DISEASE RESISTANCE SCREENING PROTOCOL AGAINST RICE (*Oryza sativa* L.) BROWN SPOT (*Bipolaris oryzae* Shoem.) AND BLAST (*Magnaporthe oryzae* Hebbert Barr) DISEASES USING ABSCISSIC ACID (ABA) ASSAY

Vanica Lacorte-Apostol[1], Edna A. Aguilar[1], and Hei Leung[2]
[1] Institute of Crop Science, College of Agriculture and Food Science,
University of the Philippines Los Baños, Laguna Philippines
[2]Strategic Platform Division, IRRI, DAPO 7777, Metro Manila, Philippines
*Corresponding author: vlapostol@up.edu.ph

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ABSTRACT

Broad spectrum resistance can combat wide array or strains of a pathogen. Developing broad spectrum resistance requires understanding and screening sources of resistance. Abscissic acid (ABA), a known plant resistance response, was used to differentiate resistance and susceptibility response against brown spot and blast fungal rice diseases in IR64 wild type and mutants. To validate the association of ABA into brown spot and blast resistance, ABA assay was conducted. The study was conducted in International Rice Research Institute in 2013. ABA acid assay was effective in differentiating response of rice against brown spot and blast diseases at 7 days after inoculation (DAI). This study also suggests that IR64 can be used as resistance source against blast and brown spot diseases.

Keywords: pathogen, broad-spectrum disease resistance, mutants, ELISA

INTRODUCTION

Rice, *Oryza sativa* L., is the staple crop of two-thirds of the human population. It provides 21% of global human per capita energy and 15% of per capita protein (Leong 2004). In 2017, rice contributed 1,156.59 grams of calories per day per person in the Philippines. (PSA 2018), however, rice diseases have been one of the major constraints in rice production, causing a yield loss of 24-41% (IRRI 2012) thus, demands for rice are hardly reached. Fungal diseases like blast and brown spot reduce rice yield.

Rice blast is caused by the pathogen *Magnaporthe oryzae* Couch. (Couch and Kohn 2002) a hemibiotic fungal pathogen that gets its nourishments from living and non living tissues (Talbot 2003). The anamorph stage *is Pyricularia oryzae.* The *Magnaporthe oryzae* Couch was used as updated sstandard scientific name for rice blast pathogen as recommended by the Natural History in the University of the Philippines Los Baños, Laguna. The rice blast pathogen infects leaf blades, leaf collar, culm, culm nodes and at the panicle neck node (Webster and Gunnell 1992). The rice blast disease cause significant yield losses in India (5% to 10% from 1960 to 1961), 8% in Korea (mid-1970's), 14% in China (1980-1981), and 50 to 85% in the Philippines (http://www.knowledgebank.irri.org).

The life cycle of Magnaporthe starts when the spores adhere to the host tissue which then becomes hydrated. The attachment of the spore on the host tissue is facilitated by a special adhesive released from the tip of each spore. After germination, the spores differentiate into a specialized infection cell, appressorium at 20-24 hours. At the optimum temperature (24-48 °C), plant tissue will be penetrated after 20-28 hrs. The appressorium enters the leaf through the stomata, cuticle and leaf

surface (Ribot et al. 2008). This specialized infection cell facilitates invasion of underlying leaf tissue by generating enormous turgor pressure that ruptures the cuticle. The biotrophic stage of rice blast involves the movement of the fungus from cell to cell that occur by means of plasmodesmata. The fungus search for pit during invasive hyphae movement to adjacent epidermal cells (Wilson and Talbot 2009). Preformed barriers against the entry of the pathogen such as pectin, lignin, and phenolic compound will prevent breakage of cells, thus, can enhance resistance to the blast fungus through restriction of fungal penetration and hyphal growth. Penetration is followed by invasive growth (2-6 days) that leads to lesion formation (5-7 days after penetration). Finally, sporulation will take place and the pathogen spreads to new plants that require 6-15 days at optimal temperature.

