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About the cover: Across rice ecosystems, communities of soil microorganisms, flora, and fauna perform a wide range of functions affecting rice plant growth. In irrigated lowland, the small aquatic fern *Azolla* fixes nitrogen through its symbiotic relationship with the cyanobacterium *Anabaena azollae*; hence, providing nitrogen source for rice. The nitrogen-fixing microorganisms or diazotrophs can also benefit the plant by supplying phytohormones and enzymes, mobilizing nutrients, suppressing pathogens, and increasing tolerance to abiotic stress such as drought in rainfed lowland or upland. A thorough understanding of the mechanisms by which microorganisms benefit rice and its environment is essential to the development of environmentally sustainable agronomic practices.

Contribution from the Agronomy, Soils and Plant Physiology Division, PhilRice, Nueva Ecija.

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Full Paper

Azolla Growth along the Rice Cropping Cycle in Irrigated Lowland Fields in Laguna, Philippines

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Abstract *Azolla* is a small aquatic fern that fixes nitrogen (N) through its symbiotic relationship with the cyanobacterium *Anabaena azollae*; hence, providing N source for lowland rice. Despite its rapid biomass production, *Azolla* cannot survive long enough especially during the 1-2 months fallow period due to decrease in soil moisture. Growth of *Azolla* along irrigated lowland rice cropping cycle in Majayjay, Laguna was monitored. Field and plastic tray studies of *Azolla* in a rainout shelter with plastic roofing were conducted in Los Baños, Laguna to understand its growth in lowland rice system. Results showed that in Majayjay, Laguna, growth and sporulation of *Azolla* coincided with growth and development of lowland rice. Although *Azolla* plants dried during the fallow period when soil moisture decreased, sporophytes emerged a month after re-flooding the soil. Doubling time, the number of days required to increase biomass twice, ranged from 6.2 to 6.6 days for UPLB Hybrid 1 and other accessions, and 7.0-7.4 days for the accessions from Majayjay, Laguna and Lucban, Quezon. In terms of sporulation index, UPLB Hybrid 1 ranked higher than *A. Mexicana* #2002 and *Azolla* accession from Majayjay. In response to dry, wet, and flooded soil conditions, number of spores germinated was zero in dry soil but exponentially increased from wet to flooded soils.

Keywords: *Azolla* sp., Doubling Time, Lowland Rice Cropping Cycle, Sporulation.

Introduction

Azolla Lam. is an aquatic fern that fixes N through its symbiotic relationship with the cyanobacterium (used to be called blue-green algae but a prokaryote) *Anabaena azollae* inside the leaf cavities (Lin and Watanabe, 1988; Peters and Meeks, 1989). Symbiotic N fixation is part of a mutualistic relationship in which plants provide a niche and fixed carbon to bacteria in exchange for fixed N (Mus et al., 2016).

The rapid growth of *Azolla* in water and its accumulation of biologically fixed N make it a good green manure for rice under flooded culture. *Azolla* can be an efficient fertilizer alternative or supplement in flooded rice cropping system (Fosu-Mensah et al., 2015). Several studies have shown the impact of *Azolla* as biofertilizer on rice yield (DA-UPLB-NAAP, 1988; Kannaiyan, 1987, 1993; Kulasooriya et al., 1987; and Zhuang-ta et al., 1987). The biomass of *Azolla* covering the floodwater of a hectare rice paddy was estimated to be at least 20 t/ha containing about 30 kg N ha^{-1} (DA-UPLB-NAAP, 1988). In 1982, the Philippine government implemented a program to use *Azolla* as biofertilizer in rice production. Survival of *Azolla* in rice paddies was a major constraint and it appeared that the *Azolla* distributed to farmers was dependent only on vegetative growth. Azolla multiplied asexually by branching and fragmentation, but some varieties reproduced sexually through spores that formed when condition was favorable. *Azolla* with asexual reproduction alone died when the rice paddies dried up during the fallow period. Production of spores can ensure the continued survival of *Azolla* in rice paddies during an adverse environmental condition such as drought. Previous studies on sporulation focused on large scale cultivation of sporelings (Shuying, 1987;

Quing-Yuan, 1987) but did not assess persistence of Azolla along the rice cropping cycle in paddy field.

Previous evaluation of spore production of *Azolla* in the Philippines was sporadic and occurred within a limited period during the cooler months in the highlands (Payawal et al., 1994). The importance of improving the sporulating ability of *Azolla* for easy transport and distribution prompted the researchers of the University of the Philippines Los Baños (UPLB) to conduct hybridization of *Azolla* (Payawal et al., 1994). There were hybrids that produced spores under the warm growing conditions in Los Baños, Laguna. In rice paddies in Barangay (Bgy.) San Isidro Ilawod, Malilipot, Albay; Bgy. Tinamnam, Lucban, Quezon; and Bgy. Taytay, Majayjay, Laguna, *Azolla* appeared during the cropping cycles, which was associated with the production of spores under a favorable condition. In view of the utilization of *Azolla* as a biofertilizer for lowland rice and attendant concern for N fertilizer economy and the environment (Kannaiyan, 1993; Fosu-Mensah et al., 2015), it would be relevant to re-evaluate the growth of *Azolla* hybrids (Payawal et al., 1994) and strains (Watanabe et al., 1992) along the cropping cycle in lowland rice areas in Laguna.

This study assessed the growth and sporulation of *Azolla* along the cropping cycle of rice in lowland field in Majayjay, Laguna. Field experiments were conducted to assess spore production of *Azolla* accessions under well-watered condition. Plastic tray experiments in a rainout shelter were conducted in Los Baños, Laguna to assess vegetative growth of *Azolla* accessions under well-watered condition, and germination of spores under varying soil moisture conditions.

Material and Methods

Field Sites and Tests

The rice farming community was located in Bgy. Taytay, Majayjay, Laguna, Philippines. The site was 529 m above sea level and located at latitude 14° 6' 58.27" N, longitude 120° 30' 9.97" E and 45 km away from Los Baños, Laguna. The soil in the field site originally belonged to Luisiana clay series characterized by having deep soil that developed in place through weathering of basaltic rocks among other igneous materials. The soil belonged to the Soil Order Ultisol, typically clayey (>60%) dominated by kaolinite and oxides of Fe and Al, acidic in pH (pH 5.5), Bray P2-extractable P content of 2.4 ppm with 8.70% organic matter, and 0.44% total N content.

The terraced rice paddies were supplied with cool irrigation water (18 to 20°C) from the highlands near Mt. Banahaw. The water temperature at the site ranged from 20 to 25°C from January to May and 18-22°C from October to February. The wet season crop was established in June and harvested in October while the dry season crop was established in December and harvested in April. Water from the communal irrigation system was available throughout the year and distributed to clusters of farmers to synchronize with their planting schedules. Carabao-pulled moldboard plow and toothed harrow were used. Farmers soaked their paddies 3 to 4 weeks before plowing and harrowing once with a hand tractor or carabao. Wetbed-raised rice seedlings were transplanted at 3-5 seedlings per hill in straight rows 20 cm apart. After crop establishment, a 2-4 cm floodwater depth was maintained until crop maturity. Manual weeding was done 2 weeks after transplanting prior to topdressing with 2 bags per hectare of complete fertilizer containing 14 kg N ha^{-1}, 14 kg P_2O_5 ha^{-1}, and 14 kg K_2O ha^{-1}.

The paddy selected for observation was the first to receive water from the main irrigation canal. This ensured that the *Azolla* in the paddy did not come from the other paddies.

The disappearance and reappearance of *Azolla* were monitored during the dry and wet season rice crops and fallow period in 2015. The site was visited weekly in dry season and every other week in wet season to observe growth of *Azolla* in terms of floodwater surface area covered and rice crop growth. Two 1 m x 1 m enclosures were randomly set up in the paddy to obtain data on growth and sporulation of *Azolla*.

Biomass of the spore-bearing *Azolla* was obtained from the test site in Majayjay, Laguna and

the spores were grown in the field in Los Baños, Laguna in 2015 to further assess the growth and sporulation of *Azolla*. In Los Baños, Laguna soil was Maahas clay and climatic conditions were similar to Majayjay, Laguna. *Azolla* biomass was processed and dried spores were collected following the method proposed by DA-UPLB-NAAP (1988). The fresh biomass was kept in a 40-L plastic container until fully decomposed, sun-dried, and screened to separate the finely decomposed material containing spores from the coarse organic fragments. Thirty grams of the fine fraction containing spores were spread over the surface of the puddled soil in a rectangular plastic tray. The number of germinated spores (sporophytes) was recorded daily until the spores developed into sporelings. The sporelings, having two to three branches in the main rhizome, were collected and transferred to a 1 m x 1 m plot before transferring to 4 m x 6 m plots located in a field along the Pili Drive, UPLB, Los Baños, Laguna. The number of days when the biomass fully covered the entire plot and number of days when *Azolla* first sporulated were recorded. One hundred plants were randomly sampled weekly for spore count.

Sporulation index was calculated using the following formula:

$$\textbf{Sporulation Index (SI in \%)} = \frac{\textit{Total number of plants bearing spores}}{\textit{Total number of plants collected}} \; X \; 100$$

The number of microsporocarp (MCS) and megasporocarp (MGS) was obtained from the 100 *Azolla* plants sampled. The population of *Azolla* in the plots was considered harvestable when no appreciable additional sporulation was observed. The period from the population's first sporulation until harvesting was recorded. SI at harvest and the ratio of the number of MGS to MCS was calculated. Field observations were replicated twice.

Eight accessions of *Azolla* were released in the field to test sporulation using a completely randomized design (CRD). The field was located along the Pili Drive, UPLB Campus, College, Los Baños, Laguna. Ambient temperature, floodwater temperature, and solar radiation were monitored during the conduct of experiments. The accessions evaluated were *Azolla mexicana* #2024 (UPLB Hybrid 1), *Azolla* sp. collected from Majayjay, Laguna, *Azolla mexicana* #2002, Azolla caroliniana #3002, *Azolla caroliniana* #3005, *Azolla mexicana* #2028, *Azolla pinnata* var. *pinnata* #7004, and *Azolla pinnata* var. *imbricata* #0005. Except for the *Azolla* sp. From Majayjay, Laguna, the accessions recommended and distributed during the operation of National Azolla Action Program (NAAP). Release of the accessions in the field was based on the projection that the 4 m x 6 m ponds were fully covered before November when the highest peak of sporulation was observed (Payawal et al., 1994). The period when the majority of the plants attained their peak of sporulation was recorded. Plants (total of 100) were randomly sampled weekly to count the spores. Likewise, SI, MGS, and MCS counts were obtained. Data were analyzed using analysis of variance (ANOVA). Treatment means were compared using Tukey's Procedure.

Plastic Tray Experiments in a Rainout Shelter with Plastic Roofing

The *Azolla* plant samples from Majayjay, Laguna and Los Baños, Laguna and UPLB Hybrid 1 were grown in plastic trays (33 cm x 27 cm x 10 cm) with 3 replications in the rainout shelter covered by plastic roofing. The experimental design was CRD. Additional *Azolla* plant samples were obtained from Malilipot, Albay; Bacacay, Albay; and Lucban, Quezon. The morphological features and sporulating behavior of the additional samples of *Azolla* were similar to *Azolla* sp. from Majayjay, Laguna. The trays were filled with 2-cm thick puddled Maahas clay soil from UPLB Central Experiment Station and flooded to a depth of 1 cm. With an initial biomass of 5 g, growth of each *Azolla* sp. was assessed by measuring biomass accumulated weekly. Doubling time (DT) is the number of days required for the fronds to increase their weight (Kannaiyan, 1993). DT in days was calculated using the exponential growth equation:

$$N(t) = N(0)e^{rt}$$

Spores were collected from the sporulation pond in Majayjay, Laguna and inoculated in dry, wet (above field capacity), and flooded soil

where

$$Growth\ rate = \frac{Natural \log N(t)}{N(0)} \div t$$

when

$$\frac{N(t)}{N(0)} = 2, log2 = 0.69$$

$$Doubling\ Time\ (DT) = \frac{0.69}{r}$$

Where:

N(t) = biomass at time t

N(0) = biomass at time=0

r = growth rate

conditions contained in separate plastic trays in a rainout shelter with plastic roofing for 30 days. Thirty grams of dry spore samples were contained in each of the treatment tray. The number of days to germinate and the number of spores that germinated (sporophytes) were recorded daily. The experiment were replicated thrice using CRD to validate the field observation that flooded soil condition triggered germination of spores. Data were analyzed using ANOVA and treatment means were compared using Tukey's Procedure. Other parameters like fertilization of the female spores by the male spores, germination of fertilized spores, and development of newly emerged sporelings were examined under the dissecting microscope.

Results

Field Observations on Azolla Growth in Majayjay, Laguna

The morphological features of *Azolla* sp. in Majayjay, Laguna (Figure 1) were similar to A*zolla mexicana* 2001, one of the parents of UPLB Hybrid 1 (Payawal et al., 2004). UPLB Hybrid 1, *Azolla* sp. from Majayjay and other accessions were used in our field and plastic tray studies in Los Baños, Laguna. During the dry season rice cropping in Majayjay, Laguna, spores of *Azolla* germinated within a month after flooding the paddies prior to land preparation. Before the final harrowing, patches of *Azolla* grew profusely and covered 75% of the paddies. However, during the harrowing operation, *Azolla* plants were buried in the mud and reduced *Azolla* cover by 20%. The remaining patches of *Azolla* consisted of a mixture of sporophytes and young *Azolla* plants. Growth and sporulation of *Azolla* coincided with the growth and development of the rice crop during the two cropping seasons (Figure 2). At transplanting time, *Azolla* patches occupied about 40% of the paddies due to their fast growth. The patches consisted of fragmented *Azolla* plants with two to three branches in the main rhizomes. Three weeks after transplanting, the *Azolla* covered more than 50% of the paddy surface and consisted of fully matured *Azolla* plants and newly-fragmented *Azolla* plants from the main fronds.

Azolla covered the entire paddy before the maximum tillering stage of the rice crop. At this time, some *Azolla* plants had reached physiological maturity based on morphological features of the fronds. The *Azolla* patches found near the dikes were oriented in the direction of the prevailing wind and bore spores while those in the interior of the paddies in the opposite side appeared too young to bear spores. *Azolla* in the paddies reached reproductive stage 40 days after transplanting (DAT) or near the maximum tillering stage of the rice crop. After the production of spores, *Azolla* senesced in water and spores germinated to form the sporophytes at 60 DAT or toward the flowering stage. Sporulation of *Azolla* was again observed during heading or early flowering stage of rice. The *Azolla* fronds appeared sturdy with several fruiting bodies attached beneath the fronds (Figure 1). A month after flower emergence or at harvest stage, only small patches of *Azolla* were observed as most of the *Azolla* plants senesced after bearing spores. The remaining few *Azolla* patches clung to the sides of the dikes where soil was still moist. During the fallow period, dried *Azolla* patches were observed on the soil surface. The paddy was again irrigated for the dry season cropping a month after the fallow period. Sporophytes were observed during this period. The sporulation cycle of *Azolla* in dry season followed that of the wet season cycle (Figure 2).

The major sporulation of *Azolla* (with a sporulation index of > 50) was observed twice in each cropping season, i.e., before maximum tillering and after flowering. After harvesting the wet season rice crop, fields were left fallow for a month from October until the start of the dry season crop in December. Desiccated patches of *Azolla* were observed in the field during the fallow period. Soil dehydration during the fallow period after harvesting the wet season crop did not

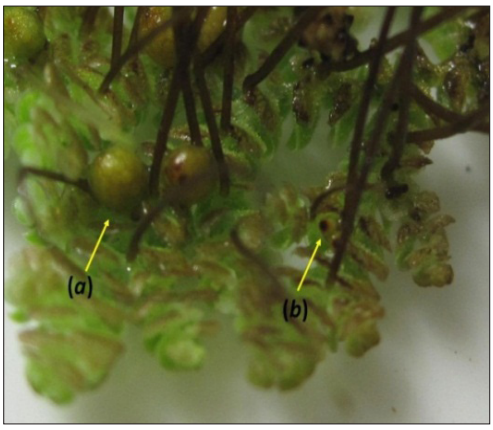

Figure 1. *Azolla* plants bearing spores underneath the fronds with microsporocarps (a) and megasporocarps (b) (10x magnification of *Azolla* in abaxial position) before draining the paddies or two weeks before harvesting the rice crop in Bgy. Taytay, Majajay, Laguna.

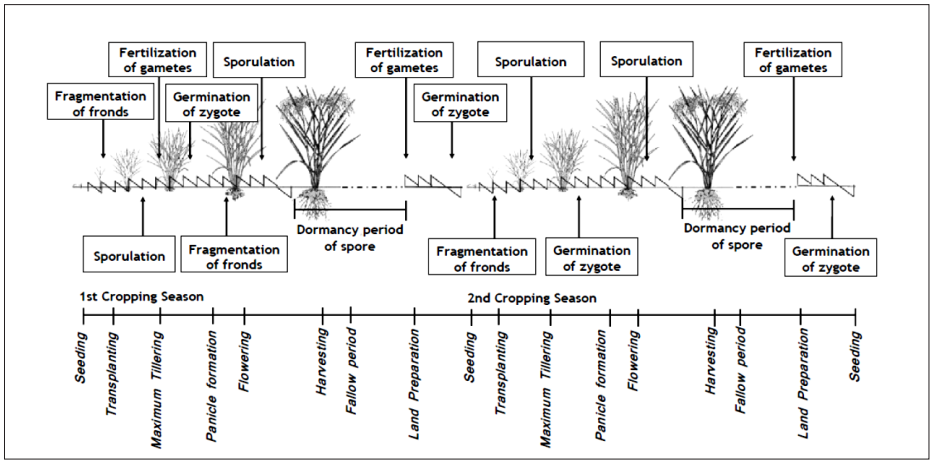

Figure 2. Growth and sporulation of *Azolla* coinciding with the growth and development of irrigated lowland rice with a fallow period between two rice crops in Bgy. Taytay, Majayjay, Laguna.

result in excessive soil cracks and soil moisture was far above the permanent wilting point. However, *Azolla* desiccated even under such soil moisture status (Figure 3a). Longer fallow period between the dry and wet season crops resulted in longer period of desiccation for *Azolla* (Figure 3b). Spores remained on the surface of the dry soil during fallow period and sporophytes emerged a month after re-flooding the field. The sporophytes became complete young plants with roots a month after flowering of the rice crop. At this time, *Azolla* plants had roots attached to the main rhizome with two to three branching of fronds.

Biomass and Spore Production of Azolla sp. *from Majayjay*

Data on biomass and spore production obtained from the sampling area in the paddy in Majayjay, Laguna were close to the biomass and spore production data obtained from the field experiments in Los Baños, Laguna for the *Azolla* sp. from Majayjay. Based on data from both field set-ups, it was observed that with full *Azolla* coverage, the 1 m x 1 m sampling area produced 1.75 kg fresh biomass. The 1.75 kg fresh biomass contained 19 g dried spores that produced 16,250 sporophytes. The sporophytes continued to grow and occupied the 1 m x 1 m plot size for 2-3 days. The first sporulation was observed 20 days after the *Azolla* fully covered the plot. Table 1 shows the duration from inoculation of spores to

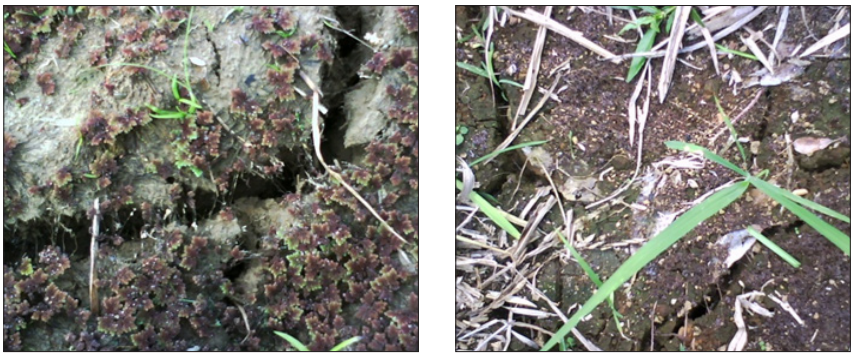

Figure 3. Drying of soil during the fallow period between wet and dry season rice cropping (A) and fallow period between the dry and wet season rice cropping (B) and observed patches of *Azolla*.

Table 1. Number of days from spore inoculation, germination, first sporulation until harvest of spore-bearing *Azolla* biomass, sporulation index, and sporocarp ratio of *Azolla* sp. from Majayjay, Laguna. Number of replications = 3.

Parameter	Mean
Number of days from spore inoculation to germination	10
Number of spores that germinated	16,294
Number of days from spore germination in plastic trays to transfer of sporelings in 1 x 1 m plot	32
Number of days from the release of sporelings in 4 x 6 m field plot until the azolla covered the entire plot	21
Number of days from azolla full coverage of the plot until the first sporulation	20
Number of days from first sporulation to harvest of spore-bearing azolla biomass	21
Sporulation index (SI) of azolla	65
Ratio of megasporocarp to microsporocarp	0.44

germination in flooded soil in plastic trays and other parameters leading to sporulation for the *Azolla* sp. from Majayjay. For example, the number of days from first sporulation to harvest of spore-bearing *Azolla* biomass was 21 and the sporulation index was 65.

Vegetative Growth of Azolla Accessions

In the plastic tray experiment under a rainout shelter with a plastic roofing, vegetative growth of *Azolla* accessions from Majayjay, Laguna and Lucban, Quezon were lower than the vegetative growth of UPLB Hybrid 1 and the other *Azolla* accessions (Figure 4). Mean doubling times (DTs) were 11.2 days for Majayjay accession in March and 7.4-10.6 days for Lucban accession from April to September.Across the 12-month period, average DTs of UPLB Hybrid 1 and accessions from Bacacay and Malilipot in Albay, and Los Baños, Laguna ranged from 6.2 to 6.6 days. Average DTs were 7.0 and 7.4 for accessions from Majayjay, Laguna and Lucban, Quezon, respectively.

