the shoot systems and were thoroughly cleaned. A root scanner coupled by Winrhizo v. 2005 (Regent Instruments, Quebec Canada) was used to measure root length (cm), volume($cm^3$), and classify root diameter classes (0mm<diameter<1mm). Percent lateral roots were computed using the formula below:

$$\textbf{\% Lateral roots} = \frac{(\textit{sum of root length with diameters ranging up to } 0.200mm)}{\textit{total root length } (cm)} \textbf{ x 100\%}$$

**Root Porosity**

Porosity, or the relative volume of internal gas spaces in root tissue, is important in determining resistance to flooding (Visser and Bogemann 2003). This parameter can also be adopted in investigating root response to drought. Root porosity was estimated using the buoyancy method (Raskin 1983, Thomson et al. 1990, Visser and Bogemann 2003). From the root base, a 3-cm nodal root was cut into 3 segments and placed in a small bottle containing distilled water. The soaked roots were blotted dry using absorbent paper for 2 seconds to remove adhering water. The dried roots were weighed and then placed in a 1.5 mLglass tube. The glass tubes containing the roots were then placed in a vacuum chamber for 45 minutes. The roots were removed from the bottle and were rapidly blotted with absorbent paper. The roots were then weighed. Using the weight (g) of the root before(g, w1) and after (g, w2)the vacuum, the porosity of the roots was calculated as:

$$\textbf{\%Root Porosity} = \frac{(w2-w1)\,x\,\textit{Specific Weight } (SW) \textit{of water}}{w2} \textbf{ x 100\%}$$

Root porosity may not be an entire representation of aerenchyma formation, as rice roots grown in a well-drained condition typically show 10-12% porosity (Armstrong 1971). Hence, this

measurement is prone to overestimation. However, since there are no established methods to estimate aerenchyma formation for the entire root system, both the buoyancy and pixel methods were used.

**Root Cortical Aerenchyma**

In a procedure, similar to that of Zhu et al. (2010) 1-cm nodal roots from the base were cut freehand. A thin cross section was made using blade under a dissecting microscope with three replicates made per sample. Sections were viewed under DP 70 Olympus microscope at 10-20x magnification, depending on the size of the section. Images were taken and analyzed using GIMP v 2.8.14. The area of the stele, whole root section, and aerenchymatous regions were measured using GIMP v.2.8.14 (GNU Image Manipulation Program). The number of pixels (px) for each parameter was then recorded. Percent root cortical aerenchyma was computed using the formula below.

$$\%RCA = \frac{aerenchyma\ area\ (px)}{whole\ root\ area\ (px) - stele\ area\ (px)} \times 100\%$$

**Biomass**

Both the shoot and the root systems were placed on separate envelopes and were dried for five days at 60 ˚C. The samples were then weighed using top loading balance.

**Statistical Analyses**

Data were analyzed in R v. 3.1.2 (R Development Core Team 2014) using Pearson correlation, analysis of variance (ANOVA), and Tukey's Honest Significant Difference (HSD) for mean comparison of parameters.

## RESULTS AND DISCUSSION

**Aerenchyma Formation under DS and WW Conditions**

Root cortical aerenchyma can be induced through a variety of conditions, such as NPK-deficiency (Postma and Lynch 2011), hypoxia (Evans 2004) and mechanical impedance (Striker et al. 2007). Rice lines under drought were also observed to have less aerenchyma (Henry et al. 2012, Gowda et al. 2011). Mean %RCA was higher under DS (14.03%) than WW (12.98%), but was not significantly different at p=0.053 (Fig. 1). Percentage variation in RCA development ranged from 35% to 2% under DS, and from 25% to 7% under WW conditions (Fig. 2). Half of the genotypes produced more aerenchyma tissue under DS than in WW. Under DS, aerenchyma tissue increased for MG-6865 (75%) and MG-6419 (61%), while under WW, percentage RCA increased for MG-6810 (89%) and MG-6353 (77%). Percent RCA was significantly different across genotypes under DS (p=0.014), but not for WW (p=0.308). Under DS, MG-6865 had the highest % RCA (35.4%) while MG-6810 had the lowest (1.98%). On the other hand, under WW, MG-6863 had the highest % RCA (25.39%) while MG-6462 had the lowest (7.13%).

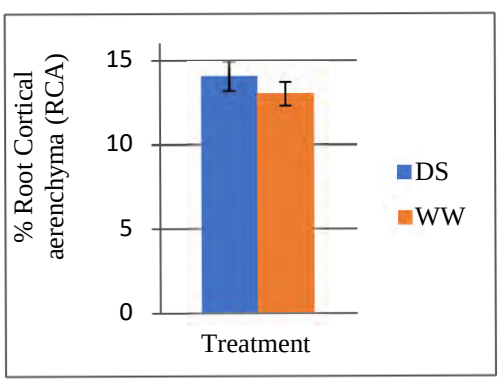


**Fig. 1**. Mean % RCA under DS and WW conditions (not significantly different at p=0.053).

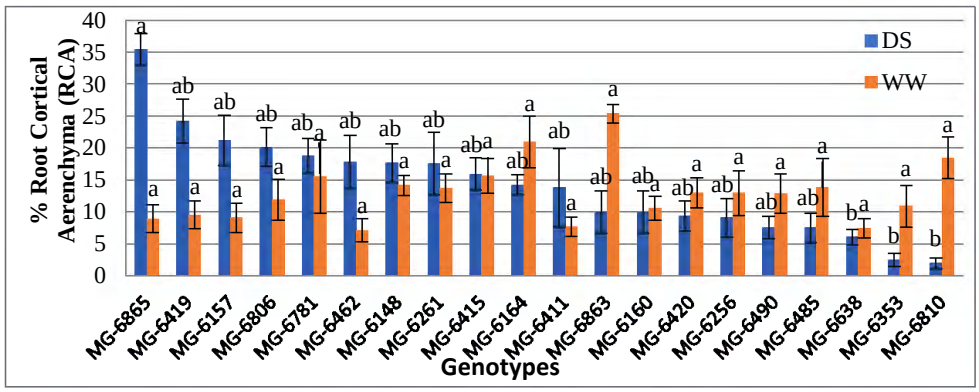


**Fig. 2.** Mean % RCA of each genotype under DS and WW conditions. Bars indicate the standard error. Means with the same letter are not significantly different at P<0.05 within treatments.

Percent root cortical aerenchyma decreases during drought (Henry et al. 2012). Representative genotypes from the global MAGIC rice population under DS had higher mean RCA values than those under WW conditions. However, results were variable as half the genotypes had greater RCA under WW than under DS conditions. The high genetic variability of the population must have contributed to such variable results (Fig.3). In maize, RCA formation reduces root respiration by converting cortical cells to gas spaces (Zhu et al. 2010).

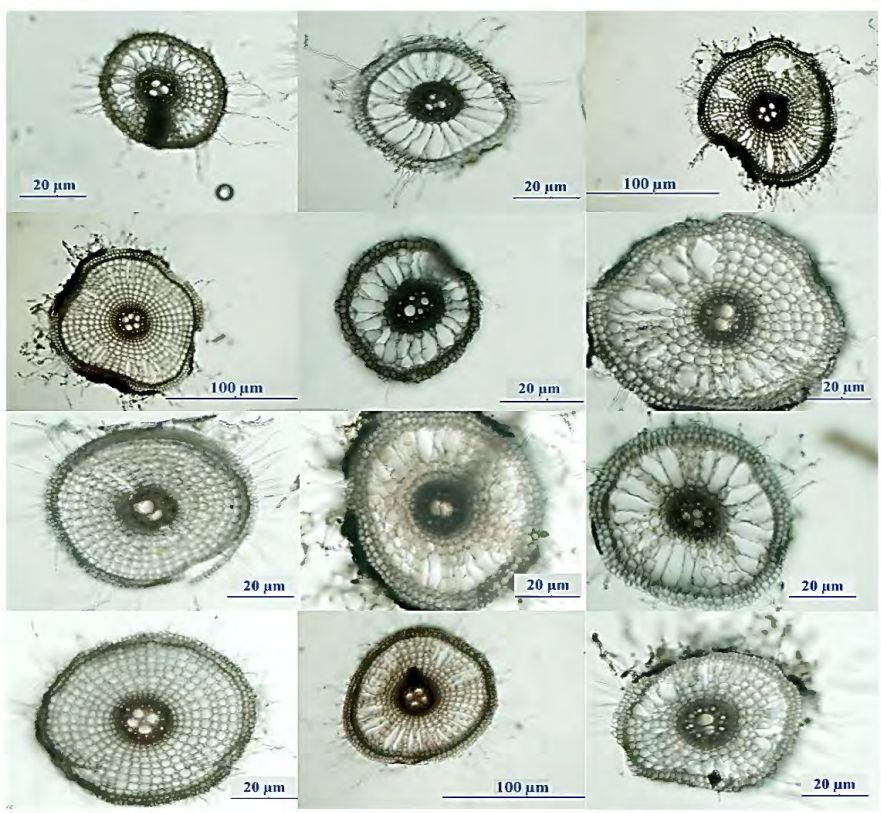


**Fig. 3.** Phenotypic variation in root cortical aerenchyma formation per genotype with three replicates: (1st row) MG-6261 (highest shoot dry weight, SDW and water uptake under DS); (2nd row) MG-6490 (lowest SDW and water uptake under DS); (3rd row) MG-6863 (highest SDW and water uptake under WW);(4th row) MG-6462 (lowest SDW and water uptake under WW).

**Relationship of Percentage RCA with Root Porosity and Percentage Lateral Roots**

Formation of root cortical aerenchyma is positively correlated with root porosity (i.e increase in percent RCA increases the volume of gas-filled spaces in roots) (Colmer 2003, Malik et al. 2003). This enhances the ability of roots, under anaerobic conditions, to transport oxygen to the shoots (Justin and Armstrong 1987, Evans 2004), but porosity may differ according to the root type, whether seminal or nodal (Thomson et al. 1990).

Mean percent porosity was higher under DS (46.7%) than under WW (45.1%) conditions, but there was no significant difference (Fig. 4). DS increased root porosity of MG-6148 by 42% and of MG-6419 by 30%; whereas WW conditions increased root porosity of MG-6806 by 18% and of MG-6420 by 17%. Percent root porosity was not significantly different across genotypes under both DS (p=0.91) and WW (p=0.24). Under DS, the highest and lowest percent porosity was present in MG-6148 (55.3%) and MG-6256 (38.9%), respectively. Under WW, on the other hand, the highest and lowest present porosity was present in MG-6353 (35.5%) and MG-6148 (31.9%), respectively. No correlation between root porosity and aerenchyma development was present (Fig. 5).

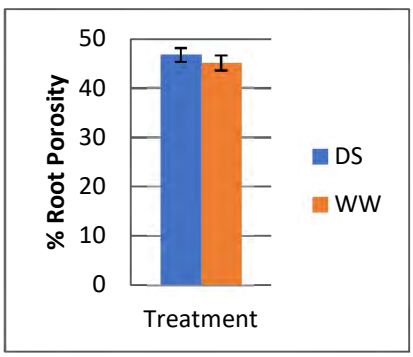


**Fig. 4.** Mean percentage root porosity values under DS and WW conditions (not significantly different at p=0.43).

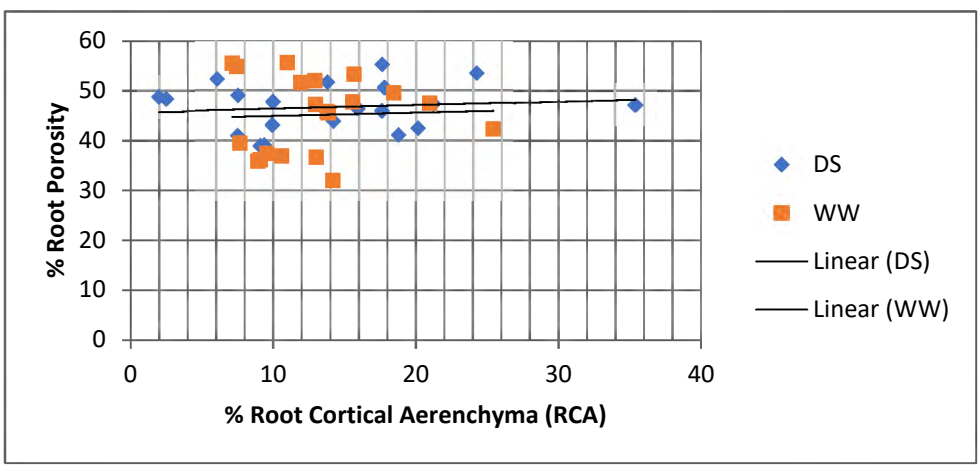


**Fig. 5.** Root porosity and percent RCA under DS and WW conditions. Correlation was not significant using Pearson's correlation at p=0.64.