Brown spot disease of rice is caused by the fungus *Bipolaris oryzae* (Breda de Haan) Shoem. Syn. *Drechslera oryzae* (Breda de Haan) Subram. and Jain *Helminthosporium oryzae* teliomorph, *Cochliobolusmiyabeanus* (Ito and Kuribayashi) Drechsler ex Dastur (Padmabhan 1973; Ou 1985). The pathogen also infects other cereal crops such as barley (*Hordeum vulgare*), oats (*Avena sativa*), wheat (*Triticum aestivum* L. em. Thell), maize (*Zea mays* L), and wild rice (*Zizania aquatica*) (Ou 1985). It has both an asexual and a sexual stage. The asexual stage is *Bipolaris oryzae* (Breda de Haan) Shoemaker (Dela Paz et al. 2006). The sexual stage is *Cochliobolus miyabeanus* (Ito and Kurib) Drechsler ex Dastur. Nectrophs like *Bipolaris oryzae* kills the host by producing toxin and lytic enzymes (Kan 2006). Brown spot reduced rice yield from 50% to 90% in Bengal (Padmanabhan 1973; Thuy 2002). Similar disease incidence was also reported in rice growing countries in South and Southeast Asia (Savary et al. 2000), America, and Africa (Ou 1985;Mew and Gonzales 2002). Symptoms of this disease are found on the rice leaves and glumes. Disease symptoms may also appear on the coleoptiles, leaf sheaths, panicle branches, but appear rarely on roots of young seedlings and stems (Ou 1985).

The disease cycle of brown spot starts with the germination of the conidia by germ tubes from the apical and basal cells. The germ tube is covered with a mucilaginous sheath which adheres to a solid surface and an appressorium is formed at the tip. After formation of appressorium, an increase in protoplasmic streaming is observed and the cell nuclei move to a position near the appressorium (Ou 1985). The germ tube may also enter the leaf through the stomata without forming appressoria (Nisikado 1926). It is then followed by the hyphae attacking the middle lamella and penetrating the cells. The middle lamella will start to separate and will caused the formation of yellowish granules. Then, 2 or 3 cells die, and mycelia develop in the cells. Appearance of minute spots follows (Ou 1985).

Using resistant varieties is one of the control measures used against brown spot and blast diseases. However, breeding for host single race-specific resistance (R) gene is only specific against particular strains or races of one pathogen species or pathovar. Moreover, the initially developed R genes can be outdone by races of pathogens, which can lose effectiveness rapidly in cases when the pathogens are capable of evading the recognition by changing its corresponding effector (also called avirulence or Avr) genes (Madamba et al. 2009; Zhao et al. 2005). These research findings led to developing plants resistant to broad-spectrum diseases.

Breeding for broad-spectrum disease resistance requires identification of germplasm containing resistance against wide array of pathogens or strains of a pathogen (Skamnioti and Gurr 2007). The objectives of these experiments were to develop and optimize a protocol that will determine resistance and susceptibility response against blast and brown spot. Efficient disease resistance screening protocol is necessary for fast and reliable screening of germplasm. Increase in abscissic acid (ABA) is one of the resistance responses of plants (Flors et al. 2009; DeVleesschauwer et al. 2010) that could be used to differentiate resistance and susceptible reactions. To validate the association of ABA into brown spot and blast resistance, sensitive and efficient ABA assay needs to be developed.

MATERIALS AND METHODS

Plant materials. The IR64 wild type was mutated by Wu et al. (2005) using diepoxybutane mutagen at the International Rice Research Institute (IRRI), Philippines. The IR64 mutants, D10808 and D6766 were used to compare with the IR64 wild type in response to brown spot and blast diseases. The utilization of IR64 mutants which have differences in mutation sites might give clues to determine the mechanism in loss of resistance to two diseases and conversely, determining the mechanism of loss of resistance to two diseases might elucidate the resistance mechanism for these two diseases.

Brown Spot and Blast Disease Inoculation and Symptom Assessment

Brown spot preparation of inoculum. Unlike blast, there are few studies on resistance against brown spot. Further, blast and brown spot differs in screening procedures including inoculum preparation. This is due to the toxin produced by the fungi *Bipolaris oryzae* that needs to be removed, thus, optimized procedures are necessary. The toxin of brown spot is not needed for the pathogenicity test of the experiment.