Spore Production of Azolla Accessions

Only 3 of 8 *Azolla* accessions that were tested had sporulated in January 2015 under lowland field in UPLB Campus, Los Baños, Laguna. These were UPLB Hybrid 1, *Azolla mexicana* # 2002, and Majayjay (Table 2). The mean ambient and floodwater temperatures in the field were 26°C and 27°C, respectively. The UPLB Hybrid 1 had a sporulation index (SI) of 100% and its megasporocarp and microsporocarp counts were significantly higher than that of Azolla mexicana # 2002 and Majayjay accession. SI of UPLB Hybrid 1 was 21% and 82% higher than that of *Azolla mexicana* # 2002 and Majayjay accession, respectively.

Germination of Spores under Different Soil Moisture Conditions

Germination of spores of *Azolla* sp. from Majayjay, Laguna was tested in dry, wet, and

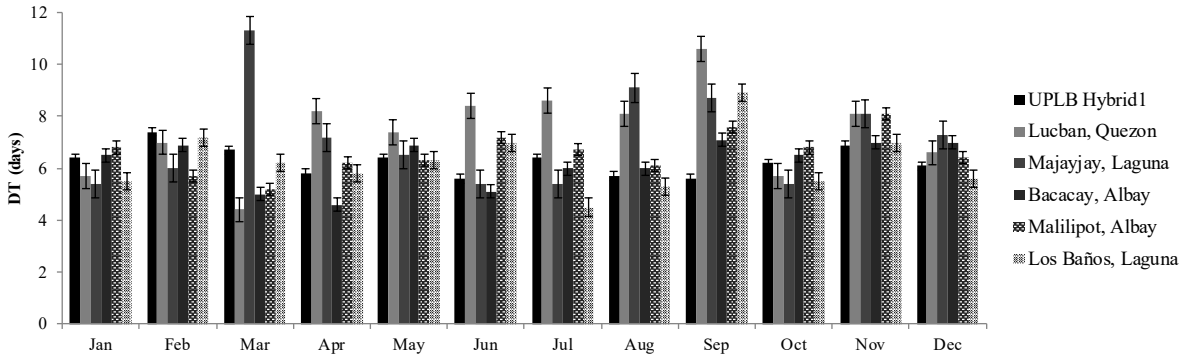

Figure 4. Doubling times (days) of *Azolla* accessions from 20 October 2014 to 28 September 2015 in PhilRice Los Baños, Laguna. Vertical bar is the standard error of each mean (± SE).

Table 2. SI, MGS, and MCS of three *Azolla* accessions in the field UPLB Campus, Los Baños, Laguna in January 2015. For MGS and MCS, means followed by a common letter are not significantly different at 5% level by Tukey's Procedure.

Source of Accessions	SI (%)	No. of MGS	No. of MCS
UPLB Hybrid 1	100	4.4b	8.8b
A. mexicana # 2002	83	1.7a	3.3a
Majayjay	55	1.3a	2.9a

Table 3. Average number of days to germinate and spores that germinated in dry, wet (above field capacity), and flooded soil moisture conditions in plastic trays in a rainout shelter with plastic roofing in Los Baños, Laguna for the *Azolla* sp. from Majayjay in 2015. For the number of spores that germinated, means followed by a common letter are not significantly different at 5% level by Tukey's Procedure.

Soil Moisture Condition	No. of Days to Germinate	No. of Spores Germinated
Dry	-	0
Wet	41	151[a]
Flooded	8	1049[b]

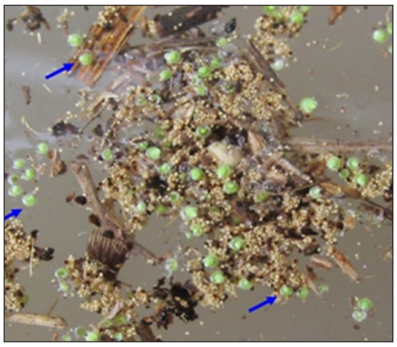

Figure 5. Sporelings or germinated spores (denoted by blue arrows) were observed a week after flooding the dry soil.

flooded soil conditions in plastic trays in a rainout shelter with plastic roofing for 30 days. Spores did not germinate under dry soil condition (Table 3). Under wet soil condition, the number of days to germinate was 41 and 151 spores germinated. Under flooded soil condition, the number of days to germinate was 5 times shorter and the number of spores that germinated was 7 times higher than in wet soil. It was observed that spores were still viable 7 days after flooding the dry soil (Figure 5).

Discussion

In Majayjay, Laguna, growth and sporulation of *Azolla* were observed along the cropping cycle of irrigated lowland rice during the dry and wet seasons. A flooded soil is critical in supporting the growth of *Azolla* and rice. A shallow floodwater depth of 5 cm or less is ideal, although *Azolla* can grow satisfactorily in greater depths (Wagner, 1997). Though *Azolla* is able to grow on a wet mud surface or wetted peat litter, it prefers to grow on free-floating conditions (Serag et al., 2000). Other factors affecting growth and development of *Azolla* (Sadeghi et al., 2013), concomitant *Azolla-Anabaena* symbiosis and impact on rice yield (Fosu-Mensah et al., 2015; Singh and Singh, 1987; Wagner, 1987) are light intensity, air and water temperature, relative humidity, wind velocity and waves, soil physical-chemical variables including pH, phosphorus, nitrogen, potassium, calcium, magnesium, salinity, and biological factors like

insects, bacteria, fungi, and viruses. For example, the optimum temperature for *Azolla* spp. is between 18° and 28°C (Tuan and Thuyet, 1979) and around 30°C for *A. pinnata*, *A. Mexicana*, and *A. caroliana* (Watanabe, 1982). In contrast to other minerals, availability of N and to some extent dissolved oxygen are considered not limiting factors for *Azolla* growth because an increase in nitrate concentration might result in low coverage of *Azolla* in the Anzali wetland (Sadeghi et al., 2013). Through its symbiosis with *Anabaena*, *Azolla* can have free access to atmospheric N for fulfilling its requirements (Costa et al., 2009). Like other photoautotrophic aquatic organisms, some nutrients such as phosphorus or P (in the form of phosphate) are limiting *Azolla* growth (Sadeghi et al., 2013). Phosphorus is an important nutrient to yield a successful growth of *Azolla* sp. (El Katony et al., 1996). If there is enough P in the aquatic environment, *Azolla* will be able to grow without the application nitrogen such as NH_4NO_3 (Costa et al., 1999). In laboratory studies, a P concentration of about 0.06 ppm (2 μM) has been reported to be adequate to sustain *Azolla* growth (Sadeghi et al., 2013). However, based on field surveys, a range between 0.3 and 1 ppm of P (10 to 33 μM) has been suggested to be adequate for *Azolla* growth. Different *Azolla* species respond to different concentrations of P (Kushari and Watanabe, 1992).

Data on biomass and spore production of *Azolla* sp. in lowland rice field in Majayjay, Laguna were similar to data on biomass and spore production of the *Azolla* sp. from Majayjay tested in Los Baños, Laguna. This could be partly attributed to some similarities in the climatic condition and clay properties in both areas. However, other physical, chemical, and biological factors that can influence the growth of *Azolla* (Sadeghi et al., 2013) in different test sites should be considered, e.g., soil N and P supplies.

Based on a 12-month study conducted in plastic trays in a rainout shelter with plastic roofing in Los Baños, Laguna, average doubling times of 6.2 to 6.6 days for UPLB Hybrid 1 and accessions from Barangays Bacacay and Mililipot in Albay and Los Banos, Laguna were less than the DT values (7.0 to 7.4) of accessions from Brgy. Majajay, Laguna and Brgy. Lucban, Quezon. The DT values in the present study were more than the DT values reported by other researchers. Based on an *Azolla* adaptability test conducted in the Philippines from 1982 to 1983, a DT of less than 3.5 days was classified as highly adaptable; 3.5 to 5 days as moderately adaptable; and greater than 5 days as a non-adaptable (Callo et al., 1985). In laboratory studies at International Rice Research Institute, *Azolla* doubled its mass in 3 to 5 days, growing in N free solution, and accumulated 30 to 40 kg N/ha in two weeks (Watanabe et al., 1977). Under ideal conditions of light and temperature, DTs of 2 days or less were reported for *A. filiculoides*, *A. caroliana*, *A. mexicana*, and *A. pinnata* (Watanabe, 1982). In studies of growth and survival of *A. filiculoides*, Janes (1998a) indicated that microclimatic effects were likely to be responsible for the discrepancy between laboratory and outdoor culture results. Hence, differences in DT values obtained in the present study and DT values from field studies reported by other researchers can be partly attributed to the smaller soil volume and other soil properties in the plastic trays and microclimatic condition inherent in our rainout shelter with plastic roofing.

Based on lowland field data in Los Baños, Laguna, the sporulation index, number of megasporocarps, and microsporocarps were significantly higher in UPLB Hybrid 1 than in *A. mexicana* # and *Azolla* sp. from Majayjay, Laguna. However, DT was less in UPLB Hybrid 1 than in the *Azolla* sp. from Majayjay, Laguna. Based on studies on 14 strains of *Azolla* in Benguet State University, Mountain Province, Philippines, DT was inversely proportional to relative growth rate (Fomeg-As and Merestela, 2014). In laboratory experiments, increasing plant density and/or P supply resulted in increased sporulation in *Azolla filiculoides* (Janes, 1998b). Also, it was estimated that a thick mat of 8 kg m^{-2} fresh biomass can produce 380,000 microsporocarps and 85,000 mgasporocarps m^{-2}. Hence, it can be assumed that DT can be inversely proportional to sporulation index. To validate this relationship, DT and sporulation index can be evaluated for UPLB Hybrid 1 and *Azolla* sp. from Majayjay, Laguna in irrigated lowland fields in Majayjay and Los Baños, Laguna under similar crop management. The interacting effects of light intensity, photoperiod, temperature, soil pH, N, and P supply regulating

sporulation (Pabby et al., 2003) will have to be considered.

Toward the end of dry and wet season cropping, water is withdrawn and field is left fallow for a month or two. During the fallow period, soil moisture is reduced and *Azolla* plants become desiccated (Figure 3). This prompted us to set up soil moisture studies in plastic trays in a rainout shelter for a month in Los Baños, Laguna to assess germination of spores of *Azolla* sp. from Majayjay, Laguna. The sensitivity of the *Azolla* sp. from Majayjay to reduction in soil moisture was evident with the significant reduction in number of days to germinate and 595% reduction in spores germinated when the wet soil treatment was compared with the flooded soil treatment (Table 3`). No spores germinated in the dry soil treatment but spores were still viable a week after flooding. It will be important to assess the germination of spores of UPLB Hybrid 1 and *A. mexicana* # 2002, *Azolla* selections (Watanabe et al., 1992; Payawal et al., 1994), and other *Azolla* spp. with some degree of tolerance to abiotic stresses (Uheda et al., 1999; Abraham and Dhar, 2010) in response to dry, wet, and flooded soil treatments, and viability of spores after flooding the dry soil.

Conclusion

This study documented the growth and sporulation of *Azolla* sp. coinciding with the growth and development of irrigated lowland rice in two cropping cycles and desiccation of *Azolla* plants during the dry fallow period between the two cropping cycles in Majayjay, Laguna.

In Los Baños, Laguna, doubling time or the number of days required to increase plant biomass twice, differed among the *Azolla* spp./accessions tested and appeared to be inversely proportional to sporulation index. This relationship should be validated during the rice cropping cycle in irrigated lowland fields in Los Baños, Laguna and Majayjay, Laguna. The interacting effects of light intensity, photoperiod, temperature, soil pH, nitrogen and phosphorus supply, and crop management influencing growth and sporulation should be considered.

For the *Azolla* sp. from Majayjay, Laguna, the number of days to germinate and number of germinated spores were strongly influenced by soil moisture condition. It is important to assess the germination of spores of UPLB Hybrid 1, other *Azolla* species, and selections with some degree of tolerance to abiotic stresses in response to dry, wet, and flooded soil conditions, and viability of their spores after flooding the dry soil.

Literature Cited

Abraham G, Dhar DW (2010) Induction of salt tolerance in *Azolla microphylla* Kauff through modulation of antioxidant enzymes and ion transport. Protoplasma **245**: 105-111

Callo Jr. DP, Dilag RT, Necesario RS, Wood DM, Tagalog FC, Gapasin DP, Ramirez CM, Watanabe I (1985). A 14-day azolla adaptability test on farmer's fields. Philipp J. Crop Sci. **10**(3): 129 -133

Costa ML, Santos MCR, Carrapico F (1999) Biomass characterization of A. filiculoides grown in natural ecosystems and wastewater. Hydrobiologia **415**: 323-327

Costa ML, Santos MCR, Carrapico F, Pereirac AL (2009) *Azolla-Anabaena*'s behavior in urban wastewater and artificial media-influence of combined nitrogen. Water Resource. **43**: 3743-3750

Department of Agriculture - University of the Philippines Los Baños - National Azolla Action Program (DA-UPLB-NAAP) (1988) Lessons on *Azolla* Technology: A Training Manual of the National Azolla Action Program (NAAP), 1st Re vision. Department of Soil Science, College of Agriculture, University of the Philippines Los Baños

El Katony TM, Serao MS, Badway AM, Mousa MA (1996) Effect of phosphorus on growth and uptake of nutrients by *A. filiculoides* Lam. Journal of Environmental Sciences **12**: 69-88

Fomeg-As DY, Merestela TM (2004) Correlation Analysis Between Doubling Time and Relative Growth Rate of *Azolla* (*Azolla* sp.) Grown in Tadian, Mountain Province. Mountain Province State Polytechnic College, Bontoc, Mountain Province. Benguet State University, La Trinidad, Benguet, Philippines. 37 p.

Fosu-Mensah BY, Vlek PLG, Manske G, Mensah M (2015) The influence of *Azolla pinnata* on flood water chemistry, grain yield and nitrogen uptake of rice in Dano, Southwestern Burkina Faso. J. Agric. Science **7**(8): 118-130. http://dx.doi.org/10.5539/jas.v7n8p118

Janes R (1998a) Growth and survival of *A. filiculoides* in Britain. I. Vegetative reproduction. New Phytol. **138**: 367-375

Janes R (1998b) Growth and survival of *A. filiculoides* in Britain. II. Sexual reproduction. New Phytol. **138**: 377-384

Kannaiyan S (1987) Use of *Azolla* in India. Azolla utilization: Proceedings of the workshop on azolla use. Fuzhou, Fujian, China. 31 March - 5 April 1985. International Rice Research Institute. ISBN 971-104-179-0. pp. 109-118

Kannaiyan S (1993) Nitrogen contribution by *Azolla* to rice crop. Proc. Indian Natl. Sci. Acad. B59(**3-4**): 309-314

Kulasooriya SA, Hirimburegama WK, Abeysekera SW (1987) Use of *Azolla* in Sri Lanka. *Azolla* utilization: Proceedings of the workshop on azolla use. Fuzhou, Fujian, China. 31 March - 5 April 1985. International Rice Research Institute. ISBN 971-104-179-0. pp. 131-138

Kushari DP and Watanabe I (1992) Differential responses of *Azolla* to phosphorus deficiency. Part II. Screening method under concentration controlled condition. Soil Science and Plant Nutrition **38**: 65-79

Lin C, Watanabe I (1988) A new method for obtaining *Anabaena*-free *Azolla*. New Phytol. **108**: 341-344

Mus F, Crook MB, Garci K, Costas AG, Geddes BA, Kouri ED, Paramasivan P, Ryu MH, Oldroyd GED, Poole PS, Udvardi MK, Voigt CA, Ané JM, Peters JW (2016) Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl Environ Microbiol **82**: 3698-3710. doi:10.1128/AEM.01055-16

Payawal PC and De Macale MAR (1994) Note: Sporulation index of *Azolla* hybrids under high land and lowland Conditions. The Philippine Agriculturist **77**(2): 223-233

P**abby A, Prasanna R, Singh PK** (2003) *Azolla-Anabaena* symbiosis- from traditional agriculture to biotechnology. Indian Journal of Biotechnology **2**: 26-37.

Peters GA, Meeks JC (1989) The *Azolla-Anabaena* symbiosis: basic biology. Ann. Rev. Plant Physiol. Plant Mol. Biol. **40**: 193-210

Quing-Yuan X, Yan-Ru S, Guang-li Y, Ke-lin P (1987) Germination of *Azolla filiculoides* Lam. sporocarps and factors affecting their growth. Azolla utilization: Proceedings of the workshop on azolla use. Fuzhou, Fujian, China. 31 March - 5 April 1985. International Rice Research Institute.

Sadeghi R, Zarkami R, Sabetraftar, Van Damme P (2013) A review of some ecological factors affecting the growth of *Azolla* spp. Caspian J. Env. Sci. **11**(1): 65-76

Serag, MS, El-Hakeem A, Badway M, Mousa MA (2000) On the ecology of *A. filiculoides* Lam. In Damietta District, Egypt. Limnologica 30: 73-81

Shuying L (1987) Methods for using Azolla filiculoides sporocarps to culture sporophytes in the field. Azolla utilization; Proceedings of the workshop on Azolla use. Fuzhou, Fujian, China. 31 March - 5 April 1985. International Rice Research Institute. ISBN 971-104-179-0. pp. 27-33

Singh AL, Singh PK (1987) Influence of *Azolla* management on the growth, yield of rice and soil fertility. II. N and P contents of plant and soil. Plant and Soil **102**: 49-54

Tuan DT, Thuyet RQ (1979) Use of *Azolla* in rice production in Vietnam. pp. 395-405 in Inter national Rice Research Institute (IRRI), Nitrogen and Rice. IRRI, Los Baños, Philippines

Uheda E, Kitoh S, Shiomi N (1999) Response of six *Azolla* species to transient high-temperature stress. Aquatic Botany **64**: 87-92

Wagner A (1997) *Azolla*: A review of its biology and utilization. The Bot. Review 63(1): 1-26

Watanabe I, Espinas CR, Berja NS, Alimagno BV (1977) Utilization of *Azolla-Anabaena* complex as nitrogen fertilizer for rice. IRRI Paper Series **11**: 1-15

Watanabe I (1982) *Azolla-Anabaena* symbiosis – Its physiology and use in tropical agriculture. Pages 169-185 in YR Dommergues and HG Diem (eds.), Microbiology of Tropical Soils and Plant Productivity. Martinus Nijhoff/W. Junk, The Hague

Watanabe I, Roger PA, Ladha JK, Van Hove C (1992) Biofertilizer Germplasm Collections at IRRI. International Rice Research Institute. ISBN 971-22-0025-6 pp. 37-51

Zhuang-Ta Z, Yu-Si K, De-Quan L, Bing-Yuan D and Xi-Lian L (1987) Utilization of Azolla in agricultural production in Guangdong Province, China. Azolla utilization; Proceedings of the workshop on Azolla use. Fuzhou, Fujian, China. 31 March - 5 April 1985. International Rice Research Institute.

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Development of New High Temperature-Tolerant Rice Genotypes through Conventional Breeding and Marker-Assisted Selection in the Philippines

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Abstract High temperatures at reproductive stage can induce floret sterility and limit rice yields. Reports show that rice yields can be reduced by 10-14% when air temperatures above 35°C coincide with reproductive stage. With the current scenario and projected increase in temperature due to climate change, new breeds of rice tolerant of heat stress are needed. Earlier, PhilRice identified genetic donors for heat tolerance and started the breeding process resulting in hundreds of lines that were developed, screened, and selected under high temperature conditions. In this study, marker-assisted selection (MAS) using a heat-tolerant marker RM3586 and conventional selection by phenotypic acceptability using the standard evaluation system were employed to identify high temperature tolerant and susceptible genotypes in early segregating populations then screened under high temperatures in the fields in PhilRice, Science City of Muñoz, Nueva Ecija and Southern Cagayan Research Station, Iguig, Cagayan. One elite breeding line selected through MAS and five lines from conventional selection were nominated to the National Cooperative Test (NCT). Of the six entries nominated to NCT, breeding lines PR42026-34-1-3-B-2, PR44500-A3-3-2-2 (MAS), and PR40330-4-2-7-1-2-1 were among the top five entries reported by NCT. Their average grain yield and percentage spikelet sterility were 3.9 and 1.9 times higher, respectively, than the tolerant check varieties N22 and Dular following exposure to heat stress at reproductive stage.

Keywords: Conventional Breeding, High Temperature Tolerance, Marker-Assisted Selection, Quantitative Trait Loci, Rice.

Introduction

Rice varietal development for high temperature conditions is an increasing concern and challenge among breeders due to global warming scenarios in the tropics. Although farmers can adapt to climate change by shifting planting dates, selecting varieties with different growth durations, or practicing crop rotation, these adjustments may result in lower yields. Developing germplasm with higher tolerance to climate-induced high temperature stresses through breeding is a sound climate change adaptation strategy. Breeding improved rice varieties by developing germplasm with higher tolerance to climate-induced stresses is a sound climate change adaptation strategy. Development of such varieties requires participation of multidisciplinary teams composed of breeders, geneticists, pathologists, entomologists, physiologists, biotechnologists, agronomists, and cereal chemists (Wassman et al., 2009).

Breeding rice varieties tolerant to high temperature has received less attention compared with other stresses such as drought and salinity (Wassmann et al., 2009). In the early 1980s (Mackill et al., 1982; Mackill and Coffman, 1983), high temperature tolerance of rice were considered within region-specific breeding programs with limited success. Through the years, however, corporation of high temperature tolerance in rice is becoming a key breeding objective in the Philippines (Manigbas and Sebastian, 2007; Ye et al., 2012). Germplasm innovation and identification of high temperature-tolerant inbred rice varieties are initiatives for variety development of susceptible high-yielding Philippine rice varieties (Manigbas et al., 2014).

