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REVITALIZING AGRICULTURAL EXTENSION SERVICES IN DEVELOPING COUNTRIES: LESSONS FROM OFF-SEASON VEGETABLE PRODUCTION IN RURAL NEPAL

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ABSTRACT

Technological advances in agriculture potentially can improve the productivity and income of farmers. However, it is unclear how rural and smallholder farmers can best learn about, adopt, and benefit from technological advances. Extension services with multiple linkages to organizations and farmers have been identified as a positive factor in successful adoption of new technologies. Participatory action research was undertaken in Kaski district of Nepal focused on introducing new (for the local context) technologies for off-season vegetable. The project sought to demonstrate best practices in agricultural extension and showcase a demand-driven, participatory, and pluralistic model of agricultural extension. It was hypothesized that doing so would enhance the productivity, income, and food security of participating rural households. Two household surveys were conducted -- a baseline survey in 2013 (before the project) and endline survey in 2015 (after the project) -- to collect data and study the impact of the intervention, if any, on household wellbeing. The results show the participatory approaches used to be associated with enhanced productivity, income, and food security for participant households. The results show that the area under off-season vegetable production, marketed vegetable volume, and income increased significantly. It appears that vegetable production and income tripled while the area of land dedicated to off-season vegetable production doubled at the end of the project. The types of crops grown by participant households also significantly increased over the project period. Furthermore, participant farmers were found to organize into groups for knowledge sharing, adopting new technology, and increasing household income. The results suggest that phase-wise learning and scaling up approach used in the off-season vegetable production can be replicated to disseminate new technologies in other contexts to strengthen extension service delivery.

Key words: Participatory action research, plastic tunnels/houses, pluralistic extension services

INTRODUCTION

Technological advances in agriculture seem to offer an opportunity to rural farmers to increase production and improve their livelihood sustainably. Adopting such technologies have contributed greatly toward the financial success of farmers through the efficient use of resources and scaling up the production at lower per unit cost. However, a majority of farmers in developing countries have not been able to adopt newly developed technologies because of their limited resources (cash, labor, time) and limited access to relevant information regarding the technology (Ghimire and Huang, 2015). Further, resource-poor farmers are often reluctant to invest in any untried technology because of their risk aversion behavior. In order to adequately address this issue, a good extension

service delivery and strong linkages between farmers, extension, research and education are prerequisites (Rivera *et al*., 2009). Public sector agricultural extension, however, is not efficient and beyond the reach of the general clients (Carney, 1998; Hoffmann *et al*., 2000).

In least developed countries (LDCs) like Nepal, public extension systems are lagging behind other nations' evolving extension system. Nepal experimented with many models of agricultural extension services in the past and most of them had pervasive bias against small and marginalized farmers. Due to the cost ineffectiveness and lack of coverage, those extension approaches were unsustainable and did not serve well as a model for national agricultural extension. Moreover, the nature of large, hierarchically structured, conventional extension approaches is unable to respond effectively to predominately small and marginal farmers' needs. Such systems of 'extension' tend to follow top-down, supply-driven approaches rather than demand-driven service delivery model and historically have had their benefits and services captured by mostly elite groups or resource-endowed farmers. Traditional and top-down approaches of extension services are hindering the diffusion of innovations among farmers (Suvedi and McNamara, 2012). Being less cost-effective and financially unsustainable, these approaches are criticized and no longer preferred and fulfilled the general needs of resource poor farmers in the rural area.

A participatory and bottom-up system, market-driven or fee-for-service systems are emerging. These emerging approaches encourage pluralistic service delivery systems (multiple organization delivering service), and decentralization of programs/project's operation at the district and village levels. Decentralization helps address problems of different agro-ecological conditions as access to agricultural production systems and markets differ significantly across regions. This has, therefore, become an agenda to reform of public sector agricultural service provision in many countries where government extension services are criticized for being inefficient. The trend is to reduce the role of the state and to promote private enterprise (Hoffmann *et al*., 2000). Privatization or outsourcing of services to Non-Governmental Organizations (NGOs), Community-Based Organizations, and Farmer Organizations are in demand.

Over the last decade, there has been a general shift in thinking about extension systems: the former via of extension as a linear, technology transfer, 'adoption of innovations' approach has given way to a recognition of extension as a system of actors with multiple roles, a wide range of actors advancing an 'innovation system' (Sandall *et al*., 2011). This shift has influenced, and has been influenced by, shifts in policies toward supporting pluralistic provision of services that are more responsive to farmer demand (Garforth, 2011). Policy makers, service providers and academics increasingly are interested in finding out whether these changes have made differences in farmers' access to extension services that support their efforts to secure improved livelihoods for themselves and their families. This paper focuses on exploring how an innovation adoption system coupled with a participatory pluralistic agricultural extension effort in rural Nepal has affected those farming households.

Some studies have recommended developing and adopting a diversified and pluralistic national strategy to promote agricultural extension and communication to advance the livelihoods (e.g., food security and income generation) of poor people in rural areas (Ghimire and Huang, 2016; Rivera and Qamar, 2003). This can be viewed from two institutional levels: extension providers and clients of extension agencies. We developed a pilot action research activity in conjunction with USAID/MEAS and Michigan State University (MSU) in Kaski district of Nepal that focused on introducing off-season vegetable production. The effort was framed to explore the roles of service providers in collaborating and the skill set for extension personnel to assist farmers with adoption a new activity -- production and post-production activities of off-season vegetables. Participatory action research has been effective in understanding and empowering community needs and societal problem

solving in Pakistan (Aziz *et al*., 2011) and management of protected areas in Nepal (Fisher and Jackson, 1998).

This paper focuses on how the application of demand-driven, pluralistic participatory extension model played a role in delivering technological innovations (extension services) for socio-economic change and improved, sustainable livelihoods of rural people in Nepal. The MSU-initiated project focused on tomato production (off-season) using tunnel plastic houses (hoop houses) in the rural Nepal. The project hoped to facilitate increased production (farm productivity), income, and household consumption of fresh vegetables. It was believed that doing so would enhance food and nutrition security of participant farm households. There are possible next steps that include scaling up of both seasonal and off-season vegetable production as well as developing village-level agricultural extension workers (leader farmers).

METHODS AND APPROACH

Methodological Framework

Various methods and tools were part of the participatory research project including technical scientific observation, research, monitoring, and evaluation in different phases. The learning-based approach adopted provided opportunities for insights for dealing with complex situations and approaches for implementing projects in situations where people do not really know where to start or what to do next (Fisher and Jackson, 1998).

A baseline survey was conducted in 2013 to analyze the preliminary production environment and socio-economic aspects of farmer households in study area. At the end of the project, an endline survey using same survey instrument (from 2013) was implemented in 2015. The project and its data collection/analyses benefited from collaborative analysis and assessment which involved the participating rural communities, Indragufa Community Development Foundation (ICDF) staff, Michigan State University, Department of Agriculture (DOA), Nepal Agricultural Research Council (NARC), Agriculture and Forestry University (AFU) and other stakeholders. Technical support and oversight of the project elements were provided by MSU researchers. The project activities were conducted in various phases as described below.

Phase 1:

Plan: Investigate off-season vegetable production in the area and set up a pilot project.

Action: Preliminary situation analysis and assessment of agricultural production environment including a baseline survey with selected farmers. Implement a pilot project of plastic tunnel houses (about 92 tunnel houses). Tunnels were constructed and off-season tomato farming piloted with additional support. Farmers received vegetable production training and some of them were provided extra training in order to develop them as leader farmers (village-level agricultural extension workers).

Observation (Conclusion): The results of off-season tomato production in the first phase were promising. It appeared to be technically, economically and socially feasible /acceptable to incorporate plastic house, off-season vegetable production with farmers and their households in the study area.

Phase 2:

Plan: After observing the appealing results from the first phase (1st year), other farmers in the region voice their desire (i.e., demand) for access to support and guidance so they too could implement off-

season vegetable production using plastic hoop houses. The MSU-team worked to scale up the program to reach an additional 200 farmers in the project's second year (2014).

Action: The project team in the research area promoted off-season tomato and increased numbers of participating farmers with project-team support built other vegetables and additional plastic tunnel houses.

Observation (Assessment): Harvesting and production of off-season tomatoes and other vegetables greatly increased. Farmers were positively motivated by the output of the previous year and many constructed additional tunnel houses on their own.

Phase 3:

Action: Off-season vegetable production technology was promoted and incentives were provided to encourage adoption. Leader farmers, in association with ICDF, MSU, DOA, NARC, AFU, NGOs and other stakeholders, help to provide agricultural extension services and inputs to their fellow farmers.

Observation (Assessment): Traditional / conventional farming of vegetables greatly reduced. Off-season vegetable production under plastic tunnel is increasingly adopted by participating and neighboring farmers (those who did not participate in the program). The income of farming households increased through the sale of fresh vegetables to local, regional markets, but the knowledge of post-harvest handling and processing of fresh vegetables seem to be inadequate, and a lack of storage facilities in the area hindered many farmers from benefits.

Phase 4:

Plan: Further scaling-up of vegetable production and post-harvest technology in the area is required. Effort to meet these demands is continuing.

Study Area

This project was undertaken in three villages in the Kaski district in western Nepal (Figure 1). The project villages (Hamsapur, Thumki and Rupakot) are located in the southeast part of the district, linked with a 25 km graveled road and a main roadway (Prithvi Highway) to Pokhara, the district headquarters. Although Kaski is a relatively well-developed district of Nepal with the nation's second largest city (Pokhara-Lekhnath Metropolitan), the study area is a rural environment in the foothills of the Annapurna range. Farm households with fertile land, forest resources, and subsistence farming are spread along the hillsides.

The study area has a unique climate, predominantly sub-tropical mid-hill zone ranging from subtropical to warm temperate with winter drought and summer rain. Mean annual rainfall is 3,979 mm, and mean annual temperature is about 20.9°C, with coldest month January (7°C) and hottest month May (30.5°C). The altitude varies from 600 to 1,200 m, with most of the area covered by sloppy hills with terraces, and practice subsistence agriculture, with average land holding of 0.54 ha (CBS, 2011). The project villages are inhabited by various ethnic groups, predominantly Brahmins, Chhetries, Gurung, Magars, Kami, and Sarki and covered in our sample.

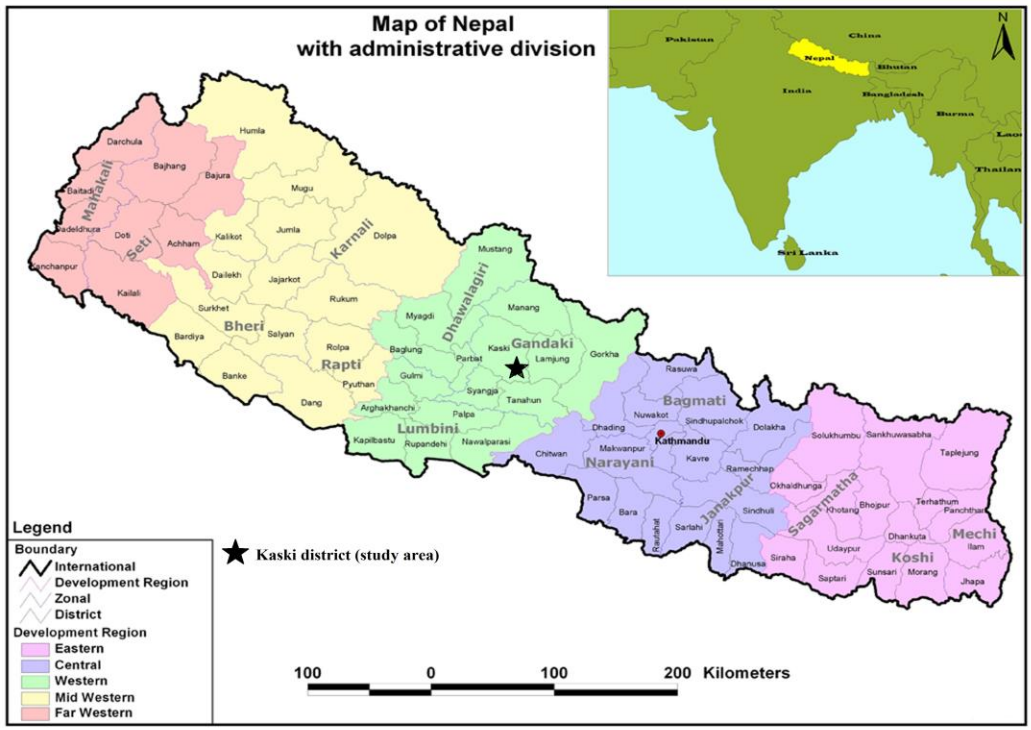

Fig. 1. Map of Nepal showing study area. (Source: MOAD, 2017 edited).

Data collection and sampling procedure

The study's population includes all farm households (mainly vegetable farmers) in the three villages. For this analysis, two surveys were conducted: a baseline survey during June-July 2013 and end-line survey was conducted during July–August 2015. Face to face, interviews of the farm household heads or principle decision maker were conducted to collect information on various aspects of household and farming systems. A multistage random sampling approach was employed in the selection of sample households. In the first stage, three villages, namely Hamsapur, Thumki and Rupakot were selected through purposive sampling. Accordingly, wards[1] 1 to 9 of Hamsapur VDC, wards 3, 5 of Thumki VDC, and ward 9 of Rupakot VDC were selected in the second stage. Finally, the total population of vegetable farmers in the area was identified from the list provided by VDC office, and then a random sample of 92 farmers for baseline survey (year 2013). For the end line survey (after the program intervention – year 2015), only 59 panel households were re-interviewed. The reasons for the loses of some observations (farm households) in the end line survey may include farmers leaving the villages for better employment opportunities elsewhere in Nepal as well as overseas employment as well as some potential respondents did not want to be interviewed.

Statistical analysis

Comparison of means (paired *t*-test) was used to estimate the impact of the project by comparing means of the responses to the same questions from the two surveys. The program impact

[1] A Ward is the lowest administrative unit at village level and all the administrative works are performed under the direct supervision of respective Village Development Committee office (VDC).

on the outcome being evaluated can be measured by the difference between the means of the samples of the two groups (before and after the program). The mean difference between the "after" and "before" values of the outcome indicators for each group was calculated and compared the treatment group 'before' and 'after' the project intervention. We used paired *t*-test because our two sets of measurements are dependent and correlated with each other, and under such conditions, the paired *t*–test gives more powerful and consistent results than a two-sample *t*–test (Hsu and Lachenbruch, 2007). Our null hypothesis was that the mean difference between paired observations is zero. When the mean difference is zero, the means of the two groups must also be equal. Because of the paired design of the data, the null hypothesis of a paired *t*–test is usually expressed in terms of the mean difference. The paired *t*–test assumes that the differences between pairs are normally distributed. We checked the normality of our data by using the histogram spreadsheet in STATA package. Some of the variables (data) were transformed in order to make sure that our data are normally distributed. The paired *t*-test is not very sensitive to deviations from normality, unless the deviation from normality is obvious.

Assumptions and variable specifications

Our assumption was that production outcomes would remain unchanged between 2013 and 2015 but for the program intervention. Although there have been exogenous changes over time period in such areas as weather, market infrastructure, information channels, and demand for vegetables, we assumed that both years (2013 and 2015) presented similar production environment, technology, and opportunities to participant farmers. This assumption is reasonable if climate conditions and the quality of other inputs such as land (e.g., soil quality), labor and machinery, are roughly the same over the period. As reported by MOAD (2014), general agricultural output for vegetables yield in Nepal during 2011 to 2014 was 13.40 tons/hectare, and in western region, it was 13.6 tons/ha. Because this trend remained similar from 2011 to 2014, we assumed that vegetables yield in 2015 will be roughly equivalent with the 2013/14 reported yields for the same area/region. This demonstrates that general equivalency in the market, environmental, and policy contexts for the research area's traditional vegetable production from 2013 to 2015.

Our project intervention focused mainly focused on using plastic tunnel houses for growing off-season vegetable production. In general, off-season vegetable production was non-existent in the region before the introduction of plastic tunnel houses. These structures allowed for vegetable production even if there was bad weather such as heavy rainfall, hailstorm, and drought, and the vegetables grew in the polyhouse remained unaffected. Kumar and Trumugam (2010) conducted a study under naturally ventilated polyhouse condition to test the suitability and influence of weather parameters on growth and yield of different vegetables including tomato in comparison with open field conditions. They reported that production and yield of vegetables were significantly increased in polyhouse over open field. Similarly, Sanwal *et al.* (2004) reported that protected controlled cultivation under tunnel house could solve the problem of low productivity during extreme weather conditions. Therefore, we expected that external factors such as weather condition that may, in fact, have differed between 2013 and 2015 not adversely affect the hoophouse production (yield) between the two years.

Regarding the outcome variables, we mainly focused on vegetable area (sq.ft.), production (kg), quantity sold (kg), income (NRs) and number of crops grown. The income variable includes only the cash income from vegetable sales. We did this because imputed value of vegetables consumed are mostly hypothetical and may lead to measurement error. To make income measures comparable, however, we changed the nominal income from vegetable sales into a real income measure by using price deflator with CPI 2010 as the base year.

RESULTS AND DISCUSSION

Descriptive statistics

Description of the study's demographic variables is presented in Table 1. In 2015, the mean age of household head was 47.79 years and 32 percent of the households were headed by female. More than two-thirds of the sample households were headed by males, which suggest the existence of some gender bias. The mean maximum years of formal education of the household head was 7.9 years and average household size was of 5.35 members. The average farm size (0.53 ha) is little lower than the national average land holdings of 0.7 ha in Nepal (CBS, 2011). More than half (59.32 %) of the households made farm decision by both (household head and spouse) where as 22% and 19% of the households made farm decision by male and female household head, respectively. About 55% of the respondents represented so-called high caste (Brahmin/Chhetri), 36% were janajati (middle-caste) and 10% represented dalit (lower caste or occupation-based caste).

Table 1. Description of demographic variables, means, percent and standard deviation (2015).

Variables	Description	Mean (n=59)	Std. Dev.
Age	Age of the HH head (years)	47.79	13.56
Education	Years of formal schooling of the HH's head	7.90	4.56
Household size	Number of family members	5.35	1.95
Farm size	Total cultivated area in the current year (hectare)	0.53	0.82
Dummy/ Categorical variables		*Frequency*	*Percent*
Gender of the Household head	Household head male = 1	40	67.80
	Household head male = 0	19	32.20
Farm decision	Farm decision made by male = 1	13	22.03
	Farm decision made by female = 2	11	18.64
	Farm decision made by both = 3	35	59.32
Ethnicity	Dalit (lower caste group) = 1	6	10.17
	Janajati (ethnic group) = 2	21	35.59
	Brahmin/Chhetri (so-called higher caste) = 3	32	54.24

Notes: n= Number of households, HH= Households.
Source: Field survey 2015.

Mean comparison test of paired data

Table 2 shows a paired *t*-test comparison of tomato production variables before and after the intervention. We included four variables in the hypothesis testing with the observed differences for the variables being significant. The mean area in production for tomato in 2013 was significantly lower than the mean area of tomato production in 2015 ($p<0.10$). Similarly, the differences for other variables such as production, quantity sold, and income (from tomato sale) were significantly different from 2013 to 2015 at the 1% level of significance. These results show that, after the project intervention, participating farmers were able to receive higher income from off-season tomato cultivation. We therefore reject our null hypothesis that the means of the tested variables in the 2013 data are not significantly different from those variables in the 2015 data. We therefore conclude that the program has positively impacted vegetable (tomato) production and household income.

Table 2. Paired *t*-test comparison of tomato outcome variables before and after intervention.

Variables	Tomato (n=59)[a]		Mean Diff. [B-A]	*t*-Stat
	2013 (Mean, SE) [A]	2015 (Mean, SE) [B]		
Area (sq.ft.)	342.08 (134.29)	598.08 (50.81)	256.01 (143.58)	1.78
Production (kg)	132.97 (40.23)	710.17 (69.93)	577.20 (80.67)	7.15*
Qty Sold (kg)	88.95 9 (31.27)	641.32 (125.69)	552.37 (129.53)	4.26*
Income (NRs)	4983.97 (1578.28)	13324.56 (1418.91)	8340.59 (2122.33)	3.93*

Source: Field survey 2013 and 2015.
Notes: * Significant at 1 % confidence level. Numbers in parentheses are the standard errors (SE). [a]n= number of observations. US$ 1 was equivalent to Nepali Rupees (NRs.) 102 during survey period (June-July, 2015)

Table 3 shows the differences between 2013 and 2015 data for other vegetables. The average production, quantity sold, and income appears to have substantially increased in 2015 while the area under cultivation just doubled. The change in mean area was statistically significant at the 10% level of significance while other three variables (production, quantity sold and income) were significantly different from the baseline data at the 1% level of significance. The mean income from vegetables increased substantially and was significantly different between 2013 and 2015. These results indicate that farmers started growing vegetables other than tomatoes and were able to increase their farm income. This would appear to help enhance food and nutrition security and the general livelihoods of rural farmers.

Table 3. Paired *t*-test comparison of other vegetables outcome variables before and after intervention.

Variables	Other vegetables (n=59)[b]		Mean Difference [B-A]	*t*-Stat
	2013 (Mean, SE) [A]	2015 (Mean, SE) [B]		
Area (sq.ft.)	1576.51 (517.01)	3070.05 (977.97)	1493.54 (785.77)	1.90
Production (kg)	219.03 (45.35)	1287.63 (225.52)	1068.59 (230.04)	4.65*
Qty Sold (kg)	128.44 (35.73)	889.17 (178.96)	760.73 (182.49)	4.17*
Income (NRs)	6021.71 (1555.47)	22654.81 (5064.86)	-16633.11 (5298.32)	3.14*
No. of vegetables grown	1.36 (0.19)	4.49 (0.29)	3.14 (0.35)	8.88*

Source: Field survey 2013 and 2015
Notes: * Significant at 1 % confidence level. Numbers in parentheses are the standard errors (SE).
[b]n= number of observations. US$ 1 was equivalent to Nepali Rupees (NRs.) 102 during survey period (June-July, 2015).

The average number of vegetable crops grown differs substantially for respondents between 2013 and 2015. For example, farmers who planted only two vegetable crops in 2013 grew about five

types of vegetable crops in 2015. This difference was significant at the 1% level. Similarly, the income from vegetable crops was about 6,021 NRs. for the sample from 2013 and 22,654 NRs. for sample households in 2015. Volume of total vegetable production was significantly higher in 2015 as compared to the year 2013. The total area covered by vegetable crop appeared to be significantly different between two data; however, the level of significance was lower than other variables (10 % level of significance). The results from comparing mean differences between 'before' and 'after' the project indicate that farm households are better off in terms of area cultivated, production, marketed volume and income at the end year of the project. It should, however, be noted that mean difference comparisons may not take into consideration other characteristics of the farmers which may compound the impact of project on the farmer's wellbeing with the influence of other characteristics (Becerril and Abdulai, 2010; Kuhlgatz and Abdulai, 2011).

CONCLUSIONS AND RECOMMENDATIONS

A pilot action research project was commenced in Kaski district of Nepal in 2013 using the introduction of off-season production to demonstrate how service providers can collaborate and examine if extension services can assist farmers with production and post-production activities of off-season vegetables. The primary focus of this study was to test if the application of demand-driven, pluralistic extension model for delivering technological innovations (extension services) can promote socio-economic change and improve the livelihood of rural people sustainably. The off-season tomato production approach using tunnel plastic houses in the rural environment did increase participants' production (farm productivity), income, and consumption of fresh vegetables as well as to enhance food and nutritional security of farm households. The project is moving forward with efforts to scale up of both seasonal and off-season vegetable production as well as develop village-level agricultural extension workers (leader farmers). These leader farmers are expected to render agricultural information to the fellow farmers and sell agricultural inputs at reasonable price with small fee-for-service (entrepreneur-cum-extension workers) at the very grassroots levels.

We examined empirically the impact of the introduction of an agricultural technology (off-season vegetable production) on participants' household farm-based production and income. We analyzed and compared both baseline 2013 data (before the project) and endline 2015 (after the program) data. To evaluate the impact of the off-season vegetable production project, we compared the paired data taken at two points in time--before and after the project implementation -- assuming all else constant. Mean differences of the tested variables -- area, production, quantity sold, income and number crops grown -- appeared to be significantly higher when the project ended supporting the conclusion that the project supported farmers' increases in production, access to market, and farm income. Interestingly, farmers constructed additional tunnel houses on their own after seeing the increased outputs of the previous year. Off-season vegetable production under plastic tunnel is being adopted by participating and neighboring farmers (those who did not participated in the program).

The project developed leader farmers, in association with ICDF, MSU, DOA, NARC, AFU and other stakeholders, who provided agricultural extension services and inputs to the fellow farmers. This collaborative effort is notable and continues. The income of farming households in the project increased through the sale of fresh vegetables to local, regional markets, but the knowledge of post-harvest handling and processing of fresh vegetables seem to be inadequate, and there appears to be a lack of storage facilities in the area. Further scaling-up of vegetable production and post-harvest technology in the area is needed and welcome. It is hoped that there will be additional effort in those areas. The success of this project demonstrates the usefulness of a demand-driven, participatory agricultural extension model for Nepal Government extension officials, NGO professionals and donor community representatives interested in rapid dissemination of proven innovative extension strategies too hard to reach, small and marginal farmers. Widening access to information, extension services,

inputs would provide producers with the means for investing in newer technology, thereby allowing for a full use of their managerial ability and for higher levels of output.

Finally, our work in these mountain villages of Nepal suggests that off-season vegetable production has strong potential for increasing household income and improving nutritional status/outcomes as opposed to the promotion of lower value, (traditional) staple crops. Getting timely and effective extension service support however remains a major challenge for such farmers. Farmers need to be empowered, not only economically, but also socially and intellectually to revive agricultural extension and sustain agricultural development. For this, adoption of bottom-up, demand-driven, pluralistic, cost recovery or fee-for-service, and participatory extension services are needed.

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CALLUS INDUCTION IN *AMARANTHUS TRICOLOR* AND *AMARANTHUS SPINOSUS*

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ABSTRACT

Amaranths are an agriculturally valuable crop, but tissue culture techniques for these species remain limited. A study was conducted at the Plant Tissue Culture Laboratory, Institute of Crop Science, College of Agriculture and Food Science from October to December 2009. This study sought to identify a combination of plant growth regulators (PGRs) to induce callus formation on hypocotyl segments of *Amaranthus tricolor* and *Amaranthus spinosus*. *Amaranthus* spp. calli were yellow to deep yellow with a few cultures exhibiting red pigmentation depending on the PGRs applied in the induction medium. PGRs also influenced the number of days before callus outgrowths became visible. Callus formation in both species was faster with a combination of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) than with α-naphthaleneacetic acid (NAA). *A. spinosus* calli were induced in 10 days with Murashige and Skoog medium (MS) + 0.5 mg L^{-1} BAP + 0.5-1 mg L^{-1} 2,4-D; callus induction took 12.5 days in MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D in *A. tricolor*. In both species, BAP treatments caused profuse callus growth, but *A. tricolor* favored NAA, while *A. spinosus* was more responsive to 2,4-D. The *A. tricolor* calli scored with profuse growth also had the greatest mass. For *A. spinosus*, the calli with the greatest mass formed in MS + 0.5 mg L^{-1} BAP + 5.0 mg L^{-1} NAA, but the larger calli formed in BAP + 2,4-D-containing media.

Key words: callus culture, hypocotyl, 2,4-dichlorophenoxyacetic acid

INTRODUCTION

Amaranth (*Amaranthus* spp.), a.k.a "kulitis", Chinese tampala or pigweed, is a member of the Amaranthaceae family (Tisbe and Cadiz, 1967) and an herbaceous and agriculturally important annual plant in Mexico, Central and South America, India and Africa. The genus *Amaranthus* includes over 60 species found across many parts of the world (Willis, 1973). Amaranths are commonly consumed as vegetables or grain crops and have high nutritional value due to the presence of lysine and calcium (Coimbra and Salema, 1994; Pant, 1983) and high amounts of riboflavin, ascorbic acid and vitamin E. Amaranths also produce secondary metabolites, particularly compounds like betalain and anthocyanin (Wink, 2000). Betalain, a natural pigment derived from tyrosine (Leathers et al., 1992), and anthocyanin, a flavonoid (Mazza and Miniati, 1993), are natural food colorants that exhibit antiradical and antioxidant effects. Anthocyanin also possesses anti-inflammatory, antibacterial/antiviral,

anticarcinogenic, antitumor-promoting and antioxidant properties (Corke et al., 2003). These nutritional and therapeutic values of amaranth have increased its potential as a crop.

Plant tissue culture in amaranths remains limited. While there are early plant regeneration studies, the techniques require refinements. Callus formation was successfully induced in *A. paniculatus* (Bagga et al., 1987), *A. caudatus*, *A. hypochondriacus*, *A. cruentus* and *A. hybridus* (Bennici et al., 1992 and 1997). Shoot formation and plantlet regeneration were also achieved in *A. paniculatus* (Arya et al., 1993). In amaranth, plant tissue culture may have some practical applications, *i.e*., it can be used for micropropagation of its related genotypes, male-sterile plants, and stress-resistant genotypes and to rescue genetic variation or even induce new varieties and select regenerated plants for increased production of protein or specific amino acids (Bennici et al., 1992; Bennici and Schiff, 1997). Furthermore, this approach can be utilized for future research focusing on its therapeutic properties and application as natural colorants for food. This study aimed to explore the effects of 6-benzylaminopurine (BAP), a-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction in *A. tricolor* and *A. spinosus* to improve tissue culture of amaranths.

MATERIALS AND METHODS

In vitro-germinated seedlings

Seeds of two *Amaranthus* species, *A. tricolor* (Acc. # 210 - Western Samar) and *A. spinosus* (Acc. # 069 – Marinduque), were sourced from the National Plant Genetic Resources Laboratory (NPGRL), Institute of Plant Breeding, College of Agriculture and Food Science, UP Los Baños, College, Laguna, Philippines. The seeds were pre-treated with 0.36% fungicide (Benlate® or Benomyl®) for 15 minutes and rinsed with tap water. Surface sterilization of seeds for both *Amaranthus* spp. followed that of Flores et al. (1982), using a 10% (w/v) calcium hypochlorite solution following the double sterilization procedure in 10 min-10 min and 15 min-15 min sequences. The seeds were rinsed four times with autoclaved distilled water after the first and second sterilization steps. The sterilized seeds were then germinated in Murashige and Skoog basal medium (MS) (Murashige and Skoog, 1962) with 2% (w/v) sucrose and 0.5% (w/v) Pronadisa agar.

Plant material, culture media, and culture conditions

Hypocotyl segments (5 mm) obtained from ten-day old *in vitro* germinated seedlings of *A. spinosus* and *A. tricolor* (Fig. 1A and B) served as explants. MS medium was solidified with 0.5% (w/v) Pronadisa agar and the pH of the media was adjusted to 5.6 before autoclaving at 15 psi for 20 minutes. The hypocotyl segments were placed on solid MS (Murashige and Skoog, 1962) basal medium supplemented with BAP (0.5 and 1.0 mg L^{-1}), NAA (1.0, 5.0 and 10.0 mg L^{-1}), and 2,4-D (0.5, 1.0 and 2.0 mg L^{-1}), singly or in combination. BAP and 2,4-D were added as required. The culture vessels contained 10 mL of the media and cultures were kept in a growth room at 27°C $\pm$ 1°C under 20 W m^{-2} continuous light supplied by cool white fluorescent lamps.

Callus weight and degree of call us formation

Calli that formed from hypocotyls for each treatment were weighed after 4 weeks of incubation using a digital Mettler balance. The weighing procedure was carried out by placing the weighing balance inside the laminar flow hood wherein each callus was taken out of the culture bottle and placed on sterile paper on top of the balance. The weight (g) for each callus was recorded. The degree of callus formation was scored based on the extent of growth of the callus cells: light (1), moderate (2), moderately profuse (3) and profuse (4) growth as recorded after 4 weeks of callus induction.

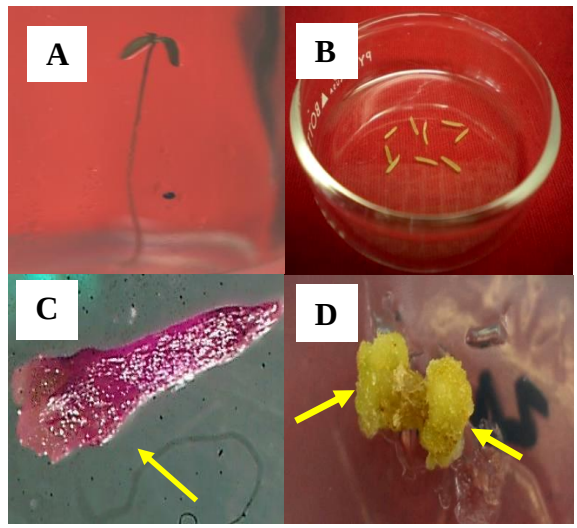


Fig. 1. **Culture initiation and callus induction. (A)** Representative ten-day old, amaranth seedling inoculated onto germination medium (MS basal salts). **(B)** Hypocotyl segments of 5 mm in length used as explants in subsequent experiments. **(C)** Swollen distal end of the hypocotyl (arrow) inoculated onto MS + 1.0 mg L^{-1}BAP. **(D)** Representative hypocotyl section of *Amaranthus* spp. inoculated onto MS + 1.0 mg L^{-1} BAP + 2.0 mg L^{-1} 2,4-D. Calli formed at the proximal and distal ends (arrows).

Experimental design and data analysis

The experiment had a completely randomized design. Twenty samples were used for each treatment and the experiment was repeated twice. The data were analyzed using ANOVA through the Statistical Analysis Software (SAS) System (SAS Institute, Cary, NC, USA). Differences in treatment means were compared using the Duncan Multiple Range Test (DMRT) at $p \leq 0.05$.

RESULTS AND DISCUSSION

Sterilization and germination of *Amaranthus* seed

Two amaranth accessions, *A. tricolor* (Acc. No. 210), a purple amaranth and *A. spinosus* (Acc. No. 069), a spiny amaranth, were used in this study. A total of 402 seeds of *A. tricolor*, and 166 seeds of *A. spinosus* were subjected to sterilization treatments (double sterilization for 20 or 30 minutes) and inoculated onto solid MS basal medium. Table 1 shows the percentage contamination, number of days to germination and percentage seed germination for both species per treatment. For *A. tricolor*, seeds sterilized for 10 min-10 min sequences gave 77.11% germination, while those subjected to a 15 min-15 min sterilization period gave 90.55%. The contamination rate was found to be 4.98% for the seeds treated to 10 min-10 min sterilization, and 3.98% for seeds treated to 15 min-15 min sterilization. Twenty-three percent (23.4%) of the total number of seeds inoculated did not germinate, which could be attributed to dormancy or loss of viability.

For *A. spinosus*, eighty-nine percent (89.16%) germination was attained from the seeds sterilized for 10 min-10 min, while 87.95% of the seeds germinated from the 15 min-15 min sterilization treatment. The remaining 11.44% of the total number of seeds could have been dormant or non-viable. The two accessions responded differently to the germination medium. For *A. tricolor*, the number of days to germination ranged from 6 to 25 for seeds sterilized for either 10 min-10 min or 15 min-15 min, whereas, for *A. spinosus*, the range was 3 - 8 days. Therefore, *A. spinosus* seeds were faster to germinate than *A. tricolor*.

Table 1. Response of two accessions of amaranth seeds to different durations of sterilization.

Treatments	Species	
	A. tricolor (n=402)	***A. spinosus*** (n=166)
10 min-10 min sequence		
% contamination	4.98	0
No. of days to germination	6 to 25	3 to 8
% seed germination	77.11	89.16
15 min-15 min sequence		
% contamination	3.98	0
No. of days to germination	6 to 25	3 to 8
% seed germination	90.55	87.95

Callus type and callus color

In this study, most calli in both *Amaranthus* species were compact and predominantly yellow (*A. tricolor;* Tables 2 and 4) or yellow-green with brown sectors (*A. spinosus*; Tables 3 and 4). However, there were regions in the calli that had either white, red, brown, light green, or yellow-green parts. Two of the four friable calli in *A. tricolor* were yellow, and the other two friable calli had yellow and brown sectors. Red and brown colored calli were observed in cultures with 10.0 mg L-1 NAA alone, or in cultures with equal doses of BAP and NAA or in BAP and 0.5 ppm 2,4-D. Friable *A. spinosus* calli were in varying degrees of yellow-green, white, or brown as seen for *A. tricolor*. Callus color may be an outcome of varying the PGRs in the callus induction medium (Yaacob et al., 2015). Yaacob et al. observed cream-colored calli produced from both leaf and stem explants of *A. cruentus* in 2,4-D and kinetin- or BAP-containing medium. A combination of GA_3 and zeatin also yielded cream-colored calli, while NAA and BAP produced cream and creamy-pink calli. However, after several weeks, only calli inoculated in BAP and 2,4-D changed to green—an indication of shoot initiation. A similar result was observed in the present study, particularly for *A. spinosus* calli, which turned green after four weeks. For *A. tricolor*, a change from yellow to green was only observed after four passages at 4-week intervals (16 weeks) in the same medium. However, shoot initiation was not observed throughout the experiment. Instead, somatic embryoids were noted (Fig. 4), which indicates that shoot formation could be achieved following an extended incubation period.

Table 2. Callus type and color of *A. tricolor* hypocotyl sections inoculated onto various media formulations consisting of MS basal medium added with BA, NAA and 2,4-D singly or in combination.

Type of Callus	Number of Callus Cultures	Number of Callus Culture (n=306)						
		Callus Color						
		Y[1]	Y+W[2]	Y+R[3]	Y+B[4]	Y+R+B	Y+W+R	Y+W+R+LG[5]
Compact	302	98	14	88	34	17	34	17
Friable	4	2	0	0	2	0	0	0

[1]Yellow, [2]White, [3]Red, [4]Brown, [5]Light green

Table 3. Callus type and color of *A. spinosus* hypocotyl sections inoculated onto various media formulations consisting of MS basal medium added with BA, NAA and 2,4-D singly or in combination.

Type of Callus	Number of Callus Cultures	Number of Callus Culture (n=219)				
		Callus Color				
		Y[1]	YG[2]	Y+B[3]	YG+B	YG+B+W[4]
Compact	214	2	17	29	125	41
Friable	5	0	0	0	2	3

[1]Yellow, [2]Yellow green, [3]Brown, [4]White

Callus induction and growth

Earlier reports have demonstrated that callus induction can occur from *Amaranthus* spp. hypocotyl segments and stem sections (Bagga et al., 1987; Bennici et al., 1992 and 1997). In a similar manner, *Amaranthus* spp. hypocotyl segments were used as explants in this study, but different species were tested. *A. tricolor* and *A. spinosus* hypocotyl segments were placed in MS-based media formulations with BAP (0.5, 1.0 mg L^{-1}), NAA (1.0, 5.0, 10.0 mg L^{-1}) and 2,4-D (0.5, 1.0, 2.0 mg L^{-1}) singly, or in combination (Fig. 1).

For both species, BAP in the media was not sufficient to promote callus formation (Figs. 2 and 3; Table 4). After four weeks, hypocotyl segments in MS medium devoid of PGRs turned reddish brown to dark brown, likely from phenolic oxidation (Figs. 2 and 3), and no callus formed. Several days after inoculation, explants in MS + 0.5 mg L^{-1} BA and 1.0 mg L^{-1} BA became swollen on either or both the proximal and distal ends of the hypocotyl sections, but also did not develop any callus. Meanwhile, media with a single synthetic auxin (2,4-D or NAA) was sufficient to promote callus growth in *A. tricolor*. This was not the case for *A. spinosus*, which required the presence of BAP in the medium along with 2,4-D or NAA to induce callus growth.

Callus outgrowths were first observed on either or both ends of the explant (Fig. 1C) after the ends became visibly swollen. Similarly, Singh et al. (2009) found that callus growth initiated on the surface or cut ends of explants during the *in vitro* propagation of sessile joyweed (*Alternanthera sessilis*), a member of the Amaranthaceae family. Likewise, in this study, extensive callus formation proceeded from the cut ends to the center of the hypocotyl segments until the entire explant became a mass of cells. Proliferative growth was sustained by regular transfer every four weeks to the same callus induction medium.

The synergistic effect of BAP and synthetic auxins on callus induction has been reported in *Amaranthus* spp., using either hypocotyl segments or stem sections with BAP and low doses of NAA or 2,4-D (Bennici et al., 1997; Guidea et al., 2012; Biswas et al., 2013). Bennici et al. (1992, 1997) reported the use of kinetin and 2,4-D with similar effectiveness in causing callus formation in hypocotyl segments. Further, the relative proportion of auxin and cytokinin in the media matters. In *A. gangeticus*, a higher BAP: NAA ratio was needed for optimal callus growth in stem explants (Amin et al., 2015), unlike in the present report where a lower cytokinin:auxin ratio was more suitable for *A. tricolor* and *A. spinosus*. The prior studies also reported differential morphogenic responses between *Amaranthus* species and breeding lines. This suggests that plant regeneration systems for *Amaranthus* may have to be developed independently, as genotype—in addition to the PGRs in the induction medium—has a pronounced influence on the *in vitro* response (Bennici et al., 1997).