The brown spot isolate maintained by the Plant Pathology and Molecular Genetics Laboratory of IRRI was used for screening. The virulent SM2 isolate was revived from stock cultures by transferring 1-2 cut filter bit with fungal growth at the center of Potato Dextrose Agar (PDA) plates. Ten plates were used to prepare inoculum for the isolate. The isolate was grown under combination of NUV and fluorescent light with 12 h photoperiod, but in a room with temperature 28 ± 2^0C as optimal for growth of fungus (Hau 1980). Preliminary experiment showed that high temperature and incubation with fluorescent light caused watering in the media thus, affecting the growth of fungi. Modification of growth chamber with screen rather than closed door to maintain optimum temperature solved this and the required alternate light and darkness to maintain the optimal growth of fungus.

To clean the spore suspension, the toxin and mycelia fragments were generally eliminated. Brown spot suspension were filtered using layers of muslin cloth. The toxin was removed because of toxicity effects on leaves. Browning, yellowing of leaves, or worse early death of plants at early stage of disease development (1-2 DAI) manifest the toxicity effect of toxin thus, hinders the study on disease development. Toxins were eliminated by collecting the spores in 50 mL Falcon tube and using sterilized glass slides to scrape the spores form the surface of the agar. Then, the spore suspension was filtered through layers of muslin cloth, transferred to a sterile tube and centrifuged at 2000 rpm for 2 minutes. Centrifugation of inoculum allowed the toxin mixed in the suspension to settle down. This method is a modification from previous brown spot inoculation procedures which did not use centrifugation. Filtering lessened the concentration of inoculum hence, evaluation of spore concentration before filtering should be done to determine the change in concentration due to filtering. Spore concentration was determined using a hemacytometer. The standardized spore concentration for this protocol was 5 X 10^4 per mL, with additional 0.02 % Tween 20 to aid in the adhesion of the inoculum to the leaves.

Blast preparation of inoculum. The inoculum maintained by the Plant Pathology and Molecular Genetics Laboratory of IRRI was prepared as described by Chen (1994). Ca89 stock cultures in paper disks were revived in prune agar slants (3 pieces prune, 5-g lactose, 1-g yeast extract, and 20-g agar bar in 1 L, pH 6-6.5). About 10-mL sterile distilled water was added to the slant and mycelial growth was macerated with a sterile needle. The suspension was poured onto prune agar plates. The plates were incubated for seven days at room temperature, after which, mycelial growth was scraped with sterilized spatula and exposed to fluorescent light for four to five days to induce sporulation. Conidia were harvested by washing each plate with 10-20 mL water. The conidial suspension was filtered through four layers of nylon mesh and the concentration was adjusted to 5 X 10^4 per mL using a hemacytometer. Approximately 0.02 % Tween 20 was added to aid in the adhesion of the inoculum to the leaves.

Inoculation using brown spot and blast inoculum. Separate prepared spore suspension of brown spot and blast were sprayed onto 18-day-old rice seedlings planted in plastic trays using a motor sprayer. The motor sprayer was washed with alcohol and rinsed with distilled water for every isolate inoculated to prevent mixing of spore suspension and contamination. The inoculated rice plants were incubated inside a moist chamber covered with jute sacks for 24 hours with an air temperature of 28°C to stimulate symptom development (Bonman et al. 1996). Then, plants were transferred to humidified room (mist room) with 25-30°C and sprinkled with water DAI for the development of lesions.

Disease assessment. Quantitative scoring was done at seven days after inoculation by obtaining Percent Diseased Leaf Area (0-82) (Fig. 1). Plants with 0-4% DLA were considered resistant while plants with 8-82 % DLA were considered susceptible.