High temperature affects all rice growth

stages, from emergence to ripening but flowering (anthesis and fertilization) and booting (microsporogenesis) are the most sensitive stages (Imaki et al., 1982; Shah et al., 2011; Tenorio et al., 2013). In general, when rice plants are exposed to temperature greater than 35°C for more than an hour at anthesis, it results in increased sterility (Jagadish et al., 2007). Temperature above 35°C at flowering stage causes high pollen and spikelet sterility, which leads to serious yield losses, low grain quality, and low harvest index (Osada et al., 1973; Matsushima et al., 1982; Matsui et al., 1997a and b; Zhong et al., 2005; Prasad et al., 2006). A major cause of spikelet sterility at flowering stage is anther indehiscence. Under high temperatures, the anthers of heat-tolerant genotypes dehisce more easily compared with susceptible ones (Satake and Yoshida, 1978; Mackill et al., 1982; Matsui et al., 1997a, 1997b; Matsui et al., 2001) due to tight closure of the locules by the cell layers that delays the opening and decreases spikelet fertility (Matsui and Omasa, 2002). High temperature affects anther dehiscence, pollination, and pollen germination that leads to spikelet sterility and yield loss (Yoshida et al., 1981).

Adaptation to high temperature stress in rice through cultural management practices is insufficient to sustain and increase yields. Thus, breeding for heat tolerance in rice is a high priority. Incorporation of biotechnology like MAS has proven to be useful in rice breeding as it shortens the breeding cycle by 2-3 years. This tool was used in the development of new heat-tolerant rice genotypes along with the conventional method. In Vietnam, Lang et al. (2015) described the use of molecular markers in identifying tolerant plants using marker-assisted backcrossing populations under high temperature environments. The advantage of using molecular markers over phenotypic data is the possibility to compare genotypes even if they are sampled in different environments and the ability to detect DNA polymorphisms through the entire genome (Sakiyama et al., 2014).

This study utilized MAS and conventional selection by phenotypic acceptability to identify high temperature tolerant and susceptible genotypes in the early segregating populations prior to their screening under high temperatures in the field.

Materials and Methods

Location

The experimental areas were located at PhilRice Central Experiment Station (PhilRice CES) in Science City of Muñoz, Nueva Ecija and Southern Cagayan Research Center (SCRC) in Iguig, Cagayan. PhilRice CES, under the Department of Agriculture, is situated in Central Luzon, Region 3, 15.6758°N and 120.8903°E. Farm areas were mostly irrigated and dry season planting time was from December to January. SCRC is in Northeast Luzon, Region 2, 17.7499°N, 121.7537°E. Farm areas were irrigated and transplanting rice was from December to February. Late planting from January to February was critical because high temperatures (38 to 40°C) occurred during April-May and usually large areas were affected.

Breeding and Selection

Hybridization and field selection were done at PhilRice CES and several segregating populations were advanced and selected using pedigree method. Five selected backcrossed populations were used for marker-assisted backcrossing during dry (DS) and wet (WS) seasons. All populations were screened and selected in the field under high temperatures in two sites in unreplicated conventional breeding with an initial 4,000 plants in the F_2 population of each cross and generation advance in the F_3-F_6. Selection of desirable phenotypes under high temperature conditions was done during the DS as temperature reaches up to 38°C in April to May. Every season, each population was grouped according to flowering dates so that all breeding lines would flower at the same time during high temperature months. Selection was based on phenotypic acceptability (IRRI SES, 2014). Criteria used were erect leaves, high tillering, short to intermediate plant height, good panicle exsertion, 100-115 days maturity, high number of filled spikelets, and long panicles with intermediate to long grains. At harvest, percentage spikelet sterility/fertility was determined by counting filled and unfilled spikelets of the main panicle of 10 sampled healthy plants of each advanced breeding line. This

protocol was also followed at SCRC. After undergoing generation advance, selection under high temperature conditions and yield tests, promising lines were nominated to the National Cooperative Test (NCT). The NCT is the final testing of the breeding lines prior to varietal release. The conventional breeding scheme of backcrossing and the use of MAS as a tool are shown in Figure 1.

Phenotyping of BC_2F_4 Population

The BC_2F_4 population was evaluated from heading to maturity. Heading dates were recorded as basis for the number of days to maturity. Duration of heading was determined from start of panicle exsertion to full exsertion. To monitor the time of flowering, temperature at dehiscence, and relative humidity (RH) at flowering, the time and date on the MINCER micrometeorological equipment and digital clock were synchronized to facilitate marking of a phenotype. At physiological maturity, the number of filled and unfilled grains were recorded to determine percent spikelet sterility/fertility. Grain yield was also recorded. The mean spikelet sterility of three panicles was used to evaluate the heat tolerance of the progenies.

Quantitative trait loci (QTL) analysis was done using composite interval mapping with IciMapping 4.0 software to identify the genetic loci responsible for the variation of study traits. Linkage groups were identified using command group with logarithm of odds (LOD) > 4.0, and this LOD value was used to check linkage among the SSR markers. Permutation test was done at 1,000 iterations to increase the precision of putative QTLs or reduce the probability of finding false marker-QTL association. The QTL that could meet the new threshold set by permutation was selected based on the significance level at $p > 0.05$ and LOD score of 4.0.

DNA Preparation

Six mapping populations with heat tolerance donors N22 and Dular crossed with popular high-yielding varieties but heat-susceptible were assembled. Genomic DNA from leaf samples were extracted using the Modified Cetyltrimethylammonium Bromide (CTAB) method (Perez et al., 2012) and dissolved in TE buffer with RNAse. PCR amplification was carried out using 6.82 µL for each reaction well containing the following: sd-H_2O, 5x PCR buffer, 10 mM $MgCl_2$, 1 mM dNTP, 10 µM forward and reverse primers, and 5 U Taq polymerase. The PCR cycling regime was 94°C for 5 min, followed by 30 cycles of 1 min denaturing at 94°C, 1 min primer annealing between 55°C to 67°C, 2 min primer extension at 72°C, plus a final extension of 5 min at 72°C completed the cycle

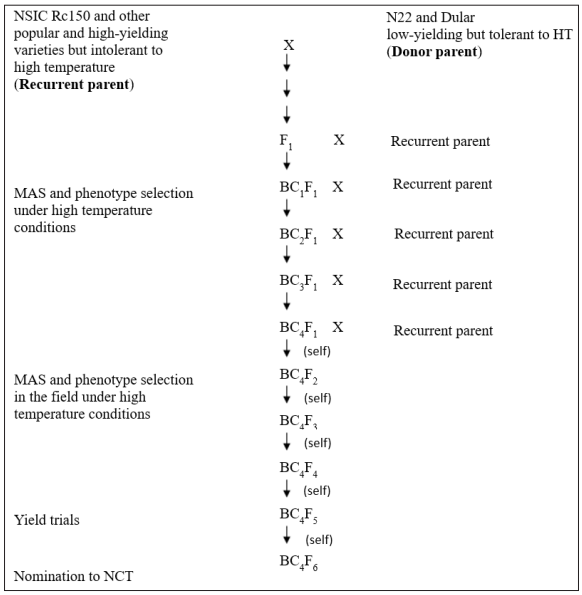

Figure 1. Schematic diagram for the development of heat-tolerant rice genotypes using marker-assisted backcrossing at different filial generations and selection prior NCT nomination. HT = high temperature; MAS = marker-assisted selection.

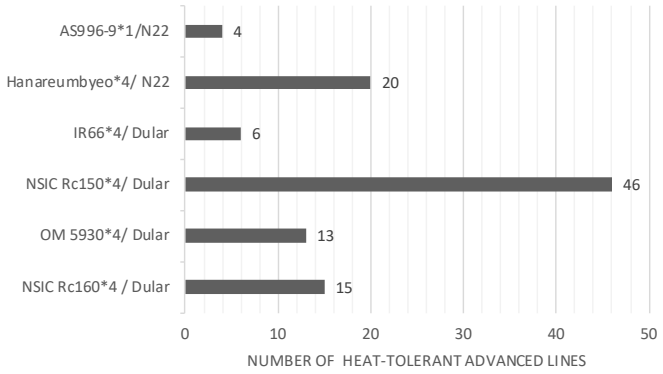

Figure 2. Number of heat-tolerant advanced lines developed through MAS and selected in the field under high temperature conditions (37 -38ºC) at PhilRice CES and SCRC.

performed in programmable thermal cycler PTC® 100, MJ Research Inc.

The PCR products were loaded in 8% non-denaturing polyacrylamide gel with 1x TBE buffer. The samples were prepared by adding 6 µL of loading dye (3x STR dye) and loaded 3.5 µL samples on the gel. The gels were electrophoresed at 100 V for 1 h, stained with 5% SYBR® Safe DNA Staining solution for 10 min and viewed in Molecular Imager® Gel Doc™ XR System with Image Lab™ Software.

Marker-Assisted Selection

MAS was used in six BC_4F_2 mapping populations that showed polymorphism (Figure 2). These populations were selected based on the genotypes using molecular marker RM3586 that was associated with heat tolerance and tightly linked to a major QTL on chromosome 3 (Zhang et al., 2009; Lang et al., 2015). After band scoring and marker data analysis, susceptible plants based on the marker data were discarded. Only the tolerant and heterozygous plants were grown in the field in a regular unreplicated pedigree nursery with 3 rows at 25 plants per row. Populations were grouped according to flowering date and planted in four batches so that they would flower at the same time under high temperatures in the field. Heterozygous plants that segregated with susceptible alleles in the next generations were discarded. Plants were not selected due to susceptibility to pests and diseases, poor plant type, highly sterile panicles, and less number of grains per panicle. At harvest, percent spikelet sterility/fertility was assessed for selected individuals. Selected plants were advanced to the next generation and repeatedly selected until uniformity of the population was achieved in the F_5-F_6 and in the backcrossed populations. Most of the selected lines were either tolerant or heterozygous based on the marker and the susceptible genotypes were discarded. Plants susceptible to high temperature, insect pests, and diseases were also dispensed.

Spikelet fertility should be >82% (or <18% spikelet sterility) to be considered tolerant, 60-81% (or 19-40% spikelet sterility) for intermediate tolerance, and <59% (or >41% spikelet sterility) for susceptible. This percentage was based on results from an experiment, which used MAS in the field (Manigbas and Madrid, 2013). Figure 2 shows the number of high temperature-tolerant advanced lines selected using MAS and field methods. Outstanding lines with heat tolerance trait generated were from cross of NSIC RC150*4/Dular with 46 lines. This was followed by Hanareumbyeo*4/Nagina22 or N22 with 20 lines, NSIC RC160*4/Dular, 15 lines; OM 5930*4/ Dular with 13 lines; IR66*4/ Dular with 6 lines; and AS996-9*1/N22, 4 lines. Breeding lines with grain yields of more than 4 t ha^{-1} and have intermediate resistance (IR) to resistance (R) against insect pests and diseases were included. Moreover, those with acceptab;e grain qualities and crop maturity were studied.

Figure 3. MINCER (Micrometeorological Instrument for Near Canopy Environment of Rice) installed at the start of the reproductive stage of the rice plant to record temperature and RH every 2 min for 24 h.

Figure 4. Portable camera installed in the field to take photo every 3 min from 8 am to 1 pm during flowering of the rice plants.

Temperature and Relative Humidity

In DS when temperature was expected to rise, a MINCER (Micrometeorological Instrument for Near Canopy Environment of Rice) was placed above the rice crop canopy to record the temperature and RH every 2 min for 24 h (Figure 3). The instrument was installed at booting stage until near maturity to monitor the temperature and RH during the reproductive stage and the maximum temperature at the onset of flowering. The instruments were useful in evaluating the breeding lines that flowered at different temperature regimes. A Ricoh camera (Model Wg-30) was installed to monitor the start, peak, and end of anthesis every 3 min from 8 am to 1 pm (Figure 4).

Yield Trials

As part of the conventional breeding approach, advanced and promising lines were evaluated in the Observational Nursery for uniformity, phenotypic acceptability, number of productive tillers, spikelet sterility/fertility, and grain yield. Uniform lines with excellent plant type and high yield (>5 t/ha) and low spikelet sterility (<40%) or more than 60% spikelet fertility were selected. These advanced lines were tested in Preliminary Multi-Environmental Yield Trial outside PhilRice while others were tested at PhilRice CES and at SCRC in 2015 DS and WS. Promising lines that passed the criteria were nominated to Multi-Environmental Trials (MET), Advanced Yield Test (AYT), MET 1, MET 2, and the NCT. Only the best advanced and promising lines were selected and presented in this paper as a result of the different yield tests conducted. In the MET, AYT, MET 1, and MET 2 tests, entries from other breeding institutions and groups with reported heat tolerance were evaluated. In the field test, the checks used were the heat-tolerant varieties N22 and Dular and heat-susceptible varieties IR52 and NSIC Rc160. NSIC Rc222, a high-yielding variety popular among farmers, was also tested in the field.

Results

Field Selection

All breeding lines from segregating populations up to advanced/promising lines, either selected through MAS or conventional breeding, were screened in the field for high temperature tolerance, and reaction to pests (i.e., insect pests and diseases). Nearly 4,000 plants were planted in the F_2 nursery and advanced in the next generation with approximately 10% selected for tolerance for high temperature and pests. Each generation, especially during DS, was subjected to high temperature stress in the field and planting was staggered so that flowering coincided with the highest temperatures in April to May. Temperature and RH from 2013 to 2015 DS were recorded using the MINCER. Figure 5 shows measurement of temperature and RH above the rice crop canopy in the field at 2 min interval for 24 h d^{-1} wk^{-1} in April-May 2015 in PhilRice CES. The maximum temperature during these months exceeded 35°C

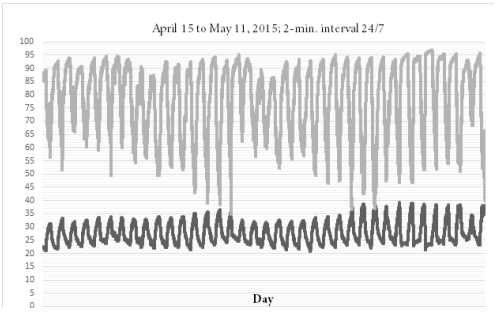

Figure 5. RH (gray in percent) and air temperature (black in ^{0}C) readings by the MINCER (Micrometeorological Instrument for Near Canopy Environment of Rice) at 2-min interval for 24 h from 15 April to 11 May 2015 in PhilRice CES. Similar trends were observed in SCRC.

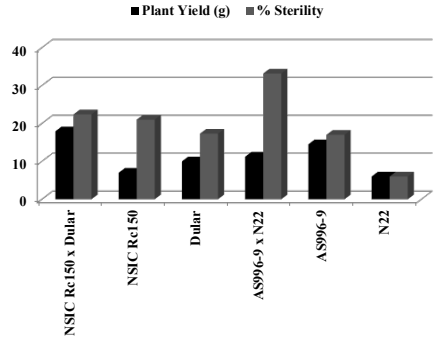

Figure 7. Plant yield and percent spikelet sterility of the different crosses and their parents following exposure to high temperature.

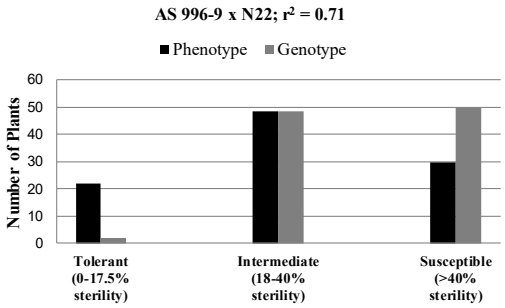

Figure 6. Reaction of a backcross population of AS996-9 (susceptible to high temperature) with N22 (tolerant to high temperature) for the tolerant, intermediate, and susceptible groups and their ranges of spikelet sterility. The phenotype and genotype relationship had a coefficient of determination of 0.71.

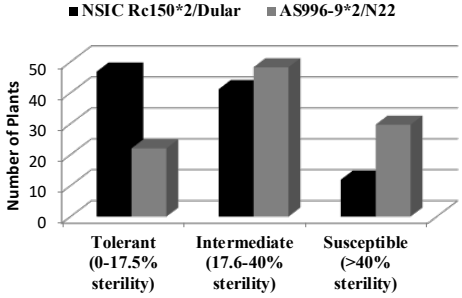

Figure 8. Number of plants in the BC_2 populations of selected populations in NSIC Rc150*2/Dular cross and AS996-9*2/N22 cross for the tolerant, intermediate, and susceptible groups and their spikelet sterility ranges. Note: *2 means recurrent parent in the second backcrossing (BC_2).

during reproductive stage and caused heat stress during anthesis, pollination, and grain development. When air temperatures exceeded 35^{o}C, average RH was 69.1% (Figure 5). Using a time-lapse camera (Figure 4) at 3 min interval, the start of anthesis was generally at 9:15 am and ended at nearly 12 noon. Similar trends in temperature and RH were observed in the other field-testing site in SCRC.

A sample phenotype and genotype relationship was obtained after crossing a susceptible variety AS996-9 with a high temperature-tolerant variety N22 (Figure 6). Based on the SSR marker RM3586 linked to heat tolerance, the coefficient of determination (R^2) was 0.71. In the intermediate tolerant types, the phenotype and genotype had similar number of plants and spikelet sterility ranged from 18 to 40%. However, there was a mismatch between the phenotype and genotype for the tolerant and susceptible types. This was also observed in other populations.

Figure 7 shows that the progeny of the cross between NSIC Rc150 and Dular had higher plant yield and spikelet sterility than the parents. However, the progeny of the cross between AS996-9 and N22 had lower yield than the parent AS996-9 but lower yield than the other parent N22. Spikelet sterility of the cross was higher than those of the parents.

In terms of frequency of the breeding lines or the number of plants in the BC_2 populations, crosses NSIC Rc150*2/Dular and AS996-9*2/N22 produced an almost equal number of intermediate tolerant types with spikelet sterility ranging from 17.6 to 40% (Figure 8). The frequency of breeding lines from both crosses was more variable for the tolerant and susceptible types. There were variations in terms of the cross combination between susceptible and tolerant parents indicating good combining ability of particular genotypes. Both crosses produced an almost

similar number of intermediate tolerant types with spikelet sterility of 17.6-40%.

Marker-Assisted Selection

Selected parents, check varieties, and backcrossed populations were screened using the SSR marker RM3586. The tolerant and susceptible checks had distinct banding patterns and were easily scored (Figure 9). The genotypic data was used to identify and confirm tolerance and susceptibility of segregating plants under high temperature conditions. In some cases however, few individuals showed tolerant reaction based on the marker but had high percent spikelet sterility in the field. It was therefore necessary that selection in the field under high temperature stress be done to confirm the genotypic data using the marker.

Performance of Breeding Lines in the Field

The following were evaluaed in the field (Table 1): 42 promising heat-tolerant lines from conventional method, 8 promising lines selected through MAS, a popular high-yielding variety NSIC Rc222, two tolerant check varieties (N22 and Dular), and two susceptible check varieties (IR52 and NSIC Rc160). These genotypes were evaluated for grain yield, % spikelet sterility, reaction to insect pests and diseases, grain quality, and maturity following exposure to high temperature stress. Breeding lines that attained grain yields close to 6 t ha^{-1} and above were nominated to NCT. NCT is a branch of the National Seed Industry Council (NSIC) under the Department of Agriculture that is responsible for evaluating promising breeding lines prior to recommending the release of varieties. In the 2016 DS NCT field performance report, three of the breeding lines PR42026-34-1-3-B-2, PR44500-A3-3-2-2 (MAS), and PR40330-4-2-7-1-2-1 (Table 1) were in the top 5 of the 25 entries from the different breeding institutions. Yields and spikelet sterility of the 3 breeding lines ranged from 5.8 to 6.8 t ha^{-1} and 7.8 to 34.0%, respectively. Yields and spikelet sterility of the tolerant check varieties N22 and Dular ranged from 1.2 to 1.9 t ha^{-1} and from 6.8 to 20.0%, respectively. Yields and spikelet sterility of the susceptible checks IR52 and NSIC Rc160 ranged from 2.1 to 3.2 t ha^{-1} and from 42.8 to 45.6%, respectively. With such yield levels, pest damage to the crop should be minimum. Amylose content ranged from low to intermediate levels. Grain chalkiness ranged from grade 1 to premium. Crop maturity ranged from 122 to 129 days from date of sowing. The other promising breeding lines were used for further improvement.

Discussion

Figure 5 shows that maximum air temperatures exceeded 35°C during reproductive stage and caused heat stress during anthesis, pollination, and grain development in field-testing sites in PhilRice CES and SCRC. As almost all breeding lines planted in the field were subjected to high temperature stress during the critical reproductive stage, selection pressure was achieved. Selection of desirable genotypes under high temperature conditions is the best strategy to screen breeding materials that possess heat tolerance. Considering that high air temperatures usually occur in April-

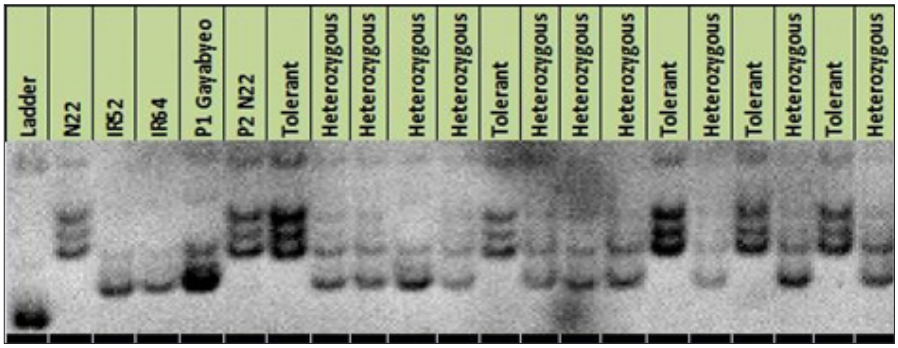

Figure 9. Reaction of selected parents (N22-tolerant, Gayabyeo-intolerant), tolerant check variety N22, susceptible varieties IR52 and IR64, tolerant and heterozygous individual plants in segregating population using the SSR marker RM3586.