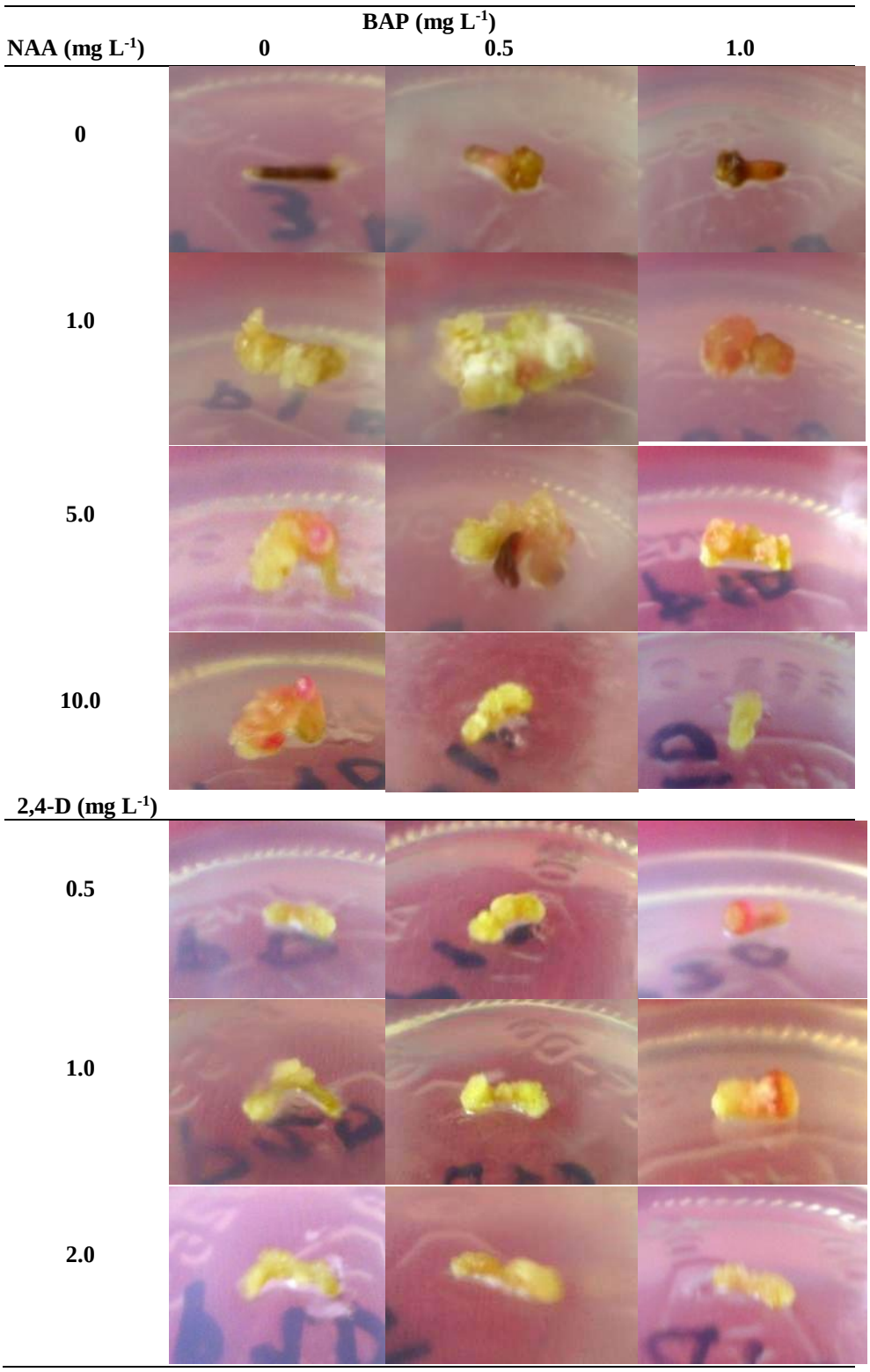


Fig. 2. Degree of callus formation and type of callus formed from *A. tricolor* hypocotyl sections inoculated in various media formulations four weeks after inoculation.

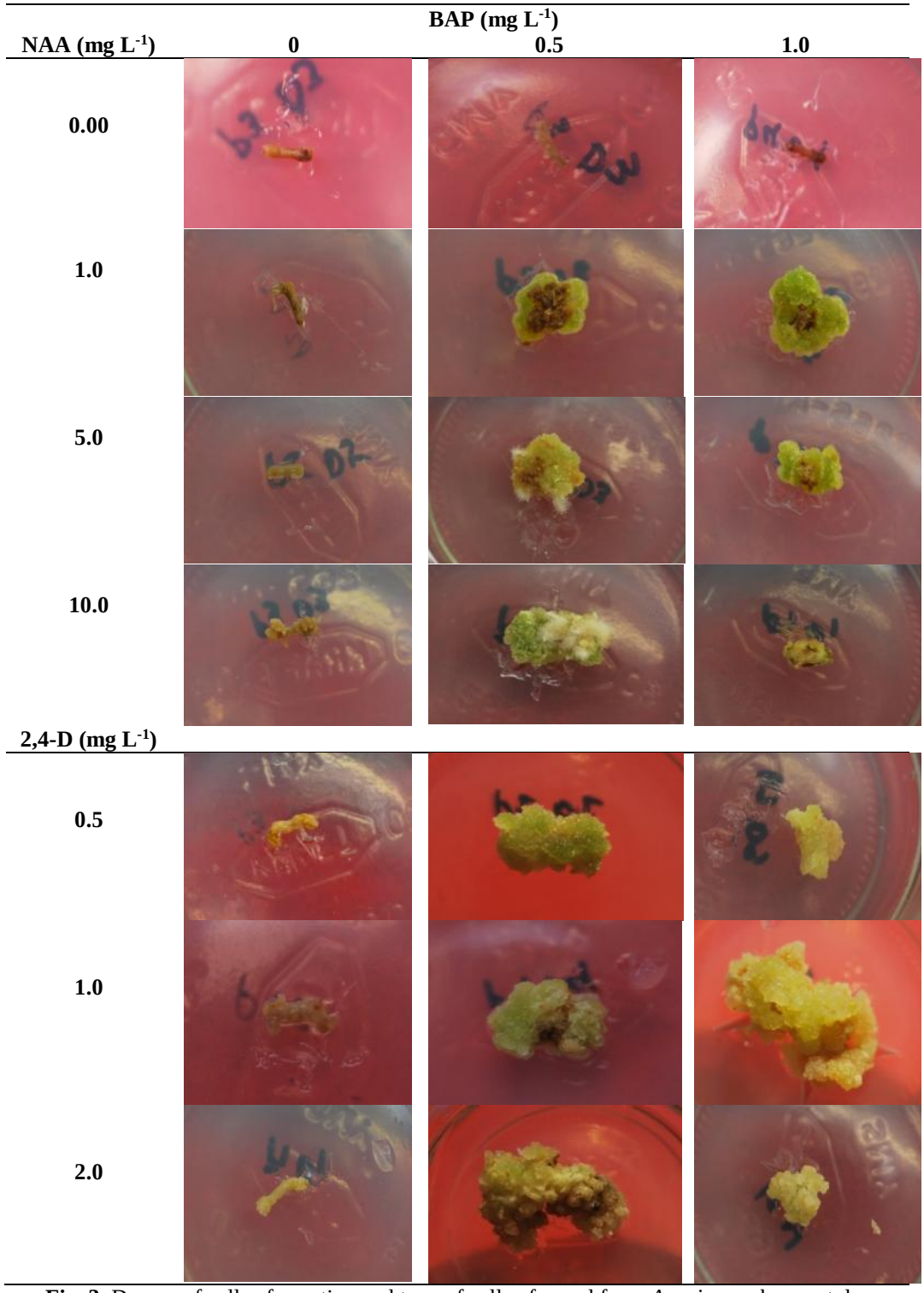


Fig. 3. Degree of callus formation and type of callus formed from *A. spinosus* hypocotyl sections inoculated in various media formulations four weeks after inoculation.

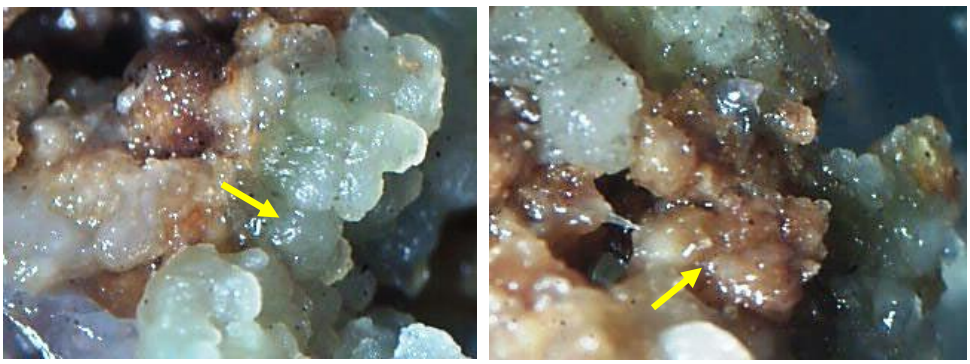


Fig. 4. Somatic embryo-like structures (left) that eventually turned brown (right).

Number of days to callus formation

The composition of the medium affected the time of callus initiation in addition to the color of the callus. Medium with 0.5 mg L^{-1} or 1.0 mg L^{-1} BAP in combination with any of the three levels of 2,4-D or NAA promoted earlier callus formation from hypocotyl segments of *A. tricolor* and *A. spinosus* than medium with 2,4-D or NAA alone (Fig. 5). If 2,4-D or NAA were added singly to the medium it took more than 30 days before callus formed in *A. tricolor*. Faster callus induction was possible in *A. tricolor* by combining 2,4-D or NAA and BAP, although the relative proportion of BAP and either auxin could vary. In general, 10.0 mg L^{-1} NAA or 0.5-1.0 mg L^{-1} 2,4-D and 0.5-1.0 mg L^{-1} BAP achieved the most rapid callus induction. However, while both 2,4-D and NAA were favored by *A. tricolor*, only 2,4-D with BAP yielded a shorter induction period for *A. spinosus*. In fact, auxins alone were unable to induce callus formation in *A. spinosus*.

Callus weight and degree of callus formation

For *A. spinosus*, the highest average callus weight (0.30 g) was obtained in cultures inoculated onto MS supplemented with 0.5 mg L^{-1} BAP + 5.0 mg L^{-1} NAA. More profuse growth, however, was recorded in 2,4-D containing media with BAP. In *A. tricolor*, the heavier calli grew in MS + 0.5 mg L^{-1} BAP + 10.0 mg L^{-1} NAA (2.54 g) and 1.0 mg L^{-1} BAP + 5.0 mg L^{-1} NAA (2.19 g). However, unlike in *A. spinosus*, the largest calli were also from the same treatment combination that gave the highest weight (Table 4).

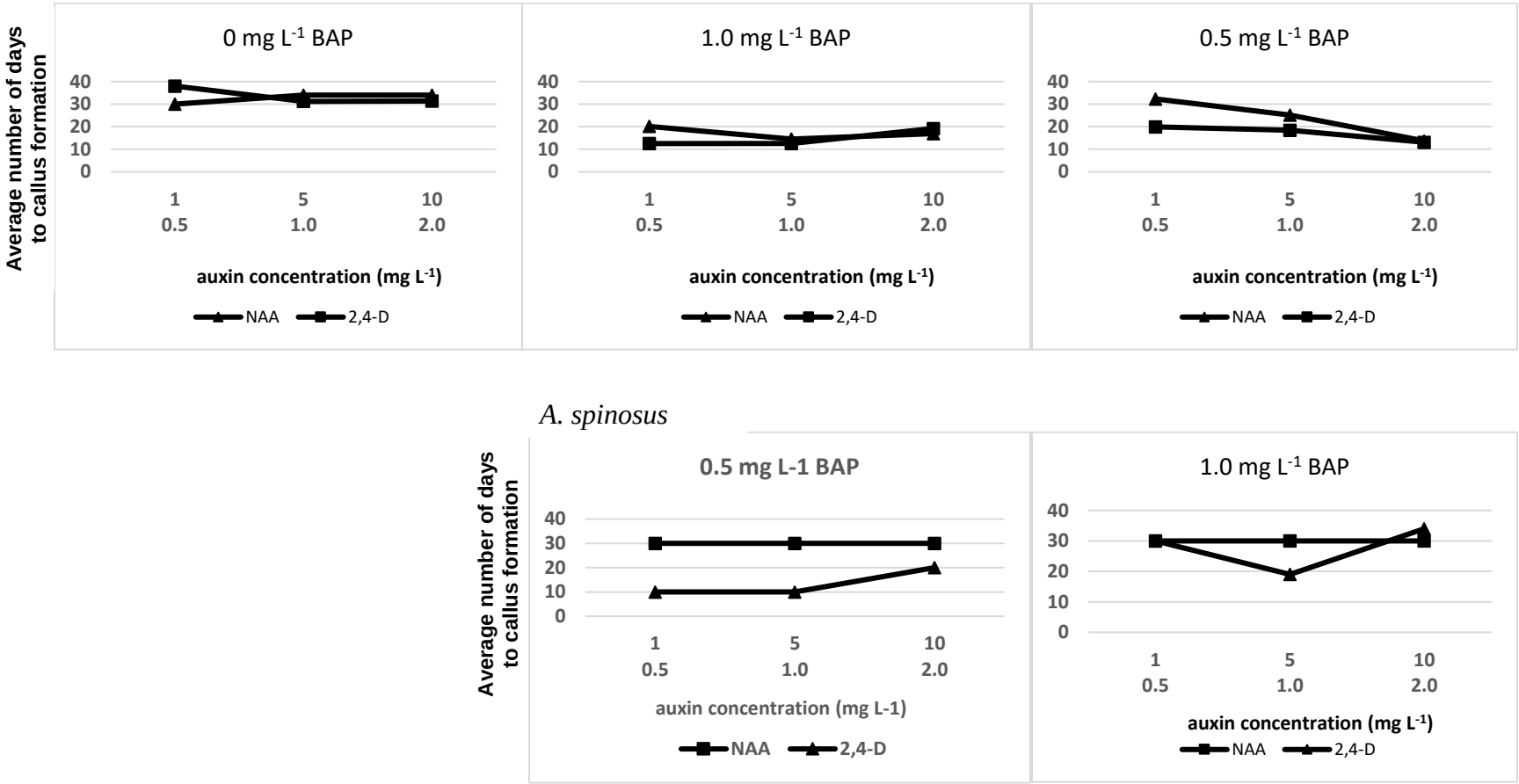
Root formation in hypocotyl explants

Root formation was observed in some hypocotyl explants of *A. tricolor* and *A. spinosus*, particularly near the distal end (Fig. 6). Root formation was observed in sections of *A. tricolor* inoculated onto MS alone or MS supplemented with 0.5 and 1.0 mg L^{-1} BAP alone or supplemented with 0.5 mg L^{-1} 2,4-D. Similarly, MS medium supplemented with proportional levels of BA and NAA (1.0 mg L^{-1}) induced root formation. Other media formulations did not induce root formation in the hypocotyl sections of *A. tricolor*. In contrast, all media formulations containing NAA singly or in combination with BAP at various concentrations induced root formation in hypocotyl sections of *A. spinosus*. Medium with the highest concentration of NAA (10.0 mg L^{-1}) combined with 1.0 mg L^{-1} BAP resulted in the highest number of *A. spinosus* root-forming hypocotyl cultures. These results indicate that NAA supplementation in the culture medium singly or in combination with BAP can induce root formation in *A. spinosus*, whereas MS basal medium alone induces root formation in *A. tricolor*. This result indicates that *A. tricolor* may have a sufficient level of endogenous auxin, and that exogenous application may have resulted in a supra-optimal concentration that led to the inhibition of root formation (Salisbury and Ross, 1992).

Table 4. Average callus weight and degree of callus formation in *Amaranthus* spp. in BA-containing media with varying levels of 2,4-D and NAA*.

TREATMENTS (mg/L)	A. Tricolor		A. spinosus	
	Average Callus weight (g) (n=40)	**Degree of Callus Formation** (n=40)	**Average Callus weight (g)** (n=40)	**Degree of Callus Formation** (n=40)
MS	-	-	-	-
MS + 0.5 mg/L BAP	-	-	-	-
MS + 1.0 mg/L BA	-	-	-	-
MS + 1.0 mg/L NAA	0.04 gh	1	-	-
MS + 5.0 mg/L NAA	0.15 efgh	1	-	-
MS + 10.0 mg/L NAA	0.31 defgh	2	-	-
MS + 0.5 mg/L 2,4-D	0.14 fgh	1	-	-
MS + 1.0 mg/L 2,4-D	0.46 defg	2	-	-
MS + 2.0 mg/L 2,4-D	0.02 h	1	-	-
MS + 0.5 mg/L BAP + 1.0 mg/L NAA	0.62 cd	2	0.15 c	2
MS + 0.5 mg/L BAP + 5.0 mg/L NAA	1.25 b	3	0.30 a	2
MS + 0.5 mg/L BAP + 10.0 mg/L NAA	2.54 a	4	0.12 cd	3
MS + 1.0 mg/L BAP + 1.0 mg/L NAA	0.36 defgh	2	0.24 b	2
MS + 1.0 mg/L BAP + 5.0 mg/L NAA	0.90 bc	3	0.08 def	2
MS + 1.0 mg/L BAP + 10.0 mg/L NAA	2.19 a	4	0.04 f	1
MS + 0.5 mg/L BAP + 0.5 mg/L 2,4-D	0.46 defg	2	0.08 def	3
MS + 0.5 mg/L BAP + 1.0 mg/L 2,4-D	0.35 defgh	2	0.07 ef	3
MS + 0.5 mg/L BAP + 2.0 mg/L 2,4-D	0.45 defg	2	0.23 b	4
MS + 1.0 mg/L BAP + 0.5 mg/L 2,4-D	0.58 cde	2	0.08 def	2
MS + 1.0 mg/L BAP + 1.0 mg/L 2,4-D	0.48 def	2	0.21 b	4
MS + 1.0 mg/L BAP + 2.0 mg/L 2,4-D	0.31 defgh	2	0.09 de	2

*Means followed with the same letters are not significantly different using DMRT at α=0.05.



Fig, 5. Average number of days to visible callus outgrowth as influenced by BAP, 2,4-D and NAA in *A. tricolor* (top panels) and *A. spinosus* (bottom panels). Callus did not form from *A. spinosus* hypocotyl explants without BAP. The values in the x-axis represent concentrations of NAA (upper) and 2,4-D (lower)

Fig. 6. Representative culture of hypocotyl section that formed callus at the distal end of the shoot inoculated onto MS + 1.0 mg L^{-1} NAA.

CONCLUSION

This study defined the combinations of plant growth regulators able to induce calli in *A. tricolor* and *A. spinosus*. Our results support earlier findings and add empirical evidence about the known effects of PGRs on callus induction in *Amaranthus* spp. The plant growth regulator combinations tested in this study did not induce shoots, and future research is necessary to explore the conditions needed to promote shoot formation and development. The differential varietal response observed in this study and in studies of other *Amaranthus* spp. indicates that genotype is a critical overriding factor influencing their *in vitro* morphogenic response. This suggests that independent plant regeneration systems are compulsory for *Amaranthus* spp.

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DEVELOPMENT OF ONE-STEP IMMUNOCHROMATOGRAPHIC STRIP FOR THE DETECTION OF TOTAL AFLATOXIN IN CORN SAMPLE BASED ON MONOCLONAL ANTIBODY CLONE 4G6

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ABSTRACT

Aflatoxins are cancer-causing chemicals produced primarily by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B1 (AFB1) is the most commonly found aflatoxin in improperly stored staple commodities such grain and feed. Its presence in the food supply, can be carried over to animal products such as meat, liver, kidney, pig blood and milk. A specific and sensitive detection method is required for preliminary screening of these samples. This research sought to develop a detection kit for total aflatoxin by immunochromatographic technique using monoclonal antibody (MAb) from the hybridoma cell line 4G6. The experiments were conducted at the Serology and Diagnostic Laboratory, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom province during 2014-2016. The MAb is composed of IgG_{2b} isotype and lambda light chain. Its specificity recognized four aflatoxins including AFB_1, AFB_2, AFG_1 and AFG_2 with cross reactivity at 100%, 89.2%, 82.6%, and 72.7%, respectively by direct competitive enzyme-linked immunosorbent assay (dcELISA). *In vitro* propagation of the hybridoma was carried out using an Integra CELLine Culture System and the antibody was purified by affinity column chromatography. The conjugate probe was prepared by comparing two sizes of colloidal gold particles at 20 and 40 nm in diameter for the conjugation with the MAb. The MAb conjugate with 40 nm colloidal gold was selected and sprayed onto the conjugate release pad (CRP). The target cut-off value for the developed immunochromatographic strip (ICS) was 20 ng/mL according to a regulation limit in Thailand. The study on the appropriate conditions for this strip showed that aflatoxin B_1 conjugated to bovine serum albumin (AFB_1-BSA) and goat anti-mouse immunoglobulin (GAM) should be immobilized at the test line and control line at the same concentrations of 0.25 mg/mL. The testing sample was extracted with 70% methanol and further diluted 1:4 with Tris buffer saline with 0.05% Tween-20 (TBST) before application on the sample application pad (SAP) and the reaction could be visualized within 15 min. The analysis of 5 naturally contaminated corn samples (n=7) indicated that 2 samples contained ≥ 20 µg/kg and 3 samples contained < 20 µg/kg. Five samples, analyzed by dcELISA, showed contamination levels at <4, 9.6, 19.9, 10.5 and 39.7 µg/kg which delivered a good correlation to the results from ICS analysis.

Key words: rapid test kit, mycotoxin, toxin analysis, lateral flow assay

INTRODUCTION

Aflatoxins (AFT) are a group of carcinogenic secondary metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus*. These are listed as group I carcinogens by the International Agency for Research on Cancer (IARC, 2002). Several types of aflatoxins are produced in nature, however, aflatoxin B1 (AFB_1) is the most common in food and feed as well as among the most potent genotoxic and carcinogenic aflatoxins. Therefore, exposure through food should be kept as low as possible. When the toxin enters human or animal bodies, it would be absorbed in the alimentary canal and spread rapidly through tissues but may take time to be excreted through urine and stool. For these reasons, AFT might be carried over to the animal product such as meat, liver, kidney, pig blood and milk (Kaushal and Bhatnagar, 1998).

European Communities (EC) have different regulations on AFT quantity in children's food and cow's milk, which should not exceed 4 µg/kg and 0.05 µg/L, respectively. In Thailand, the regulation limit of all food commodities is 20 µg/kg according to the Notification of Ministry of Public Health of 1986 (Ministry of Public Health, 1986), however, the percentage of major aflatoxin contamination observed in peanuts, dried seafood, poultry tissue and corn were reported to be 51.4%, 58.6%, 48.67%, and 54.39%, respectively (Tangmunkhong *et al*., 2011; Mahakarnchanakul *et al*., 2011; Anukul *et al*., 2013). These data indicate the need for more efforts to establish the control measures of the toxin in these commodities, especially for very low levels of AFT in food products, which requires a specific and sensitive method. Although the most acceptable and popular AFT analysis is high performance liquid chromatography (HPLC) with cleanup (Chu, 2004), it is expensive, time-consuming and requires highly skilled and professional personnel. Moreover, large quantities of chemicals are needed which poses extreme danger of used chemicals and cost of disposal. Currently, immunological methods such as quantitative enzyme-linked immunosorbent assay (ELISA) is acceptable to the Association of Official Analytical Chemists Research Institute (Lupo *et al*., 2010). Another method for rapid screening is an immunochromatographic assay (ICA) which is a qualitative analytical test recommended for preliminary screening. Its utilization has been increasing because it is convenient and allows qualitative or semi-quantitative determination of mycotoxin within a few minutes. In Thailand, the production of AFT-specific immunochromatographic strip (ICS) with the cut-off value at 40 ppb was reported and it took 20 min for color development (Biotechnology gallery, 2006). This research sought to develop the ICS for total AFT analysis, using monoclonal antibody from our previous research, with the required cut-off value at 20 ng/mL, according to Thailand regulation limit for food, by conjugating the antibody with nanogold particles. The success of this research would help to reduce import cost of commercial ICS and support agriculturist or trader needing raw material screening and decreasing the risk of consumption of food contaminated with aflatoxin.

MATERIALS AND METHODS

Materials

Hybridoma clone 4G6 from the previous research (Hongprayoon *et al.*, 2009) producing specific antibody to AFT was used for the ICS development. The upscale production of the hybridoma was carried out in a CELLine CL1000 (INTEGRA Biosciences, Switzerland) as described by the manufacturer, with Complete medium (RPMI1640 medium + 15% fetal calf serum). The antibody was harvested and further purified by HiTrap Protein G together with AKTAprime plus chromatography system (GE Healthcare, Sweden). Nanogold particles were purchased from DCN Diagnostics (USA). Goat anti-mouse Ig (GAM), Aflatoxin B1-bovine serum albumin conjugate (AFB_1-BSA) and AFB_1, AFB_2, AFG_1, AFG_2 standards were purchased from Sigma-Aldrich Company (USA). High-flow nitrocellulose membrane, fiber glass and CF6 absorption material were purchased from GE Healthcare (Sweden). Phosphate buffer saline (PBS, pH 7.4) (137 mM NaCl, 27 mM KCl,

10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and Tris buffer saline (TBS, pH 7.2) (25 mM Tris base, 0.15 M NaCl) were prepared according to the standard protocols (Sambrook and Russell, 2001).

Nanogold particle conjugation with specific antibody against aflatoxins (MAb-gold conjugate)

Conjugation of the MAb with 20 nm and 40 nm nanogold particle was carried out according to Faulk and Taylor (1971). Briefly, 10 ml of the gold suspension was adjusted to pH 7.3 by 0.2M K_2CO_3. One hundred microliter of previously dialyzed MAb in 0.5x PBS (1 mg/mL) was gradually added to the suspension. The mixture was stirred slowly at room temperature (~25°C) for 1 h. One milliliter of 10% BSA in distilled water was prepared and filtered through a syringe filter (0.45 micropore size nylon membrane) prior to adding into the mixture. Stirring at room temperature was carried on for 30 min and the suspension was centrifuged at 10,000xg for 10 min. A total of 500 µL of gold dilution buffer, pH 7.4 (0.02 M Na_2HPO_4, 0.2% NaN_3, 1% BSA) was used to resuspend the MAb-gold conjugate precipitate in a microtube. One hundred milligram of sucrose was then added and mixed by inverting the tube. The concentration of MAb-gold conjugate was determined using NanoDrop 8000 (Thermo Scientific, USA) at 520 nm (O.D. 1 = 9.0 x 10^{10} particles/ml) (Haiss *et al.*, 2007).

Development of immunochromatographic strip and selection of a sample buffer

ICS components were layered on a protecting plastic sheet adhesive backing as follows; nitrocellulose membrane (NCM, Prima 40), conjugate releasing pad (CRP, 33 glass fiber), sample application pad (SAP, 33 glass fiber), and absorption pad (AP, CF6 cotton linter) with 1 mm overlapping for each component (Fig. 1). A test line and a control line were applied onto the NCM by immobilizing toxin conjugate and goat anti-mouse IgG (Sigma, USA), respectively. Five microliter per centimeter of MAb-gold conjugate ($O.D._{520}$ = 0.5) was applied to the CRP. The Biojet and Airjet: XYZ Dispensing System (BIODOT, USA) was used to manufacture the testing strips. After the reactive lines on NCM and the CRP had been conducted, it was allowed to dry at 37ºC for two hours.

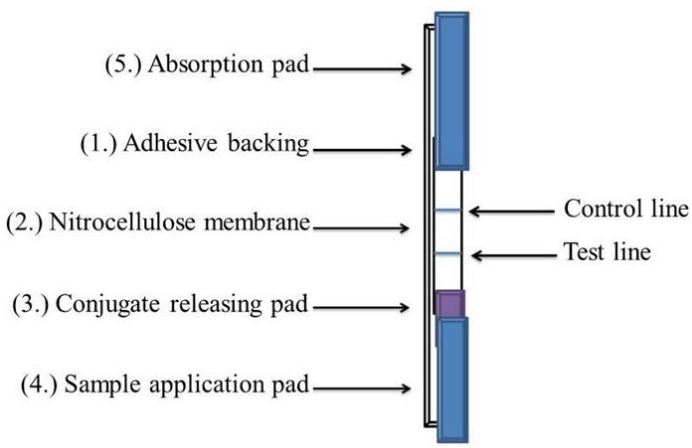

Fig. 1. Schematic diagram of the immunochromatographic strip (ICS). Numbers shown in the figure indicates orders of the component layers.

Four kinds of buffer solution including PBS, pH 7.4; PBST (PBS+ 0.05% Tween-20), pH 7.4; TBS, pH 7.4; and TBST (TBS + 0.05% Tween-20), pH 7.4 were compared to 70% (v/v) methanol (control) for their capillary action on the strip without non-specific reaction. The test was performed by dropping 50 µL of each buffer on the SAP and the reactions at both lines had been observed within 15 minutes.

Determination of the cut-off value of the ICS using aflatoxin standards dissolved in 70% methanol

Toxin standards for each aflatoxin (B_1, B_2, G_1 and G_2) were prepared in 70% methanol at the final concentrations of 10, 20, 50, and 100 ng/mL. Each standard preparation was diluted 1:4 with the sample buffer (TBST) before testing with the ICS. Completion of color development was determined 15 min after dropping the sample onto the SAP.

Analysis of spiked corn samples by the developed ICS

Spiked corn samples were prepared to evaluate the developed ICS. Dry ground corn was analyzed by HPLC at the Molecular Biology and Toxin Laboratory, Kasetsart University Research and Development Institute. The aflatoxin-negative sample was then spiked with each aflatoxin (B_1, B_2, G_1, and G_2) at the required concentrations; 0, 10, 20, 50, and 100 µg/kg, and left overnight at 4ºC. Twenty grams of the ground sample was extracted with 40 mL of 70% methanol for 20 min at room temperature and shaken at 200 rpm. To allow matrix precipitation, the extract was filtered through filter paper No.1 (Whatman, USA) after 5-10 min (Kladpan *et al.*, 2004). The sample extract was diluted 1:4 with TBST prior to analysis to decrease matrix interference and methanol concentration. Color development was determined 15 min after dropping the sample onto the SAP.

Analysis of naturally contaminated corn samples by the developed ICS

Five samples of animal feed raw material were received from the Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus. The toxin was extracted as described above and diluted 1:4 with TBST. The sample solution from each preparation was applied to the sample pad until color development which was at least 15 min. The results of 7 replicates were compared with the results from direct competitive ELISA (dcELISA) using a commercial test kit, AgraQuant® ELISA Total Aflatoxin (4-40 ng/mL) (Romer Labs, Singapore) and operated by the Veterinary Diagnostic Center. The absorbance of the samples was compared to the absorbance of the standards and an interpretative result was determined. A dose-response curve was constructed using the five standards and the unknowns were measured by interpolation from this standard curve.

RESULTS AND DISCUSSION

Development of immunochromatographic strip (ICS) and selection of a sample buffer

The monoclonal antibody from hybridoma cell line 4G6 was used in the development of ICS, which was IgG_{2b} containing lambda light chain as characterized previously. The MAb specificity was evaluated by cross reactivity (CR) with four aflatoxins including AFB_1, AFB_2, AFG_1 and AFG_2 via dcELISA (Fremy and Usleber, 2003). Its specificity to aflatoxins were as follows; 100%, 89.2%, 82.6%, and 72.7% with B_1, B_2, G_1, and G_2, respectively without cross reaction with zearalenone and ochratoxin A. These characteristics supported that the MAb possesses good affinity to total aflatoxins and could be applied to develop the ICS for the detection of total aflatoxins. Twenty four milligrams of the purified antibody was obtained from the upscale production in the Integra CELLine system. The main purpose of this research was to develop an ICS which provides results that can be visualized by the naked eye, specifying a cut-off value at 20 ng/mL following Thailand regulation for food. It was important that the color intensity of the test line was visible enough to be seen and enable a clear distinction between negative and positive results. Therefore, the appropriate density of MAb-gold conjugate, toxin conjugate and anti-species antibody were major considerations. The conjugate probe was prepared by conjugating nanogold particles with the antibody because the particles are commonly used as an immunospecific probe with good biocompatibility and label of biomolecules (Liao and Li, 2010). Comparison of the two sizes of the colloidal gold particles at 20 and 40 nm in diameter showed the better signs on the test and control lines when using 40 nm size and offered maximum visibility owing to the least steric hindrance in the IgG conjugation (Chandler et al., 2000). Experimental results showed that the optimal amount of the MAb-gold conjugate applied onto the CRP was 5 µL/cm. The

optimal immobilized AFB_1- BSA was determined to be at the concentration of 0.25 mg/mL, which was the same concentration as the control line for anti-species antibody.

Optimization of the ICS assay condition

Even though the extraction of AFT from the sample can be performed in several solvents, such as water-saturated chloroform, aqueous methanol, acetonitrile, acetone as well as polar solvents, the most widely used has been 70% methanol which corresponded to our results. However, several obstacles can also be found in the extract interference with the binding between the toxin and the MAb conjugate (Kaushal and Deepak, 1998). Thus, other factors affecting the reaction on the assay strip such as sample buffer and methanol density in the system were also investigated, in order to get strong and clear reaction line. In the experiments, four kinds of buffer solutions were compared to 70% methanol and the results showed that TBST gave the best lines for both test and control zones when the analyte was absent in the buffer. We also compared three dilutions of methanol extract in TBST, e.g. 1:2, 1:3 and 1:4. The results showed that when 1:2 diluted extract was applied to the strip, signals at both lines were not observed whereas these could be clearly visualized at 1:3 and 1:4 dilutions (especially at 1:3 dilutions). Thus, 1:3 dilution was chosen for the succeeding experiments and the complete reaction could be achieved within 15 mins.

Determination of the cut-off value of the ICS using aflatoxin standards dissolved in 70% methanol

The cut-off value of the developed strip was determined by testing the strip with four aflatoxins in 70% methanol and diluting these at a ratio of 1:4 with TBST. In the absence of aflatoxin, the binding of the MAb-gold conjugate to the solid-phase AFB_1-BSA gave a strong red band. In contrast, the color band disappeared when the test solution contained any of AFB_1, AFB_2 and AFG_1 above 20 ng/mL except for AFG_2 where the test line would disappear at 50 ng/mL of the analyte (Fig. 2). This result corresponded to the specificity of the MAb mentioned above. Nevertheless, this characteristic did not affect the strip performance since AFG2 contaminated in a natural sample is a small ratio comparing with other aflatoxins (B1: B2: G1: G2 = 4:2:1:1) (Trucksess et al., 2008).

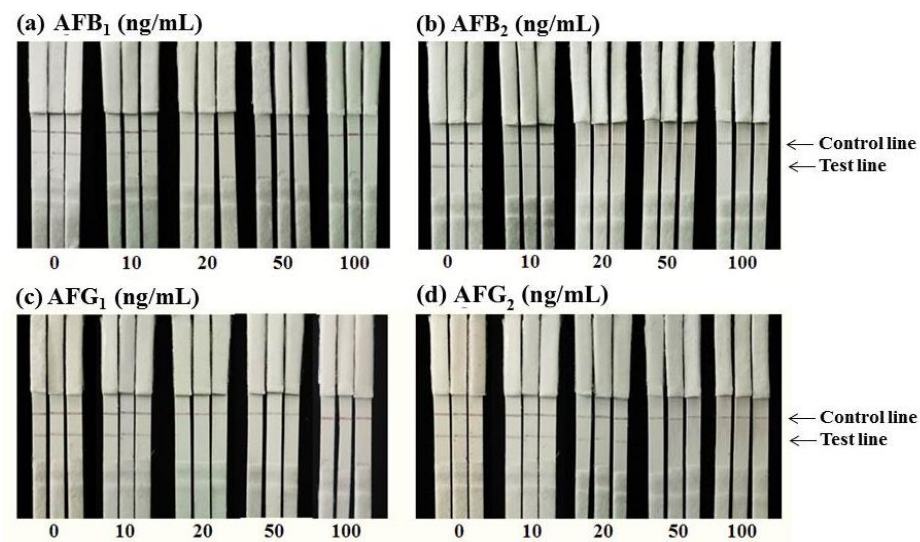

Fig. 2. Detection limit of standard aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) dissolved in 70% methanol determined by the developed ICS. A concentration exceeding 20 ng/mL of AFB_1, AFB_2, AFG_1 led to disappearance of a red line at the test zone whereas the cut-off value for AFG_2 was at 50 ng/mL (n=3).

Analysis of spiked corn samples by the developed ICS

Corn samples were spiked with each aflatoxin at a concentrations of 10, 20, 50 and 100 µg/kg and analyzed. The results demonstrated that the strip successfully determined the cut off at 20 µg/kg for three aflatoxins including AFB_1, AFB_2 and AFG_1 whereas AFG_2 cut-off value was at 50 µg/kg. The disappearance of the colored line at the test line at 20 µg/kg and higher, for AFB_1, AFB_2 and AFG_1, showed the presence of the aflatoxin. For AFG_2, it was at 50 µg/kg (Fig. 3).

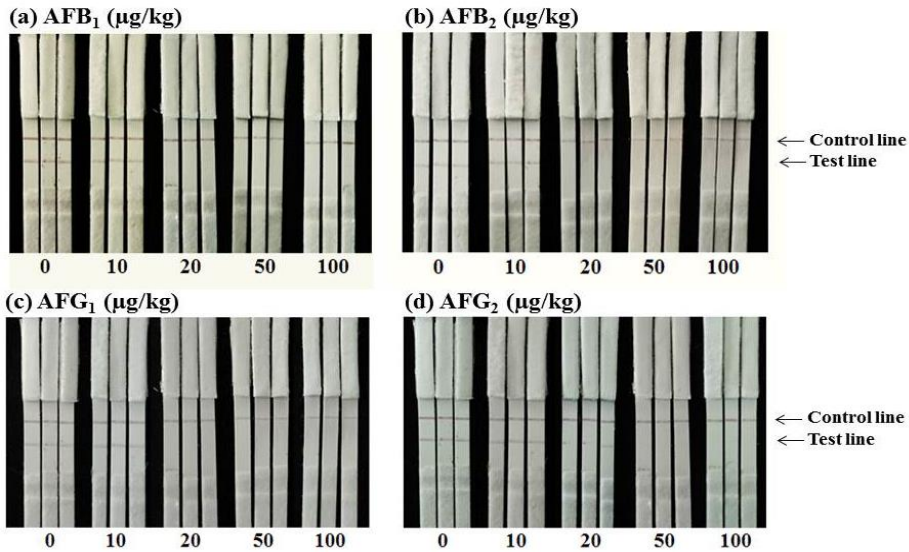

Fig. 3. Analysis of the spiked corn samples with each aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) at the concentrations of 10, 20, 50 and 100 µg/kg, respectively (*n*=3). The strip successfully determined the cut-off value at 20 µg/kg for three aflatoxins including AFB_1, AFB_2 and AFG_1 whereas the cut-off value for AFG_2 was at 50 µg/kg where the test lines disappeared.

Analysis of naturally contaminated corn samples by the developed ICS

Five naturally contaminated corn samples were extracted and analyzed using the developed ICS and the results were compared with the values determined by dcELISA using the commercial test kit (Fig. 4). Comparative analysis by both methods conformed with each other (Table 1) where 2 samples contained ≥ 20 µg/kg and 3 samples contained < 20 µg/kg aflatoxin contamination.

Table 1. Aflatoxin analysis of five naturally aflatoxin-contaminated corn samples by the ICS compared to dcELISA.

Sample no.	ICS (*n*=7)	dcELISA (*n*=3)
1	(-)	<4 µg/kg
2	(-)	9.6 µg/kg
3	(+)	19.9 µg/kg
4	(-)	10.5 µg/kg
5	(+)	39.7 µg/kg

Remarks: (-) means less than 20 µg/kg aflatoxin contamination.
(+) means equal or more than 20 µg/kg aflatoxin contamination

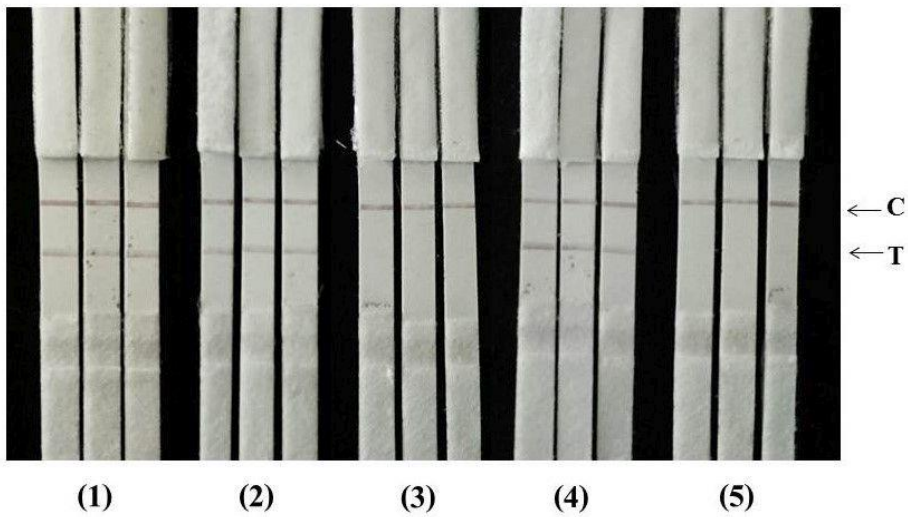

Fig. 4. Evaluation of the ICS reactivity (*n*=7) by analysis of five naturally aflatoxin-contaminated corn samples (1-5) at the concentrations of <4, 9.6, 19.9, 10.5 and 39.7 µg/kg determined by AgraQuant® ELISA Total Aflatoxin (4-40 ng/mL) test kit (*n*=3).