Percentage lateral roots ranged from 76-84% under WW and from 76-82% under DS condition, with a significance level of p<0.001. Lateral roots represent a larger portion of the entire root system (Wang et al. 2009); thus, their density may affect root system functioning, especially in "improving contact with shrinking water columns in the soil" (Henry et al. 2012). Moreover, aerenchyma development influences lateral root production under waterlogged-drought conditions

(Niones et al. 2013). However, there was no correlation between percentage of lateral roots and percent RCA (Fig. 6). Furthermore, either an increase or decrease in percent root cortical aerenchyma did not affect percentage of lateral roots for all genotypes studied (Fig. 7). Hence, RCA formation was not associated with lateral root development under DS for this study.

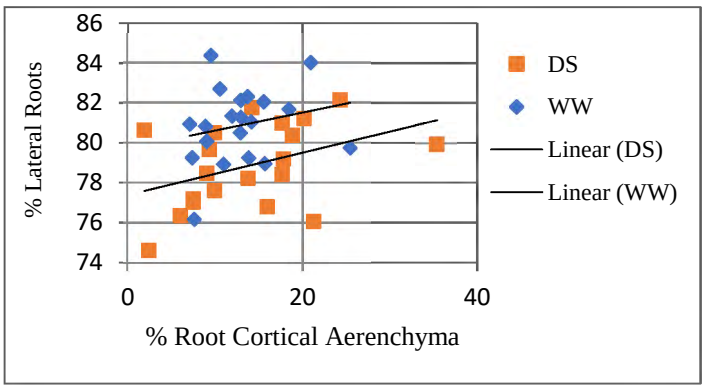


**Fig. 6.** Percent lateral roots and % RCA under DS and WW conditions. Correlation not significant using Pearson's correlation. (not significantly different at p=0.67).

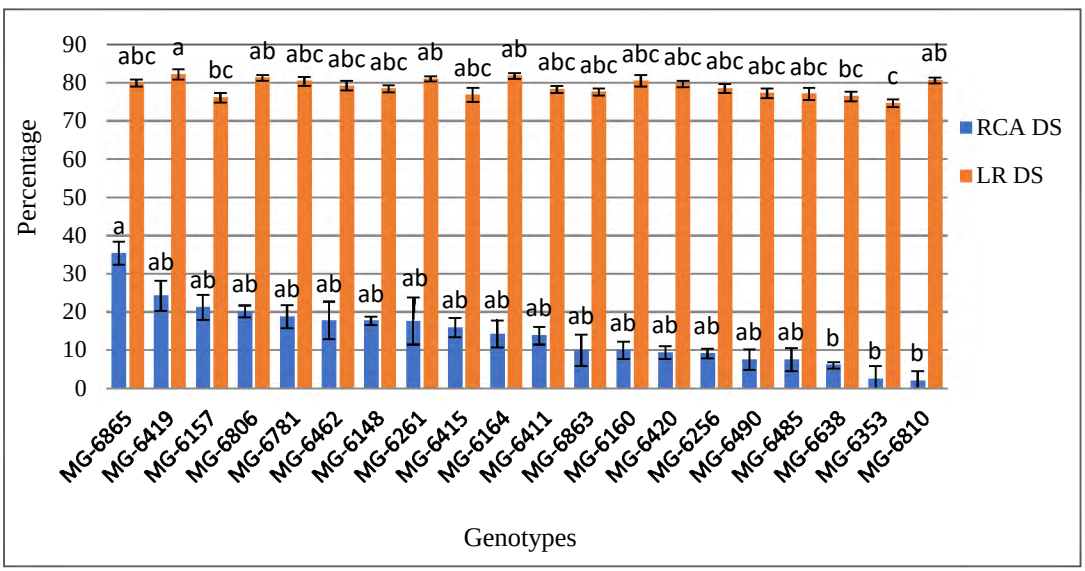


**Fig. 7.** Mean % RCA against mean % lateral roots (LR) for each genotype under DS treatment. Bar indicates the error data. Means with the same letter are not significantly different at P<0.05

**Relationship of Percent Root Cortical Aerenchyma (%RCA) with Total Water Uptake and Shoot Biomass**

Shoot dry weight (g) and total water uptake were both highly significant across all genotypes under both DS (P<0.001) and WW (p<0.01) and in between treatments. WW genotypes exhibited 54% greater shoot biomass than genotypes under DS. Shoot biomass responds significantly to varying soil moisture conditions with genotypes under DS exhibiting 70-79% reduction in shoot dry weight reduction (Suralta and Yamauchi 2008). Genotypes under WW treatment exhibited 48% more total water uptake than under DS. These results indicate RCA formation was not correlated with either water uptake and biomass accumulation under DS and WW treatments (Fig.8).

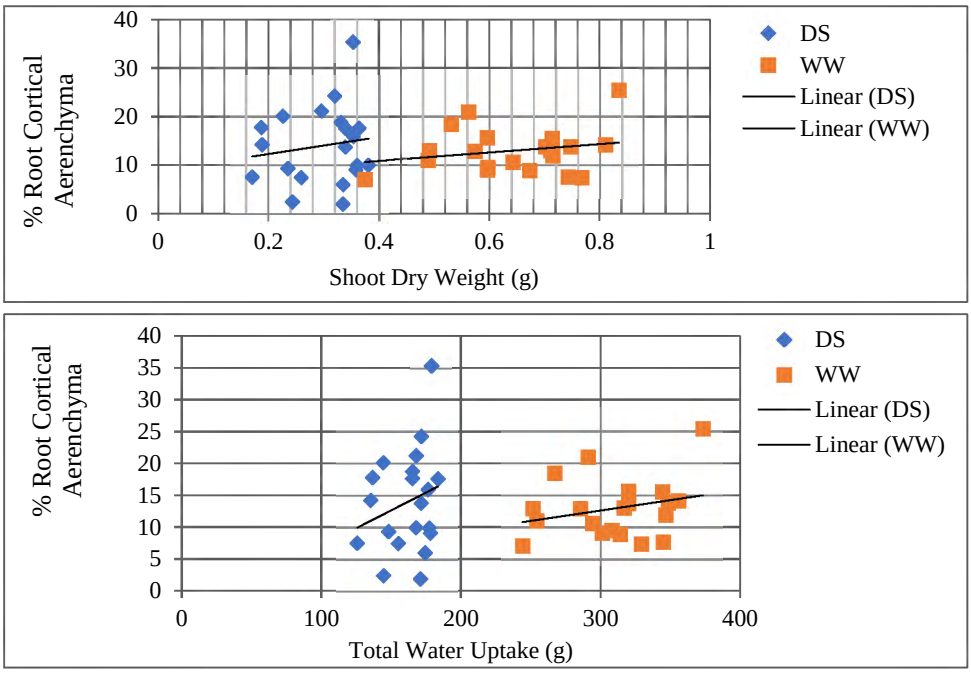


**Fig. 8**. Relation between % RCA and total water uptake (TWU) and between % RCA and shoot dry weight (SDW). SDW and % RCA are not correlated under DS ($p=0.43$) and WW ($p=0.11$). TWU and % RCA are also not correlated under DS ($p=0.75$) and WW ($p=0.18$). Data was analyzed using Pearson Correlation at $p>0.05$ significance level.

Most studies on the relationship between biomass and RCA support a strong positive correlation between the two, especially under nutrient deficient and hypoxic conditions. The efficient use of metabolic resources impacts biomass, soil exploration, and resource acquisition (Lambers et al. 2002). RCA formation in soybean adapts nitrogen metabolism and biomass partially during prolonged periods of flooding (Thomas et al. 2005). In phosphorus-deficient soils, RCA development is induced to reduce the plant's phosphorous requirements and root respiration (Fan et al. 2003). Under nitrogen-deficient soil, RCA enhances root depth by 15 to 31%, increases shoot biomass by up to 66%, and boosts grain yield by 58% (Saengwilai et al. 2014). Under drought, high RCA in maize hybrid lines exhibited high biomass and yield compared togenotypes with low RCA (Zhu et al. 2010). Under drought-to-waterlogged conditions, RCA in rice hybrids promotes lateral root production, which contributes to high dry matter production (Niones et al. 2013). RCA formation decreases water uptake in rice plants under drought conditions, as it impedes the radial transport of water (Yang et al. 2012). As our results show, RCA formation in the global MAGIC rice genotypes did not affect total water uptake, shoot biomass accumulation, and the production of lateral roots. Under seedling-stage severe drought stress, the formation of aerenchyma was observed to be a mere response to the absence of water through cell lysis. Therefore, other root traits besides RCA may be more correlated with drought-tolerance for the genotypes studied.

## CONCLUSION

Earlier studies suggest that aerenchyma tissue is formed in roots to decrease the metabolic costs of root respiration, and to increase the root system capacity for uptake, by inducing lateral root formation. In this study, aerenchyma formation in rice roots under drought was not correlated with root porosity, lateral root development, total water uptake, and shoot biomass. The high aerenchyma

formation observed in genotypes exposed to drought may have been due to cell lysis, as a response to the absence of water. Hence, aerenchyma formation does not aid the studied genotypes in adapting to drought stress. Thus, the variable results in the traits observed may be due to the population's high genetic diversity. High RCA-producing lines in the population need to be subjected to further drought studies to improve our understanding on the dynamics of aerenchyma formation in rice roots exposed to drought stress.

## RECOMMENDATIONS

As the study is an initial report on aerenchyma formation under drought stress for the global MAGIC rice, it is therefore recommended to conduct seasonal replications, to further establish the various parameters measured. In addition, identified genotypes with high RCA formation should be studied further on their drought response.

## ACKNOWLEDGMENT

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# TRANSMISSION OF EPISOMAL *BANANA STREAK VIRUS* BY MEALYBUGS OF DIFFERENT HOST PLANTS

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## ABSTRACT

The B genome of *Musa* cultivars contain several infectious endogenous sequences of Banana streak virus (BSV) that constrains bunch growth and harvest. BSV can be easily transmitted into the banana plant by mealybugs (*Pseudococcidae*) which are known to subsist on banana and other plantain. Several species of mealybugs from different host plants were collected to assess the efficiency to transmit BSV. The study was conducted at the Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños. The results showed that episomal BSV can be transmitted to uninfected banana not only by mealybugs of *Musa* sp. (*Pseudococcus elisae* and *Dysmicoccus brevipes*) but also by mealybugs of *Manilkara zapota* (unreported), *Anonna muricata*, *Ananas comosus* (*Dysmicoccus brevipes*) and *Nephelium napaceum*. The mealybugs from pineapple and rambutan had the highest transmission efficiency (100%). The inoculated test plants exhibited typical BSV symptoms at 115 and 267 days post-inoculation when mealybugs from pineapple and rambutan were used respectively. Multiplex immunocapture PCR assay detected four episomal BSV species from the test plants either singly or in mixed infections. Single BSMYV infection elicited the most severe symptoms. Samples that yielded negative results to species identification suggest the presence of new species of BSV in the Philippines

**Key words**: *Musa, Pseudococcidae,* banana streak disease, immunocapture, virus screening

## INTRODUCTION

Banana streak disease (BSD) is the most widely distributed among the viral diseases infecting banana and plantain worldwide. The disease was first reported in 1974 in banana fields in Southern Morocco and has been then reported in Africa, Asia, Central and South America, and Oceania (Lockhart and Jones 2000). Many spontaneous disease outbreak have been reported over the years but with no confirmed epidemic worldwide apart from the reported 1996 Uganda epidemic in East Africa, where BSV was classified endemic (Tushmereirwe et al. 1996; Iskra-Caruana et al. 2014).

BSD symptoms are characterized by yellow to white, broken or continuous chlorotic streaks and spindle-shaped lesions. These chlorotic lesions progressively turn necrotic on the leaves and may produce black streak appearance in older leaves. The symptoms are erratically distributed on the plant and are not shown on all leaves. Additionally, pseudostem-splitting, reduction of plant height, fruit malformation and size reduction, delay in bunch emergence and maturation and plant collapse were observed (Lockhart 1986; Natsuaki and Furuya 2007). The causal agent of BSD was initially referred to as *Banana streak virus* (BSV). However, at present, the International Committee on Taxonomy of

Viruses (ICTV), has recognized nine distinct BSD-causing viruses, all belonging to the genus Badnavirus of the family Caulimoviridae. These viruses were referred to as BSV species (BSV's) which were distinguished based on sequence information, genetic and serological analyses (Lockhart 1986; Geering et al. 2000; Jones 2000; Harper et al. 2004 and 2005; Geering et al. 2005a and b; Geering and Parry 2011; Lheureux et al. 2007; Bhat et al. 2016).