Table 1. Percentage spikelet sterility, grain yield, reaction to pest and disease, grain quality, and maturity of breeding lines developed through MAS and conventional method, tolerant and susceptible checks, and a popular high-yielding variety NSIC Rc222 after exposure to heat stress. The last column shows the breeding lines nominated to the NCT.

Designation	% Spikelet Sterility under Heat Stress	Grain Yield (t ha^{-1})	Reaction to Pest & Disease	Grain Quality		Maturity (Day)	
				Amylose	Chalk		
PR42026-34-1-3-B-2	34.0	6.8	--	I	Pr	129	NCT
PR42130-M-1-B-6-2-B-7	29.9	6.4	IR-Blast	L	G1	116	NCT
PR42130-M-1-B-3-2-B-9-B	37.8	7.0	--	--	--	115	
PR44500-A3-3-2-2 (MAS)*	25.0	6.2	--	I	G1	124	NCT
HTVN-13-9-2-1-1-B	49.3	6.0	IR-Blast	VL	Pr	121	NCT
PR42130-M-1-B-3-2-B-5-B	28.2	6.0	--	--	--	115	
PR 40330-4-2-7-1-2-1	7.8	5.8	--	L	G3	122	NCT
PR42130-M-1-B-2-1-B-4-B	34.4	5.8	--	--	--	115	
PR44499-A15-9-2-3-1-B (MAS)	14.8	5.8	--	--	--	106	
PR42131-M-1-B-8-1-B-5-3	14.7	5.7	--	--	--	130	
PR42130-M-1-B-8-3-B-4-B	25.5	5.6	--	--	--	122	
PR42132-M-3-B-B-B-B-9	26.4	5.6	R-Blast	--	--	120	
PR42130-M-1-B-3-2-B-1-B	27.8	5.5	--	--	--	118	
PR42132-M(I)-1-B-8-B-B-6	14.3	5.5	IR-BLB	L	Pr	118	NCT
PR42132-M-1-B-B-B-B-17-B	19.5	5.5	R-Blast	--	--	115	
PR44499-A14-5-2-3-6-B (MAS)	17.7	5.5	--	--	--	106	
PR42130-M-1-B-6-1-B-2-B	36.2	5.4	--	--	--	112	
PR42130-M-1-B-8-3-B-9-B	21.9	5.4	IR-Blast	--	--	118	
PR 42079-42-1-3-1-2-B	15.1	5.4	--	--	--	130	
PR44499-A14-6-2-2-2-B (MAS)	14.5	5.4	--	--	--	105	
PR42130-M-1-B-6-3-B-9-B	40.5	5.3	--	--	--	118	
PR42131-M-1-B-8-1-B-5-1	16.3	5.2	--	--	--	130	
PR 42086-16-1-4-1-1-B	15.2	5.1	--	--	--	115	
PR42130-M-1-B-10-3-B-7-B	31.9	5.0	R-Blast	I	Pr	124	
PR 42082-2-3-1-2-3-B	16.2	4.9	--	--	--	126	
PR42129-M-4-B-4-B-B-7	12.3	4.9	IR-Blast	--	--	126	
PR42130-M-1-B-6-3-B-1-B	22.4	4.9	R-Blast	--	--	120	
PR 42075-4-2-5-1-1-B	8.3	4.9	--	--	--	118	
PR42032-26-6-1-B-1	37.4	4.7	--	--	--	122	
PR42130-M-1-B-1-2-B-8-B	35.9	4.6	IR-Blast	--	--	123	
PR44498-A2-1-3-3-3-B (MAS)	16.7	4.6	--	--	--	108	
PR42130-M-1-B-10-2-B-8-B	29.4	4.6	--	--	--	120	
PR42130-M-1-B-1-2-B-9-B	40.0	4.6	IR-Blast	L	G1	118	
PR42130-M-1-B-2-1-B-4-B	15.1	4.6	R-Blast	--	--	119	
PR42032-26-6-1-B-2	12.0	4.6	--	--	--	123	
PR44499-A9-5-2-2-5-B (MAS)	15.6	4.5	--	--	--	106	
PR44499-A16-1-3-3-2-B (MAS)	13.3	4.5	--	--	--	104	
PR42130-M-1-B-8-3-B-3-B	19.6	4.5	R-Blast	L	Pr	121	
PR40780-58-2-1-2-1-2-B	14.5	4.4	--	--	--	115	
PR40780-58-2-1-2-1-2-B	14.5	4.4	--	--	--	115	
PR42130-M-1-B-3-2-B-1-B	16.7	4.4	--	--	--	121	
PR 40788-16-1-1-1-1	24.8	4.4	--	--	--	129	

Table 1. Percentage spikelet sterility, grain yield, reaction to pest and disease, grain quality, and maturity of breeding lines developed through MAS and conventional method, tolerant and susceptible checks, and a popular high-yielding variety NSIC Rc222 after exposure to heat stress. The last column shows the breeding lines nominated to the NCT. (**cont.**)

Designation	% Spikelet Sterility under Heat Stress	Grain Yield (t ha^{-1})	Reaction to Pest & Disease	Grain Quality		Maturity (Day)
				Amylose	Chalk	
PR40780-42-3-1-1-1	15.5	4.4	--	--	--	121
PR42132-M-1-B-B-B-B-15	16.0	4.3	--	L	G1	126
PR42130-M-1-B-8-B-B-7-2	13.4	4.2	--	--	--	104
PR 44538-11-6-3	49.0	4.2	--	--	--	125
PR42130-M-1-B-3-2-B-5-B	16.8	4.2	IR-Blast	--	--	120
PR42127-M-B-B-B-B-B-9	36.0	4.1	--	--	--	121
PR44499-A16-3-1-3-4-B (MAS)	13.1	4.1	--	--	--	106
PR42128-M-4-B-9-3-B-15-B	14.0	4.0	--	--	--	119
PR42130-M-1-B-2-1-B-2-B	12.4	4.0	--	--	--	122
NSIC Rc222	40.0	4.2	--	--	--	114
N22 (Tolerant Check)	6.8	1.2	--	--	--	82
Dular (Tolerant Check)	20.0	1.9	--	--	--	102
IR52 (Susceptible Check)	42.8	2.1	--	--	--	104
NSIC Rc160 (Susceptible Check)	45.6	3.2	--	--	--	107

Legend: R (Resistant to blast); IR (Intermediate resistance to blast); IR-BLB (Intermediate resistance to bacterial leaf blight); Grain Quality - Amylose: I (Intermediate), L (Low), VL (Very Low); Chalk: Pr (Premium), G1 (Grade 1), G2 (Grade 2), G3 (Grade 3); Maturity - from sowing to physiological maturity in days; *MAS- selected through SSR marker RM3586; (--) No data.

May in the Philippines, breeding lines are made to flower within this period to efficiently evaluate their performance through grouping of flowering dates and staggered planting. The camera set to take photos every 3 min is useful in evaluating and recording the start, peak, and end of anthesis. This way, tolerance or escape can be determined. Tolerant genotypes escape high temperature stress by flowering early morning while others are inherently tolerant similar to N22. For example, the early morning flowering types have been observed in wild rice such as *Oryza glaberima*.

The average RH was 69.1% during the periods when air temperatures exceeded 35^{0}C. Wassman et al. (2009) noted that processes close to the meiotic stage during tetrad formation and young microspore stage were most sensitive to high temperature during microsporogenesis, similar to drought and cold stress. A significant reduction in pollen production at 5^{0}C above ambient air temperature was attributed to impaired cell division of microspore mother cells.

Spikelets that are exposed to temperature greater than 35^{o}C for about 5 days during flowering period are sterile and set no seed. If there is an interaction between temperature and duration, then the response of spikelet fertility to temperature has a cumulative temperature response above the threshold temperature (Prasad et al., 2006). Spikelet sterility was approximately 5% under normal conditions while field surveys revealed that sterility increased up to 15% when heading stage coincided with high-temperature days (Hasegawa et al., 2009). The temperature increase of 1°C shortened the number of days from sowing to heading by 4-5 days for some genotypes (Nakagawa et al., 2001). The peak of flowering of spikelets on a panicle occurs about 5 days after heading and lasts for 11 days regardless of temperature, cultivars, and flowering distribution (Nguyen et al., 2014).

The tolerant cultivars Dular and Nagina 22 or N22 showed high temperature tolerance and thus, are excellent sources of genes for high temperature tolerance (Manigbas et al., 2014). Tenorio et al. (2013) found that under screenhouse condition, Dular was heat-tolerant at booting stage after exposure to 39°C. Flowering and anthesis in most *Oryza sativa* genotypes occurred over a 5-day period, with most spikelets reaching anthesis

between 10:00 am and 12:00 pm (Nishiyama and Blanco, 1980; Prasad et al., 2006). This was also observed in this study but anthesis started earlier at 9:15 am. Exposure to high temperature stress for 1 h at anthesis is sufficient to induce spikelet sterility (Jagadish et al., 2007), but sterility does not occur when spikelets flower 1 h prior to the high-temperature treatment, suggesting that spikelets have achieved a considerably high tolerance after the completion of fertilization. Exposure to 41°C for 4 h at flowering causes irreversible damage and plants become completely sterile (Satake and Yoshida, 1978).

Rice has the ability to monitor and control the rate of flowering as an escape mechanism under high temperature. The concept of spenders and savers with reference to rate of flowering in rice has been reported (Jagadish et al., 2007), in which a 20% increase and 36% decline in the rate of flowering was observed in cultivars IR64 and Azucena, respectively, at 38°C and 60% RH over three consecutive days. Likewise, rice plants exposed to high temperatures during critical stages can avoid heat by maintaining their microclimate temperature below critical levels through transpiration cooling. The effect of high temperature is closely related to the ambient RH; hence, the level of transpiration cooling is determined by vapor pressure deficit or dryness of the atmosphere than temperature per se. Using ultra thin copper constantan thermocouples, Jagadish et al. (2007) recorded spikelet tissue temperatures of 29.6, 33.7, and 36.2°C, i.e., 0.4, 1.3, and 1.8°C below ambient air temperatures of 30, 35, and 38°C, respectively. Similar differences were observed in rice (Satake, 1995).

The results of the correlation between genotype and phenotype selections in Figure 6 showed that genotype selection was skewed to the right (from tolerant to susceptible), having the highest number of plants with susceptibility to heat. On the other hand, phenotype selection had a bell-shaped graph indicating well distributed phenotypes. Results suggest that field phenotyping is very important under high temperature conditions to determine the tolerant and susceptible individuals. However, more molecular markers linked to high temperature stress tolerance are needed.

In Figure 7, performance of tolerant and susceptible parents and their progenies are compared in terms of plant yield and % spikelet sterility. The progeny of the cross between the susceptible NSIC Rc150 and tolerant Dular had significantly higher plant yield and slightly higher spikelet sterility than the parents. In contrast, the progeny of the cross between the susceptible AS996-9 and tolerant N22 had lower plant yield than AS996-9 and higher plant yield than N22. The decreasing trend in spikelet sterility from progeny to the parents was similar the cross between NSIC Rc150 and Dular. Yield of the tolerant variety N22 appears to be inherently lower than the yield of the susceptible parent AS996-9, but its spikelet sterility is significantly lower. Similar yield trends were obtained by Buu et al. (2014) where grain yields of most progenies from OM59 and N22 parental cross were significantly higher. This was mainly due to larger sink size, specifically increased spikelet number per panicle and larger panicle. Results suggest that breeding for heat tolerance may utilize susceptible high-yielding lines and tolerant varieties like N22 and aim for intermediate spikelet sterility (i.e., 18-40% spikelet sterility) assuming that other yield components (Buu et al., 2014) compensate to attain a certain yield level.

The % spikelet sterility of the selected progenies of NSIC Rc150*2 and Dular cross was skewed to the left (from susceptible to tolerant) with the tolerant having the highest number of plants (Figure 8). For the progenies of AS996-9*2/N22, % spikelet sterility was bell-shaped with equally distributed phenotypes and the highest number of plants having the intermediate level of percent spikelet sterility. Results suggest that the heat tolerance gene from tolerant parents has high combining ability, in which the transmission of the specific trait from the parent to offspring is high (Griffing, 1956).

The use of MAS as a tool in conventional breeding is effective in the selection of genotypes with high temperature tolerance especially in the early segregating populations where a large number of plants is evaluated. This saves time and space. Although not all known markers are

polymorphic in breeding populations, use of MAS can be specific. Continuous screening for MAS is being done (Grospe et al., 2016) to map the genes in different chromosomes using different genetic backgrounds and populations and eventually identify QTLs. This study utilized the molecular marker RM3586 associated with heat tolerance and tightly linked to a major QTL on chromosome 3 (Zhang et al., 2009; Lang et al., 2015). This work resulted in the selection of eight breeding lines with grain yields ranging from 4.1 to 6.2 t ha^{-1} and spikelet sterility ranging from 13.1 to 25.0% (Table 1). From the eight market-assisted selections, PR44500-A3-3-2-2 (MAS) with a yield of 6.2 t ha^{-1} and spikelet sterility of 15%, was nominated to NCT. While 1 of the 8 marker-assisted selections was nominated to NCT, 2 of the 42 conventional selections were nominated to NCT and both selections received good ratings. Results suggest that use of more molecular markers linked to heat tolerance can increase the efficiency of breeding for heat tolerance.

Pradhan et al. (2016) studied a set of breeding lines and landraces representing 240 germplasm rice lines in relation to high temperature stress tolerance. Their analysis of molecular variance revealed 25% variation between populations, 61% among individuals, and 14% within individuals in the set. Based on the composition of materials in the panel, there were QTLs representing the entire genome for the expression of tolerance. The strongly associated marker RM547 tagged with spikelet fertility under stress and markers RM228, RM205, RM247, RM242, INDEL3, and RM314 indirectly controlling high temperature stress were detected through both mixed linear model and general linear model TASSEL analysis. These markers can be deployed as a resource for marker-assisted breeding program of high temperature stress tolerance.

This study focused on the impact of increased daytime temperature on the reproductive growth of breeding lines. However, understanding the effects of high nighttime temperatures on rice crop growth is also important. Peng et al. (2004) examined the trends and relationships between rice yields and temperature by analyzing the 1979-2003 IRRI weather data. Annual mean maximum and minimum air temperatures increased by 0.35°C and 1.13°C, respectively. Rice grain yield and minimum temperature had a close correlation: that there is a 10% grain yield decline for every 1°C increase in minimum temperature in the DS. The decrease in radiation and increase in minimum temperature were reported to reduce yield. Morita et al. (2005) concluded that growth rate was lower in the early or middle stages of grain filling, and cell size was reduced midway between the central point and the surface of endosperm at high night temperature with 22/34°C than at high day temperature with 34/22°C (Wassman et al., 2009). Mohammed and Tarpley (2009) studied the effects of high night temperatures (HNTs) and preventive exogenous effectors (a-tocopherol, glycine betaine, and salicylic acid) on growth, development, physiology, and yield of rice plants. Plants were subjected to ambient night time temperature (ANT) (27°C) or HNT (32°C) through use of continuously controlled infrared heaters, starting from 2000 h to 0600 h. The HNT did not affect leaf photosynthetic rates; however, profound effects on chlorophyll content, leaf nitrogen content, percent pollen germination and spikelet fertility were observed. In addition, HNT hastened plant development rates, as indicated by the panicle emergence date. Plants grown under HNT showed a 90% decrease in yield compared to plants grown under ANT. Dry matter partitioning to the grains of cv. Cocodrie decreased under HNT mainly due to effects on pollen germination and spikelet fertility, but not photosynthesis. Their findings indicate that exogenous application of salicylic acid reduced the negative effects of HNT by 16%.

Fu et al. (2016) reported that superior spikelets were damaged more by heat stress than inferior spikelets. Heat-resistant and susceptible rice varieties were exposed to heat stress of 40°C at anthesis. Greater decrease in spikelet fertility and kernel weight were observed in superior spikelets due to significantly higher organ and canopy temperatures, illumination, and panicle types than inferior spikelets. They suggested that rice with upright growth habit with loose panicles might be more susceptible to heat stress resulting in higher canopy and spikelet temperatures.

Wu et al. (2016) studied the changes in phytohormones and their relationships with yield

and other attributes under heat stress in rice. Four rice varieties (Nagina22 or N22, Huanghuazhan, Liangyoupeijiu, and Shanyou 63) were grown in pots and subjected to three high temperature treatments plus control in temperature-controlled greenhouses for 15 days during the early reproductive phase. Yield reductions in Nagina22, Huanghuazhan, and Liangyoupeijiu were attributed to reductions in spikelet fertility, spikelets per panicle, and grain weight. The adverse effects of high temperature were alleviated by application of exogenous 6-benzylaminopurine (6-BA) in the heat-susceptible Liangyoupeijiu. High temperature stress reduced active cytokinins, gibberellin A1 (GA1), and indole-3-acetic acid (IAA), but increased abscisic acid (ABA) and bound cytokinins in young panicles. Correlation analyses and application of exogenous 6-BA revealed that high temperature-induced cytokinin changes may regulate yield components by modulating the differentiation and degradation of branches and spikelets, panicle exsertion, pollen vigor, anther dehiscence, and grain size. Heat-tolerant Shanyou 63 displayed minor changes in phytohormones, panicle formation, and grain yield under high temperature compared with those of the other three varieties. Their results suggest that phytohormone changes are closely associated with yield formation, and a small reduction or stability in phytohormone content is required to avoid large yield losses under heat stress.

Conclusion

Rice breeding lines were subjected to high daytime temperature stress (>35^{o}C) and RH during the critical reproductive stage in the field. Conventional breeding selection resulted in 2 out of the 42 breeding lines being nominated to the NCT. With the use of the marker RM3585 linked to heat tolerance, 1 of the 8 marker-assisted selections was nominated to NCT. Both conventional and marker-assisted selection, with grain yields ranging from 5.8 to 6.8 t ha^{-1} and spikelet sterility ranging from 7.8 to 34% (tolerant to intermediate tolerant levels) received high ratings from NCT. By using more molecular markers, the efficiency of breeding for heat tolerance can be increased.

Characteristic panicle exsertion rate, time/rate of flowering, anther dehiscence, and spikelet sterility/fertility are traits associated with heat tolerance. These morpho-physiological traits can be used as candidate screening tools in the field evaluation for heat tolerance. The linkage map constructed earlier can be utilized in fine mapping of the actual genes prior to isolation of genes via map-based cloning. The information on identified tightly-linked SSR DNA markers to heat tolerance can be used as baseline information for the selection of QTLs for MAS.

There is a need to assess the differential impact of increased daytime air temperatures and increased nighttime air temperatures on the microclimate, sterility, and other yield components of superior spikelets (located on apical primary branches) and inferior spikelets (located on proximal secondary branches). It is worthwhile to study the role of phytohormones in modulating the differentiation and degradation of branches and spikelets, panicle exsertion, pollen vigor, anther dehiscence, and grain size.