CONCLUSIONS

The ICS is a semi-quantitative assay based on color intensity of the lines and enables a clear distinction between negative and positive results through the naked eye. In the present research, the cut-off value of the developed ICS was achieved at 20 ng/mL for three aflatoxins including AFB_1, AFB_2 and AFG_1 while AFG_2 cut-off value was at 50 ng/mL. In addition, the specificity of our MAb to AFG2 is much higher than the previous report (Shim et al., 2007) which is good for the detection of total aflatoxins. To evaluate the performance of the ICS, five corn samples (n = 7) were analyzed using the developed ICS and compared to the commercial ELISA kit, which is officially approved by AOAC and GIPSA for its reliability. The results from both methods conformed with each other. The ICS developed in this study can be operated easily, conveniently, rapidly and the cut-off of 20 ng/mL is suitable for Thailand regulation. Therefore, it can be used as an on-site screening tool for detecting the contaminated aflatoxins in agricultural products at the primary screening step.

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DISEASE SCREENING AND POST-ENTRY QUARANTINE PROGRAM FOR SAFE INTRODUCTION OF SUGARCANE VARIETIES IN THE PHILIPPINES

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ABSTRACT

A three-year post-entry quarantine pathogen testing protocol for foreign sources of sugarcane was developed for the Philippines. A total of 284 varieties acquired from Australia, Bangladesh, China, France, Indonesia, Malaysia, Mauritius, Thailand, USA, Japan, Vietnam and Pakistan were subjected to pathogen testing inside the post-entry quarantine glasshouse for 24 months at the Institute of Plant Breeding – College of Agriculture and Food Science, University of the Philippines Los Baños and another 10 months under the open-field conditions at Guimaras, Visayas for the period of 2001-2012. Leaf scald, ratoon stunting disease and sugarcane mosaic were among the most common diseases that were detected on foreign varieties imported from Thailand, Malaysia, Indonesia, Bangladesh, China, France, USA, Vietnam and Pakistan. The presence of other diseases such as smut, red rot, grassy shoot, sheath rot, Pokkah boeng and yellow leaf disease were also observed on some foreign varieties from Thailand, Indonesia, China, Australia, USA and Pakistan. A cold soak and hot water treatment was found effective in eradication of pathogens associated with seed pieces received from foreign countries. Furthermore, the use of an optimized and standardized scheme of quarantine pathogen testing through antibody and nucleic acid based assays proved to be a reliable practice in checking the introduction of new pathogens in new and promising sugarcane varieties received from other countries. These preliminary quarantine practices were found effective in protecting the Philippine industry from unwanted quarantine pathogens.

Key words: pathogen testing, antibody, nucleic acid, cold soak and hot water treatment

INTRODUCTION

Introduction of foreign germplasm increases the genetic diversity and desirable agronomic traits (Croft, 1996). As a result of sugarcane germplasm exchange and plant breeding, sugarcane varieties that are high yielding, adapted to changing environmental conditions, and resistant to pests and diseases can be developed. Sugarcane (*Saccharum* spp. hybrid) is the major crop cultivated in the Western Visayas (Moog, 2006) and is an important cash and exportable crop in the Philippines along with coconut, pineapple, banana, coffee and mango (Espino and Atienza, 2001). The Philippines is second in terms of sugarcane production in Southeast Asia (ASEAN Food Security Information System, 2014). The feasibility of sugarcane as a source of special sugars, bio-plastics, bio-fertilizers, among other products, is also being studied to further increase its input to the Philippine economy (www.sra.gov.ph).

In the Philippines, diseases have always been a major biotic constraint in sugarcane production. About 75-100% loss of sugarcane yield has been recorded in fields when cuttings infected by *Sporisorium scitamineum* syn. *Ustilago scitaminea* are planted (Reyes *et al.* 1980). In addition,

there are sugarcane diseases caused by fungi, bacteria and viruses which are also important for quarantine purposes and therefore, should be regulated.

Foreign sugarcane varieties are imported into the Philippines from: Thailand, Malaysia, Indonesia, China, Australia, France (CIRAD) and Mauritius. Therefore, sugarcane germplasm exchange in the Philippines poses a risk of introducing exotic diseases. Reports are available that pathogens can be disseminated and distributed through sugarcane seed pieces, without external symptoms or in the case of latent infections (Croft, 1996).

With the implementation of the Common Fund for Commodities (CFC) Varietal Exchange Agreement in 2000, the Institute of Plant Breeding of the University of the Philippines Los Baños (IPB-UPLB), Philippine Sugar Research Institute Foundation, Inc. (PHILSURIN), and the Plant Quarantine Services of the Bureau of Plant Industry (BPI-PQS) have collaborated in studying and indexing pathogens in introduced varieties through developing pathogen testing protocols and constitution of the sugarcane quarantine program. Quarantine and pathogen testing for sugarcane have been implemented in South Africa (Bailey and Bechet, 1988) and Australia (Thompson *et al.* 2011), and these measures must also be established in the Philippines. Hence, this study was initiated to develop and optimize pathogen testing procedures for the detection of pathogens such as *Xanthomonas albilineans*, the cause of leaf scald (LS), *sugarcane mosaic virus (SCMV)*, the cause of sugarcane mosaic (SCM) and *Leifsonia xyli* ssp. *xyli (Lxx.)*, the cause of ratoon stunting disease (RSD) in foreign varieties of sugarcane, to safeguard the Philippines against these pathogens. An open-field quarantine system under natural conditions, situated on a remote location, was also employed as part of the protocol to check for possible occurrence of any other new disease which did not manifest in the post-entry glasshouse condition.

MATERIALS AND METHODS

Acquisition of introduced sugarcane varieties

From 2001 to 2012, a total of 284 sugarcane setts or seed pieces were acquired from different sugarcane growing countries including Australia, Bangladesh, China, France, Indonesia, Malaysia, Mauritius, Thailand, USA, Japan, Vietnam and Pakistan (Table 1). Upon arrival of the planting materials at the Post-Entry Quarantine in IPB-CAFS, UPLB, visual inspection was done to detect readily observable symptoms and for proper documentation. The general appearance and condition, as well as number of buds per variety, presence of initial signs and symptoms were noted.

A hot water treatment for the newly imported materials was done prior to planting following the BSES protocol (Croft *et al.* 2011). Sugarcane seed pieces were exposed to an overnight cold soak treatment at an ambient temperature with occasional addition of fresh water. Then, these were given a short hot water treatment (SHWT) for 30 min at 50°C. Five minute (5.6 g/L) Mancozeb (fungicide) dip treatment was also done following the HWT. The seedpieces were then potted in plastic containers filled with sterile soil (5:1 soil:coconut coir dust) and allowed to germinate under glasshouse conditions. Upon germination, the varieties were transferred into individual 24-liter plastic utility pails with newly sterilized soil. These were labeled separately with variety names and date of planting. Two months after germination, fertilizer application using urea (46-0-0) was done. Monitoring for occurrence of diseases was conducted on a regular basis during a two-year quarantine cycle.

Closed quarantine

Post-entry quarantine inspection of the introduced sugarcane varieties were divided into two cycles: cycle 1 and cycle 2, corresponding to the number of years the varieties were maintained under

greenhouse quarantine conditions (Figure 1a). During quarantine testing, at 4 months after planting, the plants were visually observed for LS, SCM, yellow leaf syndrome, downy mildew, smut and other diseases. Also, plants were tested for the presence of *SCMV*, *sorghum mosaic virus (SrMV)* and *Xanthomonas albilineans* using either enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR). The tests were repeated. At 12 months, the plants were ratooned and stalks were observed for the presence of reddish or pinkish discoloration as an indication of RSD. After 10-12 months of the first pathogen testing cycle, sugarcane stem-cuttings or "setts" from the ratooned plants were processed and exposed to an overnight-soak in running cold water. Following the overnight soak, setts were subjected to a long hot water treatment (LHWT) for 2h at 50°C, followed by soaking in running water for an hour, and dipping in (5.6 g/L) Mancozeb solution before planting in a sterile soil-filled plastic container. The crop plants and the ratooned plants were allowed to grow for another 12 months for the second cycle of pathogen testing. Sugarcane varieties that were tested positive for the pathogens associated with diseases such as LS and RSD in diagnostic procedures underwent cold soak and long hot water treatment again to eradicate the said pathogens. Plants infected with *SCMV* were subjected to LHWT. Moreover, plants that still exhibited symptoms of diseases even after hot water and fungicide treatment were rogued out and burned.

Fig. 1. (a) Post-entry Quarantine Greenhouse at the Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños; (b) Open-field quarantine in the province of Guimaras in the Visayas.

Open-field quarantine

After 18-24 months of pathogen testing in the post-entry quarantine glasshouse, introduced sugarcane varieties that were free from diseases were ratooned. Cane setts were subjected to cold-soak for 12 hours and LHWT (50°C for 2 hours). All the cane setts were treated with (5.6 g/L) mancozeb solution. Ratooned cane setts were transferred and planted to an isolated, open-field natural condition in Guimaras province in the Visayas located at a very remote area (Figure 1b). The island is distant from commercial sugarcane plantations; hence the probability of introducing new diseases to existing sugarcane plantations is very low. Solarization and fallowing for 2-3 months are practiced to prevent the chance of varietal contamination. During the 3rd year of pathogen testing, materials were planted and allowed to grow in the field for another 12 months. The plants were regularly observed for the occurrence of pests and diseases. Sampling of leaves to test for the presence of *Xanthomonas*

albilineans and *SCMV* was done at 5-10 months after planting, while stalk sap sampling was used for *Lxx.* detection. The presence of other important diseases was also monitored. Plants that expressed visible symptoms and/or positive to indexing were rogued out and destroyed.

Pathogen testing

An optimized and standardized procedure for the detection of disease-causing organisms for all of the 284 introduced sugarcane varieties in the post-entry and open-field quarantine was adopted with modifications (BSES Quarantine Training Manual, 2001). For the antibody-based tests, leaf sampling was done using the third to the youngest fully expanded leaf at 4, 8 and 12 months for *Xanthomonas albilineans* and *SCMV* detection. An approximate of 1x1 inch leaf portion was collected per plant. At 12 months, stalk sap sampling was done by obtaining one node per stalk per plant for *Lxx.* detection. All the cutting tools were disinfected using 10% NaOCl and 70% EtOH prior to use on each plant.

Modified enzyme-linked immunosorbent assay (ELISA) techniques such as Dot-blot immunoassay using nitrocellulose membrane for the detection of bacterial pathogens of *Xanthomonas albilineans* and *Lxx.*, and Indirect-ELISA for viruses such as *SCMV* and *SrMV* were employed. Nucleic acid-based technique (Polymerase Chain Reaction) utilizing specific primers against pathogens caused by bacteria and viruses, was also performed to ascertain the presence or absence of disease-causing microorganisms. In ELISA, locally produced polyclonal antibodies against *Lxx.* and *Xanthomonas albilineans* (Dela Cueva *et al*. 2010) were used routinely in pathogen detection while commercially available antisera from Agdia® were used to detect *SCMV* and *SrMV*.

Dot-blot immunoassay (DBIA)

For *Xanthomonas albilineans* detection, a dot-blot immunoassay technique was used (dela Cueva *et al.* 2010; Viswanathan *et al*. 1998; Wang *et al*. 1999) with the following modifications. An appropriate size (~10 cm x 10 cm) of the nitrocellulose membrane (Trans-Blot® Transfer Medium, Catalog 162-0115, Bio-Rad) was obtained. The membrane was dipped in 100 mL 1 x TBS buffer (0.05M UltraPure™ Tris, 0.15M NaCl, 100 mL ddH_2O, pH 8.0). Antigen loading was then done by acquiring 3 µl of the samples. Following 12 hours of incubation, the membrane was placed in blocking buffer (50 mL 1 x TBS buffer in 0.0005% v/v Tween-20 [polyoxyethylene (20) sorbitan monolaurate], 3% wt/v non-fat milk and 0.27M glycine [NH_2CH_2COOH]). Washing was done with 50 mL 1 x TBS-T buffer (0.05M UltraPure™ Tris, 0.15M NaCl, 0.0005% v/v Tween-20, 50 mL ddH_2O, pH 8.0) for three times at 10 min intervals, and every time the buffer solution was changed. Then, antibody loading was done in a 1:200 v/v dilution. The membrane was then incubated with the conjugate antibody, alkaline phosphatase-goat anti-rabbit IgG (GARAP) (Invitrogen) in 1:1000 v/v dilutions. After the washing step, it was incubated in BCIP (bromo chloro indolyl phosphate) and NBT (nitroblue tetrazolium) tablet (Sigma) and 10 mL of deionized water substrate solution.

Dot-blot enzyme immunoassay was also used to detect *Lxx.* causing RSD (Hoy *et al.* 1999; Gao *et al*. 2008) with the following modifications. Stalk sap samples extracted by mechanical grinding were added to TBS-T buffer (200 mL TBS-$Tween_{80}$ buffer, 0.30M NaCl, 0.10M Na_2EDTA, pH 7.4) in a 50 µL sap:50 µL buffer ratio. A portion of the nitrocellulose membrane (Bio-Rad) sufficient for the samples was soaked in PBS buffer (0.137M NaCl, 0.001M KH_2PO_4, 0.01M Na_2HPO_4, 0.003M KCl, 1000 mL ddH_2O, pH 7.4). Then, 10-20 µl per sample was applied on the assigned grids. The membrane was incubated in RSD specific (locally produced, 2nd bleed rabbit polyclonal) antiserum (1:2000 dilution polyclonal anti-*Lxx* IgG). Following the washing step, the membrane was added with 20 µL alkaline phosphatase-goat anti-rabbit IgG (GARAP) (Invitrogen).

Indirect ELISA (indirect enzyme-linked immunosorbent assay)

SCMV detection through indirect ELISA was used (Gemechu *et al.* 2004) with the following modifications. A 96-well microtiter ELISA plate was loaded with 100 µL per well of leaf extract and buffer (PBS buffer) solution. After incubation, 100 µL per well of the capture antibody anti-SCMV polyclonal antiserum (Agdia®) at 1:200 dilution in buffer solution (PBS buffer, 1% milk) was added. The wells were loaded with 100 µL per well of conjugate GARAP (Invitrogen) and buffer solution (PBS buffer, 1% milk) at 1:1000 dilution, followed by loading of the 100 µL per well substrate p-nitrophenyl phosphate (pNPP) (Sigma Aldrich) and diethanolamine buffer (diethanolamine, ddH_2O, pH 9.8) solution in 1:200 dilutions. Values were then measured in an optical density (OD) of 405 nm using an ELISA plate reader (Bio-Rad).

PCR-based techniques

PCR-based method using the specific primers PGBL1 (5'-CTTGGGTCTGTAGCTCAGG-3') and PGBL2 (5'-GCCTCAAGGTCATATTCAGC-3') with a product size of 288 bp for *Xanthomonas albilineans* detection (Pan *et al*. 1999; dela Cueva *et al.* 2010) was done. Leaf diffusate per sample was prepared and was used since the primers can work directly even without DNA extraction. PCR cocktails were prepared with a final volume of 25 µL.

For *SCMV* and *SrMV* detection, reverse transcription RT-PCR with the specific primers SCMV F3/R3 (5'-TTTYCACCAAGCTGGAA-3') (5'-AGCTGTGTGTCTCTCTGTATTCTC-3') and SrMV F3/R3 (5'-AAGCAACAGCACAAGCAC-3') (5'-TGACTCTCACCGACATTCC-3') with an expected product size of ~900 bp (Yang and Mirkov, 1997) was also conducted for confirmatory testing. PCR cocktails were assembled in a sterile microcentrifuge tube with a final volume of 50 µL.

RESULTS AND DISCUSSION

Acquired sugarcane varieties from Australia, Bangladesh, China, France, Indonesia, Malaysia, Mauritius, Thailand, USA, Japan, Vietnam and Pakistan were maintained in the PEQ facility, IPB-CAFS, UPLB from 2001 to 2012 (Table 1). A pathogen testing scheme specifically adapted and developed for the importation of sugarcane into the Philippines (Figure 2) was employed as a standard quarantine procedure to screen the samples. Hot water treatment did not affect the propagation of the planting materials. CS-HWT was effective for managing the pathogens, however, results varied depending on the varietal reaction to the treatment.

Using the protocol, pathogens of major quarantine significance such as *Xanthomonas albilineans*, *SCMV* and *Lxx.* were confirmed through ELISA and PCR in some varieties. The most common diseases observed and diagnosed on 284 sugarcane varieties were SCM (16), LS (11), Red rot (6) RSD (5) and Smut (5). Infected plants were observed to exhibit typical symptoms of LS such as white pencil-line streak that started to appear six months after planting (Figure 3a). Some plants were asymptomatic under glasshouse conditions but were also tested positive through DBIA and PCR during the first cycle, but not during the second cycle. These were treated through cold soak and long hot water treatment (CS-LHWT). The CS-LHWT could have eliminated the bacterium as PCR assay of the tested leaf samples showed negative results during the second cycle of pathogen detection.

At the open-field quarantine, symptoms of LS were noted. Some sugarcane varieties from France, though negative to *Xanthomonas albilineans* during the post-entry quarantine cycle, were found to express symptoms typical to leaf scald infection when planted in open-field quarantine. DBIA results confirmed the presence of *Xanthomonas albilineans*, hence, infected plants were rogued and destroyed. This could indicate that the natural conditions provided by the open-field quarantine may have influenced the disease to manifest from its latency period. LS has been previously reported in Thailand (Dookun-Saumtally *et al.* 2011), Malaysia (Rott and Davis, 2000), Indonesia (Ricaud *et al*. 1989). These countries are known to experience drastic changes in moisture and temperature such

as in continental climates (Ricaud and Ryan, 1989) which are conditions deemed favorable for the development of LS. Moreover, it is among the frequently reported diseases in sugarcane-growing areas in CIRAD, Montpellier, France (Daugrois *et al*. 2014; Rott *et al*. 1996). The appearance of LS in some varieties which originated from these countries may also indicate an environmental influence in disease expression. It is very serious in other sugarcane producing countries but has not been observed to occur at an epidemic level in the Philippines.

Table I. Total number of sugarcane varieties acquired and grown at post-entry quarantine greenhouse from 2001 to 2012 and types of diseases detected.

ORIGIN	YEAR ACQUIRED	NO. OF VARIETIES	DISEASES INTERCEPTED AND ELIMINATED (No. of infected varieties)	CAUSAL ORGANISM (Type of pathogen)	DISEASE INDEXING SCHEME		
					1ST CYCLE	2ND CYCLE	OPEN-FIELD
Thailand	2001	7	Mosaic **(1)**	SCMV or SrMV (virus)	x		x
			RSD **(2)**	*Leifsonia xyli* sp. *xyli* (bacterium)	x		
			Leaf scald **(1)**	*Xanthomonas albilineans* (bacterium)	x		
			YLS **(1)**	ScYLV (virus)	x		
			Pokkah boeng **(3)**	*Fusarium* spp. (fungus)	x		
			Grassy shoot **(1)**	*Candidatus phytoplasma* (phytoplasma)	x		
	2003	5	-				
	2006	6	Mosaic **(1)**	SCMV, SrMV (virus)	x		x
	2007	5	-				
Malaysia	2001	7	RSD **(1)**	*Lxx.* (bacterium)	x		
			Leaf scald **(1)**	*X. albilineans* (bacterium)	x		
			Mosaic **(2)**	SCMV or SrMV (virus)	x		x
	2003	4	-				
Indonesia	2001	6	Leaf scald **(1)**	*X. albilineans* (bacterium)	x		
			RSD **(1)**	*Lxx.* (bacterium)	x		
			Mosaic **(1)**	SCMV or SrMV (virus)	x	x	x
			Sheath rot **(2)**	*Cytospora sacchari* (fungus)	x		
	2003	5	-				
Bangladesh	2001	5	Mosaic **(2)**	SCMV or SrMV (virus)	x		x
			RSD **(1)**	*Lxx.* (bacterium)	x		
	2003	5	-				
China	2005	10	Smut **(1)**	*Sporisorium scitamineum* (fungus)	x	x	x
	2006	10	-				
	2007	10	Mosaic **(1)**	SCMV or SrMV (virus)	x		x
	2008	10	Mosaic **(1)**	SCMV or SrMV (virus)	x		x
	2009	10	-				
	2010	30	Mosaic **(3)**	SCMV or SrMV (virus)	x	x	x
			Smut **(2)**	*S. scitamineum* (fungus)	x	x	x
			Red rot **(1)**	*Colletotrichium falcatum* (fungus)	x		
	2011	20	Leaf scald **(4)**	*X. albilineans* (bacterium)	x		
			Pokkah boeng **(2)**	*Fusarium* spp. (fungus)	x		
			Red rot **(1)**	*C. falcatum* (fungus)	x		
	2012	10	Red rot **(1)**	*C. falcatum* (fungus)	x		
Australia	2002	10	-				
	2003	10	-				
	2005	10	-				
	2007	10	Smut **(2)**	*S. scitamineum* (fungus)	x		
France (CIRAD)	2005	21	Leaf scald **(1)**	*X. albilineans* (bacterium)	x		x
	2007	10	Leaf scald **(1)**	*X. albilineans* (bacterium)	x		
	2012	10	-				
Mauritius	2007	8	-				
Japan	2009	1	-				

ORIGIN	YEAR ACQUIRED	NO. OF VARIETIES	DISEASES INTERCEPTED AND ELIMINATED (No. of infected varieties)	CAUSAL ORGANISM (Type of pathogen)	DISEASE INDEXING SCHEME		
					1ST CYCLE	2ND CYCLE	OPEN-FIELD
USA	2010	9	Mosaic **(2)**	SCMV or SrMV (virus)	x		x
			Red rot **(2)**	*C. falcatum* (fungus)	x		
Vietnam	2010	10	Leaf scald **(2)**	*X. albilineans* (bacterium)	x		
Pakistan	2012	10	Mosaic **(2)**	SCMV or SrMV (virus)	x		x
			Red rot **(1)**	*C. falcatum* (fungus)	x		
TOTAL		**284**					

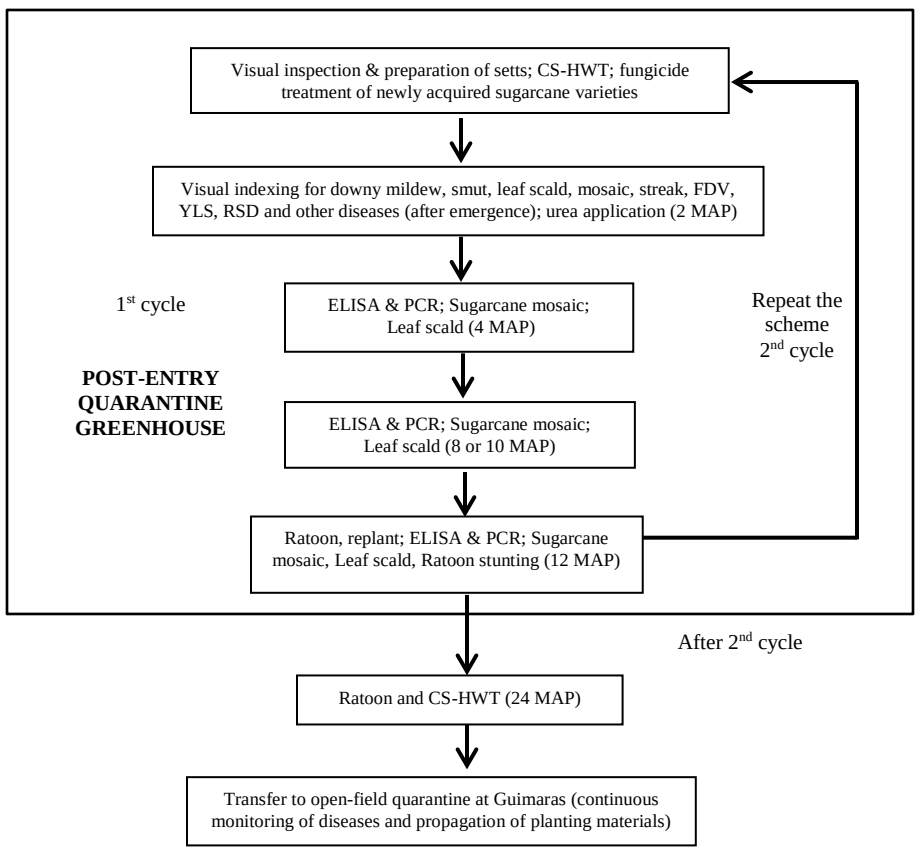

Fig. 2. Disease Indexing Scheme**.** Newly acquired sugarcane varieties were subjected to quarantine protocols for pathogen testing during the 1st cycle at the Post-Entry Quarantine Greenhouse. At first cycle, pathogen testing was done every 4, 8 and 12 MAP. Planting materials were ratooned and replanted, and the scheme was repeated for another year (2nd cycle). At 24 months, these undergone ratooning and CS-HWT, and brought to Guimaras for open-field quarantine and monitoring.

Some varieties from Bangladesh, Indonesia, and Thailand (Table 1) exhibited chlorotic and dark green discoloration on the leaf lamina (Figure 3b.1). SCMV-H strain of *SrMV* was detected among these varieties by RT-PCR. *SrMV* was detected in one variety from China which exhibited mosaic symptoms four months after emergence (Figure 3b.2). *SCMV* was also detected in varieties from Malaysia, USA and Pakistan. Presence of *SCMV* was confirmed through ELISA in some planting materials from Bangladesh, Indonesia, Thailand, Malaysia and China that showed chlorosis and dark green leaf discoloration under greenhouse conditions during the first cycle. Some varieties

from USA and Pakistan also manifested chlorotic streaks and leaf discoloration. Symptoms of SCM were not observed after exposure to CS-LHWT. This corroborates with the results of Smith (1996) where *SCMV* has been eradicated from sugarcane by serial hot water treatment of infected buds. However, the response to CS-HWT could be varietal reaction to the treatment as there were some varieties that still exhibited leaf chlorotic streaks and discoloration at the open-field quarantine. Moreover, it can be possible that the conditions provided during the two-year cycle of disease indexing inside the quarantine greenhouse are favorable for plant growth, resulting to the masking of symptoms of the viral disease. Symptoms of SCM were observed during the open-field quarantine in some varieties. All infected plants were rogued out and burned.

Mosaic disease is recorded in almost all sugarcane growing countries (Gonçalves *et al*. 2012), but only *SCMV*, *SrMV* and *SCSMV* can naturally infect sugarcane (Chatenet *et al*. 2005). The occurrence of SCM has been cited in Bangladesh (Ricaud *et al.* 1989), Indonesia (Putra and Damayanti, 2012), Thailand (Gemechu *et al*. 2004), Malaysia (Ricaud *et al.* 1989), China (Xu *et al*. 2008), USA (Ricaud *et al*. 1989) and Pakistan (Ricaud *et al*. 1989). The occurrence of SCMV strains in the Philippines has not been well-documented. Hence, *SCMV* is still considered as a disease of quarantine importance. The introduction of *SCMV* must be prevented to proscribe the spread of any strain.

The causal bacterium of RSD, *Lxx.*, was detected using DBIA in several sugarcane plants from Bangladesh, Indonesia, Malaysia, and Thailand during the first cycle. Conversely, after the second cycle and in the open-field quarantine, upon exposure of the ratoon setts to CS-LHWT, all the materials yielded negative results indicating the effectiveness of the CS-LHWT. External symptoms were not observed but reddish or pinkish discoloration on vascular bundles of the internodes were seen when the sampled stalks were sectioned (Figure 3c). Occurrence of RSD was noted in Thailand (Ricaud *et al*. 1989), Malaysia (Ricaud *et al*. 1989), Indonesia (Putra and Damayanti, 2012) and Bangladesh (Ricaud *et al*. 1989). Identification of RSD by visual inspection is often very difficult due to the fastidious nature of *Lxx.* and because the disease can be externally symptomless (Saumtally *et al*. 1996). Therefore, testing and heat treatment during quarantine are required to reduce the risk of importing infected planting materials. The results of this study also confirmed the method, as cited by Saumtally *et al*. (1996), of dual hot water treatment consisting of a SHWT followed by a three hour treatment and the CS-LHWT for eliminating the *Lxx* associated in RSD-cuttings.

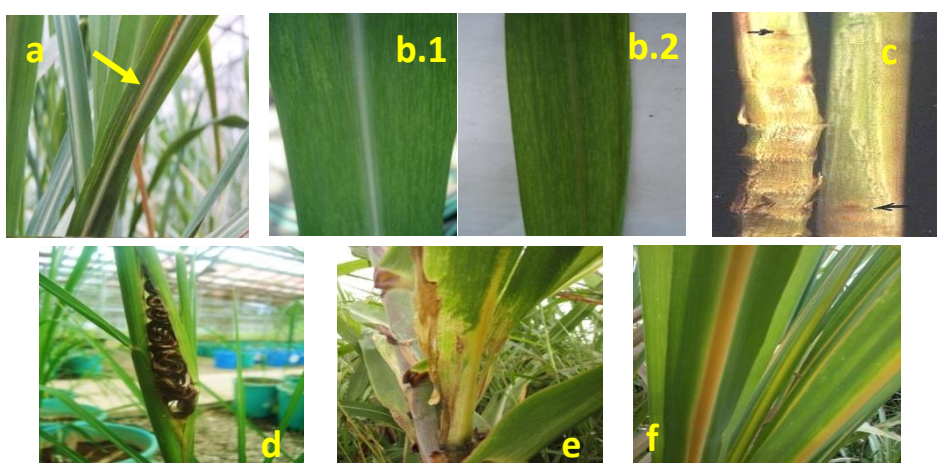

Fig. 3. Diseases of sugarcane observed in the Post-entry Quarantine, Institute of Plant Breeding - College of Agriculture and Food Science, University of the Philippines Los Baños.
(a) Leaf scald caused by *Xanthomonas albilineans* characterized by "white" pencil-line streak that developed into necrosis on leaf lamina; (b.1) *Sugarcane mosaic virus* (SCMV) with mosaic symptoms. (b.2) *Sorghum mosaic virus* (SrMv) with mosaic symptoms; (c) Ratoon stunting disease caused by *Leifsonia xyli sp xyli* characterized by pinkish discoloration as diagnostic internal symptom on infected plant; (d) Smut caused by *Sporisorium scitamineum* characterized by black, whip-like structure that developed on the spindle leaf; (e) Pokkah boeng caused by *Fusarium* spp. characterized by chlorotic discoloration at the base of young leaves and malformation/distortion of the apical shoot; (f) Yellow leaf virus (YLV) with yellowing of the midrib on the leaf underside

Visual symptoms of other diseases such as yellow leaf disease, pokkah boeng, grassy shoot, sheath rot, smut and red rot were also noted under greenhouse conditions in some varieties from Thailand, Indonesia, China, Australia, France, USA and Pakistan (Table 1). Smut was noted in some varieties from China and Australia (Figure 3d). Occurrence of Pokkah boeng disease (Figure 3e) was noted during the early stage of growth of some varieties from Thailand and China. The plants, however, recovered from infection during stalk elongation stage and the sp read of the disease inside the glasshouse was controlled by fungicide treatment. One variety from Thailand exhibited yellowing of midribs typical of *ScYLV* infection (Figure 3f). Grassy shoot disease was seen in one variety from Thailand. On the other hand, two varieties from Indonesia were observed to have sheath rot disease. All the infected plants were destroyed. The pathogens were eliminated by roguing out infected canes, and through CS-HWT of LS- and RSD-infected canes at the early stage of the disease. Smut was observed in the open-field quarantine. The infected plants were rogued out and burned.

The post-entry quarantine period inside the greenhouse provided enough time for the plants to grow and exhibit symptoms of diseases. Situation inside the greenhouse, however, sometimes did not simulate the actual field conditions. This inhibited the growth of undetected pathogens that are present in the planting materials, hence, growing of introduced materials in an open quarantine area which follows strict land management protocols, and is isolated from commercial sugarcane farms proved to be effective for disease expression. The natural environmental condition in the open-field quarantine may trigger the expression of symptoms not observed inside the post-entry greenhouse (Dodman, 1996).

CONCLUSION

Pathogen testing protocols for major diseases of quarantine importance such as LS, SCM and RSD that were employed in this study were beneficial especially for propagating clean planting materials. Likewise, using CS-HWT was a successful way to eradicate these pathogens. Since these methods were proven effective for pathogen detection and elimination, these will be used routinely for testing varieties that will be imported into the Philippines in the future, to ensure disease-free plants to increase sugarcane productivity and diversity.

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MORPHOLOGICAL DIFFERENCES IN FIVE STRAINS OF GENETICALLY IMPROVED NILE TILAPIA (*OREOCHROMIS NILOTICUS*) USING GEOMETRIC MORPHOMETRICS

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ABSTRACT

The determination of fish stock structure is important in developing optimal strategies for efficient management of aquaculture species. Morphometric analysis provides a robust, non-expensive, and statistically powerful means of stock delineation. In the Philippines, five strains of genetically improved Nile tilapia (*Oreochromis niloticus*) have been developed. This study sought to use geometric morphometrics to delineate among the five tilapia strains. Specimens were collected in June to December 2014 from various institutions in the Philippines. Images of 263 individuals were taken at four months old, and 17 landmarks were digitized. Multivariate analysis of variance (MANOVA) revealed significant shape differences between strains. The Canonical Variate Analysis (CVA) plot showed the SEAFDEC strain to be most unique in shape whereas close similarity was observed among specimens of GIFT Philippines, GIFT Malaysia and GET-EXCEL. Discriminant groupings by CVA reflect the historical relationships among the strains. Morphological traits such as the tip of the snout, insertion of the pelvic fin, ventral base of the caudal fin, and the anterior end of the dorsal fin can be used to differentiate one strain from another. Sexual dimorphism in shape was also evident. These results indicate the utility of geometric morphometrics in delineating strains of economically important fish species.

Key words: aquaculture, fish strains, genetic improvement, shape variation, stock delineation

INTRODUCTION

The tilapia is a group of cichlid fishes, which includes three economically important genera namely, *Tilapia*, *Oreochromis*, and *Sarotherodon.* It is an important commodity, ranking ninth in global aquaculture production and third in the Philippine aquaculture production (Fitzsimmons, 2000; Boyd, 2004; Fitzsimmons *et al.*, 2011). China, Egypt, Indonesia, Philippines, and Thailand are the principal producing countries of tilapia. The world tilapia production had been growing increasingly in recent years with 5.3 million metric tons in 2014 (FishstatJ, 2016). The tilapias are a great source of protein in protein-deficient inland communities (Mjoun *et al.*, 2010). Most importantly, its ability to grow fast, its large size and ease of culture are characteristics that make tilapia a desirable food fish.

In 1950, *Oreochromis mossambicus* was introduced in the country. It became a popular market fish but improper management of ponds resulted to small-sized fish (Guerrero, 1985). Introduction of another tilapia species, *O. niloticus*, in 1972 led to the expansion of tilapia industry in

the country which led to a higher demand for tilapia juveniles (Aypa, 1995; Guerrero and Guerrero, 2004). However, the greater demand for juveniles, lack of broodstock development programs, use of few broodstock in hatcheries, and introgression with *O. mossambicus* eventually led to poor tilapia production (Lal and Foscarini, 1990; Aypa, 1995).

Genetic improvement programs were initiated in the Philippines in the 1980s to improve production (Uraiwan, 1990; Eknath *et al.*, 1993; Mair *et al.*, 1997). This led to the development of five tilapia strains, namely: (1) Genetically Improved Farmed Tilapia (GIFT), which is the product of the first selective breeding program for tropical fish (Eknath *et al.*, 1993; Eknath *et al.*, 2007; World Fish Center, 2010); (2) Freshwater Aquaculture Center-selected tilapia (FaST), a product of the combination of four *O. niloticus* strains known as Taiwan, Thailand, Israel and Singapore (Bolivar and Newkirk, 2002); (3) GET-EXCEL, a cross between GIFT and FaST stock (Tayamen, 2004); (4) GIFT Malaysia, established based on the sixth generation of GIFT from the Philippines (Ponzoni *et al.*, 2005); and (5) SEAFDEC whose founding population was the Chitralada strain (Basiao and Doyle, 1999). These genetically improved tilapia strains are dispersed through national fishery agencies, foundations, research institutions and universities (Basiao and Doyle, 1999).

A fish stock is composed of individuals that are part of the same reproductive process which are contained with no immigration or emigration of individuals from one stock to another (Garcia, 2005). Determination of fish stock structure is important because it helps in the development of an optimal strategy for efficient management of fish (Coyle, 1998). Morphometric analysis is a good alternative or complement to biochemical or genetic methods of stock identification because it is cheaper and more robust than molecular techniques. External morphology, such as body shape and skin pigmentation, has been important in commercial fish farming because these can influence consumer preference (Colihueque *et al.*, 2014).

Geometric morphometrics is widely used in determining shape variation. Instead of using linear measurements, as in traditional morphometrics, data are recorded in the form of coordinates of landmark points (Adams *et al.*, 2004). Application of advanced image processing techniques has significantly enhanced stock identification and discrimination in fishes (Cadrin *et al.*, 2005). Stock identification using geometric morphometrics was recently applied on *Sebastes* spp. (Valentin *et al.*, 2014), *Gasterosteus aculeatus* (Pistore *et al.*, 2016), and *Mugil curema* scales (Ibáñez *et al.*, 2017). In *O. niloticus*, geometric morphometrics has been applied to study the effect of management (Lorenz *et al.*, 2014), to examine bone growth patterns (Fujimura and Okada, 2008), to analyze effects of temperature and salinity (Ndiwa *et al.*, 2016), and to corroborate phylogenetic relationships (Clabaut *et al.*, 2007).

This study sought to discriminate among the five strains of genetically improved *O. niloticus* using geometric morphometrics. The nature of shape variation is also characterized.

MATERIALS AND METHODS

The specimens used for this study were obtained from different institutions that maintain these. The GET-EXCEL strain was obtained from the National Fisheries Technology Training Center of the Bureau of Fisheries and Aquaculture Resource, Central Luzon State University. FaST strain was obtained from the Freshwater Aquaculture Center of the Central Luzon State University. GIFT Philippines was maintained by the GIFT Foundation of the Philippines. The GIFT Malaysia strain was from the World Fish Center in Penang, Malaysia, a stock of which is maintained by the Bureau of Fisheries and Aquatic Resources. SEAFDEC strain was from the Binangonan Freshwater Station of the Southeast Asian Fisheries Development Center. At least 50 four-month old individuals of tilapia from each of the five genetically improved strains were used in this study.

Tilapia specimens were placed in ice in order to immobilize them as adopted from Iwama and Ackerman (1994). Each specimen was then placed on a platform with white paper as background, and was assigned a code for identification. A ruler was placed next to the specimen to serve as a size standard. The body posture and fins were then teased into its natural position. Using a Nikon D60 camera with an 18-55mm lens, a photograph of each of the specimen was taken. Standard length (SL) and weight (Wt) were taken. The sex was determined by dissection and examination for the presence of either the ovaries or the testes. From the digital image, 17 landmarks (Fig. 1) from the left side of the fish were digitized using tpsDig2 (Rohlf, 2006). Landmarks were modified from those of Velasco *et al*. (1996) and Lorenz *et al*. (2014). Representative specimens are shown in Fig. 2.

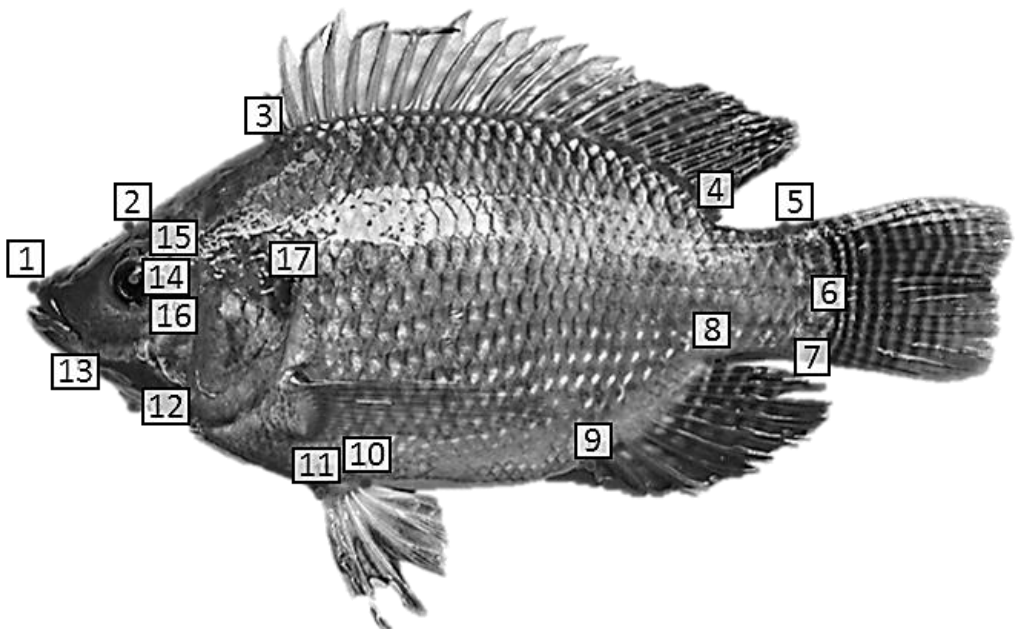

Fig. 1. Locations of the 17 landmarks used for the shape analysis of *Oreochromis niloticus.* (1) snout tip, (2) edge of the head directly above the eye, (3) anterior base of the dorsal fin, (4) posterior base of the dorsal fin, (5) dorsal base of the caudal fin, (6) base of the caudal fin at the level of the lateral line, (7) ventral base of the caudal fin, (8) posterior end of the anal fin base, (9) anterior end of the anal fin base, (10) posterior insertion of the pelvic fin, (11) anterior insertion of the pelvic fin, (12) edge of the head directly below the eye, (13) corner of the mouth, (14) center of the eye, (15) top of the eye, (16) bottom of the eye, and (17) the most posterior edge of the operculum.