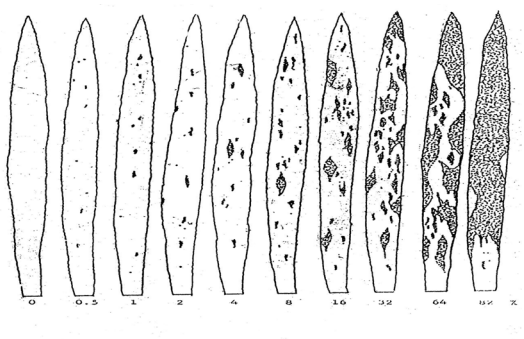


Fig. 1. Scale of disease measurement for % diseased leaf area in blast and brown spot (Roumen et al.1992)

Developing abscisic acid content assay in leaves for brown spot and blast inoculated of IR64 wild type and mutants. To associate resistance and susceptibility of ABA content on brown spot and blast response, ABA extraction and analysis assay in IR64 wild and mutants was developed. Collected leaf tissues before and at 1, 2, 3, and 7 DAI of *B.oryzae* and *M. oryzae* were ground using liquid nitrogen. Three replicates of one gram of fresh tissue per sample were homogenized with 80% (v/v) methanol and 1 mmol (220 mg)/L butylated hydroxytoluene (BHT). The extract was filtered through a C18 column and transferred to a 2 mL epi-tube. The ABA ELISA (enzyme linked immunosorbent assay kits) was used for ABA determination. One reading in three samples per treatment was quantified using UV-VIS spectrophotometer. Statistical Analysis System 9.1 (SAS) was used for data analysis. For each inoculation experiment, the RCBD with two factors (genotype x four time series) in three replicates was used. Treatment comparison was carried out using least significant difference (LSD) technique at 5% level of significance ($P<0.05$).

RESULTS AND DISCUSSION

Brown spot and blast disease assessment in leaves of IR64 wild type and mutants. The formation of typical brown spot lesions was observed in inoculated D10808 mutant plants. As for IR64 wild type and D6766 inoculated plants, small brown specks or pinhead –sized spots with halo was observed 7DAI (Fig.2.a). Based on symptoms, it can be inferred that the D10808 mutant is susceptible to the disease while IR64 wild type and D6766 are resistant. The prominent lesion type on D10808 are type 3, 4 and 5 lesions which are said to be the sporulating type of lesions while non-sporulating lesion type (type 1and 2) was observed in inoculated IR64 wild type and D6766 plants 7 DAI (Fig. 2.a). Further, a difference between D10808 and IR64/D6766 in terms of percent diseased leaf area (DLA) was observed at 7 DAI (Fig.2.a). The mutant D10808 showed the highest percent DLA (30.15) and was significantly higher compared to IR64 wild type (1.35) and D6766 (1.60) (Fig. 2). The formation of the typical brown spot lesions coupled with high percent DLA reading again suggest that D10808 is susceptible to

the disease while IR64 wild type and D6766 are resistant. Response of IR64 wild type and two mutant genotypes (D10808 and D6766) to blast infection were also observed. After 7 DAI, D10808 and D6766 showed elongated lesion with whitish center. Inoculated IR64 wild type plants, on the other hand, showed small brown lesions with grayish center (Fig.2.b). Analysis revealed that the % DLA mean (Fig.2) of three genotypes used in the experiment are statistically different from each other. D6766 showed the highest percent DLA (18.60) followed by D10808 (10.42) and IR64 wild type (6.76). Here, using the % DLA readings, it can be inferred that D6766 and D10808 in comparison to the moderately resistant plant (IR64 wild type) are susceptible to the disease. However, between D6766 and D10808, D6766 is more susceptible.

The IR64 wild type showed resistant phenotype response to both brown spot. Conversely, D10808 showed susceptible response to both diseases while D6766 had resistant response to brown spot but susceptible response to blast. These suggest that mutation is effective in disrupting resistance in blast for D6766 and D10808 mutants. The mutation in D10808 appears to be important for loss of resistance to two diseases. Comparison of the mutation sites between D10808 and D6766 appears to be important. The differences in mutation sites between D10808 and D6766 might give clues to determine the mechanism in loss of resistance to two diseases and conversely, determining the mechanism of loss of resistance to two diseases might elucidate the resistance mechanism for diseases to these two diseases.