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Literature Cited

Buu BC, Ha PTT, Tam BP, Nhien TT, Hieu NV, Phouc NT, Minh LT, Giang LH, Lang NT (2014) Quantitative trait loci associated with heat tolerance in rice (*Oryza sativa* L.) Plant Breed. Biotech **2**(1): 14-24

Cao L, Zhao J, Zhan X, L,i D, He L, Cheng S. (2003) Mapping QTLs for heat tolerance and correlation between heat tolerance and photosynthetic rate in rice. Chin J Rice Sci. **17**:223-227

Chang H, Tang Y, Hayer-Hartl M, Hartl F (2007) Snap shot: Molecular chaperones, Part I. Cell 128

Chen Q, Yu S, Li Y, Mou T (2008) Identification of QTLs for Heat Tolerance at flowering stage in Rice. Sci. Agric. Sin. **41**: 315-321

Fu G, Feng B, Zhang C, Yang Y, Yang X, Chen T, Zhao X, Zhang X, Jin Q, Tao J (2016) Heat stress is more damaging to superior spikelets than inferiors of rice (*Oryza sativa* L.) due to their different organ temperatures front. Plant Sci. **7**(1637): 1-16

Griffing JB (1956) Concept of general and specific combining ability in relation to diallel crossing systems. Austr. J. Biol. Sci. **9**:463–93

Grospe JL, Abell, EA, Manigbas, NL (2016) Quantitative trait loci for high-temperature tolerance in rice (*Oryza sativa* L.). Philipp. Agric. Scientist **99** (1): 7-18

Hasegawa T, Yoshimoto M, Kuwagata M, Ishigooka Y, Kondo M, Ishimaru T (2009) The impact of global warming on rice production: Lessons from spikelet sterility observed under the record hot summer of 2007. NIAES Annual Report 2008: 23–25

Imaki T, Jyokei K, Hara K (1982) Flower opening under controlled environment in rice plants. Bulletin of the Faculty of Agriculture, Shimane University **16**: 1-7

International Rice Research Institute (IRRI) (2014) Standard evaluation system for rice, 5th edition. Los Baños, Philippines: International Rice Research Institute

Jagadish S, Craufurd P, Wheeler T (2007) High-temperature stress and spikelet fertility in rice (*Oryza sativa* L.). Journal of Experimental Botany **58**: 1627-1635

Kim M, Kim H, Lee W, Lee Y, Kwon SW, Lee, J (2015) Quantitative shotgun proteomics analysis of rice anther proteins after exposure to high temperature. Int'l J. Genomics. doi: 10.1155/2015/238704

Lang NT, Ha PTT, Tru PC, Toan TB, Buu BC, Cho YC (2015) Breeding for heat tolerance rice based on marker-assisted backcrossing in Vietnam. Plant Breeding and Biotechnology **3**(3): 274-281

Mackill DJ, Coffman WR (1983). Inheritance of high temperature tolerance and pollen shedding in a rice cross. Z Pflanzesuchtg **91**: 61–69

Mackill DJ, Coffman W, Rutger J (1982) Pollen shedding and combining ability for high temperature tolerance in rice. Crop Sci. **22**: 730-733

Manigbas NL, Madrid LB (2013) Application of molecular marker-assisted selection for heat tolerance in rice. Best Poster presented during the 40th Annual Convention of the Philippine Society of Biochemistry and Molecular Biology. December 4-5, 2013. De La Salle University, Manila. Philippines

Manigbas NL, Sebastian LS (2007) Breeding rice for high temperature tolerance in the Philippines. Paper presented in an International Workshop on Cool Rice for Warmer World. Published in the Abstracts/Proceedings of the Workshop. March 26-30, 2007. Wuhan, Hubei, China

Manigbas NL, Lambio LA, Madrid LB, Cardenas CC (2014) Germplasm innovation of heat tolerance in rice for irrigated lowland conditions in the Philippines. Rice Science **21**(3): 162-169

Matsui T, Omasa K, Horie T (1997a) High temperature induced spikelet sterility of Japonica rice at flowering in relation to air humidity and wind velocity conditions. Jpn. J. Crop Sci. **66**: 449-455

Matsui T, Namuco O, Ziska L, Horie T (1997b) Effect of high temperature and CO2 concentration on spikelet sterility in Indica rice. Field Crops Res. **51**: 213-219

Matsui T, Omasa K, Horie T (2001) The differences in sterility due to high temperature during the flowering period among Japonica rice varieties. Plant Prod. Sci 4: 90-93

Matsui T, Omasa K (2002) Rice cultivars tolerant to high temperature at flowering: Anther characteristics. Ann. Bot. **89**: 683-687

Matsushima S, Ikewada H, Maeda A, Honda S, Niki H (1982) Studies on rice cultivation in the tropics. Yielding and ripening responses of the rice plant to the extremely hot and dry climate in Sudan. Jpn. J. Trop. Agric. **26**: 19-25

Mohammad AR, Tarpley L (2009) High nighttime temperatures affect rice productivity through altered pollen germination and spikelet fertility. Agricultural and Forest Meteorology **149**: 999-1008

Morita S, Yonemaru J, Takanashi J (2005) Grain growth and endosperm cell size under high night temperatures in rice (*Oryza sativa* L.) Ann. Bot. **95**: 695–701

Nakagawa H, Takahashi W, Hasegawa T, Watanabe T, Horie T (2001) Development of a three-dimensional simulator for rice growth and development II. Accuracy of a rice phenology model to simulate heading stage and plant age in leaf number. Japanese Journal of Crop Science **70**:125-126

Nguyen D, Lee K, Kima D, Anh N, Lee B (2014) Modeling and validation of high-temperature induced spikelet sterility in rice. Field Crops Research **156**: 293-302

Nishiyama I, Blanco L (1980) Avoidance of high temperature sterility by flower opening in the early morning. Japan Agricultural Research Quarterly **14**: 116-117

Osada A, Sasiprapa V, Rahong M, Dhammanuvong S, Chakrabandho H (1973) Abnormal occurrence of empty grains of Indica rice plants in the dry hot season in Thailand. Proc. Crop Sci. Soc. Jpn. **42**: 103-109

Peng SB, Huang JL, Sheehy JE, Laza, RC, Visperas RM, Zhong XH, Centeno GS, Khush GS, Cassman KG (2004) Rice yields decline with higher night temperature from global warming. Proc. Natl. Acad. Sci. USA **101**: 9971–9975

Prasad P, Boote K, Allen L, Sheehy J, Thomas J (2006) Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in re sponse to high-temperature stress. Field Crops Res. **95**: 398-411

Perez LM, Pastor H, Domingo J, Tabanao DA, Manigbas NL (2012) DNA fingerprinting in hybrid rice: Its application in varietal purity testing. Philippine Rice Research Institute, Science City of Muñoz, Nueva Ecija, Philippines

Pradhan SK, Barrik SR, Sahoo A, Mohapatra S, Nayak DK, Mahender A, Meher J, Anandan A, Pandit E (2016) Population structure, genetic diversity, and molecular marker-trait association analysis for high temperature stress tolerance in rice. PLoS ONE **11**(8): 1-13. doi:10.1371/journal.pone.0160027

Sakiyama NS, Ramos HCC, Caixeta ET, Pereira MG (2014). Plant breeding with marker-assisted selection in Brazil. Crop Breeding and Applied Biotechnology **14**(1): http://dx.doi.org/10.1590/S1984-70332014000100009

Satake T (1995). High temperature injury. In T Matsuo , K Hoshikawa, ed, Science of the Rice Plant, Vol. 2. Physiology. Food and Agricultural Policy Research Centre, Tokyo, pp 805-812

Satake T, Yoshida S (1978) High temperature induced sterility in Indica rice at flowering. Jpn. J. Crop Sci. **47**: 6-17

Shah F, Huang J, Cui K, Nie L, Shah T, Chen C, Wang K (2011) Impact of high-temperature stress on rice plant and its traits related to tolerance. J. Agric. Sci. **149**(5): 545-556

Tenorio FA, Ye C, Redoña E, Sierra S, Laza M, Argayoso MA (2013) Screening rice genetic re sources for heat tolerance. SABRAO Journal of Breeding and Genetics **45**(3): 371-381

Wassmann R, Jagadish SVK, Heuer S, Ismail A, Redona E, Serraj R, Singh RK, Howell G, Pathak H, Sumfleth K. (2009) Climate change affecting rice production: The physiological and agronomic basis for possible adaptation strategies. Advances in Agronomy **101**: 60-110

Wu C, Cui K, Wang W, Li Q, Fahad S, Hu Q, Huang J, Nie L, Peng S (2016) Heat-induced phytohormone changes are associated with disrupted early reproductive development and reduced yield in rice. Scientific Reports |6:34978| doi: 10.1038/srep349778

Yan C, Ding Y, Wang Q, Liu Z, Li G, Muhammad I, Wang S (2010) The impact of relative humidity, genotypes and fertilizer application rates on panicle, leaf temperature, fertility, and seed setting of rice. Journal of Agricultural Science **148**:329–339

Ye C, Argayoso MA, Redona ED, Sierra SN, Laza MA, Dilla C.J, Mo Y, Thomson MJ, Chin J, Dela Vina CB, Diaz GQ, Hernandez JE (2012) Mapping QTL for heat tolerance at flowering stage in rice using SNP markers. Plant Breeding **131** (1): 33-41

Yoshida S, Satake T, Mackill DJ (1981) High temperature stress in rice (review). IRRI Res. Paper Series **67**: 5

Zhang T, Yang L, Jiang K, Huang H, Sun Q, Chen W, Zheng J (2008) QTL mapping for heat tolerance of the tassel period of rice. Mol. Plant Breed. **6**:867-873

Zhang GL, Chen LY, Xiao GY, Xiao YH, Chen XB, Zhang ST (2009) Bulk segregant analysis to detect QTL related to heat tolerance in rice (*Oryza sativa* L.) using SSR markers. Agric. Sci. China **8**(4): 482-487

Zhong L, Cheng F, Wen X, Sun X, Zhang G (2005) The deterioration of eating and cooking quality caused by high temperature during grain filling in early-season Indica rice cultivars. J. Agron. Crop Sci. **191**: 218-225

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Time and Temperature Effects on Leaching, Thermal, and Morphological Properties of Limewater-Cooked Rice

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Abstract Starchy materials like rice can be soaked for days or weeks in alkali to modify their physicochemical and textural properties. However, a systematic study on process time and temperature to hasten the process is lacking. We treated rice with slaked lime $(CaOH)_2$ to determine the effect of time and temperature on the extent of physicochemical changes using a 3 x 4 x 2 design (3 temperatures: 50, 60, 70°C; 4 cooking time: 22, 44, 66, 88 min in 2 conditions: limewater-cooked and water-cooked). Leaching of amylose, starch, and protein from the grains into the wash water was determined using colorimetric analyses. Thermal and morphological properties of the grains were also evaluated. Alkaline cooking of rice resulted in 0.2%, 2%, and 3% leaching of amylose, starch, and protein, respectively. Temperature was the only significant parameter in alkaline cooking. Cooking at 70°C selectively promoted amylose leaching but not starch or protein. Morphologically, both limewater-treated and water-cooked rice showed partially gelatinized cross sections but with less pore development in alkaline-cooked grains. Alkaline-cooked rice retained more nutrients and exhibited thermal properties than hydrothermally-treated rice. Results of this study may be used to develop a fast and inexpensive process to selectively modify rice composition and produce a starch-based ingredient for food processing.

Keywords: Alkali, Amylose, Leaching, Limewater, Protein, Rice, Starch.

Introduction

Alkaline cooking is a process commonly applied to maize in the processing of tortillas, pozole corn chips, and other *masa*-based products (Guzmán et al., 2011). This process can also be applied to rice and other starchy products to facilitate milling, reduce mycotoxins, and impart desirable textural attributes (Reepholkul and Charoenrein, 2013). When subjected to alkaline treatment under mild temperature conditions, starch can leach and undergo cold gelatinization (Cai et al., 2014; de Souza et al., 2016). Recently, there is an interest in improving the extractability of amylose from broken rice grains to yield low-amylose rice flour as corn starch alternative (Cardoso et al., 2007; Setyawati et al., 2016). Alkaline treatment is an effective step to solubilize glutelin, the major protein present in rice and aids in extracting starch (Anupapsamosorn and Charoenrein, 2015).

Alkaline treatment of rice focused on the use of sodium-based alkali such as caustic soda, borax, and sodium carbonate (Anupapsamosorn and Charoenrein, 2015; Bello et al., 2004; Cai et al., 2014; Reepholkul annd Charoenrein, 2013). Calcium hydroxide (slaked lime), although used extensively in maize nixtamalization, is seldom applied to rice. Slaked lime is relatively inexpensive and may increase concentrations of calcium in the diet and improve niacin bioavailability (Rodríguez-Miranda et al., 2011; Ruiz-Gutiérrez et al., 2012).

Leaching of components during soaking is a slow process that is limited by the extent of water diffusion (Bello et al., 2004). Modest leaching values can be obtained when rice grains are soaked for extended periods, which is similar to an annealing process (Bian and Chung, 2016; Cai et al., 2014; Nor Nadiha et al., 2010). Moreover, the diffusion rate can be affected by external factors such as operating conditions and internal factors related to structure (Setyawati et al., 2016). Temperatures below 100°C have been used in past alkaline cooking studies (Ruiz-Gutiérrez et al., 2010; Valdez-Niebla et al., 1993). However, a systematic study of time and temperature

combinations is lacking. Thus, the objective of the present investigation was to evaluate the effect of time and temperature combinations on the release properties of amylose, starch, and protein as well as the thermal and morphological properties of the alkaline-cooked rice.

Materials and Methods

Materials

Rice grains (Super Angelika) were purchased from a commercial supermarket and stored at −10°C prior to analyses. The following analytical-grade reagents were used: calcium hydroxide (Techno Pharmachem, Haryana, India), anthrone reagent (HiMejia Lab., Pvt., Ltd., Mumbai, India), anhydrous glucose and 98% v/v sulfuric acid (RCI Labscan Ltd., Bangkok, Thailand), and Bradford reagent (Merck, Darmstadt, Germany). The following chemicals were obtained from Sigma-Aldrich Co., St Louis, MO: ammonium chloride, potato amylose type V, and bovine serum albumin. Ethanol 95% (v/v), perchloric acid 26% (v/v), iodine, potassium iodide, sodium chloride, and sodium hydroxide pellets were sourced from Univar, Ajax Finechem Pty., Ltd., NSW, Australia. Saturated limewater (0.16 % w/v) was prepared at room temperature using distilled water that had been boiled, cooled, and mixed with slaked lime powder thoroughly until the powder dissolved. The resulting pH was 10.6.

Methods

Starch, Protein, and Amylose Content of Rice Grains

A revised iodine-amylose colorimetric assay of Juliano et al. (2012) was adopted. Color was read at a wavelength (λ) of 620 nm within 20-60 min. A standard curve (R^2 = 0.95) was generated with potato amylose V at concentrations of 0, 5, 15, and 25 mg/100 mL of 0.09 molar sodium hydroxide. Results were presented as % by mass of milled rice. The anthrone-based method of Clegg (1956) was used to estimate the starch content of rice grains, as reported in other papers (Kale et al., 2015; Pachuau et al., 2017). Ground rice was mixed with 0.5 mL of 95% (v/v) ethanol and 5 mL of 26% (v/v) perchloric acid in a 100-mL volumetric flask. The mixture was allowed to stand for 1 h with occasional manual stirring every 15 min before adding distilled water. The mixture (0.1 mL) was transferred into a test tube and mixed with 2 mL 26 % (v/v) perchloric acid. The sample was placed in an ice bath before 2 mL of freshly prepared anthrone reagent (0.2% w/v in concentrated sulfuric acid) was added. The solution was mixed well, capped with glass marbles, heated in boiling water bath for 10 min, and cooled to 25°C. Absorbance was read at λ of 630 nm using a UV Mini 1240 spectrophotometer (Shimadzu Corp., Kyoto, Japan). A standard curve (R^2 = 0.94) was generated after measuring glucose concentrations of 0, 20, 40, 60, 80, and 100 ppm. The blank consisted of dilute (6.76% v/v) perchloric acid. Results were reported as % by mass of milled rice. Starch content was calculated by multiplying the glucose concentration with a factor of 0.9. The Bradford micro assay was used (Bradford, 1976). Ground rice (25 mg) was suspended in 0.15 M sodium chloride. The mixture (0.5 mL) was added to 0.5 mL of the Bradford reagent and the absorbance was read within 2-5 min at λ of 595 nm. A standard curve (R^2 = 0.95) was generated after measuring different concentrations (1, 5, 7.5, 10 g/L) of bovine serum albumin. Results were presented as % by mass of milled rice.

Alkaline Cooking Procedure

A 3 x 4 x 2 experimental design was used with three treatment temperatures (50, 60, and 70°C), four treatment times (22, 44, 66, and 88 min), and two concentrations (limewater and distilled water). The combinations were based on preliminary tests. The tubes containing 2 g of the rice samples and 6 mL of liquid (limewater or distilled water) were immersed in a temperature-controlled water bath (Taitec Personal-10 incubator, Tokyo, Japan). After each run, the tubes were removed and steeped for 18 h and allowed to cool gradually at room temperature (30°C). Then samples were washed with distilled water repeatedly until pH was approximately 7.0. The wash water (~200 mL) was collected in polyethylene terephthalate bottles and stored at −20°C. The grains were dried at 60°C for 6 h to attain moisture contents between 11 and 15 % on a wet basis. The dried grains were ground using mortar and pestle, packed in polyethylene bags

and stored at –20°C.

Wash Water Analysis

Starch, protein, and apparent amylose contents were determined using the methods described earlier. Owing to the low concentration of soluble sugars in rice grains (Singh and Juliano, 1977), the total sugars obtained using the anthrone method can be considered to primarily consist of starch. Wash water samples were thawed at room temperature and the clear supernate was collected and diluted appropriately prior to each test. Results were presented as mg of test component per g rice grains.

Differential Scanning Calorimetry (DSC)

The thermal properties of cooked and dried rice grains were analyzed using a differential scanning calorimeter (DSC 400 Perkin-Elmer, Norwalk, VA) installed at the Thomas Aquinas Research Complex, University of Santo Tomas, Manila, Philippines. The sealed sample (~9 mg) and the empty aluminum reference pans were heated from 35 to 120°C at a heating rate of 10°C/min. The enthalpy of gelatinization, onset, peak and conclusion temperatures were recorded. Duplicate runs were conducted.

Light and Scanning Electron Microscopy

Light microscopy images of cooked and dried rice grains were obtained with a USB digital microscope (RoHS, China) equipped with 1.3 megapixel sensor, a USB 2.0 interface and powered by 5V DC from USB port. Surface morphology images were obtained using a Hitachi TM3000 scanning electron microscope (Hitachi High-Technologies Corp., Tokyo Japan) installed at the Thomas Aquinas Research Complex, University of Santo Tomas. The microscope was operated at an environmental mode and an accelerated voltage of 5kV. Backscatter detector was turned on.

Statistical Analysis

Triplicate runs were conducted at each time-temperature test condition. Means were analyzed using SAS System for Windows, Version 9.1 (Cary, NC). The PROC GLM procedure was used for the analysis of variance. Tukey's honestly significant difference test was used post hoc. Means were considered significantly different at 5% level of significance.

Results

Leaching of components was significantly different for limewater-treated samples ($p<0.05$). Cooking in water resulted in greater leaching of components than alkali treatment. Compared with the control, cooking in alkali resulted in 45%, 33%, and 25% mean reduction in leached amylose, starch, and protein, respectively. Data were subsequently analyzed separately for limewater-treated samples and control (Table 1). Temperature was the only source of variation for limewater-treated samples, while time and temperature-time interaction effects were also significant for the control. Overall, temperature directly influenced the concentration of the components in the wash water. Heating at 70°C resulted in 4 to 9 times increase in released amylose compared with 50 and 60°C. Protein and starch leaching behavior was 27 to 35% higher at 60 and 70°C than at 50°C. Compared with the lowest values recorded, soaking for 44 min resulted in 80%, 39%, and 39% greater release of amylose, starch, and protein, respectively. Furthermore, leaching behavior of components in the alkaline-cooked rice samples was similar for two temperatures (50 and 60°C) with significantly greater lixiviation of proteins and starch compared with samples treated at 70°C. Mean amylose leaching at 70°C was 3 to 4 times greater than at 50 or 60°C. Leaching of starch and protein was 57% lower at 70°C than at the other two temperatures.

The uncooked rice grains were found to contain 16% amylose. Across treatment time and temperatures, wash water from control samples were found to contain an average of 0.64 mg amylose/g rice grains while limewater-cooked samples had 0.35 mg/g. Soaking in water resulted in maximum leaching of 1.70 mg amylose/g (Figure 1) or roughly 1% of the total amylose content. The amount of released amylose for limewater-treated samples varied between 0.16 and 0.69 mg/g of rice grains for the entire temperature range with coefficient of variation (CV) ranging from 24 to 39%. CV of the control ranged from 0 to 12%. A similar increasing trend

Table 1. Analysis of variance and concentration trends of wash water from alkali-treated and water-soaked rice.

Amylose content							
Source	df[a]	Alkali-treated		Water-soaked		Concentration (C) trend	
		Mean Squares	F Value *b*	Mean Squares	F Value	Alkali-treated	Water-soaked
Temperaturec (°C)	2	1.0x10-2	25.77***	5.6x10-2	1481.62***	$(C_{50}=C_{60})<C_{70}$	$C_{60}<C_{50}<C_{70}$
Timed (min)	3	8.7x10-4	2.18	2.4x10-3	64.06***		$C_{22}<C_{66}<(C_{44}=C_{88})$
Temperature x Time	6	3.9x10-4	0.97	3.8x10-3	102.43***		
Starch content							
Source	**df**	**Alkali-treated**		**Water-soaked**		**Concentration (C) trend**	
		Mean Squares	**F Value**	**Mean Squares**	**F Value**	**Alkali-treated**	**Water-soaked**
Temperature (°C)	2	1.85	5.70***	1.01	29.75***	$C_{70}<(C_{50}=C_{60})$	$C_{50}<(C_{60}=C_{70})$
Time (min)	3	0.46	1.43	0.77	22.64***		$(C_{66}=C_{88})<C_{22}<C_{44}$
Temperature x Time	6	0.27	0.85	0.86	25.23***		
Protein content							
Source	**df**	**Alkali-treated**		**Water-soaked**		**Concentration (C) trend**	
		Mean Squares	**F Value**	**Mean Squares**	**F Value**	**Alkali-treated**	**Water-soaked**
Temperature (°C)	2	2.28	5.70***	1.25	29.75***	$C_{70}<(C_{50}=C_{60})$	$C_{50}<(C_{60}=C_{70})$
Time (min)	3	0.57	1.43	0.95	22.64***		$(C_{66}=C_{88})<C_{22}<C_{44}$
Temperature x Time	6	0.39	0.85	1.06	25.23***		

a df=degrees of freedom; b Values with * = p<0.05, ** = p<0.01, *** p<0.001; c Temperatures used: 50, 60, and 70°C; d Treatment time used: 22, 44, 66, and 88 min

with temperature was observed for the control that was heated for 22 min. The trend across the control samples indicated a proportional upsurge in amylose content with increasing soaking temperatures and time. Reduction in leached amylose at 50°C for alkali-treated samples was more significant at specific heating times (i.e., 44 and 88 min). At 70°C, amylose release was inhibited by alkali at all heating times.

The initial starch content of the uncooked rice grains was 84.66%. Alkali treatment resulted in inhibited release of starch (~2% of total), compared with 3% after hydrothermal treatment (Figure 2). Across alkali-treated samples, starch concentration decreased inversely with temperature while CV ranged from 2 to 44% and 0 to 18% for the control. The opposite trend was observed when samples were heated in water for 22 min. Between 60 and 70°C, soaking in water resulted in a rapid release of starch within 22 to 44 min followed by a substantial decrease. The ascent to the peak was hastened by an increase in heating temperature. Peak value for the control was obtained after heating at 60°C for 44 min. At heating times less than 88 min, an increase in temperature resulted in proportional increase in leached starch. Beyond this value, starch content of the wash water decreased, which was similar to the alkali-treated samples.

The unprocessed rice grains had an average protein content of 7%. After alkali and hydrothermal treatments, the initial amount leached by about 3% and 4%, respectively (Figure 3). Across all treatments, protein leaching behavior was similar to starch.