BSV is a plant pararetrovirus with circular dsDNA which is approximately 7.2 to 7.8 kbp long and encodes reverse transcriptase (RT) for replication. It is encapsidated into bacilliform particles which are not only transmitted horizontally but also vertically as homologous integrated BSV sequence within the *M. balbisiana* genome (LaFleur et al. 1996; Harper et al. 1999; Ndowora et al. 1999; Lheureux et al. 2003; Gayral et al. 2008; Chabannes et al. 2013; Iskra-Caruana et al. 2014). These endogenous BSV sequences (eBSVs) are partial and rearranged sequences with various number of copies which are integral into the banana genome as a result of illegitimate recombination between the virus and the plant genome possibly during repair of breaks in plant DNA (Natsuaki and Furuya 2007, Gayral et al. 2008, Liu et al. 2012, Iskra-Caruana et al. 2014). Viral replication can occur without introgression into the banana genome (Natsuaki and Furuya 2007). Under known stress conditions, such as the use of *in vitro* tissue culture or interspecific breeding, eBSVs can escape spontaneously from the banana genome and reconstitute *de novo* infectious particles or episomal BSV. Consequently, activated eBSVs or episomal BSV sequences causes the Banana streak disease (Ndowora et al. 1999; Dallot et al. 2001; Lheureux et al. 2003; Gayral et al. 2008; Cote et al. 2010; Iskra-Caruana 2010; Chabannes et al. 2013; Chabannes and Iskra-Caruana 2013).

The majority of plant viruses are vector-borne. Seventy six of plant viruses are transmitted by arthropods, nematodes and fungi (Chi-Wei et al. 2010). Banana streak virus is a mealybug-transmitted member of the pararetrovirus genus *Badnavirus*. Although BSV can be readily transmitted experimentally to banana by mealybugs, attempts to transmit the virus by mechanical inoculation have failed and this was attributed to the very high levels of phenolic compounds, latex and other inhibitory substances present in bananas (Lockhart 1986 and 1995).

Several mealybug species known to transmit BSV in the field were reported according to the geographic location of the mealybug populations. *Planoccocus musa* was reported in BSV-infected fields of Nigeria while *Dysmicoccus* spp. was reported in West Africa and South America. Earlier experimental transmission of episomal BSV was done using *Dysmicoccus brevipes*, *Planococcus citri*, *Planococcus ficus* and *Pseudoccocus longispinus*. Out of which, only *P. longispinus* was the only vector reported that does not transmit BSV (Meyer 2006). Therefore it is necessary to identify all mealybug species that are possible vectors and efficient transmitters of the virus in order to apply effective management strategies for the disease.

In the Philippines, many of our bananas (*Musa sp)* are planted along other crops such as: rambutan (*Nephelium napaceum)*, chico (*Manilkara zapota*), guyabano (*Anonna muricata)*, papaya (*Carica papaya*), pineapple (*Ananas comosus)* and others. Several mealybug species have already been identified. *Pseudococcus elisae* and *Dysmicoccus brevipes* on banana, *Dysmicoccus brevipes* is also present in papaya and pineapple, while *Planococcus lilacinus* was recorded on guyabano and rambutan (Lit and Calilung 1994 a and b). Subsequent studies reported *Pseudococcus lepelleyi* and *Dysmicoccus neobrevipes* on guyabano and *Cataenococcus hispidus* on rambutan (Lit 1997).

This study was conducted to evaluate the transmittability of BSV to banana by mealybugs of other plant species: *A. muricata*, *C. papaya*, *A. comosus*, and *N. napaceum*, assess the relationship between the mealybugs from other plant species and BSV symptom severity, and identify the BSV species transmitted.

## MATERIALS AND METHODS

**Source of BSV infected plants.** Banana plants showing typical symptoms of BSV were collected from Laguna (Bay, Cavinti, Majayjay, Pasong Kipot), Batangas (Tanauan), Quezon (Lucban, Lucena, Pagbilao) and Davao del Sur (Hagonoy) (Fig. 1), and were kept in insect-proof cages. Collected plants or corms were individually planted in a 24-liter plastic pails containing sterile soil.

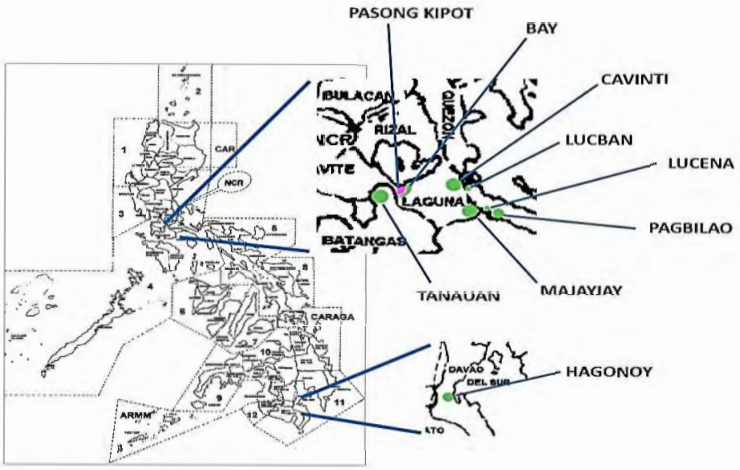


**Fig. 1.** Geographical representation of the collection sites for symptomatic banana samples (www.fao.org).

**Crude sap extraction.** The sap extraction method and immunocapture procedure was conducted following the method published by Thomas (2008) and Geering et al. (2000) with some modifications. Sample collection was done by cutting approximately 0.5 gram of banana leaf samples from the second youngest fully expanded leaf of the plant. For sap extraction, samples were individually placed in plastic collection bags containing 4 ml of sap extraction buffer (0.05 M Tris-HCl, 2.5% skim milk, 0.5% sodium sulfite). The collected samples were properly labelled and temporarily stored in a cooler with ice packs and were immediately brought to the laboratory for homogenization. Otherwise, samples were stored in freezer (0 to -5 °C). One ml of homogenized samples were individually transferred onto 1.5 ml Eppendorf tubes, tightly capped and spun for five minutes at 10,000 rpm using Centrifuge 5424 (Eppendorf, Germany).

**Detection of Endogenous BSV.** In this study, all polymerase chain reaction (PCR) assays were done twice. *Musa* virus indexing procedures by Su (1999), Geering et al. (2000) and Thomas (2008) were modified and optimized. Endogenous BSV was detected using a pair of primers BSV F1 (5' – CAACTCAAGAGCCTAGTATGC – 3') and BSV R2 (5' – TACCTCCGACCGTATTTCCAG – 3') (Su 1999) with an expected product size of 220 bp (Geering et al. 2005a and b). A total reaction mix volume of 15 µl [1X PCR buffer (Invitrogen), 1. 5 mM $MgCl_2$ (Invitrogen), 0.20 mM dNTPs, 0.10 µM of each primer pair, 1.2 units of Taq polymerase (Vivantis) and 2 µl of sap template] was assayed in Mycycler (Bio-Rad Laboratories Inc., USA) with the following PCR conditions: 1 cycle of denaturation at 94°C for 4 min, annealing at 50°C for 1 min, extension at 72°C for 2 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min; and 1 cycle of final extension of 10 min at 72°C.

**Detection of Episomal BSV.** Immunocapture Polymerase Reaction (IC-PCR) was used to detect episomal BSV. The viral protein was captured by a mixture of *Sugarcane Bacilliform Virus* (SCBV) antibody (AGDIA Co.) and carbonate coating buffer with a recommended dilution of 1:200 (Thomas

2008). Fifty (50) µl of the SCBV-carbonate coating buffer mixture were pipetted onto sterile 0.2 ml microcentrifuge tubes, incubated overnight at 4 °C, and discarded. Fifty (50) µl of the antigen (samples) were then loaded onto the tubes individually and incubated for 3-4 hours at 37 °C. Afterwards, the tubes were washed thrice with 1X PBS-T buffer, allowed to stand for three minutes every time and a final wash of distilled water. Episomal BSV infected *Musa* were then used as donor plants and positive controls for subsequent activities.

**Rearing of transmission vector.** Mealybugs used in this study were obtained from banana (*Musa paradisiaca* Linn.), chico (*Manilkara zapota*), guyabano (*Anonna muricata*), papaya (*Carica papaya*), pineapple (*Ananas comosus*), and rambutan (*Nephelium napaceum*) (Fig. 2). The mealybugs from these plants were reared separately on detached immature squash fruits. The other mealybug species were directly introduced into the banana seedling.

**Fig. 2.** Mealybugs used as the vector of banana streak virus (BSV).

**BSV Transmission set-ups.** Endogenous sequences can be found in both *M. acuminata* (A genome) and *M. balbisiana* (B genome) banana. However, episomal sequences were found to be associated only to B genome *Musa* (Ndowora et al. 1999; Geering et al. 2000; Lheureux et al. 2003; Gayral et al. 2008). Hence, in this study, B genome *Musa* tissue culture-derived banana plants were used. Available tissue culture materials were potted in 3x3x5 inch plastic bags with sterile soil mixed with coir dust and kept in the greenhouse for 2 months to acclimatize the plantlets before the virus transmission procedure. All plants were maintained in the greenhouse of the Institute of Plant Breeding (IPB, UPLB). The transmission set-up consisted of six treatments: (i) transmission from episomal BSV infected banana using *Musa* mealybugs to B genome tissue culture-derived recipient plants; (ii) transmission using *M. zapota* mealybugs; (iii) transmission using *A. muricata* mealybugs; (iv) transmission using *C. papaya* mealybugs; (v) transmission using *A. comosus* mealybugs; and (vi) transmission using *N. napaceum* mealybugs. The B genome tissue culture-derived *Musa* that served as recipient plants were obtained from the *M. balbisiana in vitro* collection of the institute.

**Acquisition and inoculation period.** Transmissions using the mealybugs from the different host plants were done separately in the insect-proof screenhouse of IPB-UPLB. Adult mealybugs were starved for 4 hours and were placed on the leaves of the infected field samples (donor plants) previously tested for the presence of episomal BSV using IC-PCR. Each species of mealybugs were allowed to feed on the BSV infected *Musa* plants (donor) for 3 days. After the acquisition period, 20 mealybugs from each plantain were removed from the donor plant and were transferred to tissue culture-derived B genome *Musa* plantlets (potted-out approximately 2 months prior to experiments).

**Disease assessment.** The inoculated plants were closely observed for the appearance of the characteristic symptoms of BSV infection such as chlorotic streaks (Natsuaki and Furuya 2007). The days starting from insect transmission to the appearance of symptoms were recorded. All banana plant samples were checked for virus activation (episomal BSV infection) at three months and six months period after inoculation using IC-PCR. Disease severity rating was done on the youngest fully expanded leaf of each sample and was quantified using a modified scale of 0 to 5 (Karanja et al. 2013;

Wambulwa et al. 2013), where 0 is no symptoms, 1 is localized flecks, 2 is scattered discontinuous streaks, 3 is continuous streaks covering moderate portion of lamina, 4 is continuous chlorotic streaks, and 5 is necrotic streaks.

**BSV strain identification using Multiplex PCR (Mp-PCR).** Strain identification of samples tested positive for episomal BSV was accomplished using the same crude sap extraction method and PCR cocktail concentration except for the final concentration of 0.40 µM BSV strain-specific primer mix (Table 1) used and the use of a multiplex touchdown condition for the assay. The multiplex touchdown PCR assay condition is as follows: 1 cycle of initial denaturation at 94°C for 30 s; followed by 2 cycles of denaturation at 94°C for 20 s, annealing at 64°C for 30 s, extension at 72°C for 1 min; 2 cycles of denaturation at 94°C for 20 s, annealing at 62°C for 30 s, extension at 72°C for 1 min; 10 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, extension at 72°C for 1 min; 25 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 30 s, extension at 72°C for 1 min and 1 cycle of final extension of 3 min at 72°C. Multiplex IC-PCR was done following the same multiplex touchdown conditions mentioned with the application of the previously mentioned immunocapture method for BSV (Le Provost et al. 2006).

**Table 1**. BSV Strain-specific primers used to distinguished BSV sequences.

| Target Virus | Name of Primer | Primer Sequences | Product Size |
|---|---|---|---|
| BSOLV* | RD-F1 | 5'-ATCTGAAGGTGTGTTGATCAATGC-3' | 522 |
| | RD-R1 | 5'-GCTCACTCCGCATCTTATCAGTC-3' | |
| BSGFV* | GF-F1 | 5'-ACGAACTATCACGACTTGTTCAAGC-3' | 476 |
| | GF-R1 | 5'-TCGGTGGAATAGTCCTGAGTCTTC-3' | |
| BSMYV* | Mys-F1 | 5'-TAAAAGCACAGCTCAGAACAAACC-3' | 589 |
| | Mys-R1 | 5'-CTCCGTGATTTCTTCGTGGTC-3' | |
| BSIMV** | 914-F1 | 5'-TGCCAACGAATACTACATCAAC-3' | 384 |
| | 914-R1 | 5'-CACCCAGACTTTTCTTTCTAGC-3' | |

*Geering et al. 2000, **Chabannes et al. 2013

**Visualization of PCR Products.** An electrophoregram of 5µl PCR products was achieved by gel electrophoresis using 1.5% w/v Agarose (Vivantis) in 0.5X TAE (Tris-Acetate-EDTA) buffer, stained in GelRedTM solution and visualized using the GelDoc XR+ documentation system (Bio-Rad Laboratories Inc.). The amplicons were quantified through the comparison of their product sizes against the 1 Kb plus DNA marker (Invitrogen, USA).