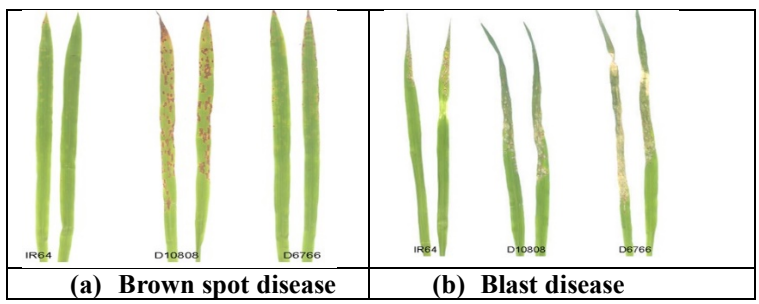


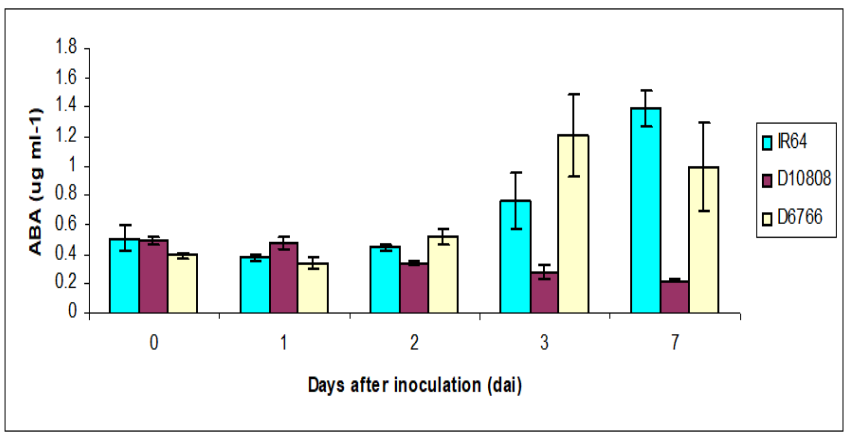
Fig. 2. Diseased leaf Area (DLA) of IR64 wild type, D6766, and D10808 infected with (a) brown spot and (b) blast at 7 DAI.

Abscisic acid content after brown spot inoculation. When plants are exposed to biotic and abiotic stresses, several plant components and processes are involved to mitigate the effect of diseases. The mean ABA of the three genotypes at 0, 1, 3, and 7 days after *B. oryzae* inoculation is presented in Figure 3.a. Baseline ABA levels of the three genotypes were not significantly different. Significant differences on the ABA levels of the 3 genotypes tested were measured starting at 3 until 7 DAI with brown spot pathogen. ABA levels of brown spot resistant genotypes IR64 wild type and D6766 were significantly higher than the ABA of susceptible mutant D10808 at 3 until 7 DAI. Increased ABA activity in the leaf from 3 DAI (colonization stage) until 7 DAI (disease symptoms) appears strongly and positively associated with resistance response of the host. Conversely, the inability of D10808 mutant to increase its ABA level soon after successful penetration of the pathogen (1 DAI) appears associated with its loss of resistance to brown spot.