Thermal and Image Analysis

Microscopy images of rice grains showed translucent and vitreous regions in control treated at 50°C (Figure 4A) with more opaque and slightly yellowish grains after alkali treatment (Figure 4B). Treatment at 70°C showed greater surface erosion and appearance of extensive cracks for the control (Figure 4C), but less erosion, less cracks, and deeper yellow grains for the limewater-cooked grains (Figure 4D). Figure 5 shows the electron microscopy images obtained for the four samples. After soaking in water at 50°C for 22 min, the surface structure of the grain shows intact, un-gelatinized regions with substantial pore development (Figure 5A). Intact cell walls were clearly seen. Polyhedral-shaped starch granules

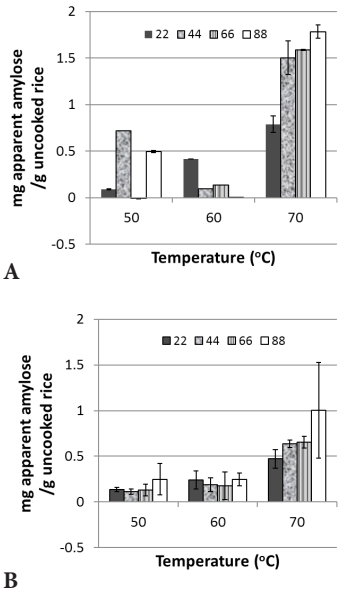

Figure 1. Apparent amylose content (mg/g uncooked rice) of wash water (n=3) after soaking in water (A) and limewater (B) at different cooking times (i.e., 22, 44, 66, and 88 min). Cooking time significantly affected leaching in water-soaked rice grains but not in limewater-treated samples. Vertical bar is the standard error of the mean (± SE).

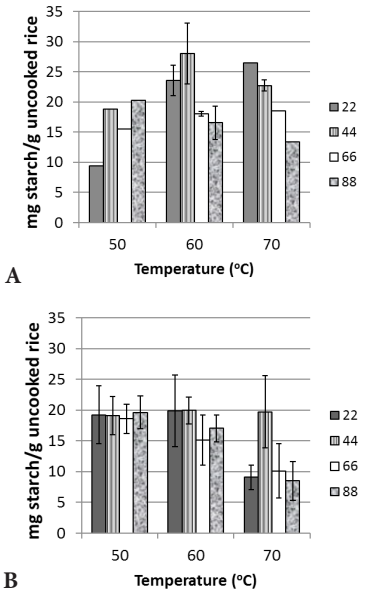

Figure 2. Starch content (mg/g uncooked rice) of wash water (n=3) after soaking in water (A) and limewater (B) at different cooking times (i.e., 22, 44, 66, and 88 min). Vertical bar is the standard error of the mean (± SE).

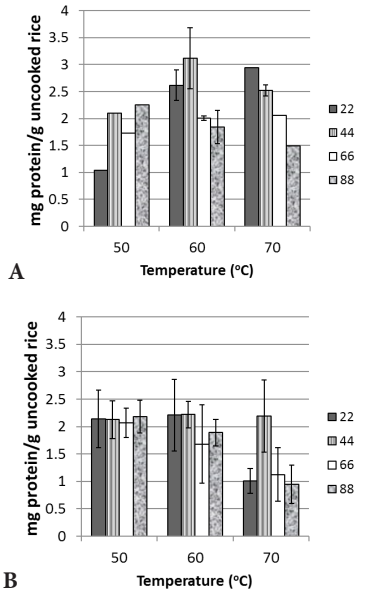

Figure 3. Protein content (mg/g uncooked rice) of wash water (n=3) after soaking in water (A) and limewater (B) at different cooking times (i.e., 22, 44, 66, and 88 min). Vertical bar is the standard error of the mean (± SE).

were abundant in porous regions. At the same test conditions, alkali-treated grains had less un-gelatinized surfaces and more extensive cracks with starch granules (Figure 5B). At 70°C, soaking in water resulted in larger pores (~20μm), twice bigger than pores observed at 50°C, and likely resulting in the accumulation of starch granules at the surface (Figure 5C). Cell walls were still visible although the structures were mostly open. Alkali treatment at 70°C resulted in less pore development (Figure 5D). Cross-sections of samples treated at 70°C showed internal fissures (Figures 5E and 5F). However, starch granules were more abundant throughout the region for the control (Figure 5E). After alkali treatment, the un-gelatinized starch granules tended to accumulate at the core, resulting in a smoother surface towards the edge (Figure 5F). Figure 6 shows the thermal properties of the selected samples based on shared characteristics among temperatures (i.e., 50 and 60 °C for limewater-treated samples and 60 and 70°C for control). Overall, the values recorded were not statistically different across treatments, although slightly lower peak gelatinization temperatures were observed for samples prepared at 70°C. Onset, peak, and conclusion gelatinization temperatures were 34-36°C, 67-77°C, and 107-115°C, respectively. Large values of enthalpies of gelatinization (i.e., 105 to 174 J/g) were obtained.

Discussion

Alkaline cooking with limewater generally results in total gelatinization of the external starch layers, partial gelatinization of the inner layers, solubilization of proteins, saponification of lipids, and increase in calcium content if limewater is used (Cornejo-Villegas et al., 2013; Mondragón et al., 2004). The concentration of alkali used can also influence the extent of water absorption in the grains. For instance, grains soaked in 1% sodium hydroxide solution were found to contain higher moisture content than the control (soaked in water), while grains soaked in 0.1% sodium hydroxide contained even less moisture than the

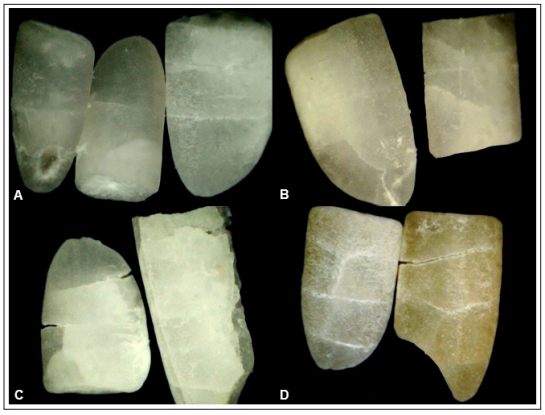

Figure 4. Light microscopy images of rice grains after soaking in: (A) distilled water, 50°C for 22 min, (B) saturated limewater, 50°C for 22 min, (C) distilled water, 70°C for 88 min, and (D) saturated limewater, 70°C for 22 min. Magnification used = 60X

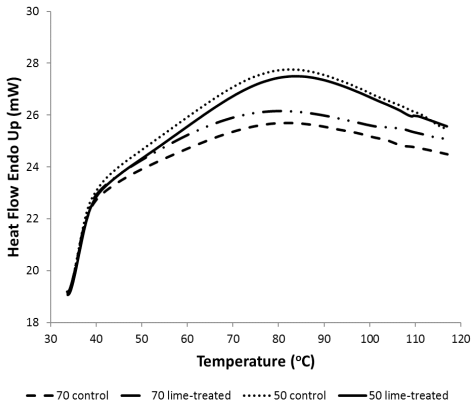

Figure 6. Thermograms of limewater-treated and water-soaked rice grains for selected treatment temperatures and times: 50°C and 22 min; and 70°C and 88 min (n = 2).

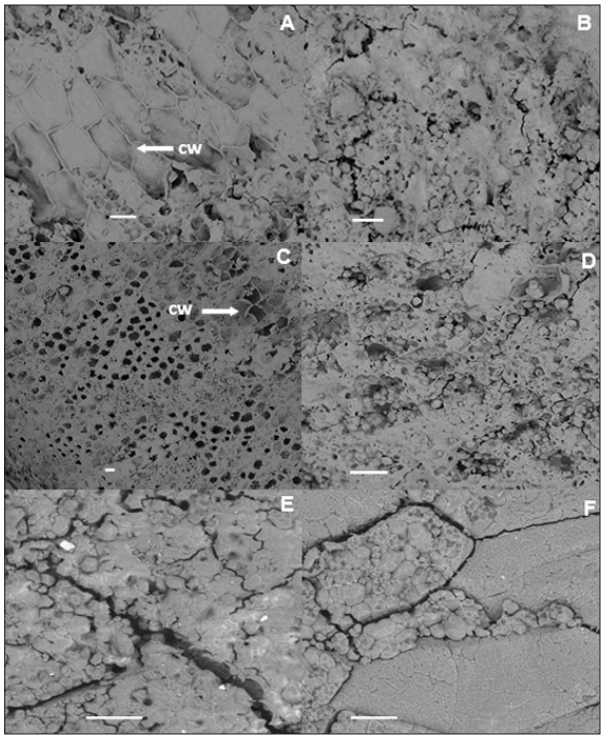

Figure 5. Scanning electron microscope images of rice grains after soaking in (A) distilled water, 50°C for 22 min, (B) saturated lime-water, 50°C for 22 min, (C) distilled water, 70°C for 88 min, and (D) saturated limewater, 70°C for 22 min. Cross-sections of grains after treatments (C) and (D) appeared as images (E) and (F), respectively. White scale bar = 20 μm> cw = cell wall.

control (Bello et al., 2004). Mechanistically, starch release from cells is enhanced when glutelin, prolamin, albumin, and globulin solubilize (Guzmán et al., 2011; Rodríguez-Miranda et al., 2011). The solubilized proteins in the liquor are consequently removed during washing. On the other hand, water soaking leads to expansion of the starch granules, disintegration of protein bodies, and leaching of rice components in the wash water (Kale et al., 2015; Tester and Morrison, 1990). The steeping process employed in alkali treatment can be associated with an annealing treatment, especially if the temperature is kept below gelatinization temperature (Bian and Chung, 2016). Both processes can further promote leaching of amylose and increase the gelatinization temperature, narrow the gelatinization temperature range, and lower the enthalpy of gelatinization (Bian and Chung, 2016; Cai et al., 2014; Figueroa et al., 2013).

The main difference between alkaline treatment and soaking in water is the reduced extent of leaching of rice components, especially amylose. Annealed rice starch can undergo 2% reduction in amylose (Bian and Chung, 2016; Reepholkul and Charoenrein, 2013), compared with a maximum of 0.6% obtained in limewater-treated samples in this study. Steeping in alkali inhibits the release of amylose (Figure 1), and this may be due to hindrance offered by cross-linked starch layers, calcium-protein interactions, and formation of amylose-lipid and amylose-protein complexes (Guzmán et al., 2009; Mondragón et al., 2004). Protein fractions solubilize with alkali, but they can also form insoluble calcium bridges (with a range of molar masses) that may be more difficult to disrupt than disulfide bridges that may form from water

treatment (Guzmán et al., 2011). The effect of alkali treatment, however, is not uniform across starch sources. For instance, lower apparent amylose contents were observed on alkali-treated sago and potato but not on corn starches (Nor Nadiha et al., 2010). Cai et al. (2014) reported that treatment of rice grains with caustic soda resulted in lower apparent amylose content of the grains (higher amylose concentrations in the wash water) than control.

Though limewater can induce lixiviation, the resulting calcium-protein and calcium-starch interactions may have hindered further pH-induced protein solubilization and starch leaching. This resulted in wash water with significantly lower starch and protein contents than control (Figures 2 and 3) and consequently, grains with higher nutritive values. For comparison, soaking of high-amylose rice in water at 65°C resulted in grains with about 5% and 8% reduction in starch and protein, respectively (Kale et al., 2015). Although calcium can interact with starch, results imply that interactions vary between amylose and amylopectin.

Despite incomplete gelatinization with both treatments, alkali-treated samples showed less surface pore formation than water-soaked samples (Figure 5), in support of observations reported by Guzmán et al. (2011). A "glue-like" system may also form among amylose, amylopectin, and proteins that may link the un-gelatinized starch with the intact cells of the endosperm (Cai et al., 2014; Guzmán et al., 2009). This may explain the formation of fewer cracks in the cross-section of the limewater-cooked samples (Figure 5F) and on the grain surface (Figure 4D). The surface morphology of water-soaked samples, which showed pores of various sizes (<20μm). Across control and limewater-treated samples, Figure 4 showed increase in opaque regions of the grain, formation of cracks, swelling and erosion, as a result of higher temperatures which promoted gelatinization. Results suggest that below gelatinization temperatures, more starch and protein leach, which gradually decrease inversely with temperature. Nor Nadiha et al. (2010) explained that alkali probably has more significant effect on amylose than on amylopectin. The proportional increase in amylose content with temperature in this study may be caused by increased mobility and diffusion of alkali and amylose.

Conclusion

The two-step process of limewater treatment (heating and steeping) of rice grains was found to inhibit leaching of components in the wash water that normally accompanies gelatinization. Thus, the cooked rice grains have comparatively higher nutritive content than water-soaked rice. Amylose leaching at higher alkaline cooking temperatures was less restricted than either starch or protein. The thermal properties of the grains were considerably similar to the water-soaked grains. Overall, the treatment was insufficient in reducing the amylose content of rice to levels indicative of waxy rice. The process may find applications in the production of an ingredient with minimal use of energy and more nutritional significance. Significant reductions in amylose but not in starch may affect the resulting texture of starch gels made with alkaline-cooked rice. The effects of alkaline treatment on the texture and cooking properties of rice are currently being investigated in the researchers' laboratory and may impact the use of alkaline-cooked rice as a food ingredient.

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Literature Cited

Anupapsamosorn S, Charoenrein S (2015) Physico chemical properties of glutinous rice in the presence of alkali and borax. Starch - Stärke **67**(11-12): 930-936. doi: 10.1002/star.201500103

Bello M, Tolaba MP, Suarez C. (2004) Factors affecting water uptake of rice grain during soaking. LWT - Food Science and Technology **37**(8): 811-816. doi: 10.1016/j.lwt.2004.02.014

Bian L, Chung H-J (2016) Molecular structure and physicochemical properties of starch isolated from hydrothermally treated brown rice flour. Food Hydrocolloids **60**: 345-352. doi: 10.1016/j.foodhyd.2016.04.008

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**: 248-254

Cai J, Yang Y, Man J, Huang J, Wang Z, Zhang, C, Wei C (2014) Structural and functional properties of alkali-treated high-amylose rice starch. [Research Support, Non-U.S. Gov't]. Food Chemistry **145**: 245-253. doi: 10.1016/j.foodchem.2013.08.059

Cardoso M. B, Putaux J-L, Samios D, da Silveira NP (2007) Influence of alkali concentration on the deproteinization and/or gelatinization of rice starch. Carbohydrate Polymers **70**(2): 160-165. doi: 10.1016/j.carbpol.2007.03.014

Clegg KM (1956) The application of the anthrone reagent to the estimation of starch in cereals. Journal of the Science of Food and Agriculture **7**: 40-44

Collado LS, Corke H (1999) Heat-moisture treatment effects on sweetpotato starches differing in amylose content. Food Chemistry **65**: 339-346

Cornejo-Villegas MdA, Gutiérrez-Cortez E, Rojas-Molina I, Del Real-López A, Zambrano-Zaragoza MdL, Martínez-Vega V, Rodríguez-García M (2013) Physicochemical, morphological, and pasting properties of nixtamalized flours from quality protein maize and its particle distribution. LWT - Food Science and Technology **53**(1): 81-87. doi: 10.1016/j.lwt.2013.01.023

de Souza D, Sbardelotto AF, Ziegler DR, Marczak LDF, Tessaro IC (2016) Characterization of rice starch and protein obtained by a fast alkaline extraction method. Food Chemistry **191**: 36-44. doi: 10.1016/j.foodchem.2015.03.032

Figueroa JD, Véles Medina JJ, Landaverde MAH, Cuevas FA, Martínez MG, Martínez EC, Willcox M (2013) Effect of annealing from traditional nixtamalisation process on the microstructural, thermal, and rheological properties of starch and quality of pozole. Journal of Cereal Science **58**(3): 457-464. doi: 10.1016/j.jcs.2013.09.005

Guzmán AQ, Flores MEJ, Escobedo RM, Guerrero LC, Feria JS (2009) Changes on the structure, consistency, physicochemical and viscoelastic properties of corn (*Zea mays* sp.) under different nixtamalization conditions. Carbohydrate Polymers **78**(4): 908-916. doi: 10.1016/j.carbpol.2009.07.024

Guzmán AQ, Flores MEJ, Feria JS, Méndez-Montealvo MG, Wang Y-J (2011) Rheological and thermal properties of masa as related to changes in corn protein during nixtamalization. Journal of Cereal Science **53**(1): 139-147. doi: 10.1016/j.jcs.2010.11.005

Juliano BO, Tuaño APP, Monteroso DN, Aoki N, Mestres C, Duldulao JBA, Bergonio KB (2012) Replacement of acetate with ammonium buffer to determine apparent amylose content of milled rice. Cereal Foods World **57**(1): 14-19. doi: 10.1094/cfw-57-1-0014

Kale SJ, Jha SK, Jha GK, Sinha JP, Lal SB (2015) Soaking induced changes in chemical composition, glycemic index and starch characteristics of Basmati rice. Rice Science **22**(5): 227-236. doi: 10.1016/j.rsci.2015.09.002

Klucinec JD, Thompson DB (1999) Amylose and amylopectin interact in retrogradation of dispersed high-amylose starches. Cereal Chemistry **76**(2): 282-291

Kong X, Zhu P, Sui Z, Bao J (2015) Physicochemical properties of starches from diverse rice cultivars varying in apparent amylose content and gelatinisation temperature combinations. [Research Support, Non-U.S. Gov't]. Food Chemistry **172**: 433-440. doi: 10.1016/j.foodchem.2014.09.085

Lobato-Calleros C, Hernandez-Jaimes C, Chavez-Esquivel G, Meraz M, Sosa, E, Lara VH, Vernon-Carter EJ (2015) Effect of lime concentration on gelatinized maize starch dispersions properties. Food Chemistry **172**: 353-360. doi: 10.1016/j.foodchem.2014.09.087

Mondragón M, Bello-Pérez LA, Agama E, Melo A, Betancur-Ancona D, Peña JL (2004) Effect of nixtamalization on the modification of the crystalline structure of maize starch. Carbohydrate Polymers **55**(4): 411-418. doi: 10.1016/j.carbpol.2003.11.006

Nor Nadiha MZ, Fazilah A, Bhat R, Karim AA (2010) Comparative susceptibilities of sago, potato and corn starches to alkali treatment. Food Chemistry **121**(4): 1053-1059. doi: 10.1016/j.foodchem.2010.01.048

Pachuau L, Dutta RS, Roy PK, Kalita P, Lalhlenmawia H (2017) Physicochemical and disintegrant properties of glutinous rice starch of Mizoram, India. International Journal of Biological Macromolecules **95**:1298-1304

Reepholkul K, Charoenrein S (2013) Effect of sodium carbonate on appearance and textural properties of glutinous rice cake. International Journal of Food Science and Technology, n/a-n/a. doi: 10.1111/ijfs.12203

Rodríguez-Miranda J, Ruiz-López II, Herman-Lara E, Martínez-Sánchez C E, Delgado-Licon E, Vivar-Vera MA (2011) Development of extruded snacks using taro (*Colocasia esculenta*) and nixtamalized maize (*Zea mays*) flour blends. LWT - Food Science and Technology **44**(3): 673-680. doi: 10.1016/j.lwt.2010.06.036

Ruiz-Gutiérrez MG, Quintero-Ramos A, Meléndez-Pizarro CO, Lardizábal-Gutiérrez D, Barnard J, Márquez-Melendez R, Talamás-Abbud R (2010) Changes in mass transfer, thermal and physicochemical properties during nixtamalization of corn with and without agitation at different temperatures. Journal of Food Engineering **98**(1): 76-83. doi: 10.1016/j.jfoodeng.2009.12.010

Ruiz-Gutiérrez MG, Quintero-Ramos A, Meléndez-Pizarro CO, Talamás-Abbud R, Barnard, J, Márquez-Meléndez R, Lardizábal-Gutiérrez D (2012) Nixtamalization in two steps with different calcium salts and the relationship with chemical, texture and thermal properties in masa and tortilla. Journal of Food Process Engineering **35**(5): 772-783. doi: 10.1111/j.1745-4530.2010.00627.x

Setyawati YD, Ahsan SF, Ong LK, Soetaredjo FE, Ismadji S, Ju YH (2016) Production of glutinous rice flour from broken rice via ultrasonic assisted extraction of amylose. Food Chemistry **203**: 158-164. doi: 10.1016/j.foodchem.2016.02.068

Singh R, Juliano BO (1977) Free sugars in relation to starch accumulation in developing rice grain. Plant Physiology **59**: 417-421
Tester RF, Morrison WR (1990) Swelling and gelatinization of cereal starches. I. Effects of amylopectin, amylose, and lipids. Cereal Chemistry **67**(6): 551-557

Valdez-Niebla JA, Paredes-López O, Vargas-López JM, Hernández-López D (1993) Moisture sorption isotherms and other physicochemical properties of nixtamalized amaranth flour. Food Chemistry **46**: 19-23

Witek M, Węglarz WP, de Jong L, van Dalen G, Blonk JCG, Heussen P, van Duynhoven J (2010) The structural and hydration properties of heat-treated rice studied at multiple length scales. Food Chemistry **120**(4): 1031-1040. doi: 10.1016/j.foodchem.2009.11.043

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Effect of Nitrogen Management on Growth Duration, Grain Yield, and Agronomic Efficiency of Applied Nitrogen of Irrigated Lowland Inbred Rice Varieties in Nueva Ecija, Philippines

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Abstract Nitrogen (N) is the most important nutrient that can influence growth duration and enhance grain yield of rice varieties. However, N fertilizer is expensive and subject to field losses due to ammonia volatilization and denitrification. Hence, it must be used efficiently and meet crop N demand. Field studies were conducted at PhilRice in 2015 dry season (DS) and wet season (WS) and 2016 DS to determine the effect of (a) "real-time" Leaf Color Chart (LCC)-based N application and (b) fixed-rate and fixed-time (FRFT)-based N application on growth duration, grain yield, and agronomic efficiency of applied N (AE_N) of rice varieties. Results showed that based on the N omission plot or control, growth duration of most rice varieties was prolonged by an average of 3 days with FRFT and LCC and partially contributed to yield increase. Grain yields were significantly higher with FRFT with total N application of 190 kg ha^{-1} than that of LCC with total N applications of 77 and 112 kg ha^{-1} in 2015 DS. AE_N of most rice varieties were significantly higher with LCC-based N application in 2015 DS and 2016 DS. With LCC-based N application, farmers can increase AE_N and save on N fertilizer, but with a yield penalty, especially in DS.