**Data analysis.** Analysis of variance (ANOVA) was done using GraphPad Instat 3.10 to determine the relationships among mealybug type/vector source, symptom expression and episomal BSV infection.

## RESULTS AND DISCUSSION

**Presence of Episomal BSV on selected materials.** The 43 plants collected from eight locations showed chlorotic and necrotic streaks (Fig. 3). Among these, only 30 plants were found to be episomal BSV positive (Table 2). Episomal BSV infected *Musa* were then used as donor plants and positive controls for this experiment. Interestingly, severe symptoms were observed in plants grown in areas with high elevation and low temperature. Infected plants exhibited symptoms from the leaves, petiole, down to the pseudostem. More samples could have been assayed if collections were not only focused on plants that showed typical symptoms of the disease on leaves. Symptoms of BSV infection were observed to be erratically distributed on the plant and may not be present on all leaves. Additionally, there have been reports of alternating symptomatic and asymptomatic stages in infected

plants even though the virus was detected at all stages (Dahal et al. 1998; Dahal et al. 2000a and b; Lockhart and Jones 2000; Harper et al. 2002).

**Table 2.** Summary of survey and collection of source plants screened using IC-PCR.

| Place of Collection | | Symptomatic plants | eBSV positive |
|---|---|---|---|
| Batangas | Tanauan | 3 | 1 |
| Davao del Sur | Hagonoy | 1 | 1 |
| Laguna | Bay | 9 | 2 |
| | Cavinti | 7 | 6 |
| | Majayjay | 4 | 3 |
| | Pasong Kipot | 12 | 10 |
| Quezon | Lucena | 4 | 4 |
| | Pagbilao | 3 | 3 |
| **TOTAL** | | 43 | 30 |

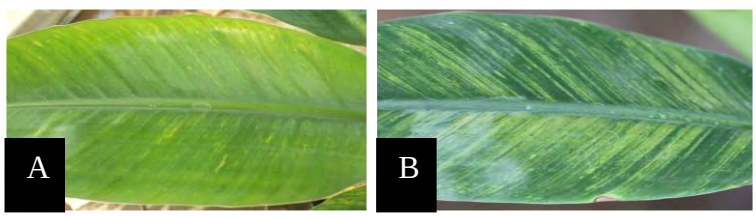


**Fig. 3**. Mild (A) and severe (B) chlorotic streaks on leaves of *Musa* sp. infected with Banana Streak Virus.

**Mealybug transmission of *Banana streak virus*.** Mealybug from banana, chico, guyabano, pineapple and rambutan transmitted eBSV to healthy *Musa* plants (Table 3). Chlorotic streaks which turn necrotic vary from 100 and 267 days after mealybug transmission. The earliest infection occurred in plants inoculated with mealybugs of banana, followed by plants inoculated with pineapple mealybugs. Plants inoculated with the virus using mealybugs from rambutan and chico exhibited typical BSV symptoms after 267 and 219 days after transmission, respectively. The results of the transmission studies show that episomal BSV can be transmitted by mealybugs from banana (*Musa* sp.), chico (*Manilkara zapota*), guyabano (*Anonna muricata*), pineapple (*Ananas comosus*), and rambutan (*Nephelium napaceum*) to episomal BSV-free *Musa balbisiana*. This suggests that the spread of activated BSV can be facilitated by mealybugs from these crops that are planted around banana growing areas. Therefore it is necessary to identify all mealybug species that are possible BSV vectors and further assess their efficiency to transmit the virus. Similar results have been reported on the grapevine leaf roll virus (GLRV) which can be transmitted by several species of mealybugs including soft-scale insects. Nine species of mealybugs were reported vectors of GLRaV (Lemaguet et al. 2012).

**Table 3.** Mean symptom expression (based on scale 0 to 5) for banana samples infected with episomal BSV.

| Mealybug Host | % of Plants infected*** | | Mean** | Days to symptom expression |
|---|---|---|---|---|
| | 3 * | 6 * | | |
| *Musa balbisiana* | 55.2 | 79.3 | 1.48±1.527 | 100 |
| *Manilkara zapota* | 83.3 | 83.3 | 1±1.637 | 219 |
| *Anonna muricata* | 60.0 | 60.0 | 1.4±1.95 | 185 |
| *Carica papaya* | 0 | 0 | 0±0 | 0 |
| *Ananas comosus* | 75.0 | 100 | 2.83±0.619 | 115 |
| *Nephelium napaceum* | 0 | 100 | 2.17±1.211 | 267 |

*months after inoculation **0 to 5 symptom expression scale (Karanja et al. 2013).
***confirmed using IC-PCR assay.

The specific identity of the BSV vector collected from banana, guyabano and rambutan needs to be confirmed because of the presence of two (banana and rambutan) and three (guyabano) mealybug species in one plant species. However, *D. brevipe*s (on pineapple) and the mealybug species from chico, are possible vectors of the banana streak virus since no other species are present/recorded on the plants. The mealybug species has not been identified yet, however, the buff mealybug *Nipacoccus nipae* reported on coconut and palms was also observed on chico and banana (Caasi-Lit et al. 2009). Nineteen species of mealybug belonging to 13 genera are known to occur on *Musaceae* (Watson and Kubiriba 2005). Experimental transmission of BSVs also has been demonstrated with the pink pineapple mealybug *Dysmicoccus brevipes* (Cockerell) by Kubiriba and co-workers (2001). Other species reported to transmit BSV are Dysmicoccus spp. in West Africa and South America, *Planococcus mu*sa in Nigeria, *Ferrisia virgata* (striped mealybug) in India (Selvarajan et al. 2006), and *Paracoccus burnerae* (Muturi et al. 2013) in South Africa.

**Symptom severity and mealybug of other plant host.** Symptom severity of the youngest fully expanded leaf of each sample was scored on a 0 to 5 modified scale (Karanja et al. 2013; Wambulwa et al. 2013). The results shown in Table 3 indicate that the symptoms were more severe in *Musa* infected using *A. comosus* mealybugs (with highest symptom expression mean of 2.83 ± 0.619 and 100% episomal BSV infection at 6 months after inoculation). The proportion of the samples infected with episomal BSV was consistent for six months using *M. zapota* and *A. muricata* mealybugs. *Manilkara zapota* mealybug infected samples expressed type 1 symptoms at 83.3% infected samples compared to *A. muricata* mealybug infected samples which expressed type 2 symptoms at a lower percentage of 60%. The mean symptom expression for samples inoculated with *N. napaceum* mealybugs followed that of *A. comosus* mealybugs at 2.17±1.211, also with 100% infection six months post inoculation. *Musa* inoculated using *C. papaya* mealybugs were negative to episomal BSV. Analysis of variance (One-way ANOVA) with regards to symptom expression showed that BSV inoculation using the six mealybug species differ significantly ($P<0.05$). The higher proportion of infected plants in the sixth month shows that virus concentration increases with plant growth. Thus, early detection and elimination of infected plants are important in mitigating the disease.

Results of this study also showed that some correlation exists between the mealybug used in the inoculation of BSV and the degree of the symptoms observed. However, since previous studies have shown that many factors influence BSV symptom expression, such as temperature (Dahal et al. 1998) and other environmental conditions (Mobambo et al. 1996; Daniells et al. 2001), the study can only be considered as a glimpse into the complex vector-virus-plant relationship.

**BSV species identification.** Species identification was done on all episomal BSV positive plants. Only 9 out of the 48 plants inoculated using mealybugs of banana showed positive results. Four episomal BSV species were detected as mixed infections in 7 plants, 4 with BSGF-MYV, 2 with BSMYV-IMV and 1 with BSOL-IMV (Table 4). While single infections of BSGFV and BSMYV were detected in two different plants.

**Table 4.** Species identification of episomal banana streak viruses in plants inoculated with BSV using mealybugs from different plants.

| Mealybugs Source | Multiplex IC-PCR | | | | | | |
|---|---|---|---|---|---|---|---|
| | **BSGFV** | **BSIMV** | **BSMYV** | **BSOLV** | **BSGF-MYV** | **BSOL-IMV** | **BSMY-IMV** |
| Banana | 1 | 0 | 1 | 0 | 4 | 1 | 2 |
| Chico | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Guyabano | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pineapple | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rambutan | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

The BSMYV infected test plant was observed to have the most severe symptoms in the study at category 4 (continuous and conspicuous chlorotic streaks) followed by the BSVGFV infected sample with category 3 symptoms (continuous streaks covering moderate portion of the lamina) according to Wambulwa et al. 2013. Symptom severity remain high in plants with mixed infections of BSMY-IMV that either fall under category 2 (scattered discontinuous streaks) or in between categories 2 and 3. While the BSVGF-MYV infected plants fall under category 3.

More severe symptoms were observed in samples with mixed infections, such that the more the number of isolates, the more severe the symptoms (Karanja et al. 2008). This is, however, in contrast with the observations made in this study. The more severe symptoms were found on single infections of BSMYV and BSGFV, and an exemption of BSGF-MYV. The severe symptoms caused by the Mysore isolate can be attributed to the more immunogenic epitopes of BSMYV compared to other isolates (Wambulwa et al. 2013). This contradiction on observed severity of symptoms between single and mixed infections calls for further research.

Multiplex-IC-PCR was not able to identify the BSV species infecting the remaining episomal BSV positive plants inoculated using mealybugs from chico, guyabano, pineapple, and rambutan (Fig. 4), this suggests that new BSV species may be present in the Philippines.

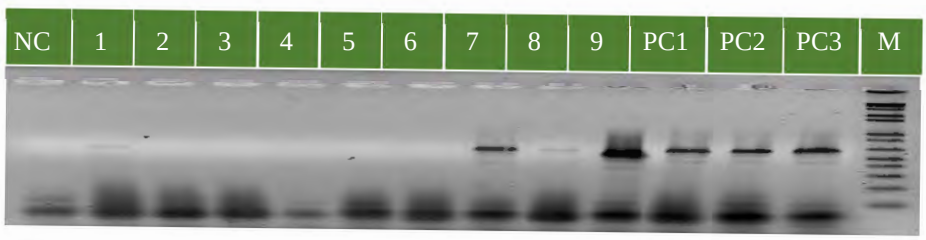


**Fig. 4.** Agarose gel (0.5%) electrophoresis of products from Multiplex IC- PCR assay using primer mix of BSGFV (476 bp), BSIMV (384 bp), BSMYV (589 bp), and BSOLV (522 bp) primers. Lane NC – negative check, Lane 1-3 – B2-B4 (6 MAT*****), Lane 4-6 – G1-G3 (3 MAT), Lane 7-9 – B15, B17, B18 (3 MAT), Lane PC1-PC3 – positive check (known infected plant), and Lane M – molecular weight marker (1 kb plus ladder). *****MAT **–** months after transmission.

## CONCLUSION

Episomal BSV can be transmitted to uninfected banana by mealybugs of *Musa* sp. (*Pseudococcus elisae* and *Dysmicoccus brevipes*), *Manilkara zapota* (unreported), *Anonna muricata*, *Ananas comosus* (*Dysmicoccus brevipes*) and *Nephelium napaceum*. Virus transmission efficiency and mean symptom severity was notably highest using the vectors from the last two crops. Mealybugs play a major role in the natural spread of the virus. Since bananas in the Philippines are also planted alongside the aforementioned crops, management strategies should therefore be addressed at reducing the population of these mealybug species from other crops.

Additionally, four episomal BSV species were detected in this study either in single or mixed infections. Single infections were observed to elicit more severe symptoms in contrast to a previous report and needs further verification. Several samples that showed negative results to species identification suggests the presence of a new species of BSV in the Philippines. It is imperative therefore, to evaluate bananas from other areas for presence of BSV to avoid further spread of the disease. Host resistance to this virus and to the vectors of BSV is one of the most effective control measure for both pests. In addition, removal of infected banana plants is one of the most practical and appropriate means to control the disease.

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# ANALYSIS OF SEASONALITY IN MONTHLY PORK PRICES IN THE PHILIPPINES BASED ON X-12 ARIMA

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## ABSTRACT

Seasonal movements in prices are assumed to be predictable as they are expected to recur within one year periods. The study aimed to capture seasonality in monthly prices of pork in the Philippines in order to reveal underlying cyclical and trend movements in the economy. Secondary data on nominal monthly price series of pork at the national level covering the period 1990–2016 used were obtained from the Philippine Statistical Authority (PSA) CountryStat website. The X-12 ARIMA (autoregressive integrated moving average) method was used to seasonally adjust the monthly farm gate and retail prices of pork in the Philippines. Results show that both price series exhibited clear upward trends and normal irregular variations. F-tests for the presence of seasonality also revealed that monthly farm gate and retail prices of pork present stable seasonality along with moving seasonality at 1% level of probability. Trading day and leap year effects were found to be insignificant.