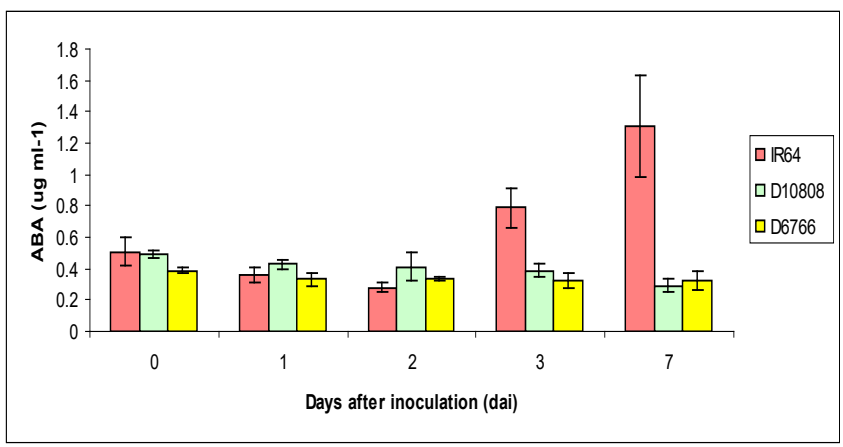
Abscisic acid content after blast inoculation. The mean ABA of uninoculated and up to 2 DAI of blast pathogen in IR64 wild type, D10808, and D6766 were not significantly different (Fig. 3.b). By 3 DAI, the ABA level of susceptible mutant D6766 and D10808 was significantly lower than IR64 wild type and by 7 DAI, the ABA content of resistant genotype IR64 wild type was significantly higher compared with both susceptible genotypes D10808 and D6766. The ABA level of D10808 and D6766 at 7 DAI were similarly low. Higher leaf ABA at 7 DAI appears strongly and positively associated with

resistant reaction to blast disease. Similarity in the inability of the two IR64 mutants to increase ABA activity at 7 DAI could have contributed to their loss of resistance to blast.

Baseline ABA levels of the three genotypes before brown spot and blast inoculation were not significantly different. When inoculated with the brown spot and blast pathogen, significant differences in the ABA levels of the 3 genotypes were observed. Increased ABA was associated positively with resistance response. Starting 3 days after inoculation (colonization stage), differences in ABA of resistant and susceptible genotypes could be detected but significant differences were observed at 7 DAI (disease symptoms appearance). This suggest that ABA assay developed could be used to differentiate and screen resistant and susceptible genotypes.



(a) *B. oryzae* inoculated



(b) *M. oryzae* inoculated

Fig. 3. Comparison of mean ABA in wild type IR64, D10808,and D6766 at different days after inoculation, (A) *B. oryzae* and (B) *M. oryzae.* Columns with the same letter in the same DAI are not significant at $p = 0.5$

CONCLUSION

The abscissic acid assay was effective in differentiating response of rice against brown spot and blast diseases. The optimized protocol are as follows: inoculation (at 18- day- old seedlings using 5 X 10^4 per mL concentration of filtered spore suspension added with .02 % Tween 20), incubation (at humidified room with 25-30ºC for 7 days), extraction (grounding of 7 DAI leaf samples using liquid nitrogen), homogenization (one gram of fresh tissue per sample homogenized with 80% (v/v) methanol and 1 mmol (220 mg)/L butylated hydroxytoluene (BHT), filtration (using C18 column), ELISA (enzyme linked immunosorbent assay kits), and quantification (using UV-VIS spectrophotometer).

The modification of brown spot inoculation protocol used in this study were successful in phenotyping for brown spot disease resistance. This adopted and modified protocol are as follows: ten-days old SM2 isolate cultured and incubated alternately for 12 hours near UV light and 12 hours darkness at 28ºC for 10 days to induce sporulation, collected spores in 50 mL Falcon tube were centrifuged at 2000 rpm for 2 minutes to lessen or removed the toxin in the inoculum, sterile distilled water with tween 20 was poured into the plates and sterilized glass slides were used to scrape the spores form the surface of the agar, the spore suspension was filtered through layers of Muslin cloth and was transferred to a sterile tube, spore concentration was determined using a hemacytometer and spore concentration was 5 X 10^4 per mL. The optimized protocol in this experiment were used in different phenotyping experiment for brown spot disease resistance studies such in 200 indica MAGIC (Multi-parent Advanced Generation Intercross) and 500 out of diverse 3k accessions from T.T. Chang Genetic Resources Center of the International Rice Research Institute. Using the effective protocol in phenotyping, these studies were able to identify putative genes associated for brown spot disease resistance in rice. Further, the protocol was used in genetic analysis and mapping study of the population generated by intermating the mutants D10808 and IR64 wild type that validated the effectivity of the protocol. Using the protocol, the different studies were able to discriminate between resistant and susceptible rice plants.

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