Digital images were assigned to their respective strain and sex. General Procrustes Analysis (GPA) was performed, superimposing landmarks into a common coordinate system, while removing variation due to size, location, and orientation (Addis *et al.*, 2010). Canonical Variate Analysis (CVA) was performed to discriminate among strains based on shape variables. GPA and CVA were conducted using programs from the Integrated Morphometrics Package (IMP; Sheets, 2003).

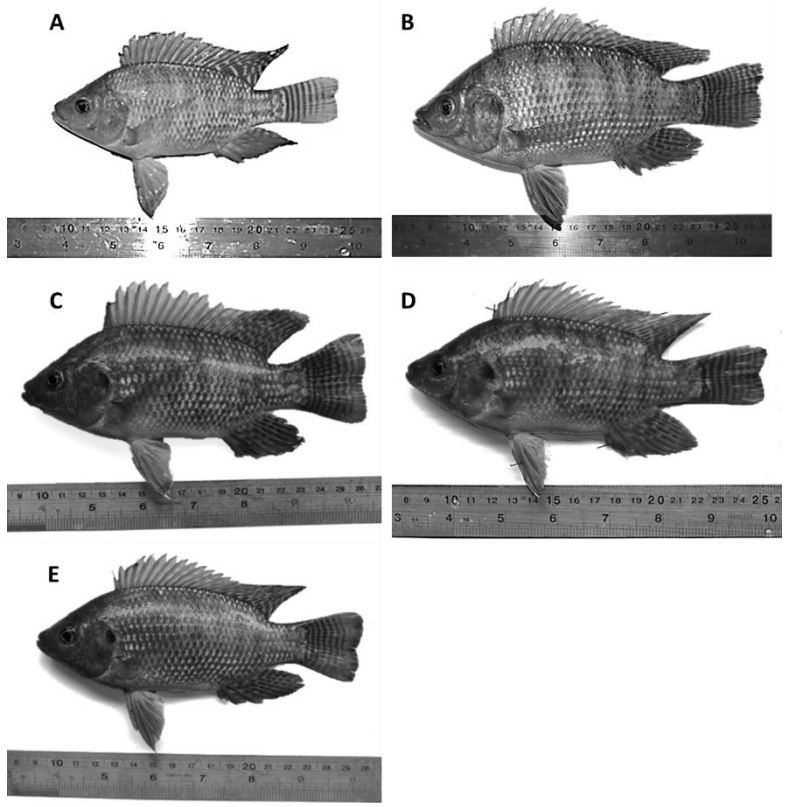

Fig. 2. Representative *O. niloticus* specimens per strain. a.) GET-EXCEL, b.) FaST, c.) GIFT Philippines, d.) GIFT Malaysia, e.) SEAFDEC

RESULTS AND DISCUSSION

Variations in SL and Wt are summarized in Tables 1 and 2. Differences in average SL and Wt were significant. The FaST strain was superior in those parameters. GIFT Malaysia was the smallest but were not significantly different from GET-EXCEL and SEAFDEC. In all strains, differences in SL and Wt between sexes were not significant (Table 2). Males were observed to be longer and heavier in GET-EXCEL, FaST, GIFT Malaysia. The opposite was observed for the GIFT Philippines and SEAFDEC strains.

For the shape variation, four distinct canonical variates were observed, indicating that the five strains can be dichotomized based on shape characters. GIFT Philippines and GIFT Malaysia showed the greatest overlap among the strains and SEAFDEC, FaST and EXCEL clustered separately (Fig. 3). Shape deformation along CV1 shows variation in the position of the ventral base of the caudal fin as well as expansion of the anterior and posterior ends. Shape deformation along CV2 also shows variation in the ventral base of the caudal fin as well as ventral compression. CV1 and CV2 contributed to 75% of the total variation. Differences between strains along CV1 and CV2 were significant as shown by one-way ANOVA (Table 1). CV1 and CV2 effectively differentiates SEAFDEC from the rest of the strains. GIFT Malaysia was significantly different from FaST based on CV1. CV2 delineates among three distinct subgroups, namely, SEAFDEC strain, the subgroup of GIFT Philippines and FaST, and the subgroup of GIFT Malaysia and GET-EXCEL.GET-EXCEL showed the highest percentage of correct classification (Table 3).

Table 1. Descriptive statistics of specimens of *O. niloticus*, comparing among strains.

Measure	Strains					F value (P value)
	GET-EXCEL (n=53)	**FaST (n=53)**	**GIFT PHILIPPINES (n=55)**	**GIFT MALAYSIA (n=51)**	**SEAFDEC (n=51)**	
SL	129.35ab±13.18	138.96c±20.22	135.49bc±21.98	125.10a±13.43	130.32ab±15.19	5.106 (0.00)
	(107.76-171.49)	(101.26-186.61)	(90.10-183.02)	(91.54-154.82)	(96.19-156.15)	
Wt	48.92ab±4.76	53.88c±8.63	51.79b±8.09	48.07a±5.74	49.19ab±6.43	6.259 (0.00)
	(38.77-64.82)	(36.95-74.59)	(36.00-71.51)	(33.64-62.01)	(33.83-63.14)	
CV1	0.0003ab±0.0038	0.0015b±0.0029	0.0007ab±0.0027	-0.0001a±0.002	-0.0025±0.0026	13.693 (0.00)
	(-0.0057-0.0074)	(-0.0034-0.0076)	(-0.0072-0.0052)	(-0.0065-0.0039)	(-0.0075-0.0018)	
CV2	0.0006b±0.0026	-0.0015a±0.0019	-0.0022a±0.0018	0.0002b±0.0017	0.0031±0.0024	49.335 (0.00)
	(-0.0054-0.0062)	(-0.0054-0.0059)	(-0.0064-0.0016)	(-0.0032-0.0038)	(-0.0018-0.0070)	
CV3	-0.0003ab±0.0023	0.0003ab±0.0032	0.0005b±0.0022	0.0006b±0.0028	-0.0010a±0.0024	3.52 (0.08)
	(-0.0054-0.0054)	(-0.0060-0.0076)	(-0.0057-0.0043)	(-0.0068-0.0066)	(-0.0075-0.0026)	

*Values are mean ± standard deviation and range (in parentheses). Means followed by the same letter are not significantly different at α=0.05 using Tukey's Post hoc analysis. n, sample size; Wt, weight (g); SL, standard length (mm); CV1, canonical variate 1; CV2, canonical variate 2; CV3, canonical variate 3.

Among the different strains, greatest overlap was observed between GIFT Philippines and GIFT Malaysia. This reflects the origin of GIFT Malaysia as the sixth generation of selection from GIFT Philippines (Ponzoni *et al.*, 2005). FaST was grouped separately because it wasn't based on the GIFT Philippines strain but rather the initial selection was based on four strains of *O. niloticus* which included Taiwan, Thailand, Israel and Singapore strains (Lester *et al.*, 1988). The GET-EXCEL strain overlapped with the cluster of GIFT Philippines and also with GIFT Malaysia and FaST because it was based on the eighth generation of GIFT Philippines strain, 13th generation of FaST, Egypt strain and Kenya strain (Tayamen, 2004). The SEAFDEC selected strain (Basiao and Doyle, 1999) showed a very distinct separate clustering from the rest of the four strains. The founding population of the SEAFDEC strain was the Chitralada strain from the National Institute of Fisheries (NIFI) in Thailand. The Chitralada strain which originally came from Egypt was a gift from the Emperor of Japan to the King of Thailand. High percent of correct assignment support significant differences of body shape among strains.

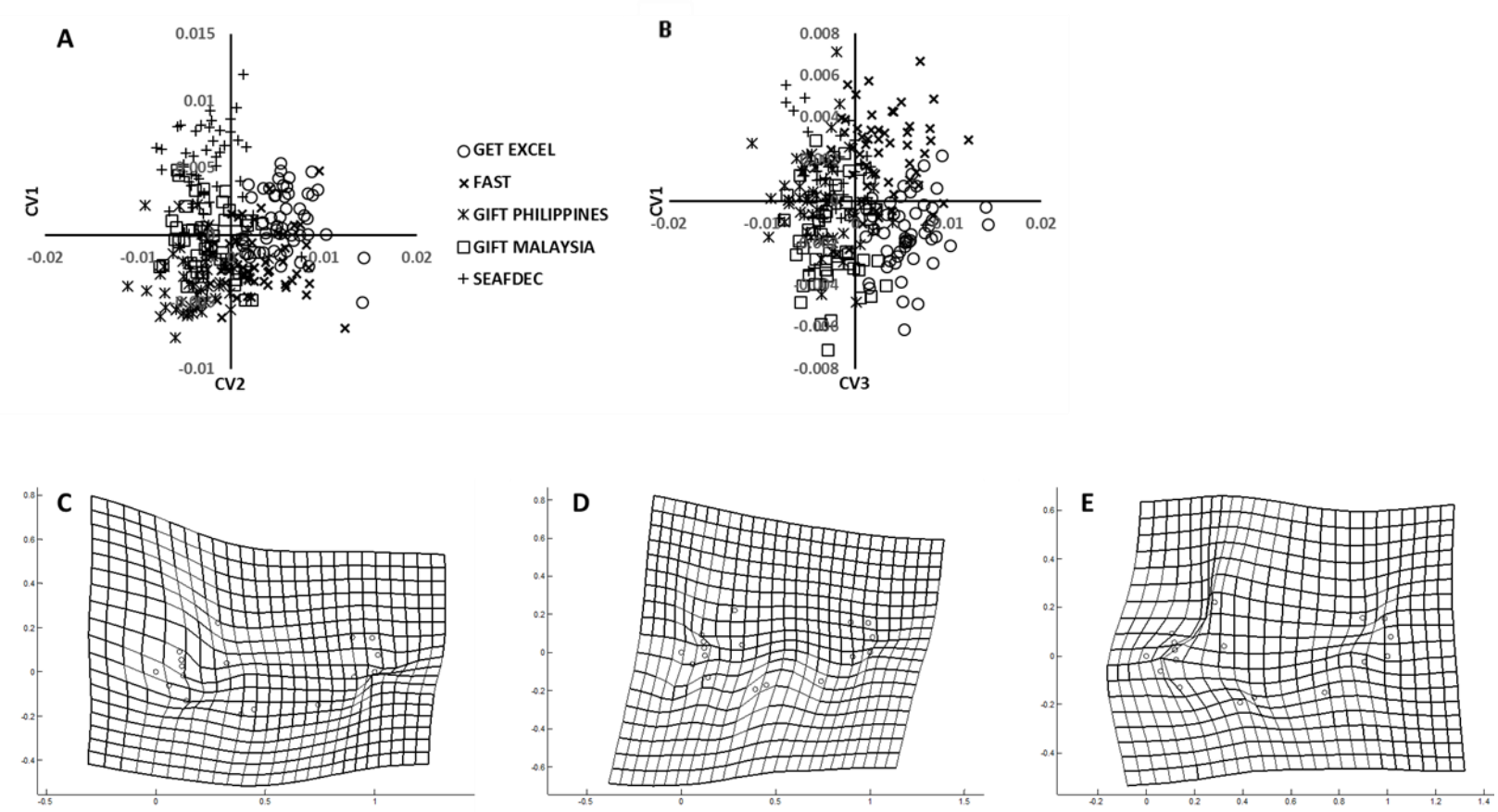

Fig. 3. Results of CVA of warp scores of both female and male samples of GET-EXCEL (n=53), FaST (n=53), GIFT Philippines (n=55), GIFT Malaysia (n=51) and SEAFDEC selected (n=51) strains of *O. niloticus*; A, CVA plot showing CV1 versus CV2; B, CVA plot showing CV1 versus CV3; C, Deformation grid showing shape change along CV1; D, Deformation grid showing shape change along CV2; E, Deformation grid showing shape change along CV3. For the deformation grids, the reference form is equivalent to mean of all configurations.

Table 2. Comparison of sexes between strains of *O. niloticus* specimens. Values are mean ± standard deviation and range (in parentheses). N, sample size; Wt, weight (g); SL, standard length (mm); CV1, canonical variate 1.

Measure	Sex		F value	P value
	GET-EXCEL			
	Male (n=26)	Female (n=27)		
SL	130.55±14.11	128.09±12.29	0.457	0.502
	(108.32-160.95)	(107.76-171.49)		
Wt	49.77±4.75	48.03±4.70	1.778	1.88
	(40.63-60.64)	(38.77-64.82)		
CV1	-0.0016±0.0062	0.0015±0.0006	324.475	0.000
	(-0.0027-(-0.0004))	(0.00020.0030)		
	FaST			
	Male (n=26)	Females (n=27)		
SL	142.31±17.64	135.73±22.30	1.359	0.249
	(101.97-164.47)	(101.26-186.61)		
Wt	55.69±8.42	52.14±8.64	2.208	0.144
	(36.95-69.66)	(35.37-39.22)		
CV1	-0.0012±0.0006	0.0011±0.0006	206.799	0.00
	(0.00-0.00)	(0.00-0.00)		
	GIFT PHILIPPINES			
	Male (n=27)	Female (n=28)		
SL	138.27±19.78	133.00±23.85	1.567	0.217
	(110.71-180.49)	(90.10-183.02)		
Wt	52.61±7.17	71.06±8.89	2.311	1.35
	(24.58-40.81)	(35.51-36.00)		
CV1	0.0015±0.0007	-0.0015±0.0010	159.624	0.00
	(0.00-0.00)	(0.00-0.00)		
	GIFT MALAYSIA			
	Male (n=27)	Female (n=25)		
SL	127.40±13.86	122.71±12.82	1.359	0.249
	(91.54-145.92)	(96.98-154.82)		
Wt	49.26±5.66	46.84±5.67	2.208	0.144
	(33.64-57.42)	(37.48-62.01)		
CV1	0.0015±0.0009	-0.0016±0.0011	121.934	0.00
	(-0.0001-0.0015)	(-0.0043-(-0.0002))		
	SEAFDEC			
	Male (n=26)	Female (n=25)		
SL	129.13±14.96	135.73±22.30	0.306	0.583
	(99.90-153.00)	(101.26-186.61)		
Wt	48.99±6.36	52.14±8.64	0.048	0.827
	(36.32-57.79)	(39.22-74.59)		
CV1	-0.0018±0.0009	0.0019±0.00059	316.261	0.00
	(-0.0034-0.0002)	(0.0009-00.3351)		

Table 3. Canonical Variate Analysis (CVA) classification for GET-EXCEL, FaST, GIFT Philippines, GIFT Malaysia and SEAFDEC populations.

A priori* assignment		***A posteriori* assignment**	
		% Correct Classification	**% Misclassification**
Males and Females combined among strains			
	GET-EXCEL	92	7
	FaST	79	20
	GIFT Philippines	74	25
	GIFT Malaysia	72	27
	SEAFDEC	82	17
Between Sexes			
GET-EXCEL			
	Female	100	0
	Male	100	0
FaST			
	Female	100	0
	Male	96	3.85
GIFT Philippines			
	Female	96	3
	Male	96	3
GIFT Malaysia			
	Female	100	0
	Male	100	0
SEAFDEC			
	Female	100	0
	Male	96	3

**A priori* assignments are based on strain and sex to which the specimen belongs while *a posteriori* assignments make use of Mahalanobis-based approach to predict the group membership of each specimen based on CVA.

Results indicate that the head region above and below the eyes can be used to differentiate among strains for both male and female samples. Additionally, the snout can be used to differentiate between strains when dealing with female samples except when differentiating between GET-EXCEL and GIFT Malaysia, further supporting the similarity of shape between these strains. It is possible that the difference observed in the snout region can be attributed to the mouth-brooding characteristic of *O. niloticus* (Tran *et al.*, 2011). The dorsal and ventral base of the caudal fin can be used to differentiate between strains when dealing with male individuals. Differences in the size of caudal fin can affect swimming performance, swimming behavior and routine activity (Plaut, 2000). A high aspect ratio caudal fin is required for steady swimming while a large caudal fin is required for unsteady swimming (Webb, 1982). Also, differences observed may be due to the differences in the growth rates of strains which was observed in Red, GIFT and Supreme strains in the study of dos Santos *et al.* (2013). SEAFDEC can be separated from the rest of the strains using all these characters, showing that this strain is distinct from the rest of the strains being studied.

CONCLUSION

Shape differences were observed among strains of *O. niloticus*. The tip of the snout, insertion of the pelvic fin, ventral base of the caudal fin, and the anterior end of the dorsal fin are traits that can best differentiate one strain from another. Geometric morphometrics is a good tool in delineating strains of important species like *O. niloticus*. This would contribute to better management of this economically important fish.

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MOTILITY AND MEMBRANE INTEGRITY OF EJACULATED BOVINE SPERMATOZOA EXTENDED AND CRYOPRESERVED IN *L*-CARNITINE TRIS-EGG YOLK EXTENDER

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ABSTRACT

This study was conducted to examine if *L*-carnitine supplementation in the Tris-egg-yolk extender can improve the motility characteristics, membrane integrity, and in vitro fertilization potential after cryopreservation of ejaculated bovine spermatozoa. It was carried out on January to May 2013 at the University of Wisconsin-Madison. *L*-carnitine at final concentrations of 0.5, 1, 10, and 30mM were added to Tris-egg yolk extender where semen was suspended at $100x10^6$ sperm cells/mL. Tris-egg yolk without carnitine served as control. Motility characteristics and functional integrity of membrane were examined by computer assisted sperm analysis and hypo-osmotic swelling test at 2, 6, and 24 hour at room temperature. The post-thaw effect of *L*-carnitine was likewise assessed. Each treatment suspension was cryopreserved in Tris-egg yolk with 7% glycerol, stored in liquid nitrogen and subjected to computer assisted sperm analysis and hypo-osmotic swelling test post-thawing. Lastly, the fertilization potential of frozen semen treated with *L*-carnitine was used for *in vitro* fertilization and cleavage and blastocyst development were assessed. *L*-carnitine was demonstrated to improve the motility characteristics in a concentration-depended manner; 1mM concentration is efficient but high concentration beyond 30mM had cytotoxic effect. A similar trend was observed on membrane integrity although significant difference was not evident. After IVF, the use of 1 mM *L*-carnitine resulted in significantly higher cleavage rate (92.4% vs. 82.8%) suggesting that *L*-carnitine supplementation at low concentration in Tris-egg yolk extender improves motility and fertilization potential of bovine sperm cells.

Key words*:* bovine semen, computer assisted sperm analysis, L-carnitine

INTRODUCTION

Semen is still the cheapest component of artificial breeding both in the Southeast Asian and other regions with artificial insemination (AI) remains the most implemented reproductive biotechnology and cryopreservation the most important procedure in order to ensure viability of male gamete and guarantee the success of AI. However, the current available methods to preserve semen as a genetic resource and its successful dissemination via AI and other assisted reproductive technologies (ARTs) are still sub-optimal (Rodriguez-Martinez, 2012a, b).

During cryopreservation, sperm cells are being subjected to stress and studies done by Chatterjee and Gagnon (2001) proved that an increase in lipid peroxidation levels during cryopreservation and thawing affects the motility of the frozen-thawed spermatozoa. Oxygen free

radicals are produced during freezing and thawing and these Reactive Oxygen Species (ROS) cause the decrease in sperm function following cryopreservation. In fact, the increased in ROS is responsible for the damage of the sperm (Li et al, 2012).

L-carnitine (levocarnitine) is an amino acid that plays a powerful role in transporting long-chain fatty acids into the mitochondria for β-oxidation (Coulter, 1995) which produces energy (ATP) needed by the cells for proper functioning (Ramsay et al., 2001; Hoppel 2003). It also facilitates the removal from mitochondria of short-chain and medium-chain fatty acids (acyl-CoA) that accumulate as a result of normal and abnormal metabolism (Rabie and Szilagyi, 1998; Arrigoni-Martelli and Caso 2001). It has antioxidant properties; it protects cellular membranes against oxidative damages resulting from peroxidation of polyunsaturated fatty acids that are component of membrane phospholipids by reducing the availability of lipids for peroxidation through transporting fatty acids into the mitochondria to generate ATP (Kalaiselvi and Panneerselvam, 1998). In processing sperm for cryopreservation, natural *L*-carnitine levels in the semen is diluted resulting to *L*-carnitine deficiency that may result to lipid peroxidation, formation of ROS, failure of fatty acid transport to the mitochondria of the sperm to produce energy, hence, depletion of normal sperm function. Since *L*-carnitine plays a vital role in sperm detoxification by maintaining the sperm membrane, in controlling lipid peroxidation and in the enhancing metabolic processing of endogenous fuel into energy (expressed as sperm motility), supplementation of it in the semen extender maybe beneficial to the quality of the frozen semen.

With the above considerations, it is worthy to examine the effect of *L*-carnitine in the semen extender before and after freezing. Though *L*-carnitine may be available in the seminal plasma, preparation and dilution for freezing must resulted to significant decrease in the level of L-carnitine necessary to support the energy production of the sperm cells and protect the plasma membrane by controlling the formation of ROS, thus, resulting to decreased motility after freezing. Further study to elucidate the effect of *L*-carnitine is needed, hence, this study.

MATERIALS AND METHODS

Semen Source

Ejaculated semen was provided by the ABS Global, Inc. USA. The study was conducted from January to May 2013 at the Laboratory of Reproduction, Department of Animal Science, University of Wisconsin-Madison. Two semen ejaculates collected a week apart from three different bulls were used. These bulls are used as semen donor for AI. Tris egg-yolk extender without glycerol containing 0, 0.5, 1, 10, 30 mM *L*-carnitine was added to ejaculated semen at least 30 to 120 minutes after collection. Semen from each bull and from each ejaculate was processed individually.

Experimental Design

Three studies were carried out; In Study-1, the desired concentration and effect of L-carnitine (0, 0.5, 1.0, 10, 30 mM) as supplement to Tris-egg yolk extender was examined. Ejaculated semen from each bull was diluted 1:1 (0 hour) with Tris-egg yolk extender containing 0, 0.5, 1, 10 and 30 mM *L*-carnitine during transport. In the lab, the semen suspensions were further diluted with the respective extenders to make 100 x 10^6 sperm cells/mL and kept at room temperature. After the 2, 6 and 24 hours from time of exposure to *L*-carnitine, sperm motility characteristics were assessed by computer assisted sperm analysis (CASA) while functional integrity of membrane was examined by hypo-osmotic swelling test (HOST). In Study-2, 5 mL of the treated sperm cells in Study-1 were cryopreserved in liquid nitrogen and post-thaw semen characteristics and functional integrity of membrane were examined after thawing. In Study-3, frozen semen treated with the effective concentration of *L*-carnitine was used for In Vitro Fertilization (IVF). Cleavage and blastocyst development of the fertilized eggs were assessed.

Semen Extender, Processing and Cryopreservation

The Tris-egg yolk extender was made 1 to 3 days before semen collection and stored in 3-5ºC. It contains 20% egg yolk, 2.42% Tris (tris(hydroxymethyl)aminomethane), 1.38% citric acid, 1 % fructose and protected with antibiotic mix containing 5.25 mg tylosin, 26.25 mg gentamycin, 15.75 mg lincomycin and 31.5 mg spectinomycin. On the day of semen collection, 10 mL of Tris-egg yolk extender were supplemented with *L*-carnitine HCl (Sigma Chemicals) at final concentration of 0.5, 1.0, 10, and 30 mM. Without *L* -carnitine served as control. Ejaculated semen was diluted 1:1 with the extender (0 hour) and transported to the laboratory at room temperature. At the laboratory, sperm concentration was determined and the sperm concentration in each treatment was adjusted to 100 x 10^6 sperm cells/mL with the respective extender and kept at room temperature. Sample of sperm from each treatment was taken at 2, 6, and 24 hour for sperm motility evaluation by CASA (IVOS, Hamiltorn Thorne) and functional integrity of the membrane by HOST. For Study 2, 5 mL of the treated semen was cooled for cryopreservation.

For cooling, semen for each treatment was kept in 15 mL centrifuge tubes submerged in water in 250 mL beaker and brought to cooled room. Slow cooling to 5°C was carried out for 2 to 2.5 hours. At 5°C, Tris-egg yolk extender containing 14% glycerol (without *L*-carnitine) was added in stepwise manner at 15 minutes interval until 1:1 ratio was attained shaking the semen suspension every after addition. This makes the sperm concentration 50 x 10^6 sperm cells/mL. Semen was then loaded on 0.5 mL straw, sealed and exposed to liquid nitrogen vapor (3 cm above LN_2) for 10 minutes then completely submerged in liquid nitrogen. Semen straws were stored in liquid nitrogen tank for storage and future evaluation.

Analysis of Sperm Motility Characteristics

Motility characteristics of the sperm cells were analyzed by Computer Assisted Sperm Analysis (CASA) using IVOS Hamilton Thorne with the following settings: Frame rate: 60 Hz; Frames required: 30 images; Minimum contrast: 50 pixels; Minimum cell size: 5 pixels; Threshold straightness: 80.0%; Medium VAP cut off (MVV): 60.0 microns/second; Low VAP cut off (LVV): 25.0 microns/second; (VSL) cut off (LVS): 10.0 micron/second; Non-motile head size: 5 micron; Non-motile head intensity: 90; Static size limit: 0.69 to 2.75 pixels; Static intensity limit: 0.28 to 1.50; Static elongation limit: 5 to 73%.

Tris-egg yolk extender provided granules that interfere with the accurate motility analysis by CASA. To avoid this, semen sample from each treatment group were diluted with pre-warmed TL-hepes (Lonza, Cambrex Bio-Sciences, Walkersville, MD) containing 22 ug/mL Na-pyruvate and 3 mg/mL BSA Fraction V to make a sperm concentration of 20,000,000/mL. Ten microliter of the sperm suspension was subjected to sperm motion characteristics by CASA and parameters observed are detailed here. 1. Overall motility (MOT, %; is the population of cells that are moving at or above a minimum speed as determined by values defined under CASA setup above. 2. Progressive motility (PROG, %; is the number of cells moving in a straight line with both path velocity greater than medium Average path velocity (VAP) cut off of 60% (VAP>MVV) and straightness is greater than threshold straightness of 80% (STR>S_0). 3. Average path velocity (VAP, µm/s; the point to point velocity on a path constructed using a roaming average. The number of points in the roaming average is 1/6th of the frame rate of video used. Cells must not be SLOW in order to be included in the average). 4. Progressive velocity (VSL, µm/s; measured in the straight line from the beginning to the end of tract. 5. Track speed or curvilinear velocity (VCL, µm/s; total distance traveled by a sperm per second. It is measured over the point to point track followed by the cell. 6. Amplitude of lateral head displacement (ALH, µm; deviation of sperm head from the average path calculated from all cell tracks that have a straightness greater than the threshold straightness (S_0=80.0%) and are not measured as SLOW. 7. Beat cross frequency (BCF, Hz; frequency with which the sperm head moves back and forth in its track across the cell path. 8. Straightness (STR, %; measures the departure of the cell path from a straight line. It is the ratio of VSL/VAP). 9. Linearity (LIN, %; measures the linearity of the

cell track. It is the ratio of VSL/VCL). 10. Rapid (%) is the fraction of all cells moving with path velocity greater than the medium VAP cut off (VAP>MVV). In all parameters examined, cells must not be SLOW to be included in the average as indicated in the CASA settings. Five views were used per slide to gather the motility characteristics.

Analysis of Plasma Membrane Integrity

The plasma membrane integrity was evaluated using the HOST described by Brito et al (2003) with few modifications. The assay was performed by incubating 0.1 ml of semen with 1.0 ml of 100 milliosmole (mOsm) hypo-osmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) at 37ºC for 60 min. After incubation, 10 µL of the sperm suspension was loaded on a glass slide and covered with coverslip. Two hundred sperm cells were evaluated under magnification 400X with Phase contrast microscopy. Sperm with swollen or coiled tails were considered viable.

Statistical Analysis.

Statistical analysis was performed using one way analysis of variance (ANOVA) to compare the sperm parameters across treatments and t-tests (LSD) was done to compare the statistical difference between treatment means. A p-value less than 0.05 were accepted as a statistically significant difference.

RESULTS AND DISCUSSION

Prominently improved sperm motility characteristic was observed at 2 hour of *L*-carnitine treatment and this was further improved until 6-hour of exposure at room temperature (Fig.1). After that, decline in motility were observed in all treatments. Interestingly, all motility parameters were improved with *L*-carnitine treatment except the amplitude of lateral head (ALH) (Table 1). Overall motility was significantly (P=<0.007) improved in 0.5 to 1 mM *L*-carnitine containing extender as compared to the control group (77.7% and 80.50% vs. 71.45%) but progressive motility was significantly increased irrespective of the *L*-carnitine concentration.

Average path velocity, progressive velocity, culvilinear velocity, and proportion of rapid spermatozoa were significantly improved by 1 mM *L*-carnitine but Straightness and Linearity was improved by 10 and 30 mM concentrations. At extended period of *L*-carnitine treatment, results remained higher with differences noted on progressive motility, Average path velocity (VAP), Progressive velocity (VSL), Curvilinear velocity (VCL), Amplitude of lateral head displacement (ALH), Linearity (LIN) and spermatozoa moving rapidly at 6 hours (Table 2) and on overall motility, LIN and spermatozoa moving rapidly on 12 hours (Table 3) of *L*-carnitine exposure. Motility characteristics in *L*-carnitine treatment were still superior in 0.5 to 10 mM *L*-carnitine at 24 hour treatment but 30 mM exhibited negative effects.

Table 1. Motility characteristics of fresh ejaculated bovine sperm cells 1 to 2 hours after exposure in *L*-carnitine supplemented Tris-egg yolk extender.

LC (mM)	Mot (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	71.45^{b}	20.50^{c}	104.01^{b}	70.86^{bc}	186.17^{b}	13.86^{a}	28.59^{bc}	66.80^{c}	38.40^{c}	53.90^{b}
0.5	77.70^{a}	24.65^{b}	107.86^{b}	75.95^{ab}	193.29^{ab}	7.69^{b}	29.74^{abc}	69.50^{bc}	40.45^{c}	60.30^{ab}
1	80.50^{a}	26,20^{b}	118.49^{a}	82.22^{a}	211.67a	8.27^{ab}	27.91^{c}	68.85^{c}	39.80^{c}	61.25^{a}
10	76.35^{ab}	31.65^{a}	102.72^{b}	83.29^{a}	174.37^{b}	8.78^{ab}	32.73^{a}	72.35^{b}	43.75^{b}	60.25^{ab}
30	71.95^{b}	33.84^{a}	83.84^{c}	66.36^{c}	144.82^{c}	5.94^{b}	31.05^{ab}	77.95^{a}	47.53^{a}	55.84^{ab}
p- value	<.0070	<.0001	<.0001	0.0005	0.0915	0.0203	<0.0001	<.0001	<.0001	0.01449

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different (P<0.05).

Table 2. Motility characteristics of sperm cells at 6 hours storage at room temperature in a *L*-carnitine supplemented Tris-egg yolk extender.

LC	Motility (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	76.80^{ab}	21.47^{c}	109.14^{b}	74.21^{b}	190.71^{b}	7.41^{b}	24.16^{b}	70.40^{b}	43.47^{a}	56.87^{c}
0.5	82.60^{ab}	26.52^{bc}	127.05^{a}	88.17^{a}	232.33^{a}	8.82^{a}	26.26^{b}	67.80^{b}	38.40^{b}	74.20^{ab}
1	79.80^{ab}	26.13^{bc}	116.04^{ab}	81.62^{ab}	203.06^{ab}	7.92^{b}	26.02^{b}	70.27^{b}	42.93^{a}	78.86^{a}
10	84.26^{a}	32.40^{ab}	110.75^{ab}	80.33^{ab}	199.47^{ab}	8.01^{ab}	27.54^{b}	71.40^{b}	41.46^{ab}	73.73^{ab}
30	74.27^{b}	35.47^{a}	105.61^{b}	82.01^{ab}	187.81^{b}	7.63^{b}	38.16^{a}	75.46^{a}	44.13^{a}	67.73^{b}
p-value	0.159	0.0004	0.117	0.235	0.074	0.016	0.001	0.002	0.071	<0.0001

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different (P<0.05).

Table 3. Motility characteristics of sperm cells at 24 hours storage at room temperature in *L*-carnitine supplemented Tris-egg yolk extender.

LC	Motility (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	67.60^{b}	27.40^{a}	93.91^{a}	70.70^{a}	175.62^{a}	7.29^{a}	26.85^{a}	73.80^{ab}	42.35^{b}	59.85^{b}
0.5	73.40^{ab}	32.80^{a}	96.97^{a}	74.75^{a}	174.35^{a}	7.28^{a}	26.78^{a}	76.25^{ab}	45.70^{ab}	62.25^{a}
1	78.80 ^{a}	35.45^{a}	99.02^{a}	75.36^{a}	176.03^{a}	7.11^{a}	27.94^{a}	75.40^{ab}	44.20^{ab}	72.40^{a}
10	76.10^{ab}	37.55^{a}	93.74^{a}	73.25^{a}	164.31^{a}	6.97^{a}	27.58^{a}	76.95^{a}	46.95^{a}	73.70^{a}
30	67.65 ^{b}	29.05^{a}	97.51^{a}	72.47^{a}	173.30^{a}	7.36^{a}	26.44^{a}	73.45^{b}	43.55^{ab}	58.35^{b}
p-value	0.0393	0.2627	0.8311	0.8952	0.7632	0.650	0.8022	0.1443	0.0914	0.0028

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different (P<0.05).

Table 4. Motility characteristics post-thawing of bovine sperm cells treated with *L*-carnitine in Tris-egg yolk extender.

LC (mM)	Mot (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	71.45^{b}	20.50^{c}	104.01^{b}	70.86^{bc}	186.17^{b}	13.86^{a}	28.59^{bc}	66.80^{c}	38.40^{c}	53.90^{b}
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p-value	<.0070	<.0001	<.0001	0.0005	0.0915	0.0203	<0.0001	<.0001	<.0001	0.01449

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different (P<0.05).

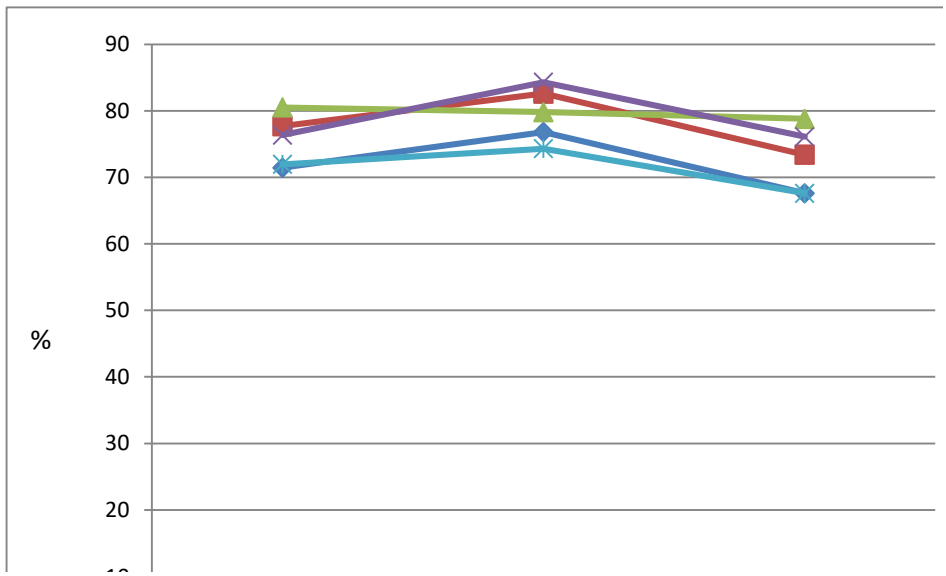

Fig. 1. Percent motility of fresh ejaculated bovine sperm cells after exposure in different concentration of *L*-carnitine in Tris-egg yolk extender and at extended time.

At post-thawing (Fig. 2), all motility characteristics were found of the same trend in all treatments but significant difference were observed on STR and LIN (Table 4) with 0.5 mM L-carnitine group superior to the Control group (80.33% vs. 77.95% and 49.60% vs. 46.56%, respectively). A high concentration of 30 mM consistently exhibited cytotoxic effect.

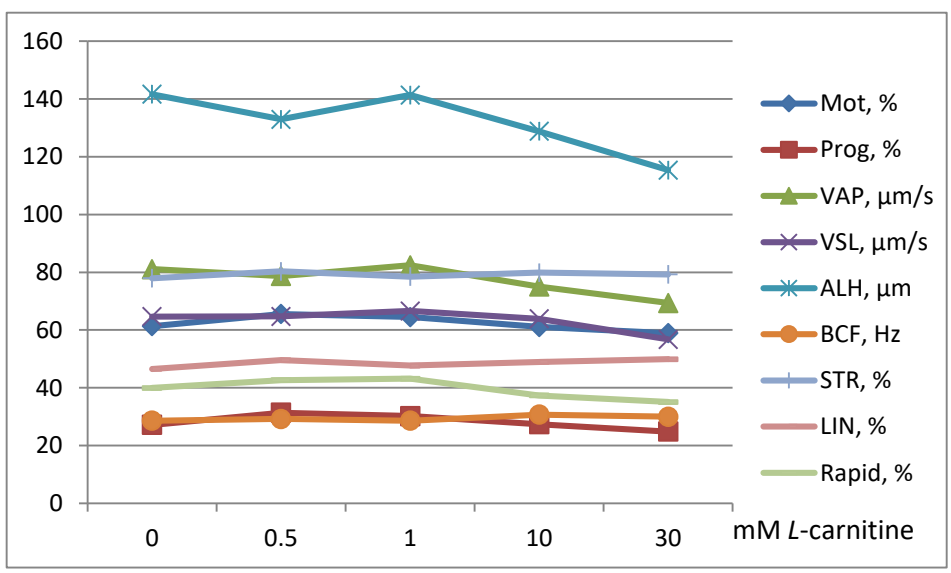

Fig. 2. Motility characteristics post-thawing of bovine sperm cells treated with different concentration of *L*-carnitine in Tris-egg yolk extender.

Functional integrity of the membrane was also improved with *L*-carnitine (Table 5) but significant difference with the Control group was not evident. Throughout the observation, lower concentration of *L*-carnitine, 1 mM, was beneficial but higher concentration, especially 30 mM, displayed negative effect at long time exposure and post-thawing.

Table 5. HOST reaction of sperm cells treated with or without *L*-carnitine in Tris-egg yolk extender before, after freezing, and at extended time.

Treatment	Pre-freezing		Post-freezing		Extended at RT, 24 h	
	N	HOST+ (%±SE)	N	HOST+ (%±SE)	N	HOST+ (%±SE)
0	1279	79.5±0.48	2013	49.5±0.30	1538	63.6±0.28
0.5	1206	81.9±0.37	1865	55.2±0.23	1149	69.1±0.30
1	1372	84.1±0.25	1781	56.9±0.30	1574	69.0±0.21
10	1338	84.2±0.20	1776	51.6±0.15	1469	70.8±0.23
30	1316	74.0±0.37	1721	53.8±0.24	1717	59.6±0.26

N: number of sperm cells; HOST +: oocytes that reacted to hypo-osmotic swelling test; RT: room temperature (P>0.05)

When sperm cells processed in the presence (1 mM) or absence (0 mM) of *L*-carnitine were used for IVF of *in vitro* matured oocytes, cleavage rate was significantly higher (P<0.05) in 1 mM group (92.44 vs. 83.74% and 82.81%, Fig. 3) but no significant difference was observed on blastocysts development and hatching rate (Table 6).

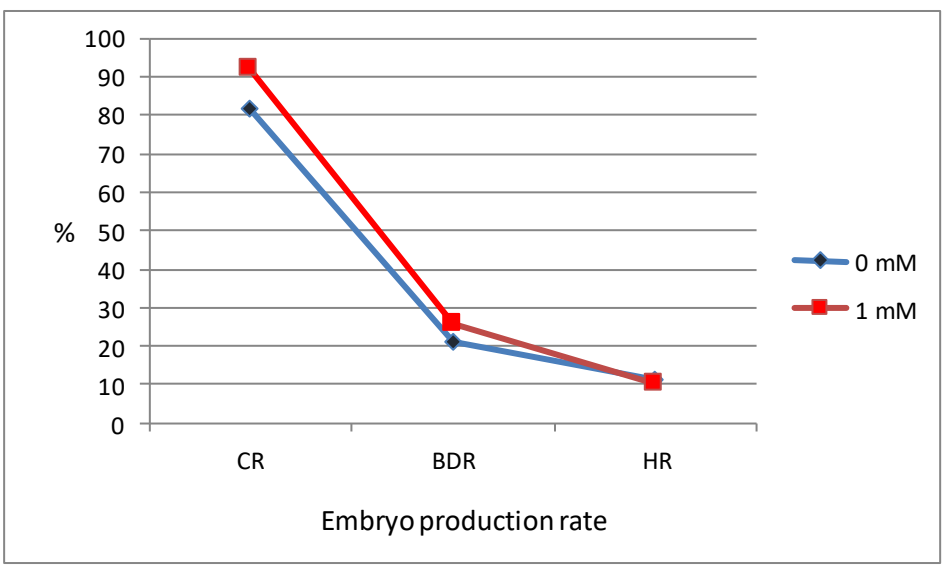

Fig. 3. Embryo development rate of *in vitro* matured bovine oocytes fertilized with semen cryopreserved with or without 1 mM *L*-carnitine in Tris-egg yolk extender

Table 6. Cleavage and blastocyst development of *in vitro* matured bovine oocytes fertilized with semen cryopreserved with or without 1 mM *L*-carnitine

***L*-carnitine**	**N**	**Cleavage Rate, CR (%±SE)**	**Blastocysts development rate, BDR (%±SE)**	**BDR/CR (%±SE)**	**Hatched/CR (%±SE)**
0 mM	193	82.8±0.5[a]	21.4±0.3 [a]	26.0±0.7 [a]	10.6±0.9 [a]
1 mM	199	92.4±0.1[b]	25.8±0.2 [a]	27.9±0.6 [a]	9.9±0.9 [a]

N: number of *in vitro* matured oocytes used for IVF, BDR/CR: developed to blastocysts out of cleaved eggs; Hatched/CR: hatched blastocysts out of cleaved eggs.
Figures with different superscript are significantly different (P<0.05) .