**Key words**: X-12 ARIMA, trend-cycle, seasonality, irregular component

## INTRODUCTION

Swine production in the Philippines is a P191 billion industry (DOST-PCAARRD 2016). Next to rice, the swine industry contributes 18.25 percent to the country's gross value added (PSA 2017). The Philippines is one of the top ten countries in the world with the fastest growing meat consumption. According to the Organization for Economic Cooperation and Development (OECD), 2017, an average Filipino consumes about 14.2 kg of pork (2 kg more than the world's average pork consumption), 11.6 kg of chicken and 3.0 kg of beef or veal. The country's present total meat consumption of 28.8 kg per year is projected to further grow given the increasing population and rising income. In 2025, the pork consumption per capita in the Philippines is projected to increase to about 14.35 kilograms per person annually (Statista).

Because of the importance of meat in the diet composition (Ompoy and Prantilla 2013) of Filipinos, especially for low-income households, understanding the behavior of meat prices, both the overall long-term trend of prices and the intra-year price fluctuations, is a major concern for the Philippine Government. Gordoncillo et al. (2016) claim that any movement in meat prices can have a significant and serious impact on household food security and well-being. The producers, consumers, policymakers, and other stakeholders directly or indirectly involved in production, consumption, and marketing of hogs, need to take into account these price variations in their decision-making process.

Agricultural prices often follow a seasonal pattern because production is seasonal and storage is costly. In some cases, seasonal demand (such as holiday consumption) may also contribute to seasonality in agricultural prices. The literature abound in long-term projections about the supply and

demand for hogs, trade and prices but the systematic analysis of seasonality in livestock prices has received less attention. This paper hopes to address the research gap by analyzing seasonality in the monthly prices of pork in the Philippines.

Seasonality in prices reflects monthly or quarterly variations caused by changes in the weather, agricultural arrangements, social traditions, among others (Zhou and Dong 2007). Seasonal movements are assumed to be predictable as they are expected to recur within one year periods, and enable making short term forecast. Understanding seasonality is essential for correct seasonal adjustment and analysis of the data, and useful for making better forecast in production and marketing decisions, and food security interventions.

The main goal of this study was to analyze the seasonality of monthly farm gate and retail prices of pork. To achieve this goal, X-12 ARIMA was used to seasonally adjust the monthly price series of pork from 1990–2016. Seasonal adjustment is a tool for highlighting the underlying trends and short-run movements of economic processes, as well as unexpected events and shocks of economic processes. Hence, seasonally adjusted data can facilitate control of various aspects of government policy. The comparison based on adjusted data is more reliable because the seasonally adjusted time series are artificial data that depict the economy unaffected by repetitive events like usual weather conditions, holidays, typical social habits and practices. They are especially helpful when comparing the periods in the presence of outliers.

## METHODOLOGY

Secondary data on monthly nominal farmgate and retail prices of pork in the Philippines from 1990 to 2016 were gathered from CountryStat of the Philippine Statistics Authority (PSA) website (http://countrystat.psa.gov.ph). The X-12 ARIMA model was used in the analysis. This model has been widely used by most leading statistical institutes world-wide to seasonally adjust data. It is comprehensive, with many options available for tailoring seasonal adjustment to each individual series, and it also provides procedures for examining trading day, holiday and some calendar effects of the time series.

### *X-12 ARIMA*

The US Census Bureau developed in 1998 the X-12 ARIMA model, which is an extended and improved version of the X-11 and X-11ARIMA/88 method of Statistics Canada (Dagum 1980, Hungarian Central Statistical Office 2007, Zhou and Dong 2012), and provided new capabilities and methods of seasonal adjustment program (Findley et al. 1998).

X-12 ARIMA consists of two linked parts: the regARIMA model for estimation of the deterministic components (mainly calendar effects), and the decomposition part of the linearized series for the stochastic components (trend-cycle, seasonality, and irregulars) performed using the X-11 filters combined with those of the ARIMA model extrapolations. According to the *Guide to Seasonal adjustment with X-12 ARIMA* (ONS 2007), in practice most economic time series exhibit a multiplicative relationship and hence the multiplicative decomposition usually provides the best fit. However, because negative values were observed in the monthly farm gate and retail prices of pork, the additive seasonal decomposition was more appropriate. The ARIMA process for both monthly price series was $(0, 1, 1) \times (0, 1, 1)_{12}$. The default 13-term moving average Henderson filter was used. Diagnostic methods include residual diagnostics and F-test for seasonality.

Fig.1 presents the flow diagram for seasonal adjustment with X-12 ARIMA. As described by Findley et al. (1998), the program runs several steps. First, the series is modified by any user-defined prior adjustments. Then the program fits a regARIMA model to the series in order to detect and adjust

for outliers and other distorting effects for improving forecasts and seasonal adjustment. The program then uses a series of moving averages to decompose a time series into three components. In the last step a wider range of diagnostic statistics are produced, describing the final seasonal adjustment, and giving pointers for possible improvements which could be made.

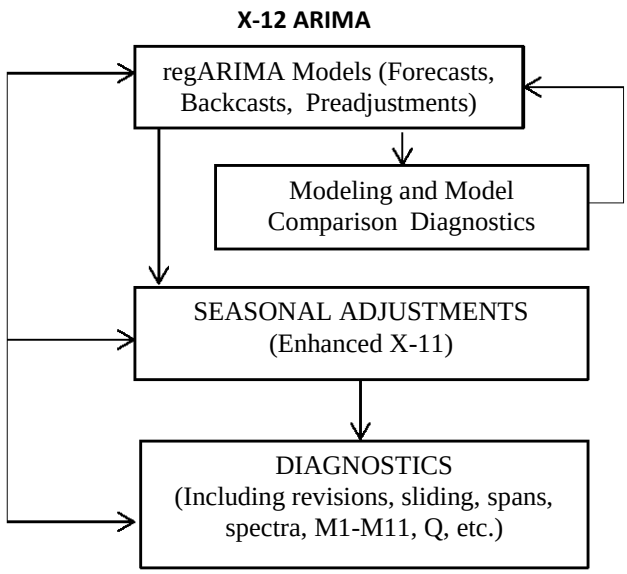


**Fig. 1**. Flow diagram for seasonal adjustment using X-12 ARIMA (Source: Findley et al. 1988)

## RESULTS AND DISCUSSION

### *The Original Price Series*

Before doing seasonal adjustment, the original series were checked for stationarity, additive outliers (AOs), and the level shifts (LSs). The ARIMA model requires the data series to be stationary. A time series is stationary if its mean, variance and autocorrelation structure do not change over time. If the series is not stationary, backcast or forecast of the series, AOs or LSs detection cannot be made available, i.e., the regARIMA part of the X-12 ARIMA would feedback false results (Zhou and Dong 2012). Outliers are observations which do not fit in the tendency of the time series observed as they differ dramatically from the typical pattern of the trend and/or seasonal components (Eurostat, n.d.). Their presence can substantially distort the estimation of the time series component and can affect the moving averages applied in X-12 ARIMA resulting in unrepresentative pattern of the price series.

The behavior of the pre-adjusted (original data series) monthly nominal farmgate and retail prices of pork is shown in Fig. 2. Both price series exhibited a seemingly upward trend with irregular short-term fluctuations. One can infer from the figure that both price series were not stationary.

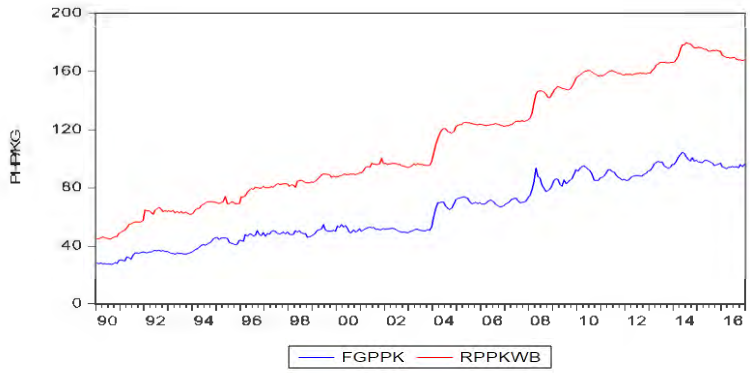


**Fig. 2.** Monthly farmgate and retail prices of pork in the Philippines, 1990-2016.

In order to achieve stationarity, the original farmgate and retail price series were transformed by differencing (*D*) the data, i.e., $y_t = x_t - x_{t-1}$. The first and second seasonal differences of the original farm gate and retail prices of pork are shown in Figs. 3a and 3b, respectively. A cursory examination of the plots indicates that the first difference already improved the stationarity of both price series such that the second seasonal difference resulted in over-differenced series.

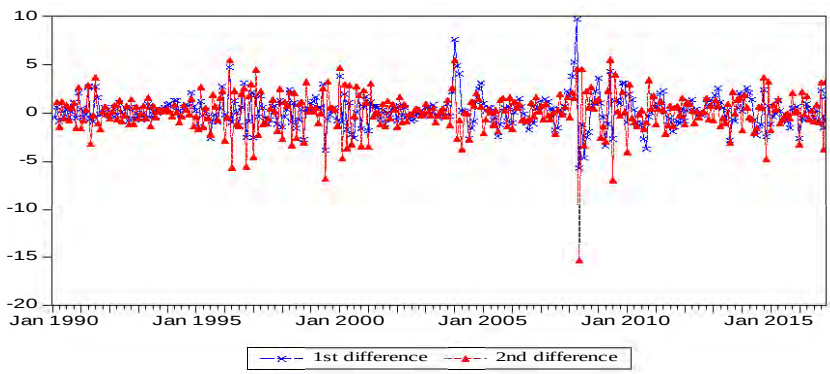


**Fig. 3a.** 1st seasonal difference $\Delta_{12}mY_t$ and 2nd seasonal difference $\Delta^2_{12}mY_t$ for the monthly farm gate price of pork.

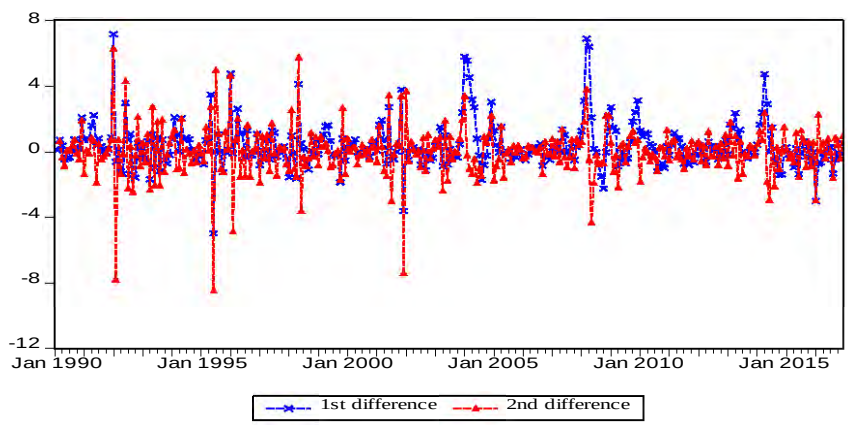


**Fig. 3b.** 1st seasonal difference $\Delta_{12}mY_t$ and 2nd seasonal difference $\Delta^2_{12}mY_t$ for the monthly retail price of pork

To check for autocorrelation, X-12 ARIMA can produce Autocorrelation Function (ACF) and Partial Autocorrelation Function (PACF) of the residuals, along with (Ljung and Box 1978) summary Q-statistics. The ACF and PACF are graphed in Figs. 4a and 4b for farmgate price and retail price, respectively. The ACF of the 1st differenced monthly farmgate and retail price series resembles (0, d, 1) case non-seasonality and (0, d, 1) case seasonality.

The correlogram suggests that (p,d,q) *x* (P, D, Q)$_s$ equals (0, 1, 1) *x* (0, 1, 1) where *d* is order non-seasonal difference, D is order seasonal difference, p and q is the order of non-seasonal autocorrelation process of the explained variable and the non-seasonal white noise's moving average process, respectively; P and Q is the seasonal autocorrelation process of the explained variable and the seasonal white noise's moving average process, respectively. The orders are supported by Akaike Information Criterion test.