Keywords: Agronomic Efficiency of Applied Nitrogen, Fixed-Rate and Fixed-Time, Grain Yield, Growth Duration, Leaf Color Chart, Rice Varieties.

Introduction

In the Philippines, rice production must increase to keep pace with population growth. Thus, increasing rice crop productivity or yield potential is imperative and can be achieved through use of improved rice varieties and better crop management. Yield potential is the yield of a variety in an environment where nutrient and water are non-limiting, with minimum pests and diseases (Evans and Fischer, 1999) and favorable weather condition. Among the plant nutrients, N is the most important element that can help attain the yield potential of modern rice varieties. It is a constituent of proteins which, in turn, are constituents of protoplasm, chloroplasts, and enzymes (Yoshida, 1981). However, N fertilizer is subject to ammonia volatilization, denitrification, (De Datta and Buresh, 1989; De Datta et al., 1991), leaching, and runoff (Choudhury and Kennedy, 2005) losses in the soil-floodwater system. In many field situations, more than 60% of applied N is lost due in part to the lack of synchrony of plant N demand and N supply (Shukla et al., 2004). Hence, there is a need to improve the congruence between crop N demand and the available N supply from soil and applied N fertilizer to increase N-use efficiency (Peng et al., 1996; Peng and Cassman, 1998). In addition, farmers must use the often expensive N fertilizer as efficiently as possible to contain the production cost, optimize grain yields, and maintain environment safety. The use of N efficient varieties (Fageria and Filho, 2001) and practical diagnostic tools to assess plant N status in the field are important complementary strategies to improve rice yield and reduce field losses and cost of production. According to Choudhury and Kennedy (2005), N fertilizer losses in the field can be reduced by (1) applying soluble chloride or nitrate salts of calcium, magnesium, and potassium; (2) using urease and algal inhibitors; (3) deep placement of N

fertilizers and use of modified forms of urea; and (4) use of slow-release fertilizers. Recently, one-time urea application into 10 cm deep holes positioned 5 cm from the rice root zone resulted in lower N fertilizer loss and higher yield and N use efficiency than the farmer's practice in sandy soils (Liu et al., 2016).

Chlorophyll content is highly related to plant N status because a large fraction of N is used for photosynthetic enzymes (Friedmann et al., 2016; Islam et al., 2007). The Minolta SPAD 502 chlorophyll meter is a practical and non-destructive tool to estimate the plant N status of rice (Peng et al., 1996; Islam et al., 2009), maize (Bullock and Anderson, 1998), and other field crops. The LCC is another practical non-destructive tool to assess the "real-time" plant need for N fertilizer for rice (Balasubramanian et al., 1998; Islam et al., 2007; Islam et al., 2009; PhilRice, 2008) and maize (Friedmann et al., 2016). The LCC is much less costly than the SPAD. Techniques that are inexpensive and easy to use may benefit individuals, small operations, and other stakeholders by managing N to enhance nutrient efficiency and environmental quality (Cassman et al., 2002).

The fixed-rate and fixed-time N application is the common N fertilizer management method followed by farmers in the Philippines (PhilRice, 1991). This is also called the calendar-based N management or the blanket N recommendations in which the N rates may vary depending on location, soil type, and cropping season. Blanket fertilizer N recommendations were mainly derived from empirical testing of N response of rice varieties to a few fixed doses applied at critical crop growth stages (Shukla et al., 2004). However, the fixed-rate and fixed-time N management do not consider the dynamic crop N requirement and the changing soil N supply during the crop growth period. This is where LCC–based "real-time" N management is useful, and N application can be synchronized with dynamic crop demand. The LCC-based N management is used to improve the existing fixed split N recommendations (Shukla et al., 2004). It also increases N use efficiency and reduce N loss (Islam et al., 2009).

The efficiency with which applied N is absorbed by the rice crop significantly varies among modern varieties and environments. Some varieties require low N rates (Hasegawa, 2003) due to efficient N utilization by plants, disease incidence, physiological problems, and other factors that limit their yields at higher N rates. There are other rice varieties that respond well to high N doses and attain maximum yield at higher N rates (Bufogle et al., 1997). Sanico et al. (1999) evaluated the nitrogen use efficiency of rice varieties developed in the Philippines since 1966 and they did not find significant differences in AE_N among the old and new rice varieties under N rates of 0, 60, 120 and 200 kg ha^{-1}.

The reported grain yields of newly-released irrigated lowland Philippine rice varieties in the National Cooperative Test (NCT) for rice were usually based on a fixed-rate and fixed-time N application of 120 kg N ha^{-1} in DS and 90 kg N ha^{-1} in WS across locations. Moreover, the growth duration, grain yield, and AE_N of popular and newly-released irrigated lowland rice varieties in response to LCC-based N management had not been assessed.

This study assessed the effects of (a) LCC-based N application and (b) fixed-rate and fixed-time-based N application on growth duration, grain yield, and AE_N of selected Philippine popular and newly-released irrigated lowland rice varieties.

Materials and Methods

Experimental Seasons and Site

This study was conducted in 2015 DS and WS and 2016 DS at the Philippine Rice Research Institute (PhilRice) Central Experiment Station (CES), Maligaya, Science City of Muñoz, Nueva Ecija (15.67°N, 120.90°E, 59m asl). The soil is classified as Maligaya clay (Montmorillionitic Ustic Epiaquerts by USDA soil taxonomy) with an estimated indigenous N supply (INS) of 45 kg N ha^{-1}. Based on previous experiments at the site, grain yields in N omission or INS plots ranged from 3.5 to 4.5 t ha^{-1}.

Experimental Design

The experiment was laid out in a randomized complete block design (RCBD) with split plot arrangement where nitrogen treatments formed

the main plots and rice varieties the sub-plots, and treatments were replicated three times. The sub plot size was 36.04 m^2.

Test Rice Varieties, Growth Duration, and Planting Dates

The rice varieties tested for this study were (a) early-maturing varieties: NSIC Rc238 (110 days from sowing to maturity), PSB Rc82 (110 days), NSIC Rc308 (111 days), NSIC Rc240 (115 days), NSIC Rc302 (115 days); and (b) medium-maturing varieties: NSIC Rc122 (121 days), NSIC Rc360 (122 days), and PSB Rc18 (123 days). Data on crop growth duration were obtained from NCT. Pre-germinated seeds were sown in well-prepared seedbed to raise rice seedlings. Urea (15 g m^{-2}) was applied on seedbed 10 days after sowing to produce healthy seedlings. Twenty-one-days-old seedlings were transplanted at a hill spacing of 20 x 20 cm with 2 to 3 seedlings per hill on January 15, 2015 (DS), July 9, 2015 (WS), and January 12, 2016 (DS).

N Fertilizer Treatments

N treatments were (N1) no N fertilizer application or N omission plot (NOP) as control; (N2) Leaf Color Chart (LCC)-based N topdressing of 35 kg N ha^{-1} in DS and 23 kg N ha^{-1} in WS when LCC reading was below 4. Before LCC-based N application, a basal application of 6 bags 14-14-14-12S (equivalent to 42 kg ha^{-1} each of N, P_2O_5 and K_2O + 12% S) in DS and 4 bags (equivalent to 28 kg ha^{-1} each of N, P_2O_5 and K_2O + 12% S) in WS at 14 days after transplanting (DAT); and (N_3) fixed-rate and fixed-time N (FRFT) application of 190 kg N ha^{-1} in DS and 95 kg N ha^{-1} in WS, applied in 3 splits i.e., 50 kg ha^{-1} at mid-tillering (MT), 100 kg ha^{-1} at panicle initiation (PI) and 40 kg ha^{-1} at first flowering (FF) for DS; and 25 kg ha^{-1} at MT, 50 kg ha^{-1} at PI and 20 kg ha^{-1} at FF for WS. In all N treatments, P_2O_5 and K_2O rates were applied at 42 kg each ha^{-1} in DS and 40 kg ha^{-1} in WS. Fertilizer $ZnSO_4$ was applied a day before transplanting at the rate of 10 kg ha^{-1} for NOP and FRFT N plots and 14 DAT for LCC-based N plots. The 4-panel PhilRice LCC (PhilRice, 2008) was used to assess the "real-time" plant N status in the present field studies.

Other Crop Management Practices

Molluscicide was applied immediately after transplanting to control golden apple snails. Pre-emergence herbicide was applied 1-3 DAT to control weeds. Supplemental hand weeding was done, when necessary. Carbofuran was applied during active tillering (18-20 DAT) and panicle initiation stage for protection against stem borer.

Data Collection

Mature plants were harvested from a 5 m^2 area located at the center of each treatment plot to determine grain yields. Grain samples were sun-dried and the yield was adjusted to 14% moisture content. For yield components, four adjacent plants were obtained from two corners of the harvest area for a total of 8 hills per plot. Straw and grains were oven-dried at 70°C for 3-4 days or until constant dry weight is achieved. During panicle initiation (PI), four hills per plot were collected for determination of plant height, number of tillers, aboveground biomass and leaf area index. The phenology of the crop, i.e., the dates of PI, flowering and physiological maturity, was recorded.

Agronomic efficiency of applied nitrogen (AE_N, kg grain kg^{-1} N) was computed in the following manner:

$$AE_N = \frac{\text{Grain yield in N fertilized plot (kg ha}^{-1}\text{) - Grain yield in NOP (kg ha}^{-1}\text{)}}{\text{Total amount of applied N fertilizer (kg ha}^{-1}\text{)}}$$

Data Analysis

Analysis of variance was performed for each parameter and treatment means were compared using Least Significant Difference (LSD) Test for N management and Tukey's Honest Significance (HSD) Test for rice variety at 0.05 probability. Statistical analyses were done using the Statistical Tool for Agricultural Research (STAR Version 2.0.1 IRRI, 2014).

Weather Data

Weather data were obtained from the Climate Change Center of PhilRice CES, Maligaya, Science City of Muñoz, Nueva Ecija. The average daily minimum and maximum air temperatures and irradiance from transplanting to crop harvest for each cropping season were recorded. Each cropping period was approximately four months.

Results

Weather Condition

The weather conditions during the three cropping seasons are shown in Table 1. The mean irradiance of 19.7 MJ m^{-2} d^{-1} in 2015 DS was close to 19.2 MJ m^{-2} d^{-1} in 2016 DS, but 11% lower in 2015 WS. However, the mean maximum air temperature of 31.8^0C d^{-1} and mean minimum air temperature of 24.5^0C d^{-1} in 2015 WS were on the average 3% and 11%, respectively, and higher than those in 2015 DS and 2016 DS. As frequently observed, there was a significantly higher amount of rainfall in WS than in DS.

N Fertilizer Application

Given the adequate amounts of phosphorus (P), potassium (K), zinc and sulfur fertilizers, the "real-time" LCC-based total N fertilizer applications were 77 kg N ha^{-1} each for NSIC Rc240, NSIC Rc302, NSIC Rc308 and NSIC Rc360, and 112 kg N ha^{-1} each for PSB Rc82 and NSIC Rc238 in 2015 DS (Table 2). Total LCC-based N applications in 2015 DS and 2016 DS were similar. N fertilizer was applied at 35 days after transplanting (DAT) or at early panicle initiation stage, and at 56 DAT or booting stage when LCC reading was below 4. In 2015 WS, LCC-based total N applications were 51 N ha^{-1} each for NSIC Rc122, NSIC Rc240, NSIC Rc302, NSIC Rc308 and NSIC Rc360, and 74 kg N ha^{-1} each for PSB Rc82 and NSIC Rc238. N fertilizer was applied at 35 DAT or early panicle initiation, 42 DAT or panicle initiation, and 56 DAT or booting stage when LCC reading was below 4.

The FRFT-based total N fertilizer applications were 190 kg N ha^{-1} each for the different rice varieties in 2015 DS and 2016 DS, and 95 kg N ha^{-1} in 2015 WS (Table 2). In WS and DS, N fertilizer was applied at 20-25 DAT or mid-tillering, 38-45 DAT or early panicle initiation stage, and 70-75 DAT or early flowering.

PSB Rc18 and NSIC Rc122 were excluded in

Table 1. Mean daily irradiance, maximum and minimum air temperatures, and rainfall during the 4-month cropping period in 2015 dry season (DS), 2015 wet season (WS), and 2016 DS in PhilRice, Nueva Ecija, Philippines. Each mean is followed by a standard error (± SE).

Cropping Season	Mean Irradiance (MJ m^{-2} d^{-1})	Mean Maximum Air Temperature (^{o}C d^{-1})	Mean Minimum Air Temperature (^{o}C d^{-1})	Mean Rainfall (mm d^{-1})
2015 DS	19.7 ± 0.4	31.5 ± 0.2	21.7 ± 0.1	0.1 ± 0.1
2015 WS	17.3 ± 0.4	31.8 ± 0.3	24.5 ± 0.1	10.8 ± 0.1
2016 DS	19.2 ± 0.5	30.3 ± 0.2	22.5 ± 0.1	0.1 ± 2.1

Table 2. Total N fertilizer applied in the LCC-based N management and fixed-rate and fixed-time (FRFT)-based N applications in 2015 DS and WS, and 2016 DS. Note: The total N rate with LCC-based N application included an initial application of 42 kg N ha^{-1} in DS and 28 kg ha^{-1} in WS from a compound fertilizer (14-14-14-12S) applied at 14 DAT. Adequate phosphorus and potassium fertilizers were applied in FRFT treatment. PSB Rc18 and NSIC Rc122 were excluded in 2015 DS due to damage by stem borer. Likewise, PSB Rc18 was excluded in 2015 WS due to damage by stem borer. PhilRice, Nueva Ecija, Philippines.

Rice Variety	Cropping Season					
	2015 DS		2015 WS		2016 DS	
	LCC (kg N ha^{-1})	FRFT (kg N ha^{-1})	LCC (kg N ha^{-1})	FRFT (kg N ha^{-1})	LCC (kg N ha^{-1})	FRFT (kg N ha^{-1})
PSB Rc18	-	-	-	-	77	190
PSB Rc82	112	190	74	95	77	190
NSIC Rc122	-	-	51	95	112	190
NSIC Rc238	112	190	74	95	77	190
NSIC Rc240	77	190	51	95	77	190
NSIC Rc302	77	190	51	95	77	190
NSIC Rc308	77	190	51	95	77	190
NSIC Rc360	77	190	51	95	77	190

2015 DS and PSB Rc18 was excluded in 2015 WS (Table 2) due to 20-33% stem borer damage to the rice crop at reproductive stage.

Crop Growth Duration

In 2015 DS (Figure 1A), across rice varieties, crop growth durations ranged from 105-118 days in the N omission plot (NOP), 109-121 days with LCC-based N application, and 111-122 days with FRFT-based N application. Growth durations with FRFT-based N application were significantly higher than growth durations with LCC-based N application for PSB Rc82, NSIC Rc238, NSIC Rc240, NSIC Rc302, and NSIC Rc308 but not for NSIC Rc360.

In 2016 WS (Figure 1B), similar trends in growth duration were observed among NOP, FRFT, and LCC-based N applications. FRFT growth durations ranged from 116 to 119 days and were significantly higher than growth durations with LCC-based N application for PSB Rc82, NSIC Rc122, NSIC Rc238, and NSIC Rc302 but not for NSIC Rc240, NSIC Rc308 and NSIC Rc360.

In 2016 DS (Figure 1C), growth durations were shorter in NOP, FRFT, and LCC-based N applications than similar treatments in 2015 DS (Figure 1A) and 2015 WS (Figure 1B). NOP growth durations ranged from 103 to 112 days and were shorter than growth durations of FRFT and LCC-based N applications for PSB Rc18, NSIC Rc302, NSIC Rc308, and NSIC Rc360. Except for NSIC Rc240, growth durations of the other seven varieties with FRFT and LCC-based N applications did not differ significantly.

Across rice varieties, average growth durations in NOP were 111 days in 2015 DS, 112 days in 2015 WS, and 106 days in 2016 DS. With LCC-based N application, average growth durations were 113 days in 2015 DS, 114 days in 2015 WS, and 107 days in 2016 DS. With FRFT-based N application, average growth durations were 117 days in 2015 DS, 116 days in 2015 WS, and 106 days in 2016 DS. Based on NOP across seasons and varieties, growth duration increased by 4 days with FRFT and by 2 days with LCC for an average of 3 days.

In 2015 DS (Figure 2A) and across rice varieties, grain yields ranged from 4.4 to 4.9 t ha^{-1} in NOP, 7.1 to 7.7 t ha^{-1} with LCC-based N application, and 6.6 to 9.5 t ha^{-1} with FRFT-based N application. N management treatments differed significantly. However, grain yields of rice varieties differed significantly with FRFT-based N application but not with LCC-based N application, and NOP. In FRFT, PSB Rc82 had the highest yield of 9.5 t ha^{-1} and NSIC Rc360 had the lowest yield of 6.6 t ha^{-1}. Although not tabulated, the harvest indices of rice varieties in NOP, LCC, and FRFT did not differ significantly and had an average 0.51.

In 2015 WS (Figure 2B) and across rice varieties, grain yields ranged from 3.9 to 4.5 t ha^{-1} in NOP, 6.1 to 6.6 t ha^{-1} with LCC-based N application, and 5.8 to 6.7 t ha^{-1} with FRFT-based N application. Yields in NOP were significantly lower than yields with LCC-based N and FRFT-based N applications. However, there was no significant difference in yields between LCC-based N and FRFT-based N applications and among yields of varieties in each N application treatment.

In 2016 DS (Figure 2C) and across rice varieties, grain yields ranged from 4.4 to 5.4 t ha^{-1} in NOP, 6.3 to 8.3 t ha^{-1} with LCC-based N application, and 6.7 to 8.1 t ha^{-1} with FRFT-based N application. Yields in NOP were significantly lower than yields with LCC-based N and FRFT-based N applications. However, grain yields with LCC-based N and FRFT-based N applications did not differ significantly. Across N applications, yields of NSIC Rc238, NSIC Rc240, NSIC Rc302, NSIC Rc308, NSIC Rc360 and PSB Rc82 were similar and ranged from 6.4 to 7.1 t ha^{-1}. PSB Rc18 and NSIC Rc122 had the lowest average yield of 6.0 t ha^{-1}.

Agronomic Efficiency for Applied N (AE_N)

In 2015 DS (Figure 3A) and across rice varieties, AE_N ranged from 23.4 and 36.3 kg grain kg^{-1} N with LCC-based N application, and 11.7 to 25.4 kg grain kg^{-1} N with FRFT-based N application. LCC-based N and FRFT-based N applications differed significantly for NSIC Rc240, NSIC Rc302, NSIC Rc308, and NSIC Rc360 but not for PSB Rc82 and NSIC Rc238.

In 2015 WS (Figure 3B) and across rice varieties, AE_N ranged from 20.8 to 44.6 kg grain kg^{-1} N in LCC-based N application, and 13.0 to 26.7 kg grain kg^{-1}N with FRFT-based N application. However, there were no significant

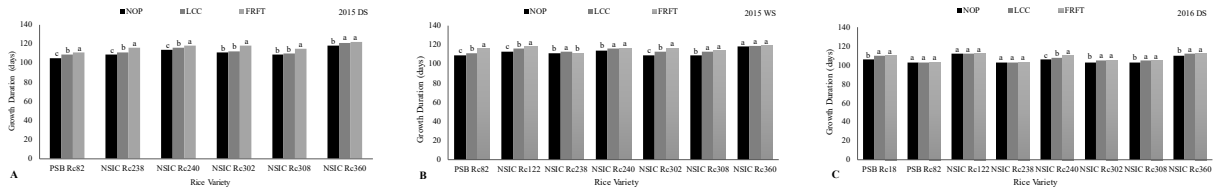

Figure 1. Growth duration (i.e., number of days from seed sowing to harvest) of rice varieties in the N omission plot (NOP), LCC-based N application and fixed-rate and fixed-time (FRFT) N applications in 2015 DS (A), 2015 WS (B) and 2016 DS (C) in Nueva Ecija, Philippines. LCC-based total N application rates were 77 and 112 kg ha^{-1} in DS and 51 and 74 kg ha^{-1} in WS. FRFT total N application rates were 190 kg ha^{-1} in DS and 95 kg ha^{-1} in WS. In the Nitrogen Omission Plot (NOP), P and K fertilizers were applied. Means with the same letter are not significantly different at 5% level by LSD.

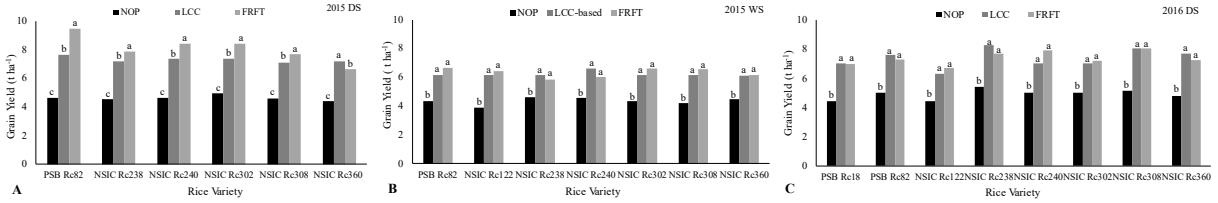

Figure 2. Grain yields of rice varieties in the N omission plot (NOP), LCC-based N application, and fixed-rate and fixed-time (FRFT)-based N application in 2015 DS (A), 2015 WS (B) and 2016 DS (C) in PhilRice, Nueva Ecija, Philippines. Means with the same letters are not significantly different at 5% by LSD.