With the above results, it was noted that a low concentration (0.5 to 1 mM) of *L*-carnitine in Tris-egg yolk extender exerts positive effect both on motility characteristics, membrane integrity and cleavage rate of *in vitro* matured oocytes *in vitro* fertilized with *L*-carnitine treated spermatozoa. Higher concentration of 10 mM and 30 mM also had positive effects but negative effect manifest at extended condition especially in 30 mM suggesting cytotoxic effect which supports earlier observations in water buffalo frozen-thawed sperm cells (Hufana-Duran et al., 2012) and in *in vitro* maturation of pig oocytes (Wu et al., 2011). These findings are also in agreement with that of Daena et al. (1984) indicating that addition of 20 mM *L*-carnitine to suspensions of ejaculated bovine spermatozoa resulted in an increase of cellular calcium transport, which inhibited the progressive motility, oxygen consumption as well as the release of the enzymes hyaloronidase and glutamate-oxaloacetate transaminase from spermatozoa but not at 2 mM concentration. High concentration of *L*-carnitine resulted to depressed motility especially at extended condition which could be explained by the earlier observations (Hamilton and Olson, 1976; Bohmer and Johansen, 1978) that *L*-carnitine inhibits respiration of ejaculated bovine spermatozoa.

The improved motility characteristics of spermatozoa observed in the present study could be due to the addition of L-carnitine which concentration in the seminal fluid was diluted during the process, into the Tris-egg yolk extender where *L*-carnitine played a key role in sperm metabolism by providing readily available energy for use by spermatozoa. Earlier reports (Matalliotakis et al., 2000) showed that *L*-carnitine mediates the transport of long chain fatty acids across the inner membrane of the mitochondria for utilization in metabolism through β-oxidation and exerts protective role against reactive oxygen species (ROS) by repairing mechanism via removing elevated intracellular toxic acetyl-coenzyme A (acetyl-CoA) and/or replacing fatty acids in membrane phospholipids (Vicari and Calagero, 2001). These properties and function of the *L*-carnitine together with its osmotic balance function (Brooks et al., 1974) and stabilization of the membrane (Daena et al., 1984) may contribute in the improvement of motility characteristics of the treated spermatozoa resulting to higher fertilization rate when used for IVF post-thawing. Tanphaichitr (1977) observed that carnitine potentiates the motility regardless of the sample's initial motility pattern. Earlier, Daena et al. (1984) reported a positive correlation between the fertility of bovine spermatozoa and the concentration of seminal carnitine which supports the results in Study 3 demonstrating higher cleavage in oocytes *in vitro* fertilized with sperm cells treated with 1 mM *L*-carnitine prior to cryopreservation. In humans, Lay et al. (2001) did not find significant difference in the concentration of carnitine between those who achieved and did not achieve pregnancy after IVF treatment. This positive effect of *L*-carnitine on cleavage rate could also be due to the *L*-carnitine's beneficial effect on DNA repair and proliferation of regenerating germ cells (Amendola et al., 1989) which may enhance better quality spermatozoa that fertilized the matured oocytes.

The observation that *L*-carnitine fails to stimulate sperm motility in washed spermatozoa and succeeds with raw semen samples suggest that it needs to be further metabolized and transported by or with factor(s) in the seminal plasma and this are present in the Tris-egg yolk extender. In ejaculated seminal fluid, most *L*-carnitine are found in the seminal plasma; very little are found in the spermatozoon itself (Bohmer et al., 1978). But washing the sperm cells from the seminal plasma would mean removal of *L*-carnitine. Similarly, dilution with extender means tremendous decrease on *L*-carnitine concentration, thus, supplementation is needed. In humans, however, Duru et al. (2000) found no improvement in motility and membrane damage after addition of *L*-carnitine to Tris-yolk buffer containing glycerol on post-thawed semen of male with primary infertility problems. This observation was in agreement to the present results showing no significant difference on the motility parameters and membrane integrity between *L*-carnitine and no carnitine treated groups after cryopreservation except on STR and LIN that may contribute in the significantly higher cleavage rate after *in vitro* fertilization. It is to be noted that the semen used in this study were from bulls used for AI and are proven fertile, hence, the semen quality is high. The beneficial effect of the *L*-carnitine could be on the provision of the needed concentration that was otherwise lost during the semen dilution, and needed by a fraction of the sperm population. This was evident by the constantly higher percentage of spermatozoa reacted in HOST than the Control group suggesting improvement on the functional integrity of the membrane in the *L*-carnitine treatment. This supports the earlier claim that *L*-carnitine has a stabilizing effect on plasma membranes (Deana et al., 1984). HOST positive sperm cells were reported to have higher fertilizing capacity (Brito et al., 2003). The significantly improved motility characteristics and better functional integrity of the plasma membrane after *L*-carnitine treatment supports the possibility that the action of *L*-carnitine is also due to osmolarity changes (Quinn and White 1969; Drevius, 1972) which *per se* could affect sperm functions (Deana et al., 1989).

The negative result observed in 30 mM *L*-carnitine is associated to earlier report indicating that *L*-carnitine at high concentration inhibits fatty acid oxidation (Hamilton and Olson, 1976) and oxygen consumption (Deana et al. 1989) by ejaculated bovine spermatozoa. In fact, higher concentrations of 60 mM have resulted to completely immobilized sperm cells after exposure (data not shown). These results suggest that *L*-carnitine is involved in the complex regulatory mechanism of metabolic pathways following cellular stimulation in bovine spermatozoa and its presence could rescue some sperm cells that require it for better functioning.

CONCLUSION

It was demonstrated that *L*-carnitine has beneficial effects on the sperm motility characteristics at lower concentration (≤10 mM) but high concentration (≥30 mM) exhibits negative effect. The exposure of bovine sperm cells in Tris-egg yolk extender containing *L*-carnitine improves the sperm motility post-thaw and resulted in improved *in vitro* fertilization of *in vitro* matured oocytes resulting in higher production of embryos *in vitro*.

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Agrobacterium-MEDIATED TRANSFORMATION OF *Cry1Ab* GENE INTO *Tectona grandis* L.(TEAK)

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ABSTRACT

A binary vector pCAMBIA1302-Ab containing*Cry1Ab* and *hygromycin phosphotransferase* (*hpt*) genes was used in the transformation of *in vitro* teak nodes and multiple shoots clumps. The explants were submerged in suspension culture of *Agrobacterium tumefaciens* strain EHA105 (OD_{600}=1.2) then were sonicated for 3 min in sonicator bath followed by vacuum-infiltration for 5 min. The co-cultivation was performed for 3 days on MS medium containing 100 µM acetosyringone. Two months after selection on 50 mgL^{-1}hygromycin containing medium, calli were formed. Total RNA was extracted from these calli and was subjected to first-stranded RT-PCR analysis of *Cry1Ab* gene. The result indicated the presence of *Cry1ab* transcript in all selected calli. A single shoot developed from a multiple shoots clump cultured on selective medium. The 4-weeks-old putative transformed shoot also showed positive results of *Cry1Ab* and *hpt* genes by PCR analysis. However, chimera tissue was observed in this shoot. The Cry1Ab protein in transgenic tissue was also determined using Bt-Cry1Ab/1Ac ELISA Kit. The expressedCry1Ab protein in putative transformed shoot was confirmed in the leaves that contained *Cry1Ab* and *hpt* genes.

Keywords*:* Genetic transformation, tree, insect resistance gene, *Agrobacterium*

INTRODUCTION

Teak (*Tectona grandis* L.) is one of the highly demanded timber-producing trees of the tropical zone. The demands for teak wood and wood products are continuously increasing throughout the world but its natural resources are rapidly depleting (Pandey and Brown, 2000). Thus, the large scale plantations are promoted beyond its native countries in Asia (i.e. Thailand, Indonesia, Malaysia) and Africa (i.e. Ivory Coast, Congo, Nigeria), and Latin America (i.e. Brazil, Costa Rica, Panama and Honduras) (White, 1991).However, mono-cropping plantation system of teak is confronted with many problems, and the big problem is infestation of various pest species. The teak defoliator (*Hyblaea puera* Cramer) and teak bee-hole borer (*Xyleutes ceramicus* Walker) are serious insect pests in teak plantation. Low growth rate and low quality of wood normally occurred when these two insect pests come into the plantation (Nair *et al*., 1984). There are many approaches that tried to solve these problems such as spraying with *Bacillus thuringiensis (Bt)* on the leaves. The main limitation of this technique, however, is the poor coverage on plant surfaces which cannot reach the teak bee-hole borer

inside the tree trunk. The conventional breeding for insect pest tolerance teak cultivar can be done but still needs long period of time to succeed (Suseno and Wibisono, 2000).

Therefore, genetic transformation in order to create an insect-resistant teak is a possible way to improve pest management in the teak plantation. A group of *Bt* genes has been proven to be effectively used in various crops to control the damaged due to the insects in the order Lepidoptera, Diptera and Coleoptera (Chilcott and Wigley, 1993). The Bt endotoxin can only be digested by the enzymes in the guts of these specific insect species and become toxic to these insects within few days and that prevent the damage done by insects. Thus, Bt toxin is not harmful to human due to the lack of specific enzymes in human body (Knowles, 1993).Thus, BT can reduce the risk of pesticides use and reduce the amount of chemical residues in agricultural produces. However, the big problem of gene transformation in teak is low transformation rate and poor regenerating ability. The introduction of useful agricultural genes into teak or woody plant species has been delayed when compared to other species. Teak transformation has been reported using both biolistic and A*grobacterium tumefaciens*-mediated methods to transform the *gus* reporter gene. Widiyanto *et al.* (2009) reported the success of transient gene expression (94.6%) after transferring *gus* reporter gene into the multiple shoots clumps of teak using *A. tumefaciens*- mediated method. Likewise, Sontikun *et al*. (2013) reported the success of transformation of *gus* reporter genes into the nodal segment tissue of teak by *A. tumefaciens*-mediated method of approximately 58.32%.For the transformation with insect resistance gene, Norwati *et al.* (2011) successfully transferred *Cry1Ab* gene into teak tissue *via* particle bombardment. In this report, we demonstrated the successful *Agrobacterium*-mediated gene transformation of *Cry1Ab* gene into teak using the multiple shoots clump explant.

MATERIALS AND METHODS

Plant materials

The *in vitro* teak clone 22c53obtained from The Forest Industry Organization, Thailand was cultured on hormone-free MS medium (Murashige and Skoog, 1962) at 25±2ºC and 16 h day^{-1} in cool light (55µmol.m^{-2}.sec^{-1}) with monthly sub-cultured. Samples of nodal segment and *in vitro* multiples shoot clump of teak, 300 each, were prepared for genetic transformation. Samples of nodal segment of teak were prepared by single node cutting with leaf blade detached. The *in vitro* multiples shoots clump (MSC) was prepared by culture of the single node on solid MS medium supplemented with 6 mgL^{-1} N_6-benzyleadenine (BA) for a month to obtain callus clump with emerging shoots. The MSC was then cut in half prior to be used as an explant for transformation.

Genetic transformation using *Agrobacterium tumefaciens*

Plasmid vector and A. tumefaciens preparations

The binary vector, pCAMBIA1302-Ab, was constructed by inserting 2.5 kb *Cry1Ab* gene under the control of 35SCaMV promoter into the *Nco*I–*Bst*EII site of pCAMBIA1302as a replacement of *mgfp* gene. The carrier plasmid also contained *hygromycin phosphotransferase* (*hpt*) gene as a selectable marker gene. The finished construct of pCAMBIA1302-Ab is shown in Figure 1.The *A. tumefaciens* strain EHA105 was then transformed with this plasmid vector by heat-shock method. After the confirmation of the existing of pCAMBIA1302-Ab, the single colony of *A. tumefaciens* was grown in LB medium containing 50 mg L^{-1} kanamycin and incubated at 28ºC and 120 rpm for 16 h to reach an optical density of 1.2 unit at 600 nm. The aliquot of 200 µL bacterial suspensions was transferred to 50 mL LB medium containing 50 mgL^{-1} kanamycin and 100 µM acetosyringone and incubated at 28ºC at 120 rpm for 16 h. Bacteria cells were then pelleted by centrifugation at 12,100rpm at 4ºC for 10 min then re-suspended in equal volume of MS liquid medium for the latter inoculation procedure.

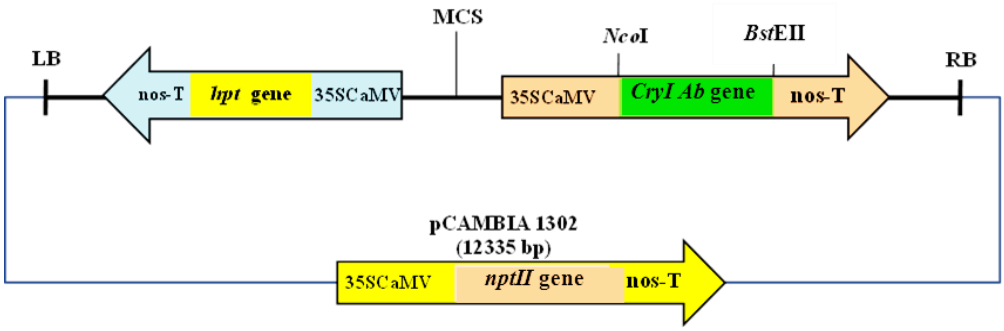

Fig. 1. The structure of the binary vector pCAMBIA1302-Ab

Genetic transformation procedure

For the step of inoculation, the *in vitro* MSC and nodal segments tissues were submerged in suspension culture of *A. tumefaciens* ($O.D._{600}$=1.2) and were sonicated for 3 min using sonicator bath (T460/H, 35kHz, Elma Hans Xchmidbauer, Germany) followed by vacuum infiltration at 600 mmHg for 5 min. Thereafter, inoculation period was allowed to perform for 1h. The MSC and nodal segments tissues were blotted with sterilized filter paper to get rid of the excess bacteria prior to co-cultivated for 3 days on solid MS medium containing 100 µM acetosyringone in dark condition at 25±2ºC. After co-cultivation, explants were washed 3 times with liquid MS medium containing 500 mgL^{-1}cefotaxime and blotted dry on sterilized filter paper. The explants were then transferred to solid MS selective medium containing 300 mgL^{-1} cefotaxime and 50 mgL^{-1} hygromycin and the transferred to the fresh medium every 2 weeks. After 4 weeks on solid selective medium containing cefotaxime, the explants were then transferred to shoot induction medium (MS supplemented with 2 mgL^{-1} BA, and 50 mgL^{-1}hygromycin) for one month. The surviving explants which were putative transformed explants were then cultured on MS medium for further investigation for the existence and expression of *Cry1Ab* gene.

Verification of *Cry1Ab* gene in putative transformed tissue

DNA level verification

The genomic DNA was isolated from the putative transformed calli and leaves using a method described by Phire Plant Direct PCR Master Mix (Thermo Scientific, Thailand). To confirm the integration of the transferred genes into the genome, the polymerase chain reaction analysis (PCR) was performed based on the amplification of 2500 bp fragment of the*Cry1Ab* gene and 800 bp fragment of the *hpt* gene using specific primers for *Cry1Ab* and *hpt* genes respectively, as following sequences:

Cry1Ab-F	5'-CATGGACAACAACCCAAACATCAACGA-3'
Cry1Ab -R	5-GTCACCTTGCTACCGAAAGTCCTCGTTC-3'
hpt -F	5'-CCTGAACTCACCGCGACG-3'
hpt -R	5'-AAGACCAATGCGGAGCATATA-3'

PCR reaction was conducted in total volume of 20 µL containing100 ng of genomic DNA, 10 µL 2x Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, Thailand) and 1µM of each primer under the following conditions: one cycle of 95°C for 5 min; 30 cycles of 95°C for 10 s, 60°C for 10 s, 72°C for 1 min; a final extension at 72°C for 1 min. The amplified products were separated by electrophoresis on a 1% (w/v) agarose in TAE gel and visualized by ethidium bromide staining.

RNA level verification

A reverse transcriptase-polymerase chain reaction (RT-PCR) was used for genetic transformation verification in RNA level. The cDNAs were synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). For the first-strand cDNA synthesis from RNA, the amount of 1µg RNA was first added to an oligo-dT primer (0.5 µgµL^{-1}) or a specific primer for*Cry1Ab* gene (15-20 pmol) and adjusted to a final volume of 12.5µL with nuclease-free deionised water. The mixture was then denatured at 65ºC for 5 min and then instantly cooled on ice. Subsequently, other reaction components were added: 4 µL5X reaction buffer; 0.5µLRNAse inhibitor (20 UµL^{-1}), 2 µLmixed dNTPs (10 mM each) and 1 µL RevertAid™ M-MulV reverse transcriptase (200 UµL^{-1}). The reaction mixture was then incubated at 42ºC for 1 h. Subsequently, the reaction was stopped by heating the tubes at 70ºC for 10 min and the products were then directly used for PCR amplification.

Protein level verification

To confirm the expression of *Cry1Ab* gene, the Cry1Ab protein was detected using enzyme-linked immunosorbent assay (ELISA) by following the protocol of Bt-CryIAb/1Ac ELISA Kit (Agdia Company). Putative transgenic, non-transgenic teak leaves and transgenic tobacco leaves were separately ground in 1×PBST wash buffer at a ratio of 1:10 (g of tissue : ml of buffer). The aliquot of 100 µL supernatants were then loaded into ELISA well that was coated with RUB6 enzyme conjugate solution and 100 µL of CryIAb specific antibody. The 100 µL of positive control (Agdia Company) and negative control (deionized water) were loaded into the appropriate test wells. The testing wells were mixed gently by swirling the plate on the bench-top shaker and incubated for 2 h at room temperature. After 2 h, the test wells were washed 7 times with 300 µL of PBS-Tween 20 (PBST) washing buffer prior to blot dry using paper towel. The aliquot of 100 µL 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added into each well and incubated for 20 min at 37ºC. The result of each test well was evaluated by ELISA reader (Labsystems/Multiskan EX) at 450 nm. The positive result that represented Cry1Ab protein expression must had the value of more than 2 folds of a non-transformed plant.

RESULTS ANDDISCUSSION

Plant transformation and regeneration

The *in vitro* MSC and nodal segments tissue were transformed with *A. tumefaciens* strain EHA105 containing the binary vector pCAMBIA1302Ab carrying*Cry1Ab*and*hpt* genes, both under the controlled of 35SCaMV promoter and Nos-terminator. After 8 weeks on selective medium containing 300 mgL^{-1}cefotaxime and 50 mgL^{-1}hygromycin, two independent putative transformed MSC and nodal segments survived (Figure 2I).The calli were formed from these surviving tissues when they were transferred onto MS medium containing2 mgL^{-1}BA, however, only one shoot regenerated from callus derived from MSC (Fig. 2II B). These calli and shoot tended to be the putatively transformed tissue because the concentration of hygromycin at 50 mgL^{-1} was proven to be the effective concentration to get rid of non-transgenic tissue (Sontikun *et al*., 2013). Therefore, the surviving tissue on this selective medium could be, at least, *hpt* putative transformed lines because they could survive and grew on the selective medium containing hygromycin. However, the molecular level analysis was needed to be performed to confidently confirm the existence and expression of both *hpt* and *Cry1Ab* genes.

For the emerging shoot, chimera may occur due to the fact that it may originate from the multiple cells. Thus, for intensively investigation, the putative transformed shoot was divided into two nodes and one shoot explants prior to subculture onto hormone-free solid MS medium to obtained 3 mature plantlets (Fig. 3). Each plantlet was then separately subjected to further molecular analysis of the transgenes, *Cry1Ab* and *hpt*.

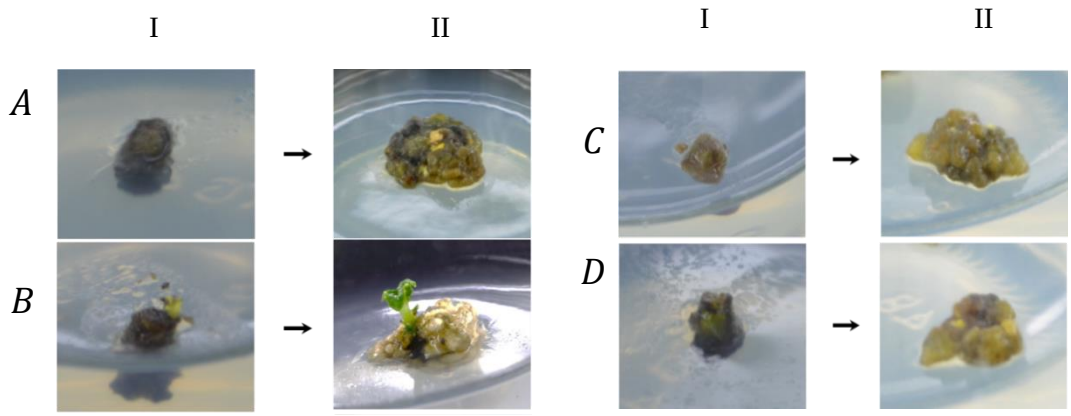

Fig. 2. The putative transformed multiples shoot clumps of teak clone 22c53 (A and B) and nodal segments tissue (C and D) after culture for 4 weeks (I) on MS selective medium containing 300 mgL^{-1}cefotaxime and 50 mgL^{-1} of hygromycin. The survive calli were form after 4 weeks on MS medium containing 2 $mgL^{-1}$of BA (II) and a single shoot emerged from MSC tissue (II B).

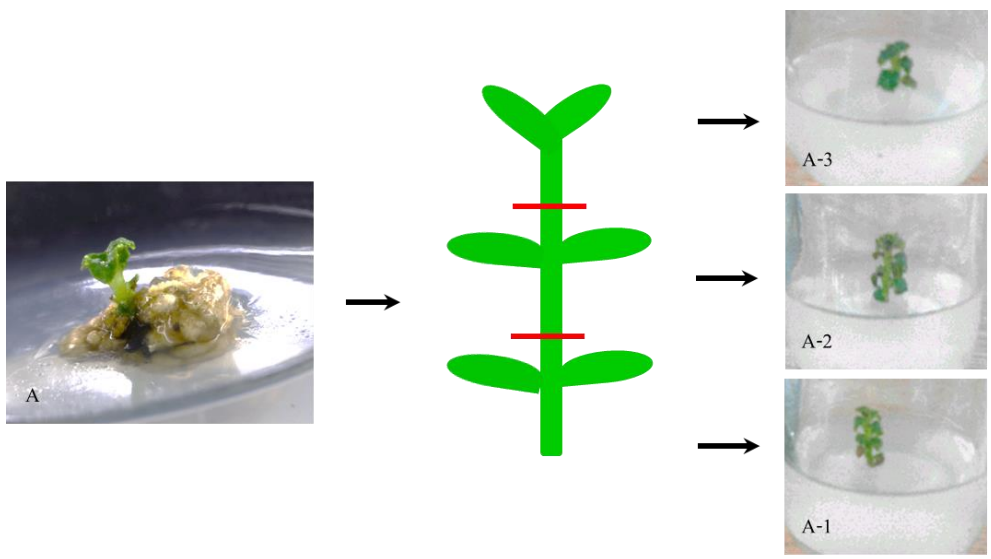

Fig. 3. The putative transformed shoot derived from multiple shoots clump was divided into two nodes and one shoot explants prior to subculture onto hormone-free solid MS medium to obtained 3 mature plantlet lines, A-1, A-2 and A-3.

Verification of the genetic transformation in putative transformed calli

DNA level verification* of *Cry1Abgene in calli

To confirm the presence of *Cry1Ab* gene in the survive calli, genomic DNAs were extracted from each callus and subjected to PCR amplification using *Cry1Ab* gene specific primers. The results demonstrated that genomic DNA of each calli produced the amplified DNA band of 2,500 bp similar to the size of the DNA band produced from pCAMBIA 1302-Abpositive control. For the PCR analysis of the non-transgenic callus (negative control), no DNA band was observed (Fig.4). These results confirmed the existence of*Cry1Ab* gene in all selected putative transformed calli.

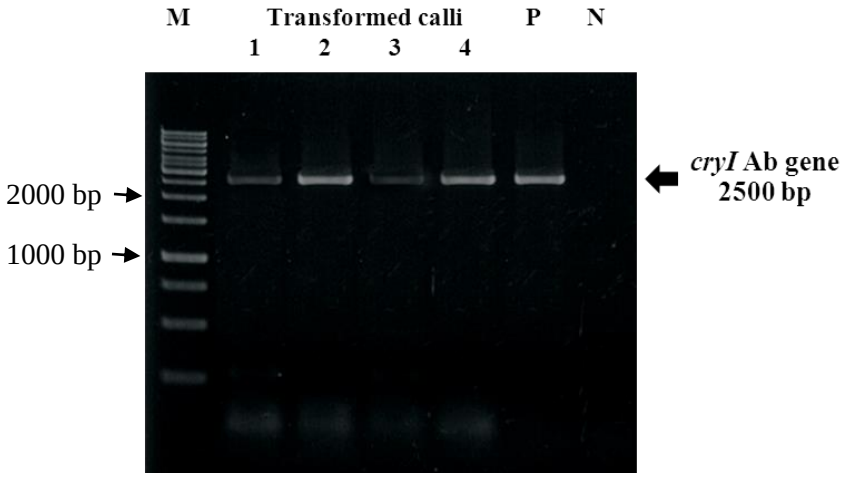

Fig. 4. PCR analysis of *Cry1Ab* gene in putative transformed calli using genomic DNA as DNA template.

M = 1 kb ladder molecular weight marker (Fermentas)
1-4 = genomic DNA from 4 putative transformed calli
P = PCR product of *Cry1Ab* gene from positive control (pCAMBIA 1302-Ab)
N = PCR product of negative control (non-transformed callus)

RNA level expression of Cry1Ab gene in putative transformed calli

The RNA level expression in the putative transformed calli was also conducted using first-strand cDNA analysis. For this technique, the first strand cDNA was generated from total RNA and then it was used as a template for PCR analysis with primers specific to the gene of interest. The positive band presented the specific cDNA band of the novel gene. The results revealed the presence of specific bands of 260 bp of *Cry1Ab* gene only in the samples of putative transformed calli (Fig. 5) but not found in the negative control (non-transformed line).This confirms the presence of *Cry1Ab* gene and at least the intermediate step of expression to RNA level in the putative transformed calli.

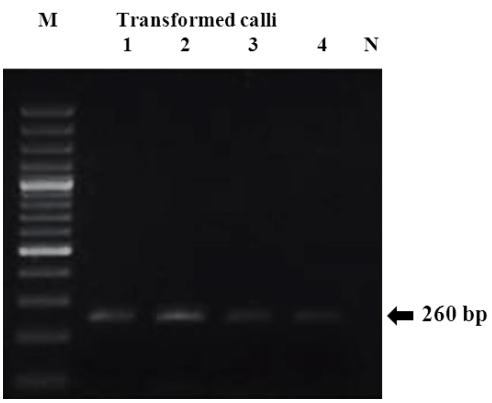

Fig. 5. PCR analysis of *Cry1Ab* gene in putative transformed calli using first-strand cDNA as DNA template.

M = 100 bp plus molecular weight marker (Fermentas)
1-4 = cDNA from each putative transformed callus
N = negative control (non-transformed callus)

The results revealed the difference in level of expression of *Cry1Ab* gene in transformed calli as the quality of the bands were observed. The initial quantity of mRNA is very important in success in the investigation of gene expression by RT-PCR technique. Unfortunately, mRNA was rapidly degraded during extraction process. This may lead to the low visualization of the amplified bands. However, this result was comparable with the previous research work of Norwati *et al.* (2011) in which Southern blot of *Cry1Ab* gene detected in the transgenic teak transformed by particle bombardment demonstrated the positive result but the low level expression of *Cry1Ab* gene was revealed by RT-PCR. Thus, beside the technical difficulties of RNA handling, the *Cry1Ab* gene may poorly express by itself in some plant species. The possibility may due to the A/T rich in 3' poly adenine tail is short and could accelerate the rate of destruction of *Cry*-mRNA, thus, quantity of *Cry*-mRNA is therefore present in small amounts (Rocher *et al*., 1998). Beside, the chimeras should be also accounted for the present of small amounts of *Cry*-mRNA in the calli as well.

Verification of the genetic transformation in putative transformed shoots

DNA level verification

The transformed shoot derived from transformed callus, was cut and sub-cultured to new hormone-free MS medium for 4 weeks to generate 3 new putative transformed shoots (A1, A2 and A3). They were analyzed to confirm the presence of *Cry1Ab*and *hpt* genes. The genomic DNA was extracted from each leaf of the plantlets and subjected to PCR analysis. The non-transgenic teak was used as a negative control, while transgenic tobacco containing *Cry1Ab* and *hpt* genes was used as positive control in this PCR analysis.

The results showed that specific bands of *Cry1Ab* (2,500 bp) and *hpt* (800 bp) genes wereobserved in the positive control sample (transgenic tobacco), but none was founded in negative control one (non-transgenic teak). For the putative transgenic samples, the results were varied (Fig. 6A and B) and were summarized in Table 1. The plantlets were chimera since some leaves in the same plantlet did not have *Cry1Ab* and/or *hpt* genes. For those leaves that failed to detect *hpt* gene, *Cry1Ab* band also absented (N, L1, 2L6, 3L2, 3L3, 3L4). However, not all of *hpt* gene was co-transformed with *Cry1Ab* gene as the results demonstrated that in 5 out of 14 leave samples (L3, L4, L7, L9, and 2L5), the *hpt* gene was detected but not *Cry1Ab* gene. On the other hand, no *Cry1Ab* gene detected without the presence of *hpt* gene. The event of non co-transformation of these 2 genes may be resulted from the event of the transfer process of *Agrobacterium* that the genes franked by LB and RB are cut and transported into the host cells. During these processes, the transferred DNA may be sheared and that cause the insertion of truncated piece (Sheng and Citovsky, 1996). However, in this experiment, the selective medium used was the medium containing hygromycin, thus at least the cells contained *hpt* gene would survive selection. Nonetheless, the escape event also may be occurred due to the large size of target tissue. In this report, in the A1 plantlet which originated from the bottom part of the emerging shoot, 9 leaves were analyzed and only one leaf demonstrated of non-transformed event, but the rests were transformed in which half of those (4 leaves, 44%) were co-transformed with both *Cry1Ab* and *hpt* genes and another half (4 leaves, 44%) were transformed with only *hpt* gene. The plantlets that obtained from the upper part (A2 and A3) demonstrated even less in the percentage of transformation. In A2 plantlet, 4 leaves out of 6 were transformed. Among these, 3 leaves (66%) contained both *Cry1Ab* and *hpt* genes while 1 leaf (16%) only *hpt* gene was detected. In the top part, A3 plantlet, 5 leaved were analyzed and only 1 contained both *Cry1Ab* and *hpt* genes while in the other 4 leaves none of the transgenes were detected.

The results indicate the chimera event of the transformed shoots in this experiment may be due to the target tissue that used was the multiple shoots clump which was a large tissue. The callus obtained at the first step may also be chimera that consisted of a mixed of transgenic and non transgenic cells but the PCR detection could not be able to distinguish that event. However, the shoot might emerge from a group of cells instead of a single cell origin, some were transformed but some

were non-transformed. Thus, the analysis results in one individual leaf tissue clearly demonstrated the chimera in all of the transformed shoots. The previous research work also reported the chimeric tissue as well. Lachance *et al.* (2007) reported the chimeric plant produced from the transformation of *Cry1Ab* in hypocotyl tissue of *Perilla frutescens* using *A. tumefaciens*. Similarly, the chimera plants also obtained in the transformation of cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm.(Lee *et al.*, 2005) and somatic embryo of white spruce (*Picea glauca*) (Prakash and Gurumurthi, 2009) with *Cry1Ab* gene *via Agrobacterium*-mediated genetic transformation. To be able to obtain a solid transformed plantlet from these chimeric plants, the number of sub-cultures and selection on hygromycin containing medium should be performed for several generations to get rid of the non-transformed cells.

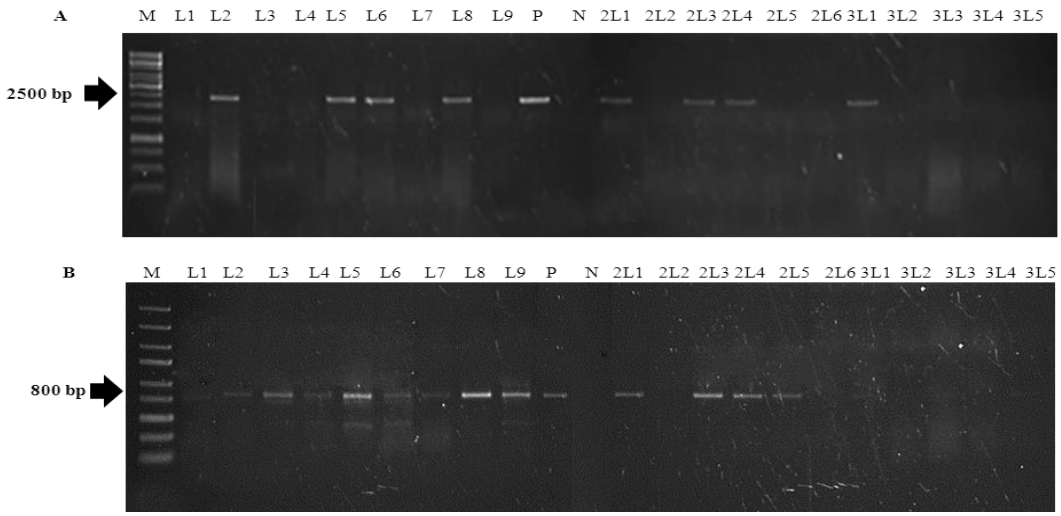

Fig. 6. PCR analysis for the presence of *Cry1Ab* gene (A: 2500 bp) and *hpt* gene (B: 800 bp) in genomic DNA of putative transformed teak leaves from plantlets A-1, A-2 and A-3.

M	= 1 kb ladder molecular weight marker (Fermentas)
L1-L9	= Leaves from A-1 plantlet
2L1-2L6	= Leaves from A-2 plantlet
3L1-3L5	= Leaves from A-3 plantlet
P	= positive control (transgenic tobacco containing *Cry1Ab* and *hpt* genes)
N	= negative control (non-transformed teak)

Table 1. The summary of the results of PCR analysis for *Cry1Ab*and *hpt* genes in leaves of A-1, A-2 and A-3 plantlets (from Figure 6) as + present / - absent.

Genes	Leaves of A-1 plantlet										
	L1	L2	L3	L4	L5	L6	L7	L8	L9	P	N
Cry1Ab	-	+	-	-	+	+	-	+	-	+	-
hpt	-	+	+	+	+	+	+	+	+	+	-
	Leaves of A-2 plantlet										
	2L1	2L2	2L3	2L4	2L5	2L6					
Cry1Ab	+	-	+	+	-	-					
hpt	+	-	+	+	+	-					
	Leaves of A-3 plantlet										
	3L1	3L2	3L3	3L4	3L5						
Cry1Ab	+	-	-	-	-						
hpt	+	-	-	-	-						

Protein level verification of Cry1Ab in transformed plantlets

The expression of *Cry1Ab* gene into Cry1Ab protein was determined by Bt-Cry1Ab/1Ac ELISA Kit, a commercial ELISA test kit specific for Cry1Ab protein, in five selected leaf samples (L2, L8, 2L1, 2L3 and3L1) that were confirmed with both *Cry1Ab* and *hph* genes as described in Table 1. This ELISA test kit visualized yellow solution after tested with standard protein (Cry1Abprotein, Agdia®) in which the OD-450 could be read by the ELISA reader. In this study, the standard positive sample (Cry1Abprotein, Agdia®) had the OD value read at 0.290 and the OD of negative sample was 0.07 (Fig. 7). The value that determined positive result should had at least two fold higher than the value of negative sample, hence, the samples that showed positive in this experiment should have OD higher than 0.14. The results from ELISA reader illustrated that the Cry1Ab protein was detected in the all of transformed leaves tested as they demonstrated the value of OD-450 in the range of 0.163-0.180 and the tobacco positive control had OD value of 0.194 which prove the expression of *Cry1Ab* gene to Cry1Ab protein. Among the transformed leaves, the leaf sample 2L1 had the highest OD value of 0.180 while the other samples had OD in the range of 0.163-0.169. The low level expression of *Cry1Ab* gene may be due to several reasons. The position effect was one of the reasons as the insertion site of the gene affects the level of transcription. The copy number of the inserted gene was another main reason involve with gene expression. However, to address this low level expression problem, several supporting molecular analysis such as Southern blot, iPCR, TAIL PCR and etc. needed to be performed.

The evidence of low OD value of the transgenic leaves was coincided with the finding of the chimera in these plantlets. The variation in OD value possibly accounted for the degree of chimera of each tissue tested. The results confirmed the study of Lachance *et al*. (2007) which transformed *Cry1Ab* gene into white spruce (*Picea glauca*) and expression of *Cry1Ab*gene to Cry1Ab protein in this species was detected by ELISA. However, a relatively high level of protein (389 ng mg^{-1}leaf fresh weigh) was later determined and the insect bioassay with eastern spruce budworm (*Choristoneura fumiferana)* also demonstrated that90% of the insect died after infestation of the transformed white spruce leaves. Therefore, the transformed teak resulted from this experiment should provide the possibility of genetic transformation with the aim to create the insect-resistant teak for the improve pest management in the teak plantation. In the insect pest management of teak plantation, more than 70% of teak was reported to be damaged by teak defoliator and bee-hole borer (Nair *et. al.,* 1984; Royal Forest Department of Thailand, 2009), thus, any figure less than that should be beneficial. On the other hand, the chimera *Bt* transgenic plant also offer an ideal integrated pest management as it allowed some percentage of refuges that concur the less stress to the insect that, in turn, leads to the fact of resistance insects.

ELISA visualization								
Sample	N	P	tobacco	L2	L8	2L1	2L3	3L1
OD-450	0.07	0.290	0.194	0.163	0.169	0.180	0.167	0.163
Analysis result		+	+	+	+	+	+	+

Fig. 7. The detection of Cry1Ab protein by Bt-Cry I Ab/1Ac ELISA Kit® in transgenic teak leaves that confirmed to contained *Cry1Ab* and *hpt* genes. The evaluation is considered positive when the absorbance is higher than two-fold of negative control.

N = negative control (protein solution from non-transformed teak leaves)
P = positive control (Cry1Abprotein, Agdia®)
tobacco = protein solution from tobacco leaf transformed with *Cry1Ab* gene
L2, L8 = protein solution from teak leaves of A-1 plantlet
2L1, 2L3 = protein solution from teak leaves of A-2 plantlet
3L1 = protein solution from teak leaf of A-3 plantlet

CONCLUSION

This report demonstrated the success of introduction of *Cry1Ab* and *hpt* genes into teak tissue by *Agrobacterium*-mediated transformation method using the *A. tumefacience* strain EHA105 containing the binary vector pCAMBIA1302Ab carrying *Cry1Ab* and *hpt* genes. The *Cry1Ab* and *hpt* genes were detected in teak tissue by PCR. The expression of *Cry1Ab*gene in leaves tissue of putative transgenic plants was confirmed by RT-PCR and ELISA. However, the plantlets obtained were chimera. Thus, to avoid the chimera problem, the optimization on tissue culture technique should be intensively explored for other plant tissue types such as somatic embryogenesis. Nonetheless, for the chimera transgenic plants obtained from this experiment, the *in vitro* screening technique can be employed in order to obtain the solid transgenic plant.

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CONSTRUCTION AND CHARACTERIZATION OF SINGLE CHAIN VARIABLE FRAGMENT-ALKALINE PHOSPHATASE FOR RAPID DETECTION OF AFLATOXIN B_1 IN AN ELISA-BASED ASSAY

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ABSTRACT

Aflatoxins are metabolites produced by *Aspergillus* spp. and can be found as contaminants in various food and agricultural products. A specific antibody is needed for the development of serological method, such as Enzyme-linked immunosorbent assay (ELISA), as a screening process to determine toxin contamination. In this study, nine clones of specific single chain variable fragments (scFv) were selected from naïve mouse phage display scFv library and their reactivity with aflatoxins were determined. The experiments were conducted in the Serology and Diagnostic Laboratory, Center of Agricultural Biotechnology, Kasetsart University from 2012-2013. The scFv gene from the recombinant phagemid clone 22A12 (scFv-22A12 gene), which gave the strongest reaction, was selected for further investigation on aflatoxin analysis. The recombinant protein product was 30 kDa. Concurrently, an alkaline phosphatase (AP) gene was amplified from *Escherichia coli* strain HB2151. The scFv-22A12 and the AP genes were ligated into pCANTAB-5E phagemid and transformed into *E. coli* TG1 and HB2151 to produce phage scFv-AP and soluble scFv-AP, respectively. Comparison on the efficiency of Phage scFv, Phage scFv-AP and soluble scFv-AP to whole molecule antibody for detecting AFB_1 was performed by ELISA. The resulst showed that the soluble scFv-AP gave highest reactivity and in accordance with those obtained from the whole molecule antibody. Cross reactivity with other aflatoxins (B2, G1 and G2) was reported to be 38.63%, 21.24 % and 9.64%, respectively. When using soluble scFv-AP to analyze ground samples of corn and groundnut spiked with 100 µg/kg of AFB1, acceptable results were obtained with 87.02 and 94.41% recovery, respectively. Analysis of the certified reference material (TMAF No.2 and TMAF No.3) showed comparable results with those analyzed through high performance liquid chromatography.