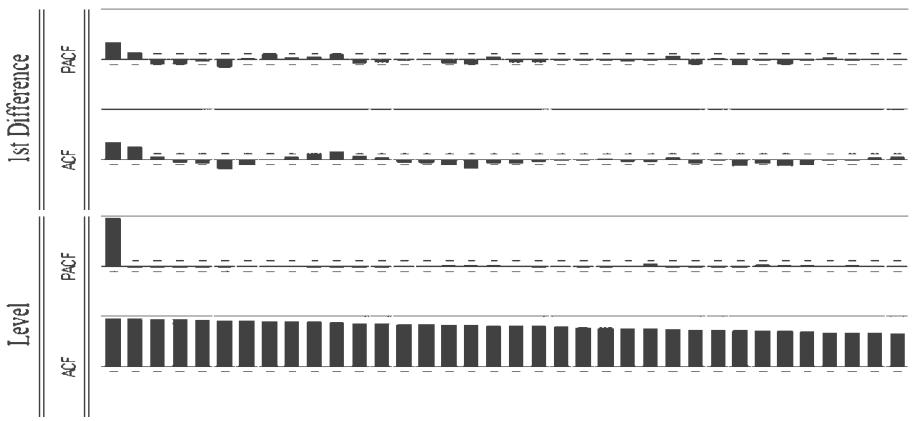


**Fig. 4a**. The correlogram of the monthly farm gate price of pork.

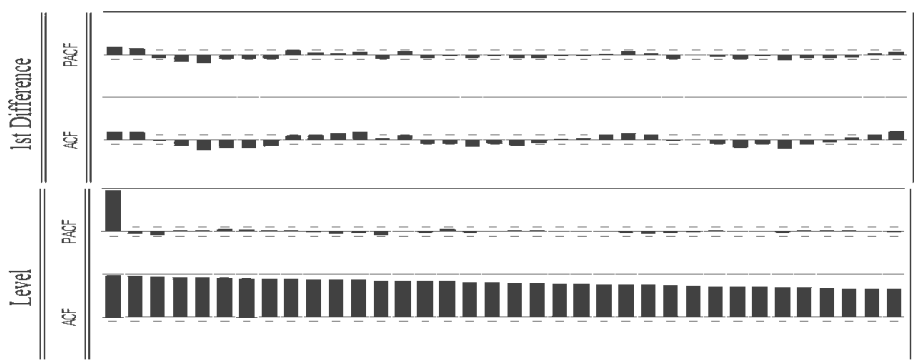


**Fig. 4b.** The correlogram of the monthly retail price of pork

The regARIMA adjusts outliers, trading day effects, holiday effects and user-specified effects using seasonal ARIMA model before the X-11 seasonal adjustments. In this study, the AO/LS outliers were tested at 1% level of probability for both farmgate and retail prices of pork. For monthly farmgate price, the largest AO t-value was 8.27 (2008 Apr) and the largest TS t-value was 5.52 (2004 Jan) suggesting that AO and LS outliers are present (the critical absolute *t*-value for AO/LS was 4.05). These outliers were automatically adjusted in regARIMA for the monthly farm gate price series. For monthly retail price, no AO or LS was detected, and the combined trading day and leap year regressors were also not significant (*p*-value = 0.57).

***The Forecast and Seasonal Adjustments***

In order to assign symmetric weights to the original farm gate and retail price series, the regARIMA model part of the X-12 ARIMA program extended the series by one year ahead forecast. The forecast values of both farm gate and retail prices are presented in Tables 1a and 1b. The monthly forecast for retail price appears to perform better than the farm gate price in terms of error ratio and confidence interval coverage.

*The seasonal adjustments*

The decomposition results include the trend-cycle (TC), the seasonal factors (SF) and the irregulars (IR). The additive decomposition components of monthly farmgate and retail prices series exhibited clear upward trends and normal irregular variations (Fig. 5a and 5b). The secular or long term trend lines for both farmgate and retail prices of pork show that the market was on a clear uptrend. Livestock production and prices are characterized by more or less regular cycles, that is, production and prices have a tendency to move up and down over some period of years and this pattern repeats itself regularly regardless of outside forces. When production is increasing, prices are decreasing, holding other factors constant. After some time, these movements will reverse themselves such that production will decline and prices will move upward. These cycles can be explained by the tendency of the producers to make future plans to produce based on the profits gained from current or more recent past operations.

The seasonal factors on the other hand appeared to be stable. As with all agricultural markets, livestock markets are susceptible to seasonal variation (Norwood and Lusk 2008). From March to May, the seasonal factors are more than the seasonal average for the farmgate price of pork. In May, the seasonal index spiked, which could be due to strong market demand to celebrate several religious festivities during the month. On the contrary, the lowest seasonal index in November means that the farmgate price of pork was lower than the seasonal average. This could have been caused by the fact that swine raisers, in anticipation of the upcoming holiday season in December, increased their animal stock such that there is already so much supply in the market for which there is still not enough demand except for the few enterprising individuals who are into processing (e.g. ham, bacon, etc.). This notion was supported by the fact that by December the index started to pick up onwards January until the start again of the annual festivities in the country. Almost the same factors can be observed for the retail price of pork.

The other critical component of the price movement is the irregular component, which is unpredictable and corresponds to the movement that appears irregularly and generally during short periods. More volatile movement of monthly farmgate price of pork over time relative to the monthly retail price can probably be due to disease outbreaks. Swine is highly susceptible to diseases especially during inclement weather. Pigs need to be protected from extreme weather conditions and typhoons carry with them, most of the time, excessive rains and cold temperature and in some cases flooding. Frequent occurrence of typhoons may result to mortality and cause morbidity among pigs.

**Table 1a.** The forecasts of Philippines monthly farm gate price of pork in 2017.

| Month | Forecast | Confidence interval (at 95%) | Std. Error |
|---|---|---|---|
| Jan | 96.47 | (93.55, 99.39) | 1.5 |
| Feb | 97.30 | (92.97, 101.63) | 2.2 |
| Mar | 98.89 | (93.50, 104.28) | 2.8 |
| Apr | 99.35 | (93.08, 105.61) | 3.2 |
| May | 99.70 | (92.67, 106.73) | 3.6 |
| Jun | 99.59 | (91.87, 107.32) | 3.9 |
| Jul | 98.41 | (90.05, 106.76) | 4.3 |
| Aug | 97.39 | (88.44, 106.34) | 4.6 |
| Sep | 96.71 | (87.20, 106.21) | 4.8 |
| Oct | 97.23 | (87.20, 107.25) | 5.1 |
| Nov | 97.19 | (86.66, 107.72) | 5.4 |
| Dec | 98.59 | 87.59, 109.58) | 5.6 |

**Table 1b.** The forecasts of Philippines monthly retail price of pork in 2017.

| Month | Forecast | Confidence interval (at 95%) | Std. Error |
|---|---|---|---|
| Jan | 168.90 | (166.43, 171.37) | 1.3 |
| Feb | 169.60 | (165.67, 173.54) | 2.0 |
| Mar | 170.82 | (165.83, 175.81) | 2.5 |
| Apr | 171.27 | (165.42, 177.12) | 3.0 |
| May | 172.38 | (165.78, 178.99) | 3.4 |
| Jun | 172.64 | (165.36, 179.92) | 3.7 |
| Jul | 172.78 | (164.88, 180.68) | 4.0 |
| Aug | 172.62 | (164.14, 181.09) | 4.3 |
| Sep | 172.31 | (163.30, 181.32) | 4.6 |
| Oct | 172.01 | (162.49, 181.52) | 4.9 |
| Nov | 172.22 | (162.21, 182.22) | 5.1 |
| Dec | 172.56 | (162.10, 183.02) | 5.3 |

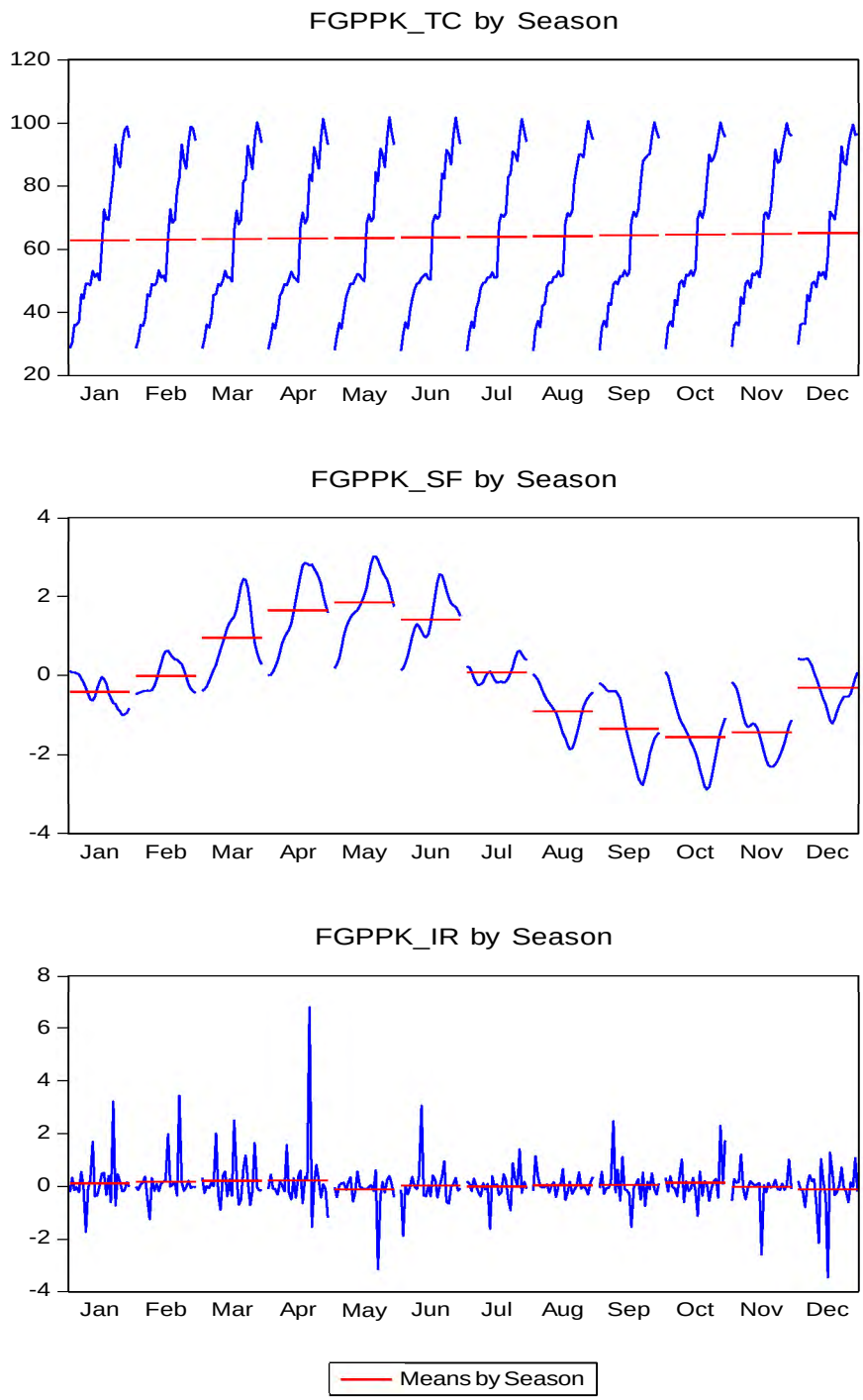


**Fig. 5a.** The additive components of monthly farm gate price of pork.

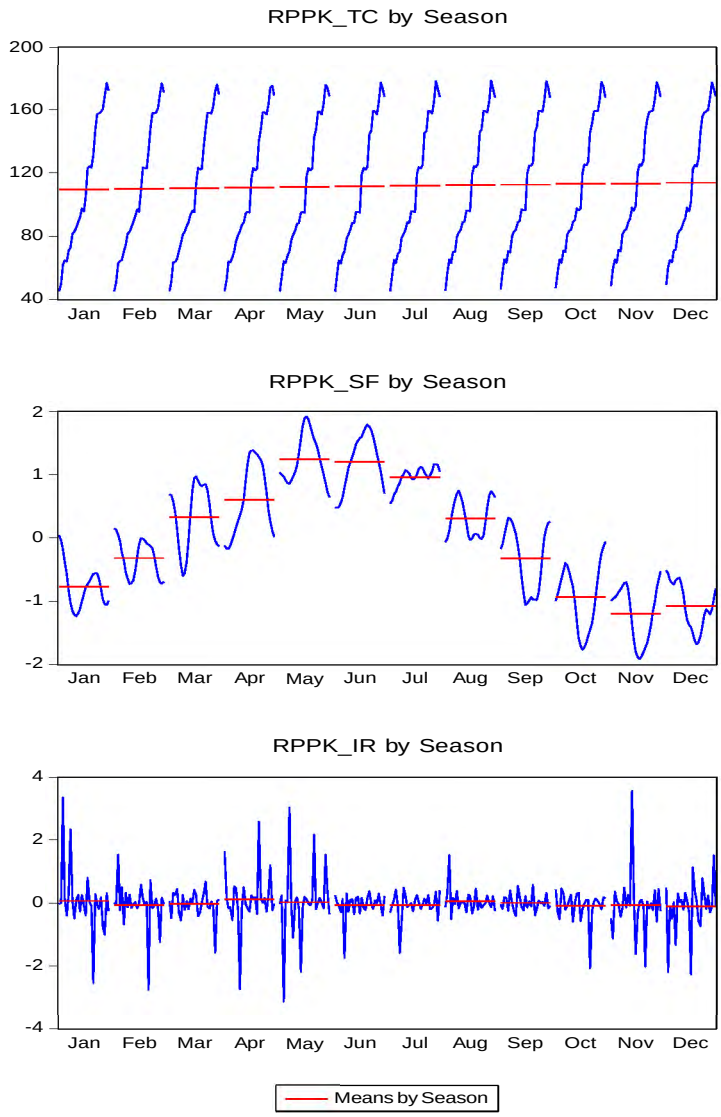


**Fig. 5b.**The additive components of monthly retail price of pork.

For seasonality to be regarded as present, the series should be identified as seasonal by using the "test for the presence of seasonality assuming stability" and "nonparametric test for the presence of seasonality assuming stability." Also, since the presence of moving seasonality can cause distortion, it is important to evaluate the moving seasonality in conjunction with the stable seasonality to determine whether the seasonality is identifiable. The test for identifiable seasonality was performed by combining the *F* tests for stable and moving seasonality, along with a Kruskal-Wallis test for stable seasonality.