Keywords: mycotoxin, phage display, recombinant antibody, scFv, serological method

INTRODUCTION

Aflatoxins, a group of mycotoxins, are produced mainly by certain strains of *Aspergillus flavus* and *A. parasiticus*. They are found contaminated in a wide range of tropical and subtropical agricultural products, with commonly contaminated food commodities including cereals, oilseeds, spices, and tree

nuts. It is difficult to eliminate these toxins because of their heat stability, therefore heating or cooking cannot be relied on to destroy these toxins. Based on their fluorescent properties under ultraviolet light and chromatographic mobility, aflatoxins are divided into six major toxins, including aflatoxin B1 (AFB_1), B_2, G_1, G_2, M_1 and M_2. However, AFB_1 is the most toxic and the most prevalent and is categorized as Group 1 by the IARC (International Agency for Research on Cancer) (IARC, 1993; Rodrigues and Schuh, 2013). Concentrations of these toxins were limited in each country, with varying concentrations in each commodity. Since fungi can grow on a wide range of commodities and because of their stability in food, control is best achieved through methods designed to prevent the contamination of crops in the field and during storage, or early detection and removal of contaminated products from the food supply chain.

Analytical methods have been developed based on Thin layer chromatography (TLC), High performance liquid chromatography (HPLC) and Enzyme–linked immunosorbent assay (ELISA), as well as available rapid screening kits. Among these, serological method, especially ELISA, is preferred over other analytical methods because of their simplicity and cost-effectiveness. Production of high quality antibodies is required for the assay development of aflatoxin estimation. High-throughput phage display technology is currently an attractive and effective method of choice to obtain single chain variable fragment (scFv) antibody, wherein affinity maturation can be modified (Hoogenboom *et al*., 1991; Smith, 1985) and the molecule can be further engineered. In addition, several serological techniques require enzyme as a label to detect measurable signal, for example alkaline phosphatase (AP), which is one of the widely used enzymes. It has a wide range of applications in diagnostics, immunology and molecular biology, serving as a biochemical marker in the quantitative measurement of analytes. In this article, the scFv specific to AFB_1 was selected from the phage display naïve mouse scFv library (Koohapitagtam *et al*., 2010) and characterized, then the scFv gene was fused with alkaline phosphatase gene. Preliminary characterization of this fusion proteins was carried out for further application in aflatoxin determination.

MATERIALS AND METHODS

Bacterial culture and reagents

Aflatoxin B_1 conjugated with bovine serum albumin (AFB_1-BSA) and soluble aflatoxins B_1, B_2, G_1 and G_2 were purchased from Sigma (USA). DNA was extracted by using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). *Escherichia coli* strains TG1 and HB2151 (Amersham Biosciences, Uppsala, Sweden) were used in phagemid manipulation and expression. PCR products of the scFv gene (light chain variable gene-heavy chain variable gene, V_L-V_H gene) were cloned into pCANTAB-5E phagemid (Amersham Biosciences, Uppsala, Sweden). The SOBAG medium (17 g tryptone, 10 g yeast extract, 5 g NaCl and 15 g bacto-agar in 1 L) plates supplemented with 100 µg/mL ampicillin and 2% (w/v) glucose were used for the selection of transformants. Anti-M13 polyclonal antibody (PAb) for phage library selection was previously produced in our laboratory (Koohapitagtam, 2010). Goat anti-rabbit antibody conjugated with alkaline phosphatase (GAR-AP) for anti-M13 detection was purchased from Sigma (USA). The detection reagents including *p*-nitrophenyl phosphate (*p*NPP), nitro blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for alkaline phosphatase assay were all purchased from Zymed (California, USA). Other reagents used in this study were at least of analytical grade.

Phage display scFv library (Phage library)

The naïve mouse scFv library in this experiment was previously constructed by Koohapitagtam (2010), which consists of fd phage carrying a second copy of phage gene VIII. The N-terminus of this gene contained a randomized sequence of cysteine codons, with a four or six codon deletion. The cysteine codons were designed to constrain by cross-linking the conformations which the peptides might adopt.

Affinity selection of phage display scFv antibody (phage scFv) from phage library by biopanning

Three rounds of biopanning were carried out on the library to select AFB_1-specific phage scFv, using AFB_1-BSA conjugate, by the method modified from Amersham Biosciences (Amersham, 1996). Four millilitre of AFB_1-BSA conjugate (10 µg/mL) in phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was coated in each immunotube. After overnight incubation (16–18 h) at 4ºC, the tubes were washed three times with PBS and blocked with PBS supplemented with 3% (w/v) skimmed milk (3% MPBS) for 1 h at room temperature. After removal of the blocking solution, the tubes were washed three times with PBS.

The library was pre-incubated with 2% (w/v) MPBS supplemented with 2% (w/v) BSA and 0.1% Triton-X100 for 1 h at room temperature, then added into the pre-coated immunotubes and continually incubated at room temperature for 2 h. Unbound phage was washed away 20 times with PBS and 3 times with PBS supplemented with 0.05% (v/v) Tween 20 (PBST). The eluted phage was recovered by infecting 1 mL of exponential growing *E. coli* TG1 and incubated at 37 ºC for 30 min without shaking. Infected cells were subjected to 10-fold-serial dilutions and spread onto SOBAG plates supplemented with 100 µg /mL ampicillin and 2% (w/v) glucose. The agar plates (inverted) were incubated overnight at 37 ºC.

Screening of AFB_1-specific phage scFv by ELISA

After the biopanning process, individual recombinant TG1 colonies were picked and grown at 37°C in a 96-well tissue culture plate (Corning, USA) for 1 h and rescued by M13KO7 helper phage to produce phage scFv for further screening. AFB_1-BSA at 5 µg/mL concentration was coated into a microtiter plate (Corning, USA) and incubated at 37°C for 1 h, while a negative control plate was coated with free BSA. Subsequently, the plates were washed three times with 200 µL of PBST and 150 µL of 3% MPBS was added to block non-specific binding. Fifty microlitre of each amplified phage was added to the plates and incubated for 2 h at 37°C. Plates were washed three times with PBST and indirect detection was carried out by adding 50 µL of rabbit anti-M13 antibody, followed by incubation with goat anti-mouse immunoglobulin G (IgG) conjugated with alkaline phosphatase (GAM-AP) at 1:30,000. In each step, the plate was incubated for 1 h at 37°C. The absorbance at 405 nm was recorded by a MultiscanEX ELISA reader (Labsystems, Finland) at the end of 1 h incubation period with 100 µL of enzyme substrate *p*NPP.

Cloning of an alkaline phosphatase gene

PCR primers were designed and synthesized for amplification of the alkaline phosphatase (AP) gene from *E. coli* HB2151. AP forward and reverse primers were as follows; 5'-GCGGCCGCTCGGACACCAGAAATGCCTGTTCTG-3' and 5'-GCGGCCGCTTTCAGCCCCAGAGCGGCTTTCAT-3', respectively. Each primer was modified to include a *Not*I restriction site (underlined). To amplify the AP gene by PCR method, 20 µL of the PCR reaction contained 2 µL of the bacterial cell suspension, 10 pmole of AP forward and reverse primers, 1x Phusion^R HP buffer, 25 mM dNTPs and 0.2U Phusion^R Hot Start II DNA polymerase. The reaction mixture was preheated at 98ºC for 30 s and 30 cycles of the amplification were carried out as follows; 98ºC for 10 s, 55ºC for 10 s, 72ºC for 45 s and then a final extension at 72ºC for seven minutes. The AP gene was purified using QIAquick Gel extraction kit according to the manufacturer and inserted into pJET1.2/blunt cloning vector (Qiagen, Valencia, USA) using T4 ligase. The ligated DNA population was introduced into *E. coli* DH5α by heat shock at 42ºC for 2 min. Transformants were grown overnight by culturing in 2YT supplemented with 100 µM ampicillin at 37ºC and the positive clones by PCR method using AP-specific primers were selected.

The positive recombinant plasmid DNA was sequenced and phylogenetic analysis was done by the distance method using CLC Main Workbench (version 5.5). The distance matrix for the aligned

sequences was clustered using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Reliability of the inferred tree was estimated by 1000 bootstrap resampling using the same program.

Construction of the recombinant phagemid carrying scFv-AP genes

Single chain variable fragment-alkaline phosphatase fusion proteins (scFv-AP) for direct serological assay was constructed according to the protocol described previously (Wozniak *et al*., 2003). Briefly, 1 µg of DNA from the recombinant phagemid clone 22A12 carrying scFv gene and pJET1.2/blunt cloning vector containing AP gene were digested by *Not*I at 37ºC for 3 h. DNA fragments were purified from 1.5% agarose gel using QIAquick Gel extraction kit. The alkaline phosphatase gene was inserted into the scFv recombinant phagemid (ratio 3:1) using T4 ligase. The ligated DNA population was then introduced into *E. coli* TG1 and HB2151 by heat shock at 42ºC for 2 min (Sambrook and Russell, 2001).

Production of phage scFv-AP and soluble scFv-AP fusion proteins

Phage scFv-AP was produced by rescuing the recombinant phage using M13KO7 helper phage to infect the recombinant TG1 strain, and determined for their AFB_1 specificity by phage ELISA. To produce soluble anti-AFB_1 scFv-AP fusion proteins, the recombinant log-phase HB2151 culture was induced by isopropyl β-D-thiogalactopyranoside (IPTG) (Amersham, 1996). A further purification step of the soluble scFv-AP was carried out by gel filtration chromatography and the catalytic activity of each fraction was determined by dot immunobinding assay (DIBA) (Wozniak *et al*., 2003).

Characterization of the soluble scFv-AP by native polyacrylamide gel electrophoresis (native-PAGE) and Western Blotting

The integrity of the scFv-AP fusion proteins was examined by native-PAGE and Western blotting (Wozniak *et al*., 2003). Briefly, 10 µL of each soluble scFv-AP protein was mixed with 2x loading buffer and loaded into 10% native-PAGE (without SDS). The electrophoresis was run under the following conditions; 70 V for 30 min and then 120 V for 90 min (Laemmli, 1970). The protein bands were then transferred onto nitrocellulose membrane by electrophoresis at 40 V for 120 min. After washing the membrane in TBST, the scFv-AP band was allowed to react with AP substrates, NBT and BCIP, for a chromogenic reaction within 10 min.

Comparison of three scFv formats to whole molecule MAb on the detection of AFB_1 by plate-trapped antigen ELISA (PTA-ELISA)

Five µg/mL of AFB_1-BSA or 1% (w/v) BSA (control treatment) in 100 µL PBS were immobilized in ELISA wells and incubated for 1 h at 37ºC. The plates were washed three times with PBST and blocked with 3% MPBS for 1 h at room temperature. One hundred microlitres of each antibody format, including phage scFv-AP, soluble scFv-AP, phage scFv and whole molecule antibody [anti-AFB_1 monoclonal antibody (MAb) clone C10 (Kladpan *et al*., 2009)] at 10 µg/mL, was added into the ELISA wells with 1 h incubation. For AP-conjugated scFv formats, direct ELISA was performed and the AP substrate was added after the incubation period of the primary antibody, while the indirect ELISA method was applied when non-AP-conjugated scFv and MAb were in use according to the procedure described above. Absorbance values at 405 nm were measured after 1 h incubation at 37ºC with the substrate in both formats.

Determination on the Cross reactivity of the scFv-AP antibody by direct competitive ELISA (dcELISA)

Cross reactivity of the scFv-AP antibody was assayed against three aflatoxins, including B_2, G_1 and G_2. Stock solution of each aflatoxin was prepared in methanol and diluted in PBS-10% (v/v) methanol (PBS-met) with two-folded dilutions from 16 to 2 ng/mL. Wells in microtitre plates were coated with 50 µL of AFB_1-BSA conjugate dissolved in a carbonate coating buffer, pH 9.6 (CB). The plates were incubated for 1 h at 37°C, emptied and washed three times with PBST. Blocking solution (3% MPBS) was added at 200 µL per well and incubated for 1 h at 37°C, and washed three times with

PBST. A mixture of scFv-AP and the prepared standard aflatoxin (1:1 ratio) at varied concentrations was added to each pre-coated well and incubated for 1.30 h with 75 rpm shaking. Unbound antibody was removed by washing three times with PBST. Following this step, the *p*NPP substrate solution was added and incubated at room temperature for 60 min. Absorbance at 405 nm was read and degree of competition was calculated using the following a formula: % AFT binding = ($Absorption_{AFT}$/ $Absorption_{PBS}$) x 100. Relative cross-reactivity was determined as % cross-reactivity = [50% inhibitory concentration (AFB_1) / 50% inhibitory concentration (competitor)] x 100 (AOAC, 2002).

Sample preparation for aflatoxin determination

Groundnut and corn ground matrices were obtained in 50 g quantities. After a thorough mixing, sub-samples were drawn (20 g for each sample) and spiked with AFB_1 at 100 ppb, then the samples were incubated overnight at 4ºC. Each sub-sample was then extracted in 100 mL of 70% methanol while shaking at 250 rpm for 30 min. The extract was filtrated through Whatman no.1 filter paper and diluted 1:5 with PBS-met before analysis by ELISA (Technical Committee CEN/TC 275, 1999).

Determination of AFB_1 contamination in spiked groundnut, corn samples and certified reference materials (CRM) by competitive ELISA

The assay was performed by competitive ELISA protocol. Fifty microlitres of AFB_1-BSA conjugate (5 µg/mL) dissolved in carbonate coating buffer, pH 9.6 was pre-incubated in the ELISA wells for 1 h at 37ºC. The samples of spiked groundnut, corn samples and CRM (Total material aflatoxins; TMAF No.2 and TMAF No.3) were extracted as described above. For scFv-AP dcELISA, the extract was mixed with the scFv-AP at 1:1 ratio and transferred into the conjugate pre-coated wells. The plates were incubated for 1 h at 37°C, emptied and washed three times with PBST. Bound scFv-AP was detected by adding the substrate. In case of the MAb, indirect competitive ELISA (icELISA) was carried out by mixing the antibody with the extract at the same ratio, incubating for 1 h, then the GAM-AP was added and incubated. Reactivity was visualized by adding the enzyme substrate. Standard curve was obtained by plotting log_{10} values of AFB_1 standard at various concentrations ranging from 2 to 16 ng/mL, against the absorbance at 405 nanometer.

RESULTS AND DISCUSSION

Affinity selection of phage scFv from phage library by biopanning

The phage clones specific to AFB_1-BSA were first selected by biopanning method and screened by ELISA for three rounds. One hundred and fourteen clones were specific to AFB_1-BSA; however, only 9 clones (13C7, 15E4, 16C5, 17F4, 17F5, 18C3, 20C3, 21A1, 22A12) were positive with AFB_1 ($O.D._{405}$ ranging from 0.426 – 0.873) without cross reaction with BSA. The scFv gene from the recombinant phagemid clone 22A12 giving the strongest reaction (0.873) was chosen for the following experiments. During the biopanning step, we noticed that the selected phage scFv contained high ratio of anti-BSA phage antibody, therefore an attempt had been made to eliminate this background by incubating the phage scFv with 2% MPBS + 2%BSA + 0.1% Triton X-100 for 1 h prior to the addition into immunotubes. The satisfactory result was achieved.

Cloning of an alkaline phosphatase gene

The AP gene from *E. coli* HB2151 was successfully amplified and the result showed a band size of approximately 1,350 bp (Fig. 1). AP nucleotide phylogenetic tree sequence (KF387511) showed the similarity of our cloned AP gene with those reported in GenBank including 99% similarity with M29664, M29665, X04586, M13345, FJ546461, EU905389 and EU905386; 98% similarity with M29669, M29670; and 97% similarity with M29668 (Fig. 2A) supported by 100% bootstrap values. On the contrary, the AP gene was dissimilar to those belonging to *Aedes aegypti* (XM_001663484 and XM_001663428) by 100% bootstrap (Fig. 2B). In place of using the commercial vector which contained alkaline phosphatase gene (Kuntalee *et al*., 2011; Pershad *et al*., 2011; Wang *et al*., 2008), we

successfully cloned it from *E. coli* HB2151 and still maintained its catalytic activity as compared to the commercial one.

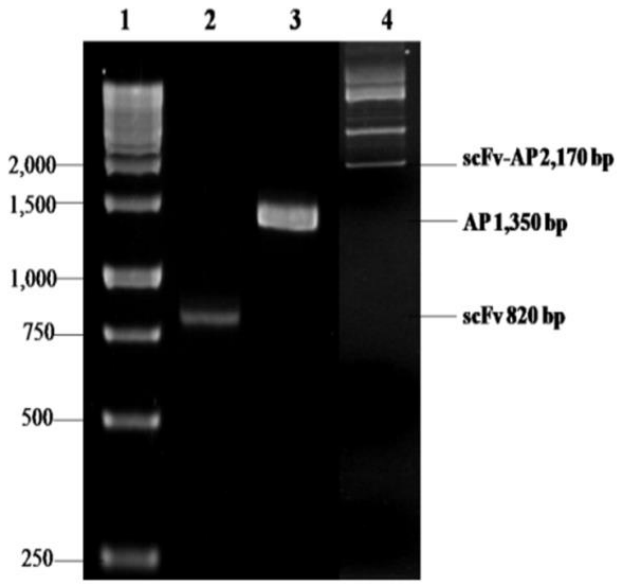

Fig. 1. Gel electrophoretic analysis of PCR fragments of scFv gene (lane 2), AP gene (lane 3), digested phagemid clone 22A12 with *Hind*III and *Not*I (lane 4) and molecular weight markers (lane 1).

Production and Characterization of the soluble scFv-AP by native-PAGE and Western Blotting

The insert carrying scFv (clone 22A12) and AP genes produced a DNA band of 2,170 bp by *Hind*III and *Not*I restriction digestion (Fig. 1). In this research, the fusion proteins were produced based on V_L-V_H construct; however, there is no consistent rule on the effect of direction, but rather it depends on the genes (Hu *et al*., 2005). The expressed recombinant fusion proteins provided a protein band of 72 kDa (Fig. 3). The purified protein from gel filtration chromatography (24 fractions) showed a catalytic activity of alkaline phosphatase in the fractions no. 4-18 by DIBA compared to phage scFv-AP and lysed cell extract of HB2151 strain.

Comparison of three scFv formats to whole molecule MAb on the detection of AFB_1 by plate-trapped antigen ELISA (PTA-ELISA)

After the successful generation of phage scFv from the recombinant phagemid clone 22A12 in *E.coli* TG1, the catalytic activity of the AP gene was determined by ELISA. Then, scFv-AP fusion proteins were produced by ligating the scFv and AP genes and subsequently transformed into *E. coli* TG1, as well as HB2151 strains. This led to the production of phage scFv-AP and soluble scFv-AP, respectively. Comparison on the efficiency of phage scFv, phage scFv-AP and soluble scFv-AP to the MAb in detection of AFB_1 were carried out by PTA-ELISA. Results showed that the soluble scFv-AP gave the highest $O.D._{405}$, which was comparable to the result obtained from the MAb. However, since the scFv-AP reaction was carried out by the direct method, therefore the consumed working period was reduced by one hour (Fig. 4). Thus, the scFv-AP could be useful and convenient as one-step detection probe for competitive ELISA. Since the scFv-AP was produced by gene fusion, its protein product was very stable and will be benefit for further use in other serological applications.

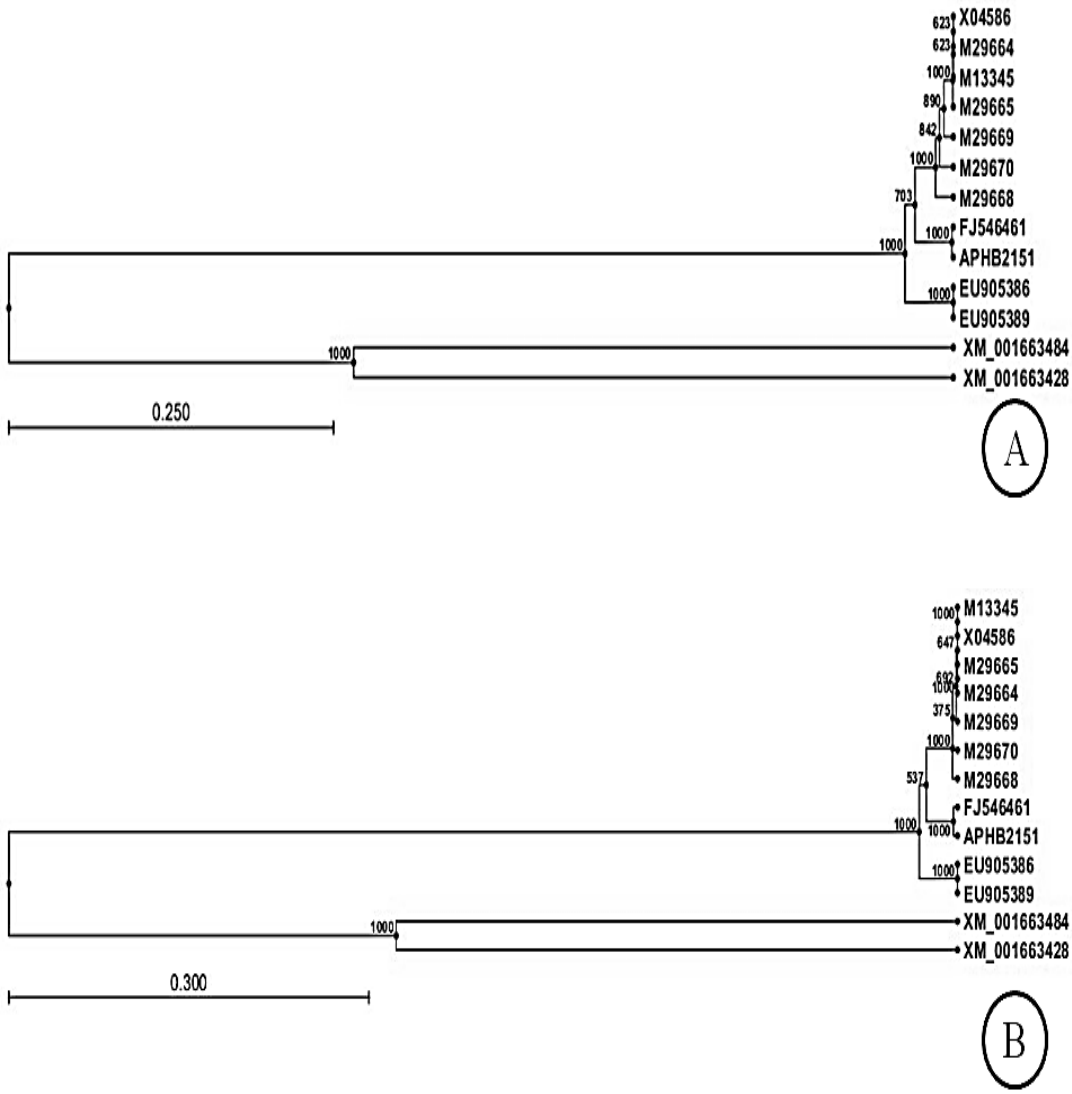

Fig. 2. Phylogenetic trees of alkaline phosphatase gene; nucleotide sequence analysis (A) and amino acid sequence analysis (B), compared with the AP genes from the GenBank databases, using UPGMA method at 1000 bootstrap replicates.

Determination of cross reactivity of the scFv-AP by dcELISA

Cross reactivity of the scFv-AP was determined by dcELISA and the results showed a degree of cross reaction with AFB_2, AFG_1 and AFG_2 at 38.6%, 21.2% and 9.6%, respectively. The competitive ELISA also showed promising outcome for using this scFv-AP raised from the naïve mouse library to detect AFB_1. In the previous report (Kuntalee *et al*., 2011), a unique human phage display library was used to generate scFvs to AFB_1 and the cross-reactivity test revealed that the scFv from the recombinant phages clones TomI-F6 cross-reacted with aflatoxins G1> B2 > G2 > M1 (68.75%, 16.92%, 13.75% and 12.94%, respectively), while YM1-C3 scFv could cross react with afaltoxins G1> G2 > B2 > M1 (70%, 29.17%, 26.92% and 0.88%, respectively). In our research, the advantage of cloning the scFv with the AP genes producing phage scFv-AP led to less time consumption in dcELISA, since the substrate could be added right after the addition of the phage scFv-AP.

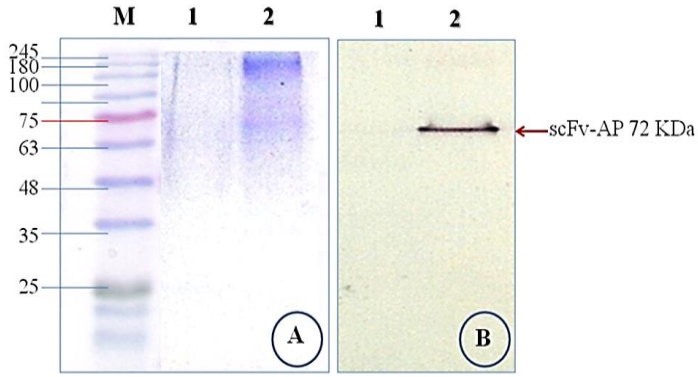

Fig. 3 Analysis of the expressed scFv-AP by native polyacrylamide gel electrophoresis (A) and Western blotting (B). Fraction no.6 from gel filtration chromatography (lane 2) showed strong reaction with alkaline phosphatase substrate compared to the lysed cell of *E.coli* HB2151 (lane 1) and molecular weight markers (M).

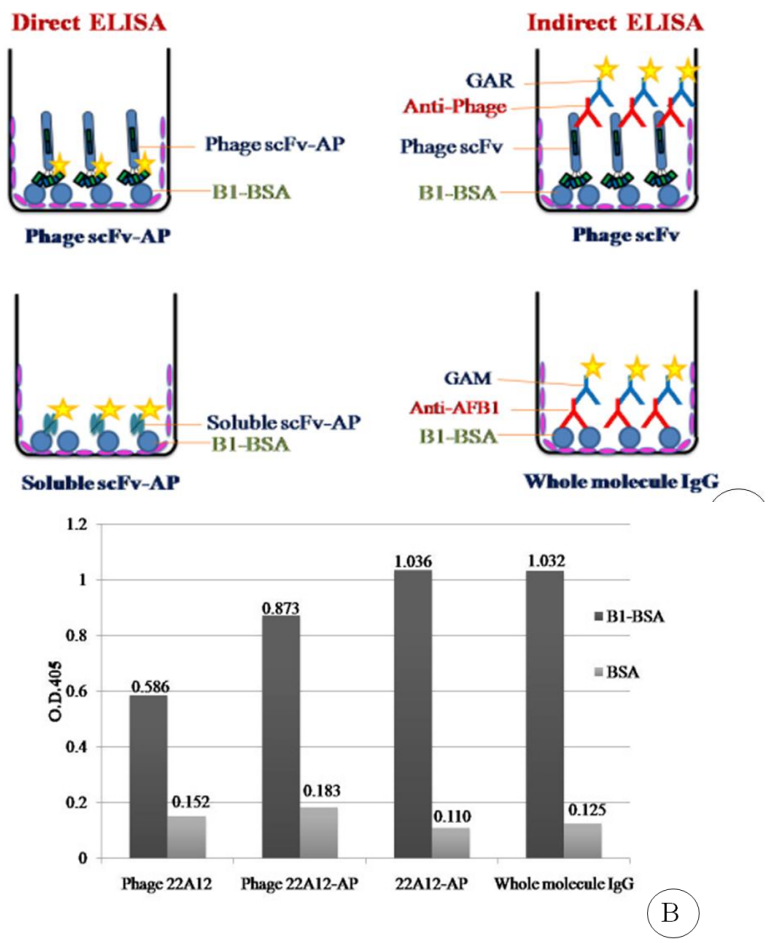

Fig. 4 Comparison on the efficiency of Phage scFv, Phage scFv-AP and soluble scFv-AP to whole molecule MAb for detecting AFB_1 by different ELISA formats (A). Result showed the absorbance values at 405 nm when reacting with AFB_1-BSA, compared to BSA negative control (B).

Determination of AFB_1 contamination in spiked groundnut, corn samples and certified reference material (CRM) by ELISA

When using soluble scFv-AP to analyze groundnut and corn spiked with 100 µg/kg of AFB_1, acceptable results were achieved with 87.02 and 94.41% recovery (Table 1), respectively. Analysis of the CRM (TMAF No.2 and TMAF No.3) showed comparable results with those analyzed by high performance liquid chromatography (Table 2).

Table 1 Analysis of groundnut and corn spiked with 100 µg/kg of AFB_1 by soluble scFv-AP antibody compared to whole molecule monoclonal antibody (MAb).

Sample	Analysis by scFv-AP		Analysis by MAb	
	AFB_1 (µg/kg)*	% Recovery	AFB_1 (µg/kg)	% Recovery
Blank corn	4.44±0.43	-	5.92±0.39	-
Corn spiked with AFB_1 100 ppb	91.46±0.44	87.02±0.07	91.19±0.17	85.27±0.22
Blank groundnut	16.44±0.43	-	18.18±0.55	-
Groundnut spiked with AFB_1 100 ppb	110.85±0.87	94.41±0.45	117.86±0.52	99.68±0.03

Note: * All samples were performed in triplicates and extracted as detailed under materials and methods. ± Standard error of each mean.

Table 2 Analysis of the certified reference materials TMAF No.2 and TMAF No.3 by competitive ELISA with HPLC.

Certified reference material	Total AFT (ppb)		
	scFv-AP/ dcELISA*	MAb/ Indirect C-ELISA*	HPLC**
TMAF No.2	58.25±0.91[a]	58.13±0.57[a]	57.11±1.18[a]
TMAF No.3	137.55±3.51[b]	133.36±0.63[b]	130.22±5.50[b]

Note: * All samples were extracted as detailed under materials and methods and performed in triplicates. ** Determined by Scientific Equipment Center, Kasetsart University Research and Development Institute. ± Standard error of each mean. Values in a column with different letter are significantly different at (P<0.05).

CONCLUSIONS

In conclusion, phage display technology has many advantages over the animal based antibody technology because it is much faster, more robust and consequent advantages are a large volume of antibody, and affinity maturation can be achieved. In our case, the scFv-AP fusion protein was successfully produced from naïve mouse scFv library and its specificity to AFB_1 is acceptable for further application in ELISA-based detection of the toxin contamination, which was focused on agricultural commodity, including corn and groundnut. Comparison of phage scFv, phage scFv-AP and scFv-AP with the whole molecule MAb showed that the efficiency of the scFv-AP was as good as MAb in binding to free AFB_1 in dcELISA. The soluble scFv-AP also gave high satisfaction for the detection of AFB_1 in spiked corn and groundnut as compared with the MAb. The results demonstrated that the soluble scFv-AP produced from this research is useful for developing rapid detection assays. Further study is required for large-scale production, and purification of the anti-AFB_1 scFv, as well as practical

application of detecting mycotoxins in food systems by serological methods, such as ELISA and lateral flow assay.

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EFFECT OF 1-MCP ON ETHYLENE REGULATION AND QUALITY OF APPLE, APRICOT AND ASPARAGUS

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ABSTRACT

The effect of 1-methylcyclopropene (1-MCP) on fruits and vegetables has always been a topic of interest. The current study was conducted to investigate the effect of 1-MCP on ethylene production and on quality of apple (*Malus domestica*) during ripening, of Japanese apricot (*Prunus mume*) harvested at green and yellow stages, of and asparagus (*Asparagus officinalis*) spears. The apple fruit, Japanese apricot fruit, and asparagus spears were treated with 1-MCP for 24 h. Ethylene, CO_2, color, firmness, and upward growth (bending upward when stored at a horizontal position due to negative geotropism) of the asparagus spears were measured sequentially at the laboratory of Tropical Horticulture, Department of International Agricultural Development, Tokyo University of Agriculture (Setagaya campus) from 2014-2015. Ethylene production reduced significantly in apple, was unaffected in Japanese apricot, and increased in asparagus. Respiration significantly decreased in apple and Japanese apricot during green and yellow stages, but no significant changes were observed in asparagus spears. Quality parameters, such as color, firmness and weight loss (apple) have improved in all the tested fruits and vegetable, suggesting that 1-MCP could maintain quality during storage under ambient conditions. Asparagus spears in a horizontal position stopped upward growth when these were treated with 1-MCP, suggesting it could improve asparagus post-harvest quality

Key words: firmness, respiration, upward growth

INTRODUCTION

Fruits and vegetables are important sources of vitamins, minerals, sugar, fiber, and other nutrients. As recommended by the World Health Organization (WHO), an adult needs to consume at least 400 g of fruits and vegetables daily (WHO, 2003). Although Southeast Asian countries have experienced significant economic progress, rapid growth still has not improved the livelihood and nutrition in these regions. These countries still deal with poor nutrition (i.e. deficiencies in carbohydrates, proteins, essential vitamins and minerals) and infectious diseases (AVRDC, 2014-16). To provide a variety of fruits and vegetables throughout the year, there are various activities which deliver adequate nutrients, from the introduction of new vegetable varieties, encouraging traditional vegetable production at home and school gardening to appropriate postharvest technologies (AVRDC, 2016).

On the other hand, maintaining quality of fruits and vegetables is critical, as they are prone to lose postharvest freshness and decay faster than other agricultural commodity. The loss of fruits and

vegetable in Southeast Asian countries are generally high, ranging from 10 to 50% (FAO, 2011). For instance, postharvest loss was reported to range from 10-30 % in Japan, 12-60% in Thailand, 28-42 % in Philippines, and 10 % in Taiwan respectively (FFTC, 1993).

Ethylene is a gaseous hormone, which has been known to promote ripening and accelerate senescence, leading to fruits and vegetables losses (Taiz and Zeiger, 2002). In order to improve postharvest freshness of fruits and vegetables in Southeast Asia, controlling ethylene metabolism with simple and effective methods might be an essential key in providing nutritious commodities for consumers, especially for infants, elderly, and/or reproductive women.

The discovery of 1-methylcyclopropene (1-MCP) as an ethylene inhibitor goes back to the 1980s by Blankenship and Sisler (Blankenship and Dole, 1993). 1-MCP is a gas, which is commercially stored as a stable powder that is dissolved in water to start gas production. It has a higher affinity to ethylene binding sites, 10 times more than ethylene itself, and is released easily. 1-MCP can be a safe and cheap alternative to promote postharvest quality of fruits and vegetables, especially in developing countries.

This research therefore sought to investigate the effect of 1-MCP on ethylene regulation on the quality of apple (*Malus domestica*), Japanese apricot (*Prunus mume*), and asparagus (*Asparagus officinalis*) to provide basic information useful for practical applications.

MATERIALS AND METHODS

Plant materials

The experiments were conducted at Tokyo University of Agriculture (Setagaya campus), under ambient temperature from 2014 to 2015. Apple c.v. "Fuji", Japanese apricot c.v. "Nanko", and asparagus c.v. "UC157" were brought to the Laboratory of Tropical Horticulture, Tokyo University of Agriculture. Forty fresh mature apples (2 treatments with 20 replications for each treatment), 80 Japanese apricot fruits (2 treatments with 10 replications for each treatment during each stage) harvested at green and yellow stages, and 20 asparagus spears (5 for each treatment) were prepared for the experiments mentioned below. Uniform materials were selected by checking initial ethylene production. All plant material was stored under ambient conditions.

1-MCP applications

1-MCP solution was prepared by dissolving 8.184 mg powdered 1-MCP into 1.56 ml of water, then placed into a 6.8-liter container. All experimental materials mentioned above were treated with 1µl L^{-1} concentration of 1-MCP using Smart Fresh® made by Agro Fresh, Inc. for 24 h, while the control was kept without any treatment in airtight chambers, as suggested by Blankenship and Dole (2003), Watkins (2006) and Guillen (2006).

Measurement of ethylene production, CO_2 release, hardness, color, weight, and rising angles

Ethylene and CO_2 production were analyzed in apple, asparagus, and Japanese apricot at 0, 1, 3, 6, 9, 12, 24 h after treatment with 1-MCP. In apples, ethylene and CO_2 production were continuously measured every 5 days by gas chromatography using a flame ionization detector (Shimadzu) and TCD (Shimadzu) using headspace method, respectively. Each material was placed inside a 550-ml glass jar and incubated in the dark for 1 h at room temperature and 1 ml headspace gas was removed using a plastic syringe for analyzing ethylene and CO_2 production. Both GCs were equipped with a Sunpack A column (Shinwa Kako). The parameters were set at: injector 180°C, column 80°C, detector 200°C for ethylene analysis, and at injector 150°C, column 40°C, detector 150°C for CO_2 analysis.

Fruit firmness was measured with a non-destructive firmness meter Multilateral Tester Model 2519-104 (INSTRON), indicating the force (N) required to press the fruit skin randomly (usually in the middle part of the fruit's skin) at 1 mm/sec using a non-destructive Φ 1 cm metal plug. The Multilateral Tester was calibrated with a computer through which firmness was calculated and presented in excel sheet.

A portable colorimeter (NR-3000, Nippon Denshoku Ind.Co.Ltd., Japan) with D65 standards illumination and a 2° standard observer were used to determine color parameters (L = lightness, a = positive values for red color intensity and negative values for green color intensity, and b = positive values for yellow color intensity and negative for blue color intensity). Weight loss for apple was recorded by an electric balance (HF-4000, Japan) from each replication in all treatments at periodical intervals, and cumulative losses in weight were calculated and expressed in percent.

Asparagus spears were usually stored in a vertical position, because spears are prone to upward growth due to negative geotropism when placed horizontally, resulting in a loss of energy and freshness. Considering this, experiments were conducted to see whether 1-MCP could help overcome such a phenomenon. There were four treatments, including a control and a 1-MCP treatment positioned horizontally, and a control and a 1-MCP treatment positioned vertically, with 5 replications each. The 20 spears treated with or without 1-MCP (5 replications in each treatments) were laid horizontally or vertically to check if it affected the rising angle of asparagus when stored horizontally. The rising angle of the asparagus spears was measured everyday using a protractor.

RESULTS AND DISCUSSION

Apple

Ethylene and CO_2 production. CO_2 production suggested that 1-MCP significantly reduced respiration (at 5%) on a continuous basis compared with the control from 1 to 25 days after treatment. Ethylene production in apple decreased with 1-MCP treatment (Fig. 1 A and B). It was significantly different at 5% from 3 h to 25 days after continuous treatment, compared with a control (Fig. 1 A). Similarly, treating apple fruit with 1-MCP reduced respiration significantly than the control at one day after treatment (Fig.1 B). Experimental results show that ethylene production in Fuji apple fruits has dramatically reduced upon treatment with 1-MCP, compared with a control.

Ethylene production was dramatically reduced in Fuji apple fruits treated with 1-MCP, as compared with a control. Usually, lower ethylene production is reported in different cultivars of apple fruits, including Gala, Fuji, Golden delicious, McIntosh, Granny Smith, Red Chief Delicious, Law Rome, Jona gold, and Empire as pointed out by Valero and Serrano (2010). We can say that 1-MCP has different effects on ethylene biosynthesis, depending on variety and stage of fruit and vegetables. Results obtained are in accordance with other studies, which showed the influence of 1-MCP on ethylene biosynthesis through feedback inhibition (Blankenship and Dole, 2003).

The effect of 1-MCP on apple respiration suggests that treatment with 1-MCP might constantly decrease respiration (CO_2), due to a decrease in ethylene sensitivity caused by inhibition of ethylene binding to its receptors by 1-MCP. The affinity of 1-MCP is 10 times greater than ethylene. Thus, the respiration of the fruits and vegetable tested were all inhibited, regardless of any positive and negative change in ethylene production upon 1-MCP treatment, since 1-MCP inhibits ethylene by blocking its receptors. Usually, lower ethylene production is reported in different cultivars of apple fruits, including Gala, Fuji, Golden delicious, McIntosh, Granny Smith, Red Chief Delicious, Law Rome, Jona gold, and Empire as pointed out by Valero and Serrano (2010).

Color, firmness and loss in weight. A significant weight loss (at 5%) in fruits treated with 1-MCP was observed from 5 to 25 days after continuous storage, compared with a control. Although apple

was harvested at maturity, a delay in color change was still observed in fruits treated with 1-MCP at 1 or 5% compared with untreated fruits (Fig. 1; C, D and E) at 1, 2, and 3 weeks after treatment as compared with a control. However, there were no significant differences between control and 1-MCP treated fruits at 4 and 5 weeks of storage (Fig. 1; C). The results on firmness showed that 1-MCP-treated fruits were significantly harder than untreated fruits at 1 to 25 days after treatment (Fig. 1 D).

In addition, the inhibition of ethylene production and its action by 1-MCP was found to cause some changes in quality parameters, such as color, firmness and loss in weight.

Color change is an important quality for customers, and several researches pointed out the effectiveness of 1-MCP on the color of apple, pear, green plum, kiwifruit, and avocado (Valero and Serrano 2010). Our results also support the same tendency, but the precise mechanism of 1-MCP on anthocyanin or carotenoid pigmentation, as well as on chlorophyll decomposition might be studied further.

Fruit softening in apple could be prevented or delayed by 1-MCP treatment. The softening process is closely associated with ethylene production (Valero and Serrano, 2010). In plum, the activity of exo-PG and EGase was lower when treated with 1-MCP (Watkin, 2006). Similarly, the effectiveness of 1-MCP on apple firmness in our experiment is consistent with previously published reports by Mir *et al.* (2001), Fan *et al*. (1999), Rupasinghe *et al.* (2000), and Watkins *et al*. (2000).

Our results suggest that 1-MCP prevents weight loss in apple. The effect of 1-MCP on weight loss has been reported in tomato (Guillen *et al*. 2007), plum (Valero *et al*. 2003), and avocado (Watkin 2006).

Japanese apricot

1. Green stage

Ethylene and CO_2: There was no significant difference in ethylene production between fruits during green stage (Fig. 2; A). Respiration records showed that fruits treated with 1-MCP significantly produced less CO_2 than the control (Fig. 2; B) from 48 to 72 h.

In case of Japanese apricot, however, our records show that there was no significant difference in ethylene production between 1-MCP treatment and control during both green and yellow stages of harvest. For tomato fruit, ethylene production in young green fruit was inhibited, but was promoted in mature pink tomato by 1-MCP treatment, indicating that 1-MCP blocks either the self-promoting effect of system I ethylene or self-inhibiting effect of system II ethylene (Poyesh *et al*. in press). The results obtained are in accordance with other studies, which showed the influence of 1-MCP on ethylene biosynthesis through feedback inhibition (Blankenship and Dole, 2003). Martinez-Romero *et al.* (2007a) reported that the effect of 1-MCP on stone fruits generally depended on the dose of 1-MCP application, so the dose-response curve should also be considered.

Data on the effect of 1-MCP on Japanese apricot respiration suggests that 1-MCP treatment may constantly decrease respiration (CO_2). This may be due to the decrease in ethylene sensitivity by the inhibition of ethylene binding to its receptors by 1-MCP. Abdi *et al.* (1998) also reported the reduction in CO_2 in plum while Watkin (2006) reported a reduction in respiration of apple fruit due to 1-MCP treatment.

Color and firmness: Delay in color change was significantly obvious (at 5%) in 1-MCP treated fruit from 24 to 72 h of continuous treatment, compared to a control (Fig. 2; C). Data related to firmness showed that 1-MCP could prevent softening of fruits, as 1-MCP treated fruits were firmer than

untreated ones (Fig. 2; D). Firmness values were significantly higher (at 5%) in fruits treated with 1-MCP than a control from 24 to 96 h of continuous treatment.

2. Yellow stage

Ethylene and CO_2: Results from ethylene production showed no significant difference between yellow fruits treated with 1-MCP and untreated fruits throughout the study (Fig 2; E). However, fruits treated with 1-MCP produced less ethylene than the control, but was not significant. On the other hand, CO_2 production was significantly less in yellow fruits treated with 1-MCP at 0, 6, 12, and 48 h after treatment while at 72 h; 1-MCP treated fruit produced significantly higher CO_2 compared with a control (Fig. 2; F). Respiration decreased due to 1-MCP treatment up to 48 hours after treatment.

Our records show that there was no significant difference in ethylene production between 1-MCP treated Japanese apricot and untreated ones during both green and yellow stages of harvest.

Color and firmness: Data on color change revealed no significant difference between Japanese apricot during the yellow treated with 1-MCP a control under ambient conditions (Fig. 2; G). However, fruits treated with 1-MCP exhibited a slight delayin color change than a control based on data at 72 h after treatment. The data related to the softening of Japanese apricot at yellow stage revealed that fruits treated with 1-MCP were significantly firmer (at 5%) than the control at 24 to 96 h after storage (Fig. 2; H).

Asparagus

Asparagus spears are transported and stored in a horizontal position. Since it rises up when it positioned horizontally, this can cause problems. Earlier research on rice seedlings showed a positive relationship between rice ethylene production with an increasing propensity towards horizontally placed seedlings (Koshio *et al*. 2010). Hence, asparagus spears were treated with 1-MCP and positioned horizontally and vertically.

Ethylene CO_2 production: Ethylene production from both horizontally and vertically positioned spears was significantly higher (at 5%) than a control from 3 to 96 h after treatment (Fig 3;A and B). No significant differences in CO_2 production between asparagus spears treated with 1-MCP and a control, positioned either horizontally or vertically (Fig 3; C and D).

Since the asparagus spears were at a mature stage, during the present experiment, 1-MCP might block self-inhibition of ethylene production to promote ethylene production. We can say that 1-MCP has different effects on ethylene biosynthesis, depending on the variety and stage of fruit or vegetable. The results obtained are in accordance with other studies, that show the influence of 1-MCP on ethylene biosynthesis through feedback inhibition (Blankenship and Dole, 2003). Results are somehow in accordance with the study of Zhang and Zhang (2007), indicating that the quality of asparagus improved after the 1-MCP application, with a reduction in respiration.

Upward growth: Asparagus spears treated with 1-MCP had significantly slower upward growth (at 1%) than the control from 24 to 96 h after treatment. In this case, upward growth refers to the plant bending upward once stored in a horizontal position due to geotropism.

The effect of 1-MCP on asparagus spears suggests that 1-MCP could affect its upward growth when placed in a horizontal positions. In the case of rice seedlings, Koshio *et al.* (2010) reported a positive relationship between rice ethylene production and upward growth of horizontally positioned seedlings. The involvement of ethylene production in expressing negative gravitropism of rice and other plants was also mentioned by Abe *et al.* (1998), Blancaflor and Masson (2003), Horton

(1993) and Lu *et al*. (2001). 1-MCP might be useful in preventing asparagus bending and lowering respiration. Hence, our results suggest that 1-MCP could preserve quality by blocking ethylene receptors, since it blocks the action of ethylene, rather that production. A similar mechanism was reported by Sisler and Blankenship (1996) and Blankenship and Dole (2003).

CONCLUSION

Our experimental results demonstrate that in apple, Japanese apricot and asparagus, 1-MCP could improve some quality-related parameters. 1-MCP is approved by the European Union as non-toxic in 2005, by the Environmental Protection Agency (EPA) for use on ornamentals in 1999, and on edibles in 2002, followed by approval by Japan and more than 40 countries by 2010. Current experimental results demonstrate that 1-MCP could prolong storage of tested fruits and vegetables, helping farmers, traders and consumers in Southeast Asian countries to overcome the postharvest problems of their products, especially when cold storage and other facilities are not available.

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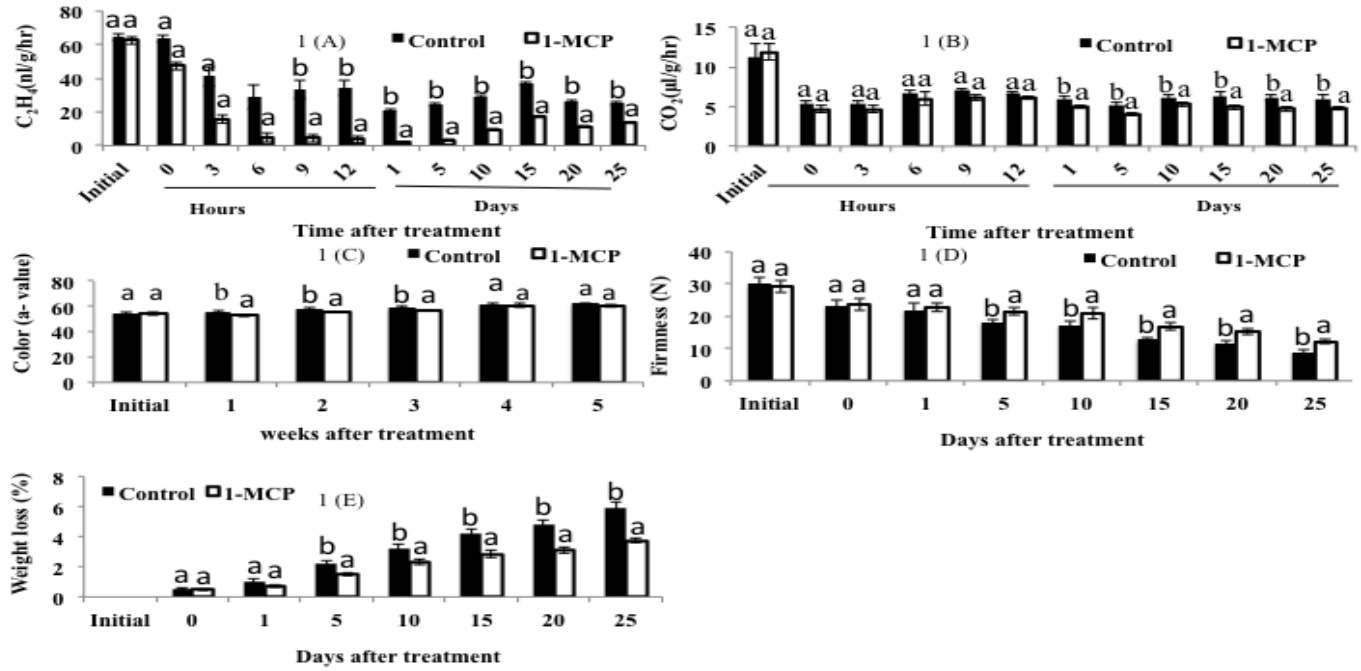

Fig. 1. Effect of 1-MCP on ethylene 1(A), respiration 1(B), color 1(C), firmness 1(D) and weight loss 1(E) on apple. Results represent means ± SE (n=10). Values with different superscripts indicate significant difference ($p < 0.05$) using Tukey test.

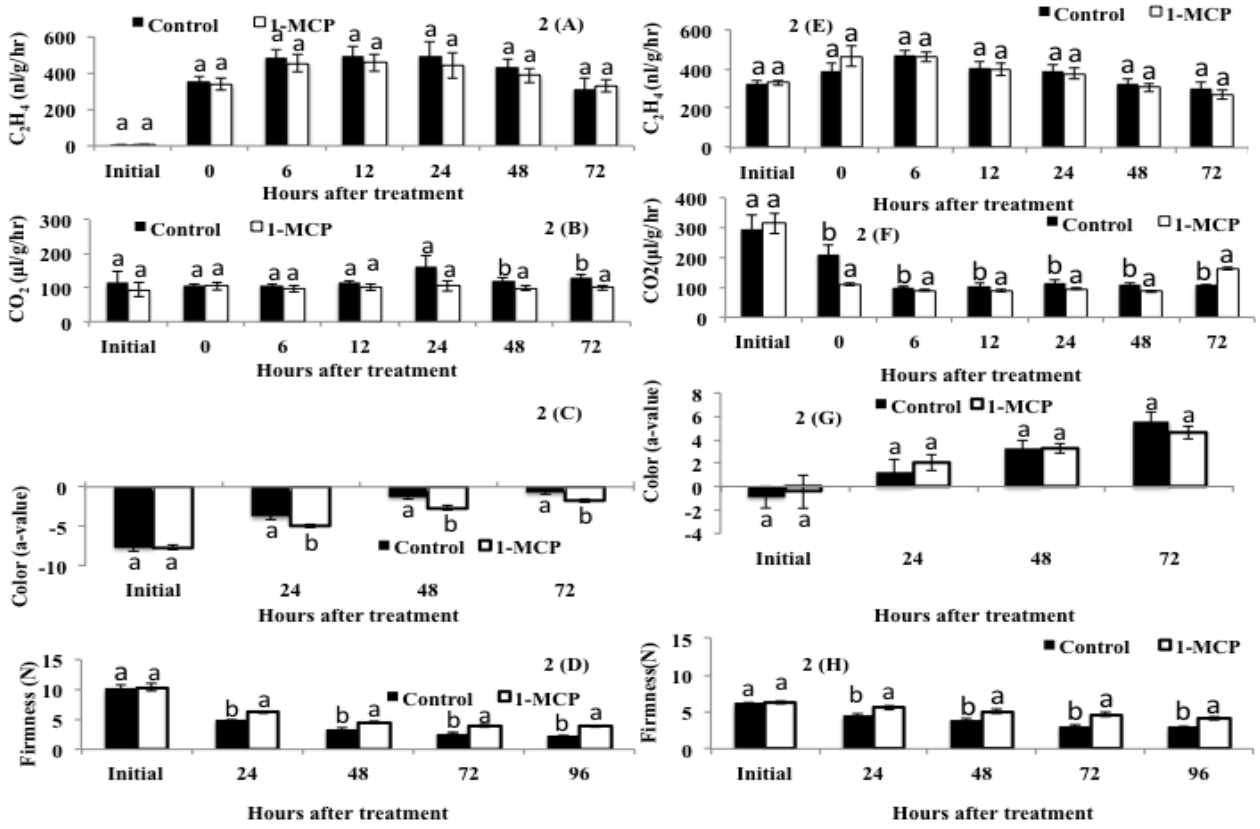

Fig. 2. Effect of 1-MCP on ethylene production 2 (A, E), respiration 2 (B, F), color 2 (C, G) and firmness 2 (D, H) on green and yellow stages of Japanese apricot. Results represent means ± SE (n=10). Values with different superscripts indicate significant difference ($p < 0.05$) using Tukey test.

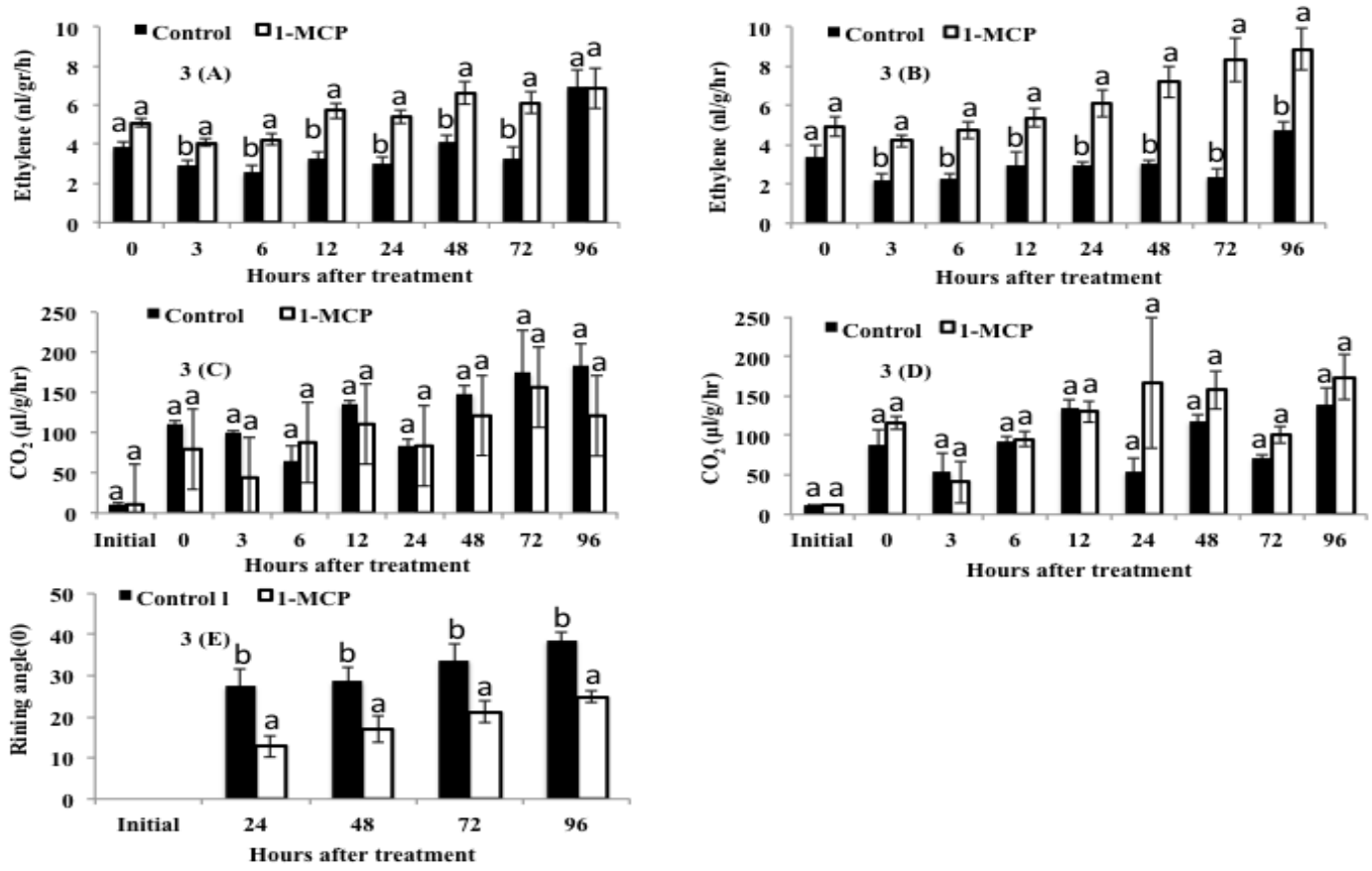

Fig. 3. Effect of 1-MCP on ethylene 3 (A, B), respiration 3 (B, C) of vertically and horizontally positioned asparagus spears and the rising angle of asparagus when horizontally positioned. Results represent means ± SE (n=10). Values with different superscripts indicate significant difference ($p < 0.05$) using Tukey test.

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SYNERGISTIC ACTIVITY OF *PIPER ADUNCUM* FRUIT AND *TEPHROSIA VOGELII* LEAF EXTRACTS AGAINST THE CABBAGE HEAD CATERPILLAR, *CROCIDOLOMIA PAVONANA*

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ABSTRACT

One of the strategies for increasing the biological activity of botanical insecticides is through the synergistic mixtures of plant extracts. In order to assess the insecticidal joint action of both *Piper aduncum* (Pa) fruit and *Tephrosia vogelii* (Tv), leaf extract mixtures were assayed on the cabbage head caterpillar, *Crocidolomia pavonana*. This study was conducted at Bogor Agricultural University, Indonesia, from October 2010 to August 2011. Pa fruit and Tv leaf powder were extracted using ethyl acetate (1:8 w/v) through maceration done four times for Pa fruit, and three times on Tv leaf. Pa and Tv extracts, and the mixture of both extracts were assayed through leaf-feeding of the second-instar larvae of *C. pavonana* with a 48 h feeding treatment. The mixtures were tested at three concentration ratios, i.e. 1:5 (Pa:Tv), 1:1, and 5:1 (w/w). Based on a comparison of LC_{50} at 72 h after treatment, mixtures of both *P. aduncum* and *T. vogelii* extracts at ratios of 1:5, 1:1, and 5:1 were 3.0, 3.1, and 4.3 times more toxic than *P. aduncum* extract, respectively. Meanwhile, the same mixtures of both plant extracts at the same ratios of 1:5, 1:1, and 5:1, was 2.4, 2.5, and 3.4 times were more toxic than *T. vogelii* extract, respectively. Based on the independent joint action model, *P. aduncum* and *T. vogelii* extract mixtures at the three concentration ratios showed a strong synergistic effect on *C. pavonana* larvae, at both LC_{50} and LC_{95} levels, in which the 5:1 mixture was the most synergistic. Thus, the use of synergistic Pa and Tv extract mixtures was more effective than individual Pa or Tv extracts in controlling *C. pavonana*.

Key words: botanical insecticides, cabbage pest, extract mixtures, synergism, tropical plants

INTRODUCTION

Endowed with rich botanical resources, Indonesia is a source of plants purportedly possessing insect control properties. Insecticidal preparations from plants, or botanical insecticides, are biodegradable and mostly safe to non-target organisms (Prakash and Rao, 1997). Because of such, they can be compatibly incorporated into an integrated pest management program. Moreover, the increase in organic farming practices, which precludes use of synthetic insecticides, has brought back the demand for botanical insecticides (Isman, 2006). Society's heightening awareness on food safety has also increased the need for safe and biodegradable insecticides, including botanicals, which are expected to leave only negligible amounts of insecticide residues, if any, in food (Koivunen, 2013).

A potential source for botanical insecticides is the fish-poison bean, *Tephrosia vogelii* (Leguminosae) (Sunarno 1997). Easily grown in Indonesia, their leaves contain the insecticidal rotenone and other rotenoids, including deguelin and tephrosin (Delfel *et al.*, 1970; Marston *et al.*,

1984). Rotenone is an important botanical insecticide commonly used in crop pest management (Cavosky *et al*., 2011).

Another abundant and potential source for botanical insecticides in Indonesia is the spiked pepper, *Piper aduncum* (Piperaceae) (Jansen, 1999). Bernard *et al.* (1995) reported an active ethanol extract from *P. aduncum* leaves against both the European corn borer, *Ostrinia nubilalis*, and rock hole breeding mosquito, *Aedes atropalpus*. Syahroni and Prijono (2013) reported an ethyl acetate extract from *P. aduncum* fruits exhibiting a strong insecticidal activity against the cabbage head caterpillar, *Crocidolomia pavonana*. A phenylpropanoid compound, dillapiole, was isolated and identified by Bernard *et al.* (1995) as the main insecticidal constituent from a *P. aduncum* leaf extract. A treatment containing dillapiole at a concentration of 0.1 ppm caused a 92% mortality in *A. atropalpus* larvae. Hasyim (2011) also reported that dillapiole was the main insecticidal component from *P. aduncum* fruit extract.

Botanical insecticides are generally applied at relatively high application rates; however, the synergistic mixtures of plant extracts can lower the application rate. Scott *et al.* (2002) reported tertiary and quaternary mixtures of piperamides from *Piper tuberculatum* exhibiting a greater-than-additive activity compared with both single compounds and binary mixtures. A mixture of both *Piper nigrum* extract and pyrethrum showed a synergistic effect on *Drosophila melanogaster*, with a synergist ratio of 11.6 (Jensen *et al*. 2006). Abizar and Prijono (2010) reported that a mixture of ethyl acetate extract containing both *T. vogelii* leaf and *Piper cubeba* fruit (5:9, w/w) was more toxic against *C. pavonana* larvae than separate extracts of both samples, furthermore, the mixture indicated a synergistic action. Also, a mixture of an ethyl acetate extract from *P. aduncum* and a methanol extract containing *Sapindus rarak* fruit (1:10, w/w) exhibited synergistic activity on *C. pavonana* larvae (Syahroni and Prijono 2013). In another study, Lina *et al.* (2013) reported a synergistic action from a tertiary mixture of *Brucea javanica*, *P. aduncum* and *T. vogelii* extract against *C. pavonana* larvae. The joint action of binary mixtures from *P. aduncum* and *T. vogelii* extracts, however, has never been reported.

Dillapiole found in *P. aduncum* inhibits the activity of a xenobiotic detoxifying enzyme called polysubstrate monooxygenase (PSMO) located in the midgut of *O. nubilalis* (Bernard *et al*., 1990). This compound contains a methylenedioxyphenyl moiety, which is common among numerous synergistic insecticidal compounds. It also inhibits the oxidative metabolism of specific insecticides through PSMO, and as such, synergist compounds can retain their insecticidal toxic activity (Bernard and Philogène, 1993). *P. aduncum* extract is thus expected to show a synergistic activity when mixed with extracts from other botanical insecticides, such as *T. vogelii*. This study sought to assess the joint insecticidal action of *P. aduncum* fruit and *T. vogelii* leaf extract mixtures on *C. pavonana* larvae.

MATERIALS AND METHODS

Collection of insecticidal plant materials

T. vogelii leaves were collected from the Agropolitan Area (6°43’23” S, 107°0’26” E, 1283 m asl) of Cipanas District, West Java, Indonesia, while *P. aduncum* fruits were gathered from the campus area of Bogor Agricultural University, Bogor, Indonesia. *T. vogelii* leaves were immediately cut to small pieces and air-dried for one week, and were protected from direct exposure to sunlight. Whole *P. aduncum* fruits were also air-dried in the laboratory for one week.

Rearing of test insects

A *C. pavonana* colony was maintained at the Laboratory of Insect Physiology and Toxicology of the Department of Plant Protection, Bogor Agricultural University, following procedures as described by Prijono and Hassan (1992). *C. pavonana* larvae were briefly fed with pesticide-free

broccoli leaves. The adults were provided with a 10% honey solution dipped in a cotton swab. The second-instar larvae were used for bioassays.

Extraction of plant material

After air-drying, both the *T. vogelii* leaves and *P. aduncum* fruit were ground separately using a blender and then sieved through a 0.5 mm mesh. After grinding, 25 g of *T. vogelii* leaves and 25 g of *P. aduncum* fruit were macerated in 200 ml ethyl acetate for 3 h, with the mixture stirred every 30 minutes. The use of ethyl acetate as an extraction solvent was based from our previous studies (Abizar and Prijono, 2010; Syahroni and Prijono, 2013). The extract was filtered with Whatman filter paper no. 41 (diameter 185 mm) and the marc was macerated again using 200 ml of ethyl acetate. The solvent was evaporated using a rotary evaporator set at 50 ºC and reduced pressure. The aforementioned maceration was repeated for two to six times and the whole extraction experiment was replicated three times.

Each extract was weighed, then the percentage yield of plant extract relative to dry weight of extracted plant material was calculated. Extract yield data was transformed to $\arcsin\sqrt{\text{proportion}}$, then the transformed data was analyzed using analysis of variance based on a completely randomized design. The Duncan's multiple range test was used to compare means.

Toxicity tests

Both *P. aduncum* and *T. vogelii* extracts were tested separately and in combination with each other for their toxicity against *C. pavonana* larvae. During preliminary tests, *P. aduncum* fruit extracts were tested at a concentration of 0.1% (w/v) while *T. vogelii* leaf extracts were tested at 0.14% (w/v), with six replications each. All bioassays were done using a leaf-dip feeding method. Each test extract was mixed with methanol, Solvesso R-100 (a light aromatic petroleum solvent, primarily C9-10 dialkyl and trialkylbenzenes), and an emulsifier Tween 80 2-[2-[3,4-bis(2-hydroxyethoxy)oxolan-2-yl]-2-(2-hydroxyethoxy)ethoxy] ethyloctadec-9-enoaate (9:1:5; final concentration 0.96% v/v), then diluted with distilled water to the desired volume. A solution containing distilled water, methanol, Solvesso R-100, and Tween 80 was used as a control.

Fresh broccoli leaf portions (4 cm x 4 cm) were dipped separately into an extract preparation until complete wetness. Control leaves were dipped into the control solution. Treated and control leaves were placed separately into glass petri dishes (diameter 9 cm) placed upside-down and lined with tissue paper, extending up to the space separating the top and bottom of each petri dish. Fifteen freshly-molted second-instar larvae of *C. pavonana* were placed into each petri dish containing either a treated or control leaf portion. Test larvae were allowed to feed on the leaves for 48 h, then were fed with non-treated leaves for the next 48 h. Dead larvae was counted daily up to 96 h after treatment (HAT). Analyses conducted on insect mortality data were the same as for extract yield data.

The number of macerations of both *P. aduncum* and *T. vogelii* extracts which provided the best yield and lethal effect was used for further testing. During bioassays with separate extracts, *P. aduncum* and *T. vogelii* extracts after a specific number of macerations were tested at six concentration levels, which were expected to result in an insect mortality range of 15% to 95%. Extract treatment procedures were the same as the preliminary tests. The number of dead larvae was counted daily until 96 HAT and insect mortality data at 96 HAT were analyzed using the probit method (Robertson *et al.*, 2002-2003).

The *P. aduncum* and *T. vogelii* extract mixtures were tested at three concentration ratios (1:5, 1:1, and 5:1 w/w). Each extract mixture was tested at six different concentration levels, which were expected to result in an insect mortality range of 15% to 95%. Procedures for treatment, insect mortality count, and mortality data analysis were the same as those conducted for separate extracts.

The joint action between *P. aduncum* and *T. vogelii* extracts was determined based on the independent joint action model. This was through calculation of the combination index (CI) at both LC_{50} and LC_{95} levels following Chou and Talalay (1984). The type of joint action for extract mixtures is classified as follows: (1) if CI < 1.00, then the joint action is synergistic; (2) if CI = 1.00, then the joint action is additive; and (3) if CI > 1.00, then the joint action is antagonistic (Chou and Talalay, 1984).

RESULTS AND DISCUSSION

Extract yield and preliminary test results

In general, yields from both *P. aduncum* fruit and *T. vogelii* leaf extracts increased with increasing number of macerations in ethyl acetate solvent. The relationship between yield and number of macerations followed a quadratic regression line with a high determination coefficient (*R*), i.e. 0.899 for *P. aduncum* extract and 0.994 for *T. vogelii* extract (Fig. 1).

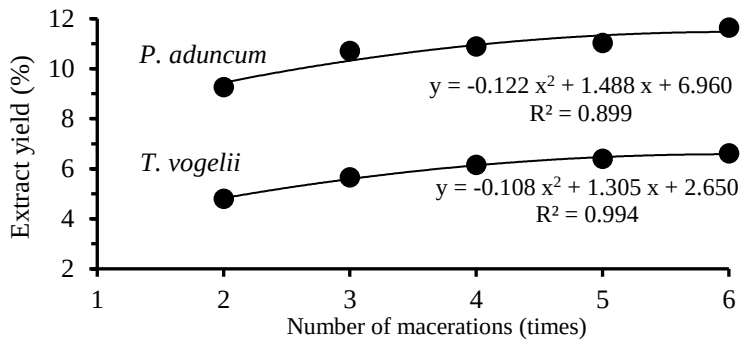

Fig. 1. Relationship between number of macerations and yield from both *P. aduncum* fruit and *T. vogelii* leaf extracts.

Yield from *P. aduncum* extracts was not significantly different among the different number of times macerated. On the other hand, yield from *T. vogelii* extract after macerating six times was significantly higher than after macerating two or three times. Extract yield after macerating four times was significantly higher than after macerating for two times, but there was not significant difference with other maceration treatments, including after macerating six times (Table 1).

Table 1. The effect of the number of macerations on the yield from *P. aduncum* fruit (0.10%) and *T. vogelii* extract (0.14%) and on larval mortality (*C. pavonana*).

Number of macerations	Extract yield (%)[a,c]		Larval mortality (%)[b,c]	
	P. aduncum	*T. vogelii*	*P. aduncum*	*T. vogelii*
2x	9.26 ± 0.99a	4.80 ± 0.26a	33.7 ± 44.7a	45.2 ± 30.3a
3x	10.71 ± 1.94a	5.65 ± 0.57ab	38.5 ± 30.6a	53.0 ± 33.2a
4x	10.89 ± 1.87a	6.15 ± 0.57bc	23.3 ± 33.9a	53.0 ± 36.0a
5x	11.03 ± 1.88a	6.39 ± 0.56bc	24.1 ± 7.2a	38.1 ± 31.6a
6x	11.64 ± 1.89a	6.62 ± 0.31c	17.8 ± 17.2a	45.2 ± 24.1a

[a]Based on air-dried weight of plant material, moisture content of *P. aduncum* fruit and *T. vogelii* leaf powder was 6.89% ± 1.42% and 7.37% ± 2.04% (n = 3), respectively.
[b]Cumulative mortality 96 h after treatment. Extract yield and larval mortality are expressed as mean ± standard deviation.
[c]Means in the same column followed by the same letter are not significantly different according to Duncan's multiple range test (α = 0.05). Data was transformed to arcsin $\sqrt{\text{proportion}}$ before analysis of variance.

Unlike yield from *P. aduncum* and *T. vogelii* extracts which exhibited an increase with number of macerations, *C. pavonana* larvae mortality was not significantly different with increasing number of macerations (Table 1). Low insect mortality was observed for both *P. aduncum* extracts obtained after more than three macerations and in *T. vogelii* extracts obtained after more than four macerations.

A probable explanation for the increase in extract yield and insect mortality with increasing number of macerations is the large proportion of active substances present upon three macerations, while further increasing the number of macerations can increase the yield of inactive substances. It seems that the proportion of active substances from the extracts was lower, and thus, the test insect mortality was also lower, as the number of macerations was done for more than three times for *P. aduncum* extract. Meanwhile, insect mortality was lower after four macerations for the *T. vogelii* extract. Based on yield and test insect mortality data, *P. aduncum* extracts after three macerations and *T. vogelii* extracts after four macerations were used for further testing.

Extract toxicity and synergism

Both *P. aduncum* and *T. vogelii* extracts both represented slow acting toxicants against *C. pavonana* larvae, with *P. aduncum* extract acting slightly quicker than *T. vogelii* extract (Fig. 2). At 24 HAT, larval mortality was generally still low with the *P. aduncum* extract treatment, and negligible in the *T. vogelii* extract treatment. Larval mortality increased sharply from 24 to 48 HAT for both extracts. After the treated leaves were replaced with untreated ones at 48 HAT, larval mortality increased only very slightly from 48 to 72 HAT for the *P. aduncum* extract, but still increased sharply in the *T. vogelii* extract. There was no increase in larval mortality from 72 to 96 HAT in *P. aduncum* extract treatment, while mortality only slightly increased for the *T. vogelii* extract. Thus, larval mortality from both test extracts has reached a constant level at 96 HAT, and during this time, larval mortality increased with extract concentration. Thus, probit analysis was performed against larval mortality data at 96 HAT to determine the quantitative relationship between extract concentration and insect mortality.

Both *P. aduncum* and *T. vogelii* extracts had strong insecticidal activity against *C. pavonana* larva, with LC_{95} values at 0.317% and 0.290%, respectively (Table 2). Based on a comparison between LC_{50} at 96 HAT, *T. vogelii* extract was 1.27 times more toxic on *C. pavonana* larvae than the *P. aduncum* extract. The difference in toxicity between the two extracts was seemingly due to the difference in toxicity and/or amount of active compounds present. The main insecticidal compounds in *T. vogelii* leaves include rotenone, deguelin, and tephrosin, which belong to the rotenoid class (Delfel *et al*., 1970; Marston *et al*., 1984). Rotenone has good insecticidal activity against various insect pests which could act as stomach and contact poison (Yu, 2008). At the cellular level, it acts by inhibiting electron transfer between NADH dehydrogenase and coenzyme Q in the Complex I of the mitochondrial electron transport chain (Hollingworth, 2001), leading to the depletion of ATP as the cellular energy carrier. This eventually immobilizes the affected insects leading to death (Yu, 2008).

Hasyim (2011) reported dillapiole as the main constituent of the most active fraction from a hexane extract of *P. aduncum* fruits, with a GC peak area of 68.8%. That active fraction had strong insecticidal activity against *C. pavonana* larvae, with LC_{95} of about 0.077%. More than 40 years ago, dillapiole was reported to possess a strong insecticidal and synergistic activity against fruit flies, *Drosophila melanogaster* (Lichtenstein *et al.*, 1974). This compound has also been reported as the main active component from *P. aduncum* leaves (Bernard *et al.*, 1995). In addition to dillapiole, myristicin was also present in the most active fraction from the hexane extract of *P. aduncum* fruits, with a GC peak area of 4.9% (Hasyim, 2011). This compound has also long been known for its insecticidal and synergistic activity (Lichtenstein and Casida, 1963).

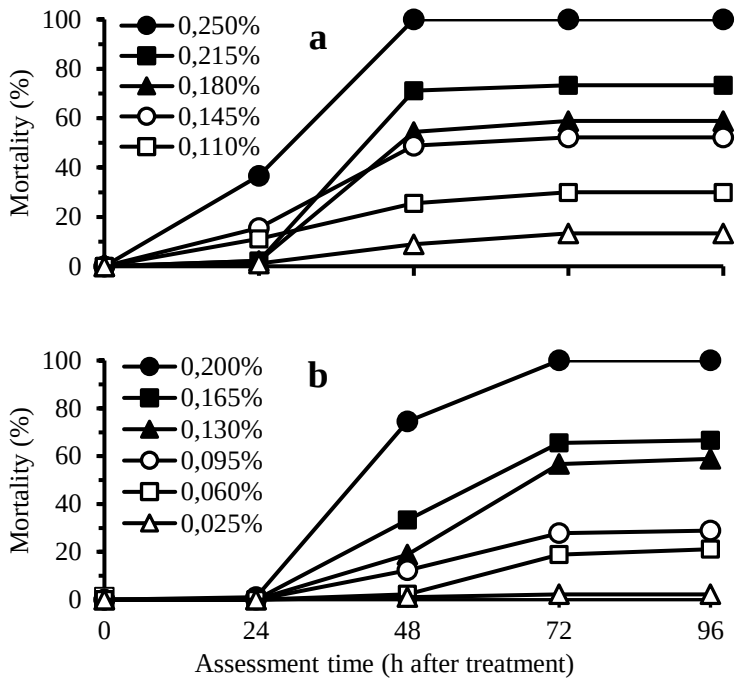

Fig. 2. Time-course mortality of *C. pavonana* larvae in *P.aduncum* (a) and *T. vogelii* (b) extracts. No mortality was observed in control larvae up to 96 h post-treatment.

Table 2. Toxicity of *P. aduncum* fruit, *T. vogelii* leaf extracts, and their mixtures against *C. pavonana* larvae 96 h after treatment.

Extract	$a \pm SE^a$	$b \pm SE^a$	LC_{50} (95% CI)[b] (%)	LC_{95} (95% CI)[b] (%)
P. aduncum (Pa)	3.982 ± 0.322	4.684 ± 0.384	0.141 (0.110–0.173)	0.317 (0.235–0.742)
T. vogelii (Tv)	3.769 ± 0.317	3.949 ± 0.330	0.111 (0.072–0.163)	0.290 (0.186–2.014)
Pa + Tv 1:5	6.896 ± 0.544	5.192 ± 0.417	0.047 (0.037–0.057)	0.097 (0.075–0.190)
Pa + Tv 1:1	6.041 ± 0.418	4.474 ± 0.324	0.045 (0.029–0.059)	0.104 (0.075–0.232)
Pa + Tv 5:1	6.647 ± 0.518	4.494 ± 0.374	0.033 (0.021–0.043)	0.077 (0.057–0.162)

[a]*a* = intercept of probit regression *b* = slope of probit regression, SE = standard error, [b]CI = confidence interval.

Table 3. Type of joint action of mixtures of *P. aduncum* fruit and *T. vogelii* leaf extracts against *C. pavonana* larvae 96 h after treatment.

Extract mixture[a]	**Combination index at**		**Type of joint action at**	
	LC_{50}	**LC_{95}**	**LC_{50}**	**LC_{95}**
Pa + Tv 1:5	0.428	0.344	Synergistic	Synergistic
Pa + Tv 1:1	0.394	0.373	Synergistic	Synergistic
Pa + Tv 5:1	0.254	0.256	Synergistic	Synergistic

Based on the LC_{50} value at 96 HAT (Table 2), mixtures of *P. aduncum* and *T. vogelii* extracts at concentration ratios of 1:5, 1:1, and 5:1 were more toxic than *P. aduncum* extract alone at 3.0, 3.1, and 4.3 times, respectively. Meanwhile, mixtures of both extracts at the same concentration ratios of

1:5, 1:1, and 5:1, were more toxic than *T. vogelii* extract alone at 2.4, 2.5, and 3.4 times, respectively. Based on the LC_{95} values, the respective figures were 3.3, 3.0, and 4.1 times more toxic than *P. aduncum* extract alone, and 3.0, 2.8, and 3.8 times more toxic than *T. vogelii* extract alone. Furthermore, based on the combination indices (CI) at both LC_{50} and LC_{95} levels, *P. aduncum* and *T. vogelii* extract mixtures at the three concentration ratios were synergistic (CI < 1.00) on *C. pavonana* larvae, with the mixture at a ratio of 5:1 (Pa:Tv) being the most synergistic (Table 3).

P. aduncum and *T. vogelii* extract mixtures at the three concentration ratios tested, i.e. 1:5, 1:1 and 5:1, were synergistic against *C. panonana* larvae. It seems that the synergistic property of the extract mixtures was contributed by dillapiole and, to a much lesser extent, by myristicin, which were present in the *P. aduncum* extract (Hasyim, 2011). Dillapiole and myristicin have a methylenedioxyphenyl (MDP) moiety in their respective molecular structures, which is typically present in various insecticide synergists (Bernard and Philogène, 1993). Compounds possessing an MDP moiety can inhibit the activity of polysubstrate monooxygenase (PSMO) enzymes that commonly break down toxic compounds or metabolites in the body (Scott *et al*., 2008), so that the compounds or other simultaneously administered toxins can still exert their action on their targets. In addition, if PSMO is inhibited, various indigenous toxic metabolic wastes will accumulate in the body leading to organismal death (Bernard *et al.*, 1995).

Bernard *et al.* (1990) reported that dilapiole inhibited the activity of PSMO in the microsomal midgut cells from *Ostrinia nubilalis*, in such a way that other insecticidal compounds present in combination with dillapiole were not degraded by PSMO. Likewise, inhibition of PSMO by dillapiole present in *P. aduncum* extracts seemed to retain the toxic activity of active compounds present in the *T. vogelii* extract, so as to impart the synergistic action of both extract mixtures. The greatest synergism between *P. aduncum* and *T. vogelii* extracts (5:1 ratio) could be due to the greatest inhibition of PSMO by dillapiole present in the *P. aduncum* extract, so that active compounds from the *T. vogelii* extract can still interact with the target site.

The synergistic *P. aduncum* and *T. vogelii* extract mixture will need a lower extract concentration or dosage to achieve a certain level of control than the two extracts applied separately. Moreover, the use of extract mixtures may increase their spectrum of activity against target pests. On the other hand, lower extract application rates may reduce the risk of poisoning non-target organisms and the environment in general (Prakash and Rao, 1997). Application of synergistic insecticides at lower rates may also reduce application cost, making it more economical (Stone *et al.*, 1988). The use of synergistic extract mixtures can also be incorporated into an insecticide resistance mitigation program (Scott *et al.* 2003). Furthermore, the use of extract mixtures will decrease the dependance on a single plant species as a source for botanical insecticides (Isman, 2006). Thus, use of a synergistic *P. aduncum* and *T. vogelii* extract mixture is more efficient than either single *P. aduncum* or *T. vogelii* extracts, and the further development of a more synergistic extract mixture is still worth pursuing.