The seasonality tests results are presented in Table 2. It can be seen that both monthly farmgate and retail prices of pork have stable seasonality along with moving seasonality at 1% level of probability. More critically, Q-statistic confirmed the seasonality for both farmgate and retail prices. Lastly, the diagnostic test for the presence of residual seasonality indicates that there was no evidence of residual seasonality in the entire series at the 1% level, nor in the last 3 years.

An interview with industry key informants revealed that in the Philippines, in the case of swine, seasonality in production is caused mainly by porcine epidemic diarrhea (PED) causing death among sows and piglets nearly depleting the existing stock and thus, practically reducing pork supply in the market. The United States Department of Agriculture (USDA, n.d.) reported that PED is most serious in neonatal piglets where morbidity and mortality can be 80 to 100%. So far, there is no effective treatment other than controlling secondary infections. The USDA has also reported that the Philippines is among the countries with cases of PED. The disease can only be directly transmitted through fecal-oral contamination but indirectly it can also spread through contaminated personnel, equipment, or any object, to susceptible herds. If chickens have avian flu, its equivalent in swine is PED. PED vaccines exist in Japan, South Korea and China but imported vaccines are not that effective in the local setting, hence the disease continues to cripple the country's swine industry. In addition, in China, because of three new PED variants discovered in 2011, the CV777-based vaccine is no longer effective (Li et al. 2012 as cited by the USDA, n.d.).

In addition, pork is one of the frequently smuggled agricultural commodities into the country such that when smuggled shipments reach the market, prices fall rendering the locally produced pork uncompetitive unless price at the farm gate is reduced contributing to fluctuations.

**Table 2.** The F-Tests for seasonality

| Price | Test for Stable Seasonality* | Test for Moving Seasonality* | Kruskal-Wallis Statistic* | Combined Seasonality | Q-Statistic | Decision |
|---|---|---|---|---|---|---|
| Farm gate | Present | Present | Present | Identifiable | 0.46 | Accepted |
| Retail | Present | Present | Present | Identifiable | 0.55 | Accepted |

*At 1% level of probability

## CONCLUSION AND RECOMMENDATION

More frequently, agricultural products like swine are produced seasonally as they have natural agro-climatic requirements. Agricultural products are also highly perishable and thus are more prone to losses compared with manufactured products. These and other factors affect their price formation and, as such, their prices vary because of these components: trend-cycle, seasonal, and irregular.

The key results of the seasonally adjustment of monthly farm gate and retail prices were:

1. *t*-values of weekend regressor and leap year regressor were not significant;
2. Both price series exhibited clear steep upward trends and normal irregular variations;
3. Stable and moving seasonality were present in monthly farmgate price; the combined test for the presence of identifiable seasonality was identified at the 1% level of probability;
4. Stable and moving seasonality were present in monthly retail price; the combined test for the presence of identifiable seasonality was identified at the 1% level of probability;
5. There was no evidence of residual seasonality in the entire monthly price series of pork at the 1% level, nor in the last 3 years.

Knowledge of seasonal price changes provides a basis for determining the period of production for a more profitable sale. Records of past seasonal variations are important for farmers in determining the behavior of prices in the near future so that they will be able to adjust production in order to avoid selling their products during low periods. At the same time, they will be able to recognize and take advantage of some favorable price conditions in the market that usually offer opportunities for profit. These kinds of information may also be useful to policy-makers in the

formulation of specific policies and programs aimed at increasing or stabilizing the supply of pork. For instance, the government can take a closer look at how it is responding to the PED that is one of the major causes of swine death and morbidity in the country eventually resulting to pork price seasonality. Funding researches for vaccine development technology is a strategic but a long term strategy for swine industry improvement.

## ACKNOWLEDGMENT

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# PROMOTING GOOD AGRICULTURAL PRACTICES (GAP) TO ENHANCE COMPETITIVENESS, RESILIENCE AND SUSTAINABILITY OF SMALLHOLD SABA/CARDABA BANANA GROWERS

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## ABSTRACT

This paper presents an overview of saba/cardaba banana production in the Philippines, the existing practices of smallhold growers vis-à-vis the expanding demand for reliable supply of fruits complying with the quality requirements of buyers. Generally, the smallhold banana sector is not yet prepositioned towards certification despite the support and subsidy by the Government program for PhilGAP and Organic Certification. The authors posit that shifting of smallhold banana growers from traditional to compliance to either of these standards, if not certification, is an important strategy to sustain the country's competitive advantage in the export market, to propel inclusive growth in the local banana industry sector and increase income and profitability of smallhold farmers. Realizing the optimistic potential of the local banana sector to increase fruit supply to feed the growing demand of both fresh and processing markets calls for a transformation of small hold banana farms into viable enterprise that is profitable, resilient, competitive, and sustainable aligned with the standards of the international market. These objectives are interconnected and need to be considered holistically. Proposed strategies to effect this shift, using already available technologies, approaches/strategies and lessons from past and on-going projects, and the Department of Agriculture, Bureau of Plant Industry's (DA-BPI) programs to strengthen support and intensify the PhilGAP program are presented.

## INTRODUCTION

### Overview of banana production and trade in the Philippines

Banana is the number one fruit grown in the Philippines in terms of volume, value and area of production. It is also one of the top agricultural exports of the country. Globally, in 2014, the Philippines ranked 2nd largest banana producer, and 3rd in the export market, 6th in fresh fruits and 1st in banana chips (PSA, 2015).

Production system of banana in the country differ starkly between the small scale grower and commercial plantations. Cavendish, the export cultivar, is produced in Mindanao in extensive plantations, high external inputs, highly efficient, productive and profitable. Dominated by multinationals, these companies are generally compliant/certified with a number of local and /or international standards accrediting bodies. While accounting for only 19% of the total area planted, Cavendish contributes 50.3% in production volume with mean yield of 53.2 t/ha, about 2.2x above the Philippine Development Plan (PDP) target yield of 24.6 t/ha for 2016 (NEDA, 2016). In 2015, though lower than previous years, 1.6 million metric tons (Mmt) or 121 million boxes of fresh fruits were exported. The banana industry paid out Php 44 Billion on wages and PhP 6.5 Billion in taxes in 2014 (PBGEA, 2015). Exported bananas comprise only 20% of the total fruits produced in 2015 (PSA, 2015).

Local banana cultivars account for 80% of banana hectarage, and about 90% of banana growers are backyard in scale and smallholders (DA-HVCC, 2007). Popular types are Cardaba and Saba (cooking types and used for chips) while Lakatan and Latundan are dessert types. Banana is one of the few locally grown fruits available year-round and is the most popular fruit consumed by Filipinos contributing 75% of the total fruit intake (FNRI, 2013). Per capita consumption of Filipinos consumption of banana by Filipinos is about 136 g/d or 50 kg/ year which is only 41% of 400g/d dietary target for fruits and vegetables (FNRI-DOST 2013). Market prospects for local banana cultivars is promising. Increasing banana consumption of Filipinos by another 50 g /day (18.25 kg/yr) will further increase the domestic demand by 1.825 Mmt/year. The many uses of Saba banana is not yet fully exploited and when harnessed could further increase demand.

Local cultivars are generally produced by small farmers spread across all regions of the country. The traditional practice of chronic neglect in growing local banana cvs. is surreptiously spreading destructive pests and diseases that threatens the sustainability of local banana industry. Exceptions are small to medium scale agripreneurs and groups adapting the plantation type management. Yields of local bananas Cardaba/Saba and Lakatan are highly variable across the country but on the average still 10 kg and 7.0 kg short of the PDP target for 2016, respectively. There is much room for improving the productivity of small hold banana growers and while technologies are already available to improve and align traditional practices towards GAP farmers adoption remains low and unsustainable.

There is an expanded, market- driven demand for local bananas particulary Cardaba and Saba, the cultivars preferred for banana chips. The high nutritional value of banana, low glycemic index (GI) rating makes it a health food gaining world acceptance in a snack food market expected to be worth up to $300 Billion by 2015 (Sta Romana, 2012). However, prevalence of low and fluctuating yields outside of Mindanao constrains bridging of demand-supply gap in the processing sector. Only SOCCSKSARGEN in Mindanao consistently exceeded the 2016 PDP yield target of 24.5 t/ha for banana (NEDA, 2016). Five other regions had yields above the national mean yield of 12.6 from 2010-2015 (PSA, 2016). Increasing yield of top 5 regions to the PDP2016 target of 24.5t/ha will increase supply by over 1M mt, nearly 40% of volume produced in 2015. Increasing yield of regions with below average yield to the national mean yield will increase supply by about 630,000 MT/yr (Fig. 1).

**Quality Requirements of banana along the various value chains *vis-a-vis* Philippine National Standards (PNS) for banana farming and fruit quality**

The private sector was quick to respond to the prospect of being part of the growing demand for healthy snack food market with increased number of large processors-exporters from 15 in 2006 to 35 in 2014, and 41 in 2016, reportedly with capacities ranging from 20-60 tons a day. Actual use, however, is only 60-80% of capacity. (Briones 2014). Fluctuating supply of raw materials is a critical constraint to this underutilization (*Personal communication* with processor, 2016). Markets in developed countries are increasingly demanding that their food suppliers be GAP certified, and in addition could impose stricter environmental and health regulations.

**Government supported certification programs**

The Philippine Good Agricultural Practices (PhilGAP) is based on the legal framework of the Food Safety Act of the Philippines (RA 10611). The integration of Quality Assurance System like Good Agricultural Practices (GAP) on food supply chain ensures the safety of harvested crops like fruits and vegetables. The Code of Practices (GAP) includes practices used to prevent or reduce the risk of hazards occurring during production, harvesting, and postharvest handling of produce (RA 10611). The government issued GAP certificate after an assessment, evaluation and verification procedure is being done in accordance with the established standard. A total of 154 certificates were

issued from 2007 to 2016 (BAFS).

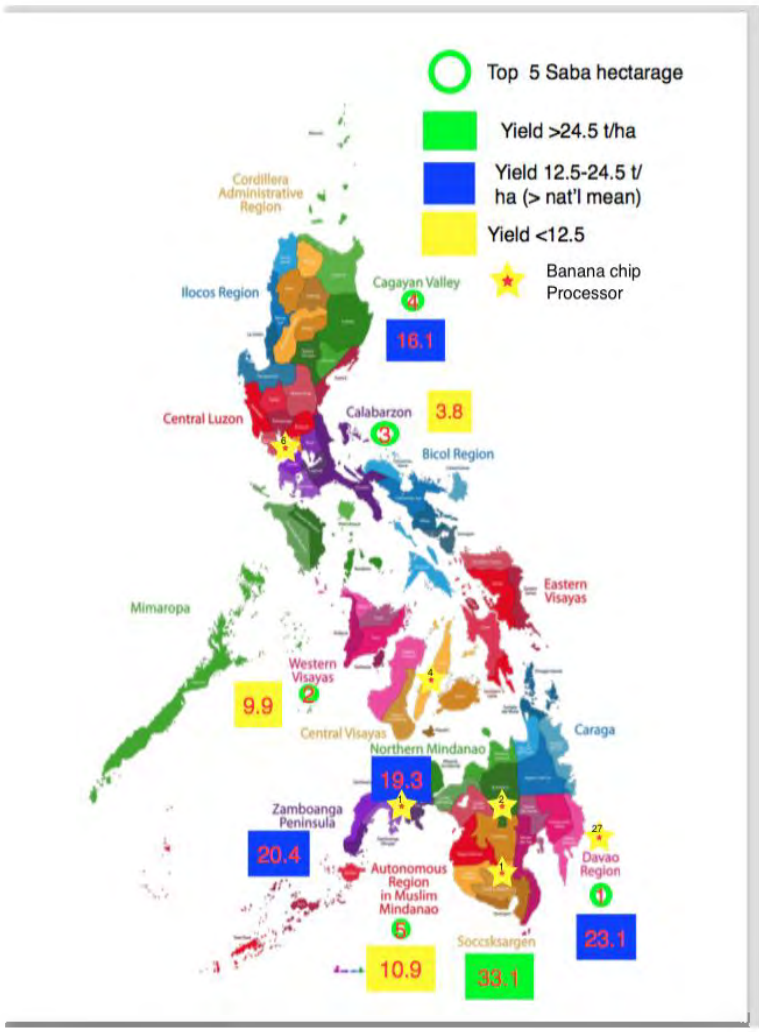


**Fig.1.** Yield (t/ha) of top five saba banana producing regions (ha) relative to location of banana processing centers.
(S*ource: philippinesmap.facts.co*)

According to the Department of Agriculture, PhilGAP will be a basis for bilateral memorandum of agreement or mutual recognition arrangement with trading partners to increase Philippine exports (de la Cruz 2007). The criteria and experiences of national GAP implementation in Malaysia, the Philippines, Singapore, and Thailand and the relevant GAP systems and guidelines from other countries and regions, on the other hand, were the bases for the ASEANGAP which aimed at preventing the risks associated with production, harvesting, and post-harvest handling of fresh fruit and vegetables but also at facilitating trade within and beyond the region (Banzon et al. 2013) Revisions of PhilGap are in the pipeline for alignment of produce quality, environmental management and workers health, safety and welfare modules with ASEANGAP.