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MARKETING PERFORMANCE OF RUBBER IN NORTHEASTERN THAILAND

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ABSTRACT

The performance of local rubber auction markets and the central network market in Northeastern Thailand was investigated. Data was randomly collected from 320 rubber farmers and 6 middlemen within the two provinces of Northeastern Thailand. Results revealed that most farmers (89.94%) had sold rubber through cooperatives or groups of farmers at local auction markets or rubber network central markets in the Northeast. Marketing costs at the farmer level were found to be 0.0240 USD/kg (rubber sheets) and 0.0179 USD/kg (cup lumps) for marketing channel 1, and 0.0323 USD/kg (rubber sheets) and 0.0250 USD/kg (cup lumps) for marketing channel 2. The local auction trader had a marketing cost of 0.0301 USD/kg (rubber sheets) and 0.0307 USD/kg (cup lumps) for marketing channel 1, and 0.0384 USD/kg (rubber sheets) and 0.0254 USD/kg (cup lumps) for marketing channel 2. Factors positively affecting rubber market participation include education, farm size, membership in farmer groups, access to credit, and distance to a market. Although groups of farmers or cooperatives are important in increasing negotiation with traders in local auction markets and the network central market, groups of farmers would need financial support to manage their rubber bidding. Therefore, the government should provide financial support with lower interests to allow stronger management of farmer groups in the rubber market.

Key words: cooperative, farmer groups, cup lump, marketing margin, marketing cost

INTRODUCTION

In the past decade, rubber trees have been grown predominately in the upper Northeastern areas, rather than in the lower Northeastern regions. The upper area plantations account for 410,120.96 ha (1 rai = 0.16 ha), with a collection area of 102,232.48 ha producing a total of 161,975 tons or an average of 1.58 tons per ha (The Office of Nongkhai Rubber Market, 2015). This has resulted from the expansion of rubber plantations in 2006 which was the government's rubber extension period under the One-Million Rai Rubber Plantation Project. The target was an extension of rubber plantations in 36 provinces from 2004-2006 to cover an area of 48,000 ha in 17 Northern provinces and 112,000 ha in 19 Northeastern provinces. Moreover, rubber prices have been relatively stable and profitable since from 2003, especially in 2011 when the price steeply increased from 1.87 $/kg to 3.31 $/kg, drawing more farmers to grow rubber trees in higher numbers than the target. In this context, the Office of Agricultural Economics (2015) stated the total national tapping area had also increased to 2.21 million ha, which represents an increase of 0.17 million ha or 8.16% from the previous year. The level of productivity per ha was 1.64 tons with an export rate of 2.95 million tons. This was a decrease from the previous year of 3.06 million tons (3.59%) because the primary consumers, such as China, USA, and Europe, were facing economic regression and required less rubber.

As above mentioned, although the trend for Northeastern rubber production is increasing, i.e., from 154,917 tons in 2008 to 504,000 tons in 2015 (The Office of Agricultural Economics, 2015), there are several problems and constraints. This is especially true in the area of marketing where middlemen take advantage of producers with respect to prices owing to the fact that raw rubber material is below the marketing price. Also, problems with low-quality rubber production have been significant, such as the result of dirty contaminants in the raw rubber sheets and an unstable rubber supply required meeting the demand placed on manufacturing factories, as well as the factor of dealing with the low yield per area. From this viewpoint, it is obvious that the problem of marketing also needs to be considered since the middlemen have often given very low prices for raw rubber sheets. Due to the rush of selling rubber cup lumps, farmers accepted all prices offered by traders; thus, the inefficiency of rubber marketing becomes the main problem for smallholder (Zakky, 2009) and the smallholder farmers have small market participation (Barret, 2008). In working towards a timely and sensible solution to these problems, the local rubber auction market of the Office of the Rubber Replanting Aid Fund (ORRAF) could be an alternative choice of a platform where the rubber farmers could sell their products by acting as an interface between buyers and sellers. The local rubber auction market has been set up to solve the problem of low prices by using the same methods as the central rubber market under ORRAF and to reduce marketing costs. Depending on rubber types, the rubber auction market of ORRAF consists of 4 systems, in general. However, the faxing auction market is the most operational in the Northeastern region (Rubberthai, 2014). The local rubber market helps farmers to increase income but not fully helps farmers as a price taker in the rubber market system (Zakky, 2009).

Most of the rubber cooperatives or farmer groups located far from the central rubber market had insufficient information on rubber marketing. This information can be the standard for trading decisions. There were insufficient officers of the central rubber market to publicize the accurate information to the farmers in specific areas. Thus, the network central market in Ubonratchathani province is established to be the trading center as a central network market of rubber which is the option for the farmers to directly access without the middleman. The central rubber market will support marketing devices, academic training, and trading system which is similar to the rubber central network market given that the farmers can sell higher costs of rubber production than the local price (Central rubber market of Burilum, 2015). Therefore, it can be said that with the collection action in market smallholders can reduce the transaction cost for input and output access (Kersting and Wollni, 2012) and can have more a bargaining power in negotiation (Marklova *et al*., 2009). The collective action in term of cooperatives or farmer groups has increased market participation (Jari and Fraser, 2009). However, the clarity of the information about this market system is not well presented, including the distinguishing market types and marketing management. In addition, many studies have been conducted on rubber technology and development. Thus, this study focused on the market performance of the local rubber auction market and the central network market in the Northeast and the estimated costs and returns from rubber marketing, as well as the marketing margin.

METHODOLOGY

In this study, data collection used comprehensive questionnaires for personal interviews by collecting information from the samples. Close-ended questions and open-ended questions were used in the questionnaires. The purposive sampling focused on provinces that had large amounts of rubber and local auction markets: Nongkhai and Ubonratchathani, provinces. The respondents by random sampling consisted of 150 households in Nongkhai province and 170 households in Ubonratchathani province, totaling 320 households and also 6 middlemen. For margin analysis, the wholesale margin is the difference between the sale price and the purchase price. The data was

analyzed by descriptive statistics, marketing costs, and marketing margins. The net market margin refers to the difference between the gross margin and the total cost in that the gross margin is the difference between the sale price and the purchase price (Tomek and Robinson, 1981).

In this study, in order to determine the factors affecting farmers' participation in rubber marketing the logit model was used and the model is specified as:

$$Y = \ln\left(\frac{P_i}{1-P_i}\right) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \varepsilon \quad (1)$$

Where

Y = dependent variable (1 = farmer participate in rubber market, 0 = otherwise)
X_1 = the farmer's gender (0=otherwise and 1=male)
X_2 = education level (years)
X_3 = farm size (ha)
X_4 = membership in marketing group farmer (0=otherwise and 1=member)
X_5 = access to credit (0=No and 1=Yes)
X_6 = distance to the market (km)
ε = the disturbance term

Positive coefficient of the independent variable (X_i) indicated the increasing participation of rubber market including male, higher education levels, access to credit, group membership, distance market, and larger farm sizes (Holloway *et al*., 2000; Conway *et al*., 2005; Hoden and Binswanger, 1998).

RESULTS

Rubber Marketing in Northeastern Thailand

Most of the farmers (89.94%) sold rubber through the cooperatives or farmer groups in the Northeast via the local auctions market. The yields were sold at the assembly point for the rubber and auctioned in order to receive the bidding price. The next day it would then be weighed and the money would be paid to the farmers under the control of ORRAF. On the other hand, the remaining 10.06% of the rubber in the local market was sold to local traders or middlemen. Farmers urgently need money to use for their households. This has led to a practice of not grading the rubber, and as a result, the farmers are being commercially exploited in terms of both purchase price and given weight rise that the farmers are not being treated fairly (Fig.1).

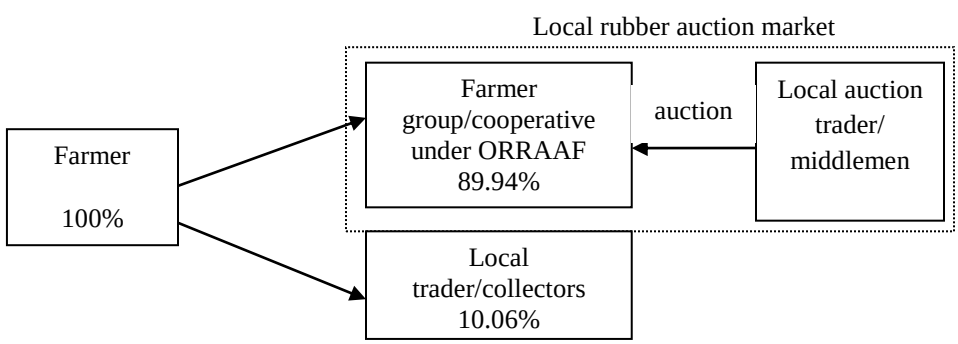

Fig. 1. Rubber marketing channel 1

Rubber Auction Market Systems in Northeastern Thailand

Most farmers sold 89.94% of their rubber to groups of farmers or cooperatives, in the form of rubber sheets (21.42%) and rubber cup lumps (68.52%). Cup lumps were sold through a local auction market, and became an outstanding point for the rubber markets in the Northeast which included a grading process, rubber quality analysis and was under the control of ORRAF. As for the local rubber market auction, the price auction is done via fax or phone. The ORRAF participates in the auction in order to increase farmers' confidence and serves as a mentor to make sure that the farmers do not receive prices lower than the market price. The ORRAF guides the market mechanism, coordinate the buyers, participates in the rubber auction and takes into consideration the welfare of the farmers. Due to the fact that the stability of the rubber prices is quite low, the government has implemented a local auction market project aimed at maintaining the stability of the rubber prices to make sure that the farmers get the fairest prices, but under the project, the farmers have to wait for 45-60 days to get their money. However, the farmers are helped with the following: 1) the weighing the rubber products, 2) participating in the auction venue, and 3) the transporting of the products. Despite the help that they receive and the fact that the prices are higher, this waiting period is a definite disadvantage of the project.

Most of the bidders are factory representatives, who participate in the auctions by trading the rubber. The bidders, who offer the highest price, are the auction winners. After the auction, the rubber is weighed and delivered to those who had won in the bidding. The marketing expenses incurred by the middleman, who came to bid at auction rubber, can also be included in all the costs of transporting the inventory to the warehouse for storage. In the case of cup lumps, farmers must bring cup lumps to the assembly point of purchase one night before in order to reduce the moisture content. Then the bidders are informed that it is time to participate in the auction. When the price is right, it will then be auctioned off. Then it is weighed again on a queue at a later date in order for the bidders to make payments to farmers. However, in the case of the rubber farmers, who have brought rubber sheets to the point of purchase prior to bidding for that day, the bidders have to know the number of rubber sheets before at 10:30 A.M. After that, when the highest bid of the auction has been made, the rubber is randomly checked for quality, and is then later weighed. After which, farmers are paid. The price for the auction from the bidders is based on the reference price from the office of Hat Yai Central Rubber Market. Yet, some middlemen depended on the prices from the local rubber factory.

Selling Through Cooperatives

The rubber cooperative in the Northeast has helped to increase the farmers' power in price bargaining in order to get the fairest deal. In this case, the farmers bring their rubber products, gather them at the rubber assembly point, and wait for an auction under the faxing auction market. The faxing auction market was developed from the general auction market by making improvements and offering greater accessibility. On the auction day, when the auction prices are known, the rubber products will be weighed and payments made to the farmers. In order to increase the farmers' confidence, the ORRAF participates in the auction. The ORRAF can be compared to a mentor who makes sure that the farmers will not receive any price lower than the market price. ORRAF guides the market mechanism by contacting and giving consideration to the buyers participating in the rubber auction. ORRAF is also responsible for the following: 1) contacting and coordinating with the agricultural cooperatives and 2) financing the cooperatives to help them maintain their liquidity and financial effectiveness. Moreover, an important role that ORRAF plays is helping with the accounting and auditing activities of the cooperatives (Figure 1). However, for small cooperatives, the farmers have to wait for 45-60 days to get their money. Although the prices are higher and the farmers are helped both in weighing their rubber products and in the auction venue, the waiting period is a weak point of the local auction market through rubber cooperative.

The PhonePhisai Cooperative in Nong Khai Province covers five areas: the Poanpisai District, the Fao Rai District, the Rattanawapi District, the PakKhat District, and the Beung Gaan district. There are 1,080 members in the cooperative. This cooperative is only opened for the purchase of rubber cup lumps, and the main target groups include farmers, who are members of cooperatives and non-members. About 500 farmer members sell cup lumps to cooperatives. The cooperative participated in rubber purchasing itself, and not through the local auction. The cooperatives have obtained a benefit averaging around 0.0691 $/kg.

The cooperatives have been responsible for providing the following: 1) the rental of transport vehicles for 96.69 $/round {10-wheel vehicle}, 2) a wage for loading rubber at 4.14 $/ton, 3) the rental of the assembly point at 55.25$/round, and 4) food and drinks for the committee at 27.62 $/round.

It opens for buying and selling twice a month. Moreover, with respect to the transportation of the rubber to a factory, it was found that there was a problem causing about 5% loss of weight of the total rubber weight per round. This situation has made it necessary for the cooperative to bear the risk because the quantity of water is not fixed. However, a benefit, given to the farmers who sell cup lumps through cooperatives, is that they are eligible for a special dividend each year: an investment credit for agriculture and an input credit. The main reason the cooperatives have chosen to become entrepreneurs is due to the need to help the member farmers. By doing so, there is a reduced risk of the farmers being exploited by the local middlemen, and furthermore, there is an increase of the marketing effectiveness for cup lumps which can be driven by market mechanisms.

Selling through Groups of Farmers

The buying and selling of rubber through the Wangluang Rubber Group in Nong Khai Province have been operated via local auctions by local merchants. The process of auctioning the cup lumps is not different from the cooperative under the Office of the Rubber Replanting Aid Fund. However, the group's only role is rubber collection in order that the auction process does not have to be financed and farm input support. Therefore, the middleman, who wins the auction, must pay the management cost for the group at around 0.0138-0.0193 $/kg and must also pay for the transportation of the rubber to the factory.

Phu Foi Lom Rubber Community Group as Central Network Market

Buriram central rubber market organized the network central rubber market for the lower northeast which was recently found at Phu Foi Lom rubber community enterprise group of Ubonratchatani province that assists the farmers in the remote area so that they can access the central market for rubber trading.

Phu Foi Lom rubber community enterprise group was found by a group of farmers for unsmoked rubber sheet trading. The network market was located in Kho Laen sub-district, Buntharik district, in 2005, and that later advanced to be the Community Enterprise Group in 2006 with the suggestion from the Faculty of Agriculture, Ubonratchathani University. In addition, the group was also supported by the Agricultural Land Reform Office that formed the group and the register of community enterprise. The Office of the Rubber Replanting Aid Fund, as a mentor, helps to promote the production, plantation, tapping, conservation, and rubber marketing. Unsmoked rubber sheet trading was the main business of the group and later, in 2008, it began to trade the cup lump, wet and dry. Recently, there was only trading of unsmoked rubber sheet and dried cup lump.

The motivation of group establishment was a far distance from the rubber farm to the market place which takes a long time to travel. Sometimes, the farmers have to ask for help from

their neighbors in case they have no car as well as to provide bargaining power to the traders for the fairness. Additionally, the motivation of assembling to be the network market of the central market was to protect and reduce the financial risks, to gain the bargaining power and the income for the farmers, to reduce the difficulty of trading price, and to reduce the transportation cost.

In 2016, there were about 630 members. The group management committee consists of 13 persons: 1 chairperson of the group, 1 vice-chairperson of the group, 1 treasurer, 1 secretary, and 9 committees divided into 1 for marketing, 6 for rubber selection, and 2 for the general committee. The trading was opened from June each year and closed in February of the following year. Recently, the trading capacity is calculated in the average of 50 tons per time, about 200 tons per month. For trading opening that is four times a month, the farmers have to take the rubber for trading on Monday. The auction is held on a Tuesday. A group will send the total weight of the rubber to central rubber market at 10 AM. The rubber is received from member group and sub-network marketing group (Fig. 2). The bidding price is announced at 11AM. The deal price goes to the bidder who offers the highest price. The bidder transfers the money to Buriram Central Rubber Market's bank account. The central market will transfer the money to the network market group. The farmers will receive the money on Wednesday through the bank. Regarding, the payment charge includes the cost of group management and divided to be the group saving money in the amount of 0.0193 $ that was managed as the committee allowance, public utility, and other expenses occurred during the period of trading. The other 0.0138 $ will be the saving of the group and allocated as compensation to the members.

There were several reasons for farmer joining the group. Firstly, there was to increase the income with the percentage of 42.1. Secondly, the group can facilitate farmers in purchasing production factors in a form of credit. With the percentage of 17.5, the farmers are able to increase the selling of products. From the above statement, price motivates the farmers to expand the production area for the unsmoked rubber sheet quantity to increase the household income. As members of the group, the farmers can find the funding source by gaining the production factors such as fertilizer and working capital through credit. The farmers are able to sell the quality products to fill the market demand that leads to the fairness of price and weighing.

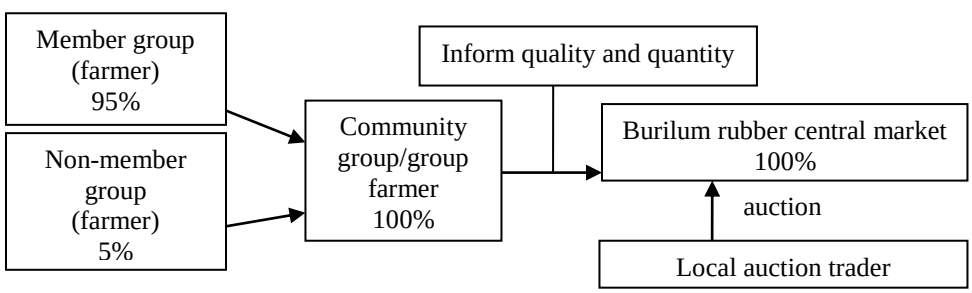

Fig. 2. Rubber marketing channel 2: Phu Foi Lom Rubber Community Group

Marketing Costs of Rubber Farmer and Local Traders for Rubber Sheets and Cup Lumps

At the farmer's level, the marketing costs were found to be 0.0240 $/kg and 0.0179 $/kg of the marketing channel 1 and 0.0323 $/kg and 0.0250 $/kg the marketing channel 2, for rubber sheet and cup lump respectively. The cost of marketing for rubber sheets was higher than the cost for the cup lumps at around 0.0061 $ and 0.0073 $ for the marketing channel 1 and 2 respectively. Also, it was found that the cost of transportation from the farm to the cooperative for rubber sheets was higher than the cup lumps (around 0.0058 $/kg for the marketing channel 1 and 0.0050 $/kg for the marketing channel 2) because the farmers needed to transport the product several times in the case

of larger amounts and needed to transport to several places in order to sell the product (Table 1). On the contrary, the local auction trader had a marketing cost of 0.0301 $/kg and 0.0307 $/kg of the marketing channel 1, 0.0384 and 0.0299 $/kg of the marketing channel 2 for rubber sheets and cup lumps, respectively. The highest cost of rubber sheets and cup lumps is the transportation cost from farm to cooperative/group, which is the highest for both marketing channels (Table 2).

Table 1. Marketing cost of rubber farmer for rubber marketing channel 1 and 2 (unit: $/kg)

Items	Mean	
	Marketing channel 1	Marketing channel 2
Marketing cost for rubber sheet	**0.0240**	**0.0323**
-Labor cost	0.0100	0.0138
-Transportation cost from farm to cooperative/group	0.0135	0.0171
-Telephone cost	0.0005	0.0014
Marketing cost for cup lump	**0.0179**	**0.0250**
- Sack	0.0053	0.0066
- Labor cost	0.0047	0.0061
- Transportation cost from farm to cooperative/group	0.0077	0.0121
-Telephone cost	0.0002	0.0002

Note: 1$ = 36.2 Baht

Table 2. Marketing cost of local auction trader for rubber marketing channel 1 and 2 (unit : $/kg)

Items	Mean	
	Marketing channel 1	Marketing channel 2
Marketing cost for rubber sheet	**0.0301**	**0.0384**
- Loading cost	0.0041	0.0022
-Cooperative/group service fee	0.0111	0.0193
-Transportation cost from farm to cooperative/group	0.0149	0.0169
Marketing cost for cup lump	**0.0307**	**0.0299**
- Loading cost	0.0050	0.0036
- Cooperative/group service fee	0.0111	0.0111
- Transportation cost from cooperative/group to factory	0.0146	0.0152

Additionally, the local auction trader purchased rubber sheets at an average price of 1.2486 $/kg for the marketing channel 1 and 1.2431 $/kg for the marketing channel 2 while at a selling price of about an average 1.3259 $/kg for both the marketing channels. The gross marketing margin was 0.0773 $/kg and 0.0828 $/kg for the marketing channel 1 and 2, respectively, and the marketing costs were 0.0301 $/kg and 0.0384 ha/kg for the marketing channel 1 and 2, respectively. Thus, the net marketing margin was 0.0472 $/kg and 0.0444 $/kg for the marketing channel 1 and 2, respectively.

For cup lumps, the purchasing and selling price was an average of 0.5829 $/kg and 0.6354 $/kg for the marketing channel 1 and 0.5801 and 0.6354 $/kg for the marketing channel 2, so the gross margin was 0.0525 $/kg and 0.0553 $/kg for the marketing channel 1 and 2, respectively

resulting in the receipt of a net marketing margin of 0.0218 $/kg and 0.0254 $/kg for the marketing channel 1 and 2, respectively. On the other hands, farmers received a real price equal to 1.2246 $/kg of the marketing channel 1 and 1.2108 $/kg of the marketing channel 2 for rubber sheet and 0.5650 $/kg for the marketing channel 1 and 0.5551 $kg for the marketing channel 2 of cup lumps (Table 3). Obviously, regarding local auction trading for cup lumps, a low purchasing price was offered at auction due to the fact that there was no quality standard to help in determining a price. Traders would evaluate cup lumps based on the percentage of moisture (an average of 45%) in the rubber, and if cup lumps were significantly dirty or contaminated, the traders would deduct 0.1381-0.2762 $/kg depending on the amount present. Most farmers in the northeast produce cup lumps with contaminants which is cheaper. Meanwhile, the rubber sheet price from traders is not different from the market price because the quality standards of the rubber sheets have already determined the price. Moreover, both auction prices in rubber sheets and cup lumps have a lower price from traders due to collusion among the traders which led to lower incomes for the farmers.

Table 3. Marketing margin of local auction trader for marketing channel 1 and 2 (unit : $/kg).

Items	Marketing channel 1		Marketing channel 2	
	Rubber sheet	**Cup lump**	**Rubber sheet**	**Cup lump**
Local auction trader				
Local rubber price (Selling price)	1.3259	0.6354	1.3259	0.6354
Auction rubber price (Purchasing price)	1.2486	0.5829	1.2431	0.5801
Gross marketing margin	0.0773	0.0525	0.0828	0.0553
Marketing cost for local trader	0.0301	0.0307	0.0384	0.0299
Net marketing margin	**0.0472**	**0.0218**	**0.0444**	**0.0254**
Farmer				
Auction rubber price	1.2486	0.5829	1.2431	0.5801
Marketing cost for farmer	0.0240	0.0179	0.0323	0.0250
Real price for farmer	**1.2246**	**0.5650**	**1.2108**	**0.5551**

Factors affecting participation in collective marketing in rubber

Around 56.3% of households are male. Most head households (50%) have the primary level of school education. The average length of education is 7.9 years. The mean farm size of rubber cultivation is 3.39 ha. The majority (64.7%) of households were members of rubber marketing group farmer. Farmers who had access to credit represented 63.4% in their marketing group farmer. The average of distance from the cultivated area to market is 7.4 km. (Table 4).

A chi-square of 140.22 percent at 5 percent level of significance that indicates a good fit to data and the log likelihood was 275.37 (Table 5). The Nagaelkerke R-square was 0.49 indicating percentage of relationship between the predictors and the predation. The variables included in the estimation were farmers' gender, education level, farm size, membership in farmer groups, and access to credit and distance to the market. The intensity of participation in the market increases with the level of household education, farm size, membership in farmer groups, and access to credit. But, the distance to the market reduces the marketing participation. Gender of households (X_1) had a positive but not significant effect: males are more likely to participate in rubber sale in marketing group.

Table 4. The characteristics of farm households participating in rubber marketing.

Variable	Mean	Standard Deviation	Percentage
Rubber marketing participation (Y)			
0 = otherwise			39.4
1 = farmers participate in rubber market			60.6
Farmer gender (X_1)			
0 = otherwise			43.8
1 = male			56.3
Education in year (X_2)	7.9	4.02	
Farm size (X_3)	3.39	4.04	
Membership in marketing group farmer (X_4)			
0 = Otherwise			35.3
1 = member			64.7
Access to credit (X_5)			
0 = No			36.6
1 = yes			63.4
Distance to the market (X_6)	7.4	7.5	

Table 5. Factors affecting a probability of marketing participation.

Variable	Coefficients	SE	Exp(B)
Constant	-1.473^{***}	0.429	0.229
X_1	0.228^{NS}	0.313	0.796
X_2	0.033^{**}	0.021	0.967
X_3	0.029^{**}	0.054	0.901
X_4	0.556^{*}	0.314	1.743
X_6	-0.044^{*}	0.025	1.045
Overall Percentage	82.2		
Chi-square	140.22^{**}		
-2log likelihood	275.37		
Nagelkerke R square	0.49		

^{NS} non-significance, *** significant at 1 %, ** significant at 5 %, * significant at 10 %

Education of household was found to have a positive and significant effect ($p< 0.05$). More educated farmers are more likely to increase the probability of participating in the market by 22.8%. The finding is in line with a study by Martey *et al*. (2012), Enete and lgbokwe (2009) but contrary to the finding of Makhura *et al*. (2001), Musah *et al*. (2014). An illiterate farmer may find it difficult to communicate with a trader and to sell in a distant market (Mawazo *et al*., 2014). Accordingly, farm size is positively related to a probability of participating in farmers marketing group. A larger farm size has a greater amount of rubber yield to sell in this group. Membership in farmers marketing group positively and significantly ($p<0.10$) impacted the amount of rubber yield sold in the market similar to Sebatta *et al*. (2014). Access to credit is positively associated with the intensity of participating in rubber marketing group. It is consistent with Lerman (2004) and Musah *et al*. (2014). The distance to the market was significant at 10 percent level with negative effect. A non-further distance from the field to the marketing group would increase a probability of market participation (Table 5). The findings are consistent with Gicheha *et al*. (2015), Gani, 2011 but contrary to the finding of Sebatta *et al*. (2014) and Bartha and Bauer (2007).

DISCUSSION

Farmers, who are members of a farmer group/cooperative, had greater confidence in terms of the rubber market and its prices because the ORRAF and the rubber central market had helped them to get fair prices and to improve their bargaining power. As a result of this, the rubber farmers have received better selling prices. Similarly, in Indonesia, the auction market can give a basic price through the provision of the local government related to the price of rubber (Zakky, 2009). In contrast, in the case of selling as an individual farmer, the farmer may be treated unfairly in terms of prices and the weighing of the rubber products. Thus, it can be said that within this kind of auction market mechanism, the farmers have the most advantages. However, despite using the rubber local auction markets, the small groups and those that are located far away would get a lesser auction price than the cooperative because the traders often refer to the transportation cost as one of many factors influenced marketing margin (Hadi, 1994). Sometimes there are only traders who are bidding, and as a result, that fact forces farmers to have to decide whether or not to sell their produce to traders at a lower auction price. Under cooperatives, the marketing of rubber is on an economic scale, influenced by the collection of rubber products and group marketing, and can lead to having more bargaining power in order to obtain a fair price for the rubber farmers (Suni, 2012). Yet, in fact, through ORRAF and rubber central market staff, the auction price process must be maintained for trading among these groups. However, sometimes no staff members have been available to help the groups because there are insufficient numbers of staff members to supervise the rubber auction process in several places at the one time. Thus, the government employs more officers, who help and manage in auction marketing process, in accordance with Zakky (2009).

The integration of farmers into groups of farmers is important to encourage the development of the local auction market. Nonetheless, the integration of farmers to create a local auction market will require working capital, which will be needed to finance the current system. Therefore, the government should encourage the groups of farmers in the Northeast into cooperatives in order that the farmers can gain more benefit from the auction market associated with Giroh *et al*. (2010). There should be support from the government to provide the groups with working capital at low-interest rates in order to strengthen and stabilize the groups or cooperatives. In order to create strength between small groups and larger groups (or cooperatives), a network of local rubber auction markets should be created in the area of each province to bolster small groups with more negotiation power and to encourage active operations of potential entrepreneurs and processors to improve market efficiency and increase the farmers' incomes. Moreover, most rubber factories in the Northeastern region produce rubber cup lumps for export, which has led the farmers to change their behaviors and to produce more rubber cup lumps, in order to increase productivity due to the simpler process and lower cost. As a result, the government should encourage areas, such as fostering the marketing of rubber cup lumps, by supporting the establishment cup lump factories.

Therefore, it can be concluded that with respect to the groups of farmers/cooperatives, it is important to encourage the development of local auction markets to provide better income so that the 'livelihoods' of the farmers can be improved through establishing fair prices in the rubber auction markets.

CONCLUSION

All factors except gender significantly influence the participation in the rubber market. The access to credit is a key factor in deciding to enter the market to sell. Thus, the government should promote auction market among smallholders that can access cheaper inputs and credits. Through the farmers' groups or cooperatives, the marketing costs at farmer level were found to be 0.0240 and 0.0179 $/kg of the marketing channel 1 and 0.0323 and 0.0250 $/kg of the marketing

channel 2 for rubber sheet and cup lumps, respectively. In comparison, the local auction traders have a marketing cost of 0.0301 and 0.0307 $/kg of the marketing channel 1, 0.0384 and 0.0299 $/kg of the marketing channel 2 for rubber sheet and cup lumps, respectively. Thus, the net marketing margin for traders was 0.0472 and 0.0218 $/kg of the marketing channel 1 for rubber sheet and cup lump, respectively and 0.0444 $/kg and 0.0254 $/kg of the marketing channel 2 for rubber sheet and cup lumps, respectively. Thus, the trader can get a good benefit from the rubber auction market at around 0.0276-0.0552 $/kg. It can be seen that rubber marketing cooperatives or groups of farmers can give a rubber farmer more bargaining power. Thus, the integration of farmers as groups of farmers or cooperatives is important to encourage the development of local auction markets. However, in order for the auction market to operate in small groups, it needs financial support to manage the auction process. Thus, the government should support the formation of groups of farmers into cooperatives. Moreover, there should be support from the government to provide the groups with working capital in the form of low-interest loans to strengthen and stabilize the groups or cooperatives.

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THE SPREAD OF GOOD AGRICULTURAL PRACTICE (GLOBAL G.A.P.) AND ITS EDUCATIONAL USE IN JAPAN

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ABSTRACT

In the world, sustainable agriculture is necessary to answer food demand due to future population increase. There is GAP as a concrete idea for that. Also in Japan, it is useful as a tool for sustainable agricultural production, but besides that, producers introducing GAP are increasing for agricultural exports and safe food supply. This paper introduces case examples of Japan's GAP (GLOBAL GAP) introduction and summarize the effect and the approach of Tokyo University of Agriculture on its farm, and discusses about the spread of GAP in the future.

INTRODUCTION

As it is well known, the world's population is increasing explosively and is said to be 9.6 billion in 2050(UN DESA). It is estimated that it is necessary to increase food production by 70% compared with the present to meet the food demand of this growing population (FAO). Agriculture, forestry, and fishery produce this food and raw materials, but this paper focuses on only agriculture to clarify the discussion.

Looked out over the world, there are many areas where agricultural water is depleted in the process of developing agricultural production to meet food demand. In 2030, it is also predicted that over 50% of the world's population will fall into water and food shortages (Antequera, 2015).

On the other hand, looking back to Japan, the situation differs significantly from overseas. Although there is abundant water, however, due to the aging of farmers and the severity of agricultural work, the Japanese agricultural sector has been tapering uneasily such as few young people are will to work in the agricultural sector.

To satisfy the increasing demand for food, it is necessary to develop economically, socially and environmentally sustainable agricultural production around the world, and various efforts are being made. One particular approach is GAP (Good Agricultural Practice). This article focus on GAP,

especially GLOBAL GAP which has the most mainstream spread in the world, organizes the development of GLOBAL GAP in Japan and discuss the future of Japanese agriculture by using GLOBALGAP.

OVERVIEW OF GLOBAL GAP

GFSI (Global Food Safety Initiative), organized by more than 400 retailers and food manufacturers operating in 70 countries around the world, is the world's largest food industry group. This organization examines how the food industry can supply safe food to consumers and sets up a mechanism to judge and approve the reliability of the certification system related to food safety. This GFSI is an arbitrary organization established under TCGF (The Consumer Goods Forum).

The idea of certification standard approved by GFSI is summarized into three points: (1) HACCP sanitation management, (2) ISO 9001 level quality control, and (3) food defense. HACCP sanitation management is to try to grasp the crisis area of food and to promote hygiene management from the perspective of the three major hazards of food. The hygiene management means control of physical hazards (foreign body contamination), biohazard (bacterial contamination), chemical pollution (residual agricultural chemicals). ISO 9001 level quality control aims to improve the management system, formulate rules for maintaining quality, and record strict compliance with rules. Food defense means to prevent malicious artifacts, producers must comply with the law, thoroughly educate employees about moral, corporate philosophy and occupational safety, and conduct traceability of their products. There are currently nine authentication standards approved by GFSI (Fig. 1). Among them, there are four standards for agricultural products, the most major standard of which is GLOBAL G.A.P. It is a concept of agricultural business management to produce sustainable and safe agricultural products, and is composed of three pillars: (1) food safety, (2) occupational safety, and (3) environmental conservation. Food safety involves hygienic food production. Occupational safety is the improvement of working conditions, while environment conservation is the establishment of sustainable agriculture.

The idea of GFSI is to realize a state where security can be secured in the process of raw material production and the process of processing and to connect the safety of the whole food chain by connecting the information. For example, if GLOBALG.A.P is in place at the agricultural site and FSSC 22000 is established at the processing phase, it establishes food safety throughout the whole continuum. It leads to social benefits of food safety.

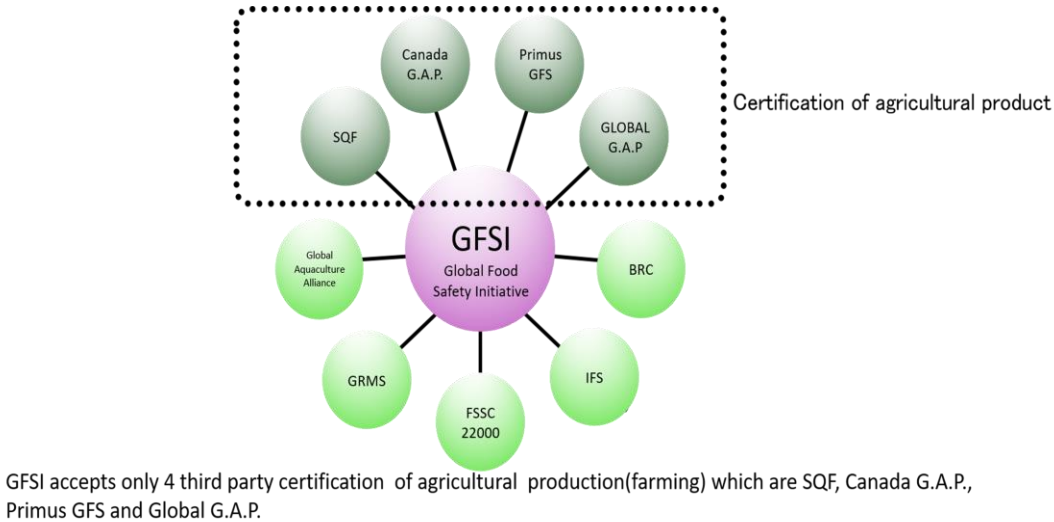

Fig. 1. Global food safety standard GFSI's certification scheme

DEVELOPMENT OF GLOBAL GAP IN JAPAN

There are many GAP systems in Japan, but none have received GFSI approval. Therefore, for producers to develop globally, one of the GFSI approved standards must be taken. Conversely, if the producer gets GLOBAL GAP, it will acquire the international level certification so that producers will be ready for global expansion. As of the end of 2015, the number of producers received GLOBAL GAP was about 200, but as of the end of September 2016, it grew almost doubled to 396 (FOOD+). This increase is thought to be due to information that GLOBAL GAP may be included as a food procurement requirement for the Tokyo Olympic Games and the Paralympic Games in 2020, although the Japanese government may be affected by the promotion of agricultural exports.

Regarding the regional characteristics of the number of certificates of GLOBAL GAP in Japan, it accounts for 52% of the number of certifications in two prefectures, Shizuoka and Hokkaido. Shizuoka Prefecture is related to the fact that the prefecture is the production area of tea, tea processing enterprises have acquired FSSC 22000 and required GFSI approval scheme. Hokkaido is the biggest producing area of Japan such as potatoes, onions, carrots, pumpkins, etc., and the shipping destination supermarket is seeking the GLOBAL GAP standard, so many producers are working on getting GLOBALGAP certification.

Some producers have acquired GLOBALGAP as export-oriented producers, but most of the producers who are exporting, however, sell to suppliers that do not need GLOBAL GAP certification. For this reason, GLOBALGAP is not currently used for exporting agricultural products in Japan.

A CASE OF MATUMOTO FARM IN KUMAMOTO PREFECTURE

Here, I would like to touch on the actual conditions of agricultural corporations that have obtained GLOBAL GAP certification. It is a Matsumoto farm in Mashiki-machi, Kumamoto Prefecture. It is an excellent producer who acquired third the certification in Japan. It is an agricultural corporation that performs annual cultivation with a production area of 50 ha, mainly root vegetables. With the opportunity to recommend EUREP GAP[1] from supplier supermarket, they studied about GAP by themselves and spent about two years to obtain certification. Initially, Matsumoto Farm was devoted to achieving the GAP checklist, and the fundamental understanding of GAP was weak. However, while updating the certification, the essence begins to appear. For example, because visualization of farm management was aimed at by thorough recording, the impossibility in management became apparent, and it began to do more efficient work than before. By proceeding with organizing, the things to look for is reduced, and it becomes possible to use time efficiently.

Matsumoto Farm also improved management quality by promoting discussion between managers and staff, establishing a mechanism to take countermeasures against the request by making a record of the discussion. As a result, the quality of agricultural products improved, leading to an increase in sales. Also, Matsumoto Farm has various effects such as being evaluated by financial institutions at the time of loan by the risk countermeasure. On the sales side, priority is given from the supermarket that has recommended GAP acquisition, and though it is a small amount for export, stable transactions can be continued. Although it did not raise the price by acquiring GLOBAL GAP, contract sales at a fixed price became possible, thereby enabling stable management to be realized. In spite of the effect of obtaining GLOBAL GAP at Matsumoto Farm was explained above, it is not intended to acquire GLOBAL GAP, but rather to use GLOBALGAP as a tool for management development, to increase its management while updating certification, this is the essential meaning of acquiring GLOBAL GAP.

THE EFFECT OF GLOBAL GAP FOR EDUCATION

In Japan, there are stereotypes that GLOBAL GAP acquisition is possible for advanced producers, but difficult for general producers. In particular, much administrative staff and agricultural cooperative staff have this idea. As a result, there are cases where farmers who do not know what GAP is doing not to tackle. However, if you check with a farmer who has already acquired GLOBAL GAP, you can hear the voice that there is a sense of cost burden, but it is not difficult to acquire. Many cases are becoming disagreeable because they do not even challenge to obtain certification from the belief that it is impossible for themselves because it is difficult. I would like Japanese agricultural producers to

[1] GLOBAL GAP was developed from EURO GAP

challenge to acquire GLOBAL GAP, but if there is a place to learn GLOBAL GAP at the educational site of the young producer, they can challenge GLOBAL GAP after the end of education. Currently, as an educational institution, GLOBAL GAP certification has been acquired at Goshogawara Agricultural and Forestry High School in Aomori Prefecture, Miyazaki University, Niigata Prefectural Agricultural College and Saitama Prefectural Agricultural College and has expanded to other schools. In fact, Tokyo University of Agriculture is working on getting GLOBAL GAP certification and experienced its certification review on Yam production at university farm. I would like to explain the educational effect of GAP from the experience of Tokyo University of Agriculture.

Initially, they aimed to acquire GLOBAL GAP for the supply of food to the Tokyo Olympic Games and the Paralympic Games in 2020 and started as a request for action on the farm site. On the scene, there are professors in charge, technical staff, technical trainees, and they started to study GLOBAL GAP. They were confused, for example, something that had to be rectified by what they had done as custom until now, but as they worked over time, the essence of GAP began to appear, and by working on GAP, they began to practice using GLOBAL GAP as a tool to improve the worksite, and they were promoted to organize everything including the warehouse. In addition, the instructor gives a new task of practicing risk assessment to the technical trainee in the Yam cultivation, so that the technical trainee can review the work of the farm, they could learn deeply about GAP through the work. The GAP judge told them that "Risk assessment was well done, for the first time in the examination, the degree of comprehension to GAP was high and excellent results".

In this way, by working on GAP not only in the classroom but also on the farm, it is possible to understand its essence. In the future, to expand this GAP in Japan as well as in the world, educational institutions will take the initiative to introduce them, and methods to teach to students through practical training will lead to further popularization.

CONCLUSION

In the world, sustainable agriculture is necessary to answer food demand due to future population increase. There is GAP as a concrete idea for that, and there are some that GSFI can recognize as authentication, such as GLOBAL GAP. Also in Japan, it is useful as a tool for sustainable agricultural production, but besides that, producers introducing GAP are increasing for agricultural exports and safe food supply. By understanding the philosophy itself rather than filling in items, this GAP has the effect of improving the management structure itself of the producer who introduced it year by year. Therefore, GAP should be introduced for future agriculture in Japan. In the introduction, by considering the next generation at the educational institution, it is expected to further spread the GAP

in Japan by doing the process of actually acquiring it and updating it with the student.

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