For Organic Agriculture Certification, BAFS grants official accreditation to Organic

Certifying bodies (OCB) according to the Philippine National Standards (PNS) for Organic Agriculture (OA) and other relevant standards. Equivalence with the ASEAN Standard for OA (AOSOA) was achieved with the revision of PNS for OA (PNS/BAFS 07:2016). Philippine National practices and products standards are benchmarked against Global GAP and aligned with ASEAN GAP. Applicable PNS for banana are the following:

*Code of good agricultural practices (GAP) for fruits and vegetable farming (PNS/BAFPS 49:2011 ICS 67.020)* This standard code of practice covers the general hygienic practices for the production and primary processing of fresh fruits and vegetables that are field-grown with or without cover, or those grown under protected facilities and cultivated for human consumption, particularly those intended to be consumed raw.

*Code of good agricultural practices (GAP) for banana production (PNS/BAFPS 129:2013 ICS 67.020)* This standard provides specific guidance to ensure the minimization of microbiological, chemical and physical food safety risks associated with the production of banana intended for fresh consumption during production, harvesting, and post-harvest handling and distribution. Also included are practices aimed towards protection of workers' health and safeguarding their safety and welfare; and environmental management. Additional and specific guidelines for banana production are provided meant to be read in conjunction with of the Code of good agricultural practices (GAP) for fruits and vegetable farming, PNS/BAFPS 49:2011.

*PNS –BAFPS 08-2004 for fresh fruit- Saba and Cardaba Banana*- standard establishes a system of grading and classifying 'Saba' and 'Cardaba' type bananas grown from *Musa balbisiana* of the Musaceae family produced in the Philippines

*PNS/BAFPS 64:2008 ICS 67.080. Fresh fruits-Banana* standard establishes a system of classifying and grading banana (generally considered table banana) grown from Musa spp., of the Musaceae family, in the mature stage, to be supplied fresh to the consumer, after preparation and packaging. Banana intended for cooking only (plantains) or for industrial processing are excluded.

*PNS/BFAD 14:2007 ICS 67.080 Recommended code of practice for the processing and handling of banana chips.* This Code of Practice is concerned with the receipt of raw materials and ingredients, preparation and processing of banana chips products to conform with the required standards in *PNS/BFAD 13:2007 Standards for Banana Chips*. The product shall be prepared from banana fruit of Musa variety used for banana chips processing. This Code is intended to provide guidelines to achieve compliance with the standards for banana chips products packed in any suitable container.

*ASEAN Standard for Banana (ASEAN Stan 12:2009, Rev.1-2012)* benchmarked against Global GAP, these specifically excludes Banana intended for cooking only (plantains) or for industrial processing.
There is a need to develop PNS standards specific for production of Saba banana (ABB) that is benchmarked against accepted International Standards.

**Changing farmers existing practices to comply to requirements of the international market means saba banana production need to transform into a sustainable and profitable agribusiness**

Majority of Saba/Cardaba farmers are small hold growers. Generally, given their traditional practices characterized by under management due to chronic neglect or 'organic by neglect", this sector is not yet prepositoned towards the government supported GAP certification programs- either hilGAP (issued by the Bureau of Plant Industry starting in 2017) or Organic Agriculture certification. The Bureau of Agriculture and Fisheries standards accredits private certifying bodies.

Whichever track the farmers would opt to go, the bottom line is, the yield and fruit quality on the production side need to improve and this can be attained only if the farmers shift from their

existing practices to technology-based good agricultural practices. GAP compliance or certification of the small hold growers is a good strategy to increase the competitiveness of their produce and to capture a bigger share of the increasing domestic market and eventually participate in the International trade to support the ASEAN Vision in 2020 by the ASEAN Economic Community 2015 "To create a stable, prosperous and high competitive, ASEAN Economic Region in which there is free flow of goods, services and investments, a freer flow of capital, equitable economic development and reduced poverty and socio-economic disparities.." Sixth ASEAN Summit, Ha Noi (December 1998)

Achieving more yield with better fruit quality that comply with the *PNS –BAFPS 08-2004 for fresh fruit- Saba and Cardaba Banana* will increase farmers' income, reduce rejects at the processor side. Increase income and profit will encourage farmers to improve existing practices. So GAP compliance, if not certification yet, is needed to sustain the competitive advantage we have in the fresh fruit and chips export market, propel inclusive growth in the local banana industry sector and increase income and profit of smallhold farmers and their communities, elevating them from poverty and the increasing concern of malnutrition.

Beyond production, major challenges for future market growth appear to be associated with a coordinated approach to managing the field-to-market-supply chain, for both fresh and processed products (FAO, 2011). Saba/Cardaba (ABB) and other local banana cvs. flow of materials is still traditional, multi layered and inefficient (Fig. 2). Mindanao to Luzon Banana Commodity Flow, based on supply chain studies by the Post –Harvest Horticulture Training Center of the University of the Philippines Los Banos, fofr example, involves up to 10 key players, multiple handling of fruits; high risk for shippers and consignees due to high losses due to inefficient, sub-optimal conditions in bulk loading, inter-island and road transports (Artes et al., 2013). Improving efficiency of commodity flow would require increasing yield and quality of fruits in and around Luzon to minimize multiple handling and transport, and integration of farmers in the higher level of the supply chain through shorter or direct link of producers to processors

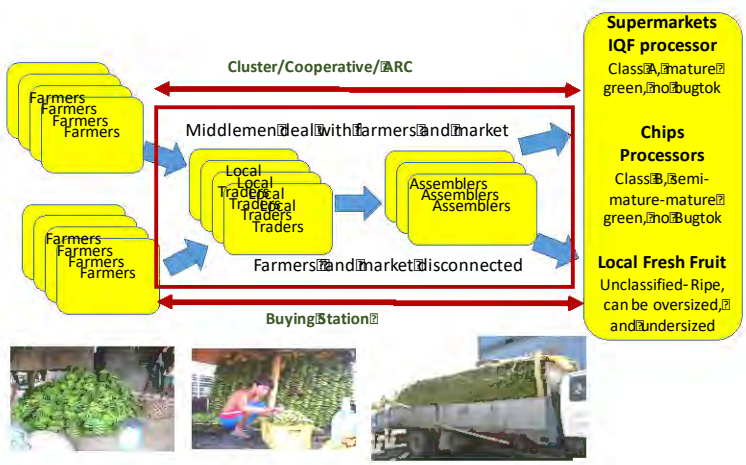


**Fig. 2**. Supply chain of Saba banana

**Way forward - learning's, technologies, government support program**

Replicate and upscale successful models wherein farmers' changed practices towards GAP alignment and integration into the banana value chain were effected. There are lessons to be learned and good strategies to adapt from previous projects such as the following:

The "good enough approach" (B-ACE project USAID and SDCAsia) of incremental

innovations and improved practices, effectively supported mobilization of public-private partnerships to upgrade initiatives in cardava production. Strategic Development Cooperation (SDC) Asia's "good enough", approach with incremental improvements in practices increased yield and profit, rather than attempting to achieve full GAP certification. Farmers' interest to learn and upgrade their practices was triggered by good prices and model farms with yields higher than the average (B-ACE 2007.) *Thus, incentives of equitable pricing for quality produce for increase farm productivity and income, is a strategy to convince farmers of the benefits of changing traditional practices and adopt new technologies.*

***The DAR CRS Misamis Oriental Agroenterprise (Banana Cardava Marketing) 2014- Linking Agrarian Reform Beneficiaries to Corporate Supply Chain*** implemented in eight (8) agrarian reform areas in Luzon and Mindanao involved 800 Agrarian Reform Beneficiaries (ARBs) comprised of 4,000 farmers/ARB households. (***https://youtu.be/9dgd-pRi030).*** The project utilized the eight steps in agro-enterprise clustering approach popularized by Catholic Relief Services (CRS) to increase the production and income of ARBs and their household through Agricultural Extension, Marketing Assistance, and Capacity-building. DAR funded the project and coordinated with the LGUs and other stakeholders. CRS provides the counterpart fund and spearheads the generation of support from the LGUs and stakeholders (DAR, 2014) Strengthened public - private sector partnership to integrate farmers in an efficient value chain while clustering and cooperative marketing was proven effective in integrating the farmers in the value-chain, thus contributing to inclusive growth.

***IFC with Unifrutti Group*** provided extension services to banana farmers in its supply chain on new farming techniques, resulting in improved social and environmental practices. The farmers received Rainforest Alliance (RA) certification. Company benefited by commanding higher prices and farmers given premium price to encourage adoption of certification standards, which helped farmers to cover costs and increase their profit (IFC, 2014)

In the models above, the provision of technical support to farmers to do incremental changes towards GAP alignment and extension support by higher value chain player enabled the farmers to comply with international certification requirements that meet consumer needs for food quality, safety and nutrition. At present, PhilGAP standard is fully aligned with Food Safety Module however, government should also focus on strengthening the alignment of the remaining 3 modules: Produce Quality, Environmental Management and Workers Health Safety and Welfare. This is critical to sustaining competitiveness of saba/cardaba banana in existing markets and in penetrating new ones.

Participatory approaches, funded by government agencies Philippine Council for Agriculture, Aquatics, and Natural Resources Research and Development of the Department of Science and Technology (PCAARRD-DOST) and the Department of Agriculture, Bureau of gricultural Research (DA-BAR) have proven effective in transforming traditional banana production to good agricultural practices, though sustainability after project phase-out is a concern.

In Infanta and Nakar, Quezon, a combination of academe-based technology development, farmer managed field adaptation trials and developing local capacities to have a cadre of technically competent staff of DA-MAO, farmer leaders and NGO's operating in the areas through training, technical support and farm cross-visits was implemented. The Local Government Units provided financial and technical support for scaling-up of project gains (Aguilar et al. 2007).

Projects promoting adoption of S&T based production technologies for local banana cultivars by PCAARRD-DOST using interpersonal (training, technical assistance and mentoring, farmer managed field trials, farmers field days and farm cross-visits) and mediated IEC (printed and audio-visual training guide, realistic and fantasy genre comics) in Cavite, Quezon and Mindoro Oriental (Aguilar, et. al. 2010). An action project implemented S&T based coconut-banana cropping

pattern in a total of 30 ha in Laguna, Bohol and Quezon. This model linked NGO facilitation of farmer organizations with the academe providing technical support for technology transfer. Immediate feedback and response to and from farmers and the academe resulted to adaptive management appropriate for the farmers' needs, skills and resources. Intercropping banana with coconut farmers' productivity and income and resilience to climate change

The DA-BAR, through its flagship programs–Community- based Participatory Action Research (CPAR) and National Technology Commercialization Program (NTCP), provided platforms to accelerate technology transfer and commercialization with successful implementation of projects on coconut-based farming systems (Lakatan+ pinakbet vegetables) in three LGUs (Magdalena, Liliw and Pagsanjan) in Laguna and Integrated Management of BTD and Bugtok diseases of Saba banana in an LGU (Macalelon) in Quezon (DA, 2014).

## CONCLUSION AND RECOMMENDATIONS

Realizing the optimistic potential of the smallhold local banana sector to increase supply of quality products to meet the growing demand of both fresh and processing markets need a transformation of these farms into viable agribusiness that is profitable, resilient, competitive, and sustainable. These objectives are interconnected and has to be considered holistically. While there are available technologies to improve and align traditional practices towards GAP, climatic disturbances, uncertainties and extremes that recurrently devastate banana farms constrain farmers from investing their limited resources in S&T based production system. Thus, improving farmers' resilience to a changing climate by diversification of livelihood sources is key to encourage farmers to invest in system innovations and new technologies. Capacity building and materials' support to intensify and diversify production and income sources to build assets and increase agrobiodiversity, conduct of Farmer Field School trainings that integrate early warning and farm advisories using localized weather forecast for adaptive management, risk transfer mechanisms such as crop insurance, immediate support to farm rehabilitation after a devastating typhoon are examples of interventions that help build farmers' resilience. Cooperative support, increased income, biodiverse and clean environment will support good nutrition and health of the farm family and community contributing further to the continuous improvement and sustainability of their farm production system. High efficiency and productivity, through clustering of farms for cost-effective inputs procurement and technical support, assurance of food safety (as increasingly being demanded by buyers) using already available GAP compatible technologies, and collective marketing will improve competitiveness. When ready, target PhilGAP certification for clustered/organized farmer groups. Sustained Government support to infrastructures, demand driven R&D and quick and effective technology dissemination. The proposed Banana Research Center should have a dedicated program to support local banana cvs.

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