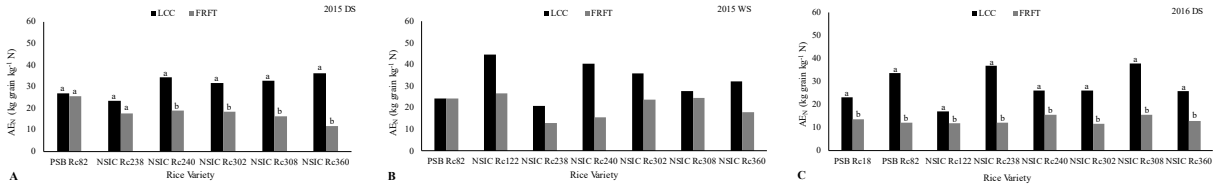

Figure 3. Agronomic efficiency of applied N (AE_N) of rice varieties with LCC-based N application and fixed-rate and fixed-time (FRFT)-based N application in 2015 DS (A), 2015 WS (B), and 2016 DS (C) in PhilRice, Nueva Ecija, Philippines. Means with the same letters are not significantly different at 5% by LSD. Note: AEN in 2015 WS showed no significant difference between LCC-based and FRFT-based N application treatments across varieties.

differences in AE_N between LCC and FRFT and among varieties in each N application treatment.

In 2016 DS (3C) and across rice varieties, AE_N ranged from 16.7-37.7 kg grain kg^{-1} N with LCC-based N application, and 11.6-15.4 kg grain kg^{-1} N with FRFT-based N application. Across rice varieties, AE_N with LCC-based N application was significantly higher than with FRFT-based N application. However, AE_N of rice varieties did not differ significantly in each N application treatment.

Discussion

Although the average irradiance was lower in 2015 WS cropping than in 2015 and 2016 DS, the average maximum air temperature was slightly higher and the minimum air temperature was 2.4°C higher in 2015 WS (Table 1). The type of clouds in WS can enhance the heating effect by transmitting solar radiation and trap some outgoing infrared radiation emitted by the earth and radiate it back downward (Graham, 1999). The 2.4°C increase in minimum air temperature in 2015 WS (i.e., 21.7°C in 2015 DS, 22.5°C in 2016 DS vs. 24.5°C in 2015 WS) was not detrimental to grain yield because average WS yield across rice varieties was high at 6.2 t ha^{-1} for either LCC-based N application or fixed-rate and fixed-time (FRFT)-based N application. However, in a climate change or global warming scenario, Peng et al. (2014) found a negative relationship between grain yield and minimum air temperature and a positive relationship between grain yield and irradiance. Since the increase in mean minimum temperature was 3-fold greater than the increase in mean maximum temperature, they concluded that rice grain yield decreased by 15% for each 1°C increase in growing-season mean temperature. It was

possible that plant maintenance respiration increased with increasing air temperature and that a greater rate of maintenance respiration reduced the amount of assimilates available for growth and yield. The differential effects of night versus day temperature could decrease tillering, leaf-area expansion, stem elongation, and grain filling.

The length of time from sowing to harvest of rice plants differs greatly and depends upon the genetic characteristics of the varieties and environmental conditions under which the plants grow (Vergara et al., 1964). In the present study, data from varieties with minimum pest damage were considered. The growth durations of early-maturing varieties (i.e., PSB Rc82, NSIC Rc238, NSIC Rc240, NSIC Rc302 and NSIC Rc308) and medium-maturing varieties (i.e., PSB Rc18, NSIC Rc122 and NSIC Rc360) did not exactly match the crop durations previously reported by NCT possibly due to differences in crop management and environmental conditions. Growth durations of most rice varieties did not differ significantly in the different N management treatments and cropping seasons. In the nitrogen omission plot (NOP) or indigenous N supply treatment, the average growth durations across varieties and seasons ranged from 103 to 118 days and were lower than growth durations in LCC-based and FRFT-based N application treatments. With LCC-based N application, growth duration ranged from 109-121 days in 2012 DS, 111-119 days in 2015 WS, and 103-112 in 2016 DS. With FRFT-based N application, growth duration ranged from 111 to 122 days in 2015 DS, 111-119 days in 2015 WS, and 103-112 days in 2016 DS. Based on NOP, the differential increase in growth duration was greater with FRFT-based N application than with LCC-based N application especially in 2015 DS. The total amount of N applied was higher with FRFT-based N application than with LCC-based N application. Hence, for the early-maturing and medium-maturing varieties tested, differences in growth duration could have been influenced by N management, indigenous N supply, and environmental conditions. Crop growth durations and grain yields in NOP were lower than growth durations and yields with LCC-based and FRFT-based N applications across varieties and seasons. Based on NOP, the average increase in growth duration of 2 days with LCC-based application and 4 days with FRFT-based N application partially contributed to yield increases in both N application treatments.

Across varieties and cropping seasons, average yields in NOP ranged from 3.9 to 5.4 t ha^{-1}. In 2015 DS, grains yields with LCC-based N application ranged from 7.1-7.7 t ha^{-1} but there was no significant genotypic variation. With FRFT-based N application, yield of the early-maturing variety PSB Rc82 was highest at 9.5 t ha^{-1} and significantly higher than yields of the other early-maturing varieties (i.e., PSB Rc238, PSB Rc240, NSIC Rc302, and NSIC Rc308) and the medium-maturing variety NSIC Rc360 that had the lowest yield of 6.6 t ha^{-1}. However, in 2015 WS and 2016 DS, there was no significant genotypic variation in yield in LCC-based and FRFT-based N application treatments. While N is the most important element that can help attain the yield potential of rice varieties (Yoshida, 1981), yields vary due to tremendous variation in soil N supply among lowland rice fields with similar soil types or in the same field over time (Cassman et al., 1998), differences in genetic characteristics, management of N and other nutrients, cropping seasons or weather, pests, and other environmental conditions. For example, grain yields were higher in DS than in WS (Figure 2). Yang et al. (2008) studied the yield gap between DS and WS from 2003 to 2004 under high-yielding tropical irrigated conditions. Results showed that higher yields were achieved in DS than in WS due to higher irradiance during grain filling and ripening stages. Moreover, the higher biomass accumulation from flowering to physiological maturity in DS was associated with higher grain yield.

For most rice varieties, the average AE_N was significantly higher with "real-time" LCC-based N application than with FRFT-based N application due to lower N rates in 2015 DS and 2016 DS. However, the average yield of varieties with FRFT-based N application (with a total of 190 kg N ha^{-1} based on 3 splits at mid-tillering, panicle initiation, and first flowering) was significantly higher than yields with LCC-based N application in 2015 DS. Similar results were

Table 3. Reported methods of N fertilizer application, total N applied, grain yields, and agronomic efficiencies of applied N (AE_N) for various rice varieties. DAT = days after transplanting.

Variety	N Application	Total N Applied (kg ha^{-1})	Grain Yield (t ha^{-1})	AE_N (kg grain kg^{-1} N)	Reference
PSB Rc82	Fixed Rate-Fixed Time with 3 splits: Midtillering: 50 kg ha^{-1} Panicle initiation: 100 kg ha^{-1} First flowering: 40 kg ha^{-1}	190 Dry Season 2015	9.5	25.4	Present study
	Fixed Rate-Fixed Time with 3 splits: Midtillering: 25 kg ha^{-1} Panicle initiation: 50 kg ha^{-1} First flowering: 20 kg ha^{-1}	95 Wet Season 2015	6.7	24.3	
PSB Rc82	Initial application of 42 kg N ha^{-1} from 14-14-14-12S at 14 DAT + LCC-based N application with a critical value of 4: 35 DAT: 35 kg ha^{-1} 56 DAT: 35 kg ha^{-1}	112 Dry Season 2015	7.7	26.8	Present study
	Initial application of 28 kg N ha^{-1} from 14-14-14-12S at 14 DAT + LCC-based N application with a critical value of 4: 35 DAT: 23 kg ha^{-1} 57 DAT: 23 kg ha^{-1}	74 Wet Season 2015	6.2	24.1	
PSB Rc82	LCC-based at critical value of 3: Early tillering: 15 kg ha-1 Early tillering: 15 kg ha^{-1} Extreme tillering: 25 kg ha^{-1} Panicle initiation: 25 kg ha^{-1} Flowering: 25 kg ha^{-1}	90 Wet season	4.6	13.9	Krishna-kumar and Haefele (2013)
IR72	4 splits: Transplanting: 60 kg ha^{-1} Midtillering: 60 kg ha^{-1} Panicle initiation: 60 kg ha^{-1} Flowering: 45 kg ha^{-1}	225 Dry Season	9.5	18.4	Cassman et al. (1993)
IR72	3 splits: Midtillering: 50 kg ha^{-1} Panicle initiation: 100 kg ha^{-1} Flowering: 40 kg ha^{-1}	190 Dry Season	9.5	15.3	Peng and Cassman (1998)

obtained by Peng and Cassman (1998) wherein they studied the upper thresholds of N uptake rates of IR72. They reported that with a total N fertilizer application of 190 kg N ha^{-1} based on 3 splits at mid-tillering, panicle initiation, and flowering in DS, total N uptake was 209 kg ha^{-1} and grain yield was 9.5 t ha^{-1}. Peng et al. (1996) reported that the best fixed-timing for N application had either greater recovery efficiency or physiological efficiency from applied N that led to relatively higher grain yield compared to "real-time" SPAD-based N application. However, SPAD-based N application had higher AE_N because of improved congruence of N supply and crop demand.

In 2015 WS, AE_N for all rice varieties did not differ significantly between LCC-based and FRFT-based N applications. In our earlier field studies, we found that "real-time" LCC-based N application had higher AE_N than FRFT-based N application in WS. Peng et al. (1996) found that AE_N of "real-time " SPAD-based N application was significantly higher than that of fixed-time N application in WS for IR72. Differences in our findings could likewise be attributed to variation in soil N supply in the same field overtime (Cassman et al., 1998).

Differences in AE_N could be due to inability of the plant to utilize higher N rates or due to rapid N losses from ammonia volatilization,

denitrification and nitrate leaching from the flood-water system (Craswell and Vlek, 1979; Fageria and Baligar, 2005; Wu et al., 2016). De Datta et al. (1991) reported that gaseous N losses due to denitrification from applied fertilizer N was 10% in lowland rice. Such N losses cause environmental problems by pollution of atmosphere, aquatic systems, and groundwater (Choudhury and Kennedy, 2005).

Nitrogen use efficiency (NUE) or AE_N has two main components: (a) the ability of crops to take up N from the soil and (b) use efficiency of absorbed N for growth and yield (Schenk, 2006; Burns, 2006). Efficiencies of these components may vary within the same crop as there are differences in plant organs, mechanisms, and environmental factors (Benincasa et al., 2011). Environmental factors such as temperature, rainfall, and soil texture can affect NUE, crop growth, development, and soil N availability through mineralization of soil organic matter and organic fertilizers (Agostini et al., 2010).

Overall, crop management, environmental factors, and abiotic and biotic stresses affect the sink-source relationship following alterations in biomass and N partitioning; hence, affecting NUE (Zvomuya and Rosen, 2002; Benincasa et al., 2011). Crop management such as crop density and spatial arrangement of plants, N fertilization and placement and timing of application, and water management also play a role in NUE (Shapiro and Wortmann, 2006; Ma and Kalb, 2006; Osborne, 2006). Liu et al. (2016) studied the effect on rice yield and NUE of basal application of 225 kg N ha^{-1} into a 10 cm deep holes positioned 5 cm from rice root in a sandy soil. This resulted in increased N-fertilizer residual and reduced N loss at harvest with higher yield increment and increased resistance to environmental threats in a sandy soil.

N-fertilizer uptake efficiency can be estimated on the basis of N uptake from unfertilized control (Greenwood et al., 1989). Genotype affects N uptake and use because it has its own morphological and functional characteristics for plant organs (Schenk, 2006). N acquisition of plants is regulated by plasma membrane-localized transporters such as AMT (ammonium transporter) super family and NRT2 (nitrate transporter) families that are regulated by AMT and NRT genes, respectively. N uptake of plants depends on the affinity of these transporters especially under low availability of N (Ludewig et al., 2007; Narcy et al., 2013; Krapp et al., 2014). Modulating root growth and architecture also plays a role in N uptake by increasing total absorptive root surface and directing growth towards nutrient-rich patches of soil (Kiba and Krapp, 2016).

Variation in AE_N can also be attributed to its method of calculation. Weih (2014) formulated a calculation tool to accurately analyze NUE or AE_N in annual and perennial crops and requires inputs such as (a) days of critical phenology stages limiting the main growth period and plant N contents at two destructive plant harvests within the main growth period for N calculation; (b) N contents of plant parts; and (c) final biomass and N yields for calculation of NUE. This is also advantageous in terms of not requiring estimates of soil N contents that is primarily linked to the usual method of calculating AE_N. The approach of Weih (2014) needs to be validated and compared with the method of calculating AE_N used in the present study.

In Table 3, grain yield and AE_N of PSB Rc82 with LCC-based N application and IR72 with FRFT-based N application reported by other researchers were compared to the results of the present study for PSB Rc82. In the present study with LCC-based N application, grain yield and AE_N of PSB Rc82 in WS were 6.2 t ha^{-1} and 24.1 kg grain kg^{-1} N respectively, and were higher than the yield and AE_N of PSB Rc82 with LCC-based N application in WS reported by Krishnakumar and Haefele (2013). Likewise, the FRFT-based N application grain yield and AE_N in WS in the present study were higher than the LCC-based yield and AEN in WS reported by Krishnakumar and Haefele (2013). In DS, the FRFT-based N application yield of 9.5 t ha^{-1} for PSB Rc82 in the present study was comparable to the yield of 9.5 t ha^{-1} for IR72 obtained by Cassman et al. (1993) and Peng and Cassman (1998), but AE_N with FRFT in the present study was higher. Overall, the relatively high AE_N of 26.8 kg grain kg^{-1} N in DS and 24.1 kg grain kg^{-1} N in WS with LCC-based N application and 25.4 kg grain kg^{-1} N in DS and 24.3 kg grain kg^{-1} N in WS with FRFT-based N

application for PSB Rc82 in the present study indicate a better congruence of N fertilizer application with crop demand for N. Because an acceptable yield of 6.2 t ha^{-1} and AE_N of 24.1 kg grain kg^{-1} N have been achieved with LCC-based total N application of 74 kg ha^{-1} in WS (Table 3), the N fertilizer application rate could be increased by 20 to 30% above the usual application rate of 35 kg N ha^{-1} when LCC reading is below the critical value 4 to achieve yields close to 9 t ha^{-1} in DS when irradiance is higher. Increasing N fertilizer application can lower AE_N. Based on Table 3, it would be reasonable to aim for an AE_N of 20 kg grain kg^{-1} N or higher and a yield target of 9 t ha^{-1} after the LCC-based N application rate is increased by 20-30% in DS. However, any substantial reduction in AE_N due to increase in N fertilization has to consider the cost of N fertilizer and its losses from the floodwater system (Cassman et al., 2002; Zhang et al., 2014).

Conclusion

Crop growth duration, grain yield, and AE_N are influenced by indigenous N supply, management N fertilizer and other nutrients, genetic characteristics, other crop management practices, cropping season or weather, pests, and other environmental conditions.

With the use of early-maturing and medium-maturing varieties and minimum pest incidence, higher AE_N was achieved with LCC-based N application than with FRFT-based N application but grain yields were lower especially in dry season for transplanted irrigated lowland rice system. An acceptable grain yield of 6.2 t ha^{-1} and AE_N of 24.1 kg grain kg^{-1} N were achieved with LCC-based N application rate of 23 kg N ha^{-1} for a total application of 74 kg N ha^{-1} in wet season. To achieve the yield target of 9.0 t ha^{-1} in dry season wherein irradiance is higher, N application rate could be increased by 20-30% above the usual application rate of 35 kg N ha^{-1} when the "real-time" LCC reading is below the critical value 4 or when the crop demands N fertilizer application, and aim for an AE_N of 20 kg grain kg^{-1} N or higher.

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Literature Cited

Agostini F, Tei F, Silgram M, Farneselli M, Benincasa P, Aller MF (2010) Decreasing N leaching in vegetable crops through improvements in N fertilizer management,. In: E. Lichtfouse (ed.). Genetic engineering, biofertilisation, soil quality and organic farming. Sustainable Agr. Rev. Vol. 4. Springer, Dordrecht, The Netherlands. pp. 147–200

Balasubramanian V, Morales AC, Cruz RT, Abdulrachman A (1998) On-farm adaptation of knowledge-intensive nitrogen management technologies for rice systems. Nutr. Cycling Agroecosyst. **53**:59–69. doi: 10.1023/A:1009744605920

Benincasa P, Guiducci M, Tei F (2011) The Nitrogen Use Efficiency: Meaning and Sources of Variation—Case Studies on Three Vegetable Crops in Central Italy. Hort. Tech **21**(3): 266-273

Bufogle A Jr., Bollich PK, Kovar JL, Machiavelli RE, Lindau CW (1997) Rice variety differences in dry matter and nitrogen accumulation as related to plant stature ad maturity group. Journal of Plant Nutrition **20** (9):1203-1224

Bullock DG, Anderson DS (1998) Evaluation of the Minolta SPAD-502 chlorophyll meter for nitrogen management in corn, Journal of Plant Nutrition **21**(4): 741-755. doi:10.1080/01904169809365439

Burns IG (2006) Assessing N fertilizer requirements and the reliability of different recommendation systems. Acta Hort. **700**:35–48

Cassman KG, Dobermann A, Walters DT (2002) Agro ecosystems, nitrogen-use efficiency, and nitrogen management. Ambio **31**:132–140. doi:10.1579/0044-7447-31.2.132

Cassman KG, Kropff MJ, Gaunt J, Peng S (1993) Nitrogen use efficiency of rice reconsidered: What are the key constraints? Plant and Soil **155**(1): 359-362

Cassman KG, Peng S, Olk DC, Ladha JK, Reichardt W, Dobermann A, Singh U (1998) Opportunities for increased nitrogen-use efficiency from improved resource management in irrigated rice systems. Field Crops Res. **56**(1-2):7-39

Chen S, Ge Q, Chu G, Xu C, Yan J, Zhang X, Wang D (2017) Seasonal differences in the rice grain yield and nitrogen use efficiency response to seedling establishment methods in the Middle and Lower reaches of the Yangtze River in China. Field Crops Research. (In Press)

Choudhury ATMA, Kennedy IR (2005) Nitrogen fertilizer losses from rice soils and control of environmental pollution problems. Communications in Soil Science and Plant Analysis **36**: 1625-1639

Craswell ET, Vlek PLG (1979) Fate of fertilizer nitrogen applied to wetland rice. pp. 175–192. In IRRI (ed.) Nitrogen and Rice. International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines

De Datta SK, Buresh RJ (1989) Integrated nitrogen management in irrigated rice. Adv. Soil Sci. **10**:143-169

De Datta SK, Buresh RJ, Samson MI, Obcemea WN, Real JG (1991) Direct measurement of ammonia and denitrification fluxes from urea applied to rice. Soil Sci. Soc. Am. J. **55**:543–548

Evans LT, Fischer RA (1999) Yield potential: its definition, measurement and significance. Crop Sci. **39**:1544-1551

Fageria NK, Baligar VC (2005) Enhancing nitrogen use efficiency in crop plants. Advances in Agronomy **88**:97-185

Fageria NK and Filho MPB (2001) In Taylor and Francis. Communications in Soil Science and Plant Analyis **32** (13-14):2079-2089

Friedmann JM, Hunt ER, Mutters RG (2016) Assessment of leaf color chart observations for estimating maize chlorophyll content by analysis of digital photographs. Agron J. **108**: 822-829

Graham S (1999) The earth's climate system constantly adjusts. http://earthobservatory.nasa.gov/Features/Clouds/07.25.2017

Greenwood DJ, Kubo K, Burns IG, Draycott A (1989) Apparent recovery of fertilizer N by vegetable crops. Soil Sci. Plant Nutr. **35**:367–381

Hasegawa H (2003) High-yielding rice cultivars perform best even at reduced nitrogen fertilizer rate. Crop Science **43**:921-926.

Islam Z., Bagchi B, M. Hossain M (2007) Adoption of leaf color chart for nitrogen use efficiency in rice: Impact assessment of a farmer-participatory experiment in West Bengal, India. Field Crops Res. **103**:70–75. doi:10.1016/j.fcr.2007.04.012

Islam MS, Bhuiya MSU, Rahman S, Hussain MM (2009) Evaluation of SPAD and LCC based nitrogen management in Rice (*Oryza sativa* L.). Bangladesh J. Agril. Res. **34**(4):661-672

Kiba T, Krapp A (2016) Plant nitrogen acquisition under low availability: Regulation of uptake and root architecture. Plant Cell Physiol **57**(47): 707-714

Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince AS (2014) Nitrate transport and signaling in Arabidopsis. J. Exp. Bot. **65**: 789-798.

Krishnakumar S, Haefele S (2013) Integrated nutrient management and LCC based nitrogen management on soil fertility and yield of rice (*Oryza Sativa* L.). Acad. Journ. **8**(41): 2059-2067

Liu X, Wang H, Zhou J, Hu F, Zhou D, Chen Z, Liu Y (2016) Effect of N fertilization pattern on rice yield, N use efficiency and fertilizer-N fate in the Yangtze River Basin, China. PLoS ONE **11**(11): 1-20. doi: 10.1371/journal.pone.0166002

Ludewig U, Neuhduser B, Dynowski M (2007) Molecular mechanisms of ammonium transport and accumulation in plants. FEBS Lett. **581**: 2301-2308

Ma CH, Kalb T (2006) Development of starter solution technology as a balanced fertilization practice in vegetable production. Acta Hort. **700**:173–185

Narcy P, Bouguyon E, Gojon A (2013) Nitrogen acquisition by roots: Physiological and developmental mechanisms ensuring plant adaptation to fluctuating resource. Plant Soil **370**: 1-29

Osborne SL (2006) Starter nitrogen fertilizer impact on soybean yield and quality in the northern Great Plains. Agron. J. **98**:1569–1574

Peng S, Cassman KG (1998) Upper threshholds of nitrogen uptake rates and associated nitrogen fertilizer efficiencies in irrigated rice. Agron. J. **90**:178-185

Peng S, Garcia FV, Laza RC, Samson MI, Sanico AL, Visperas RM, Cassman KG (1996) Increased N-use efficiency using a chlorophyll meter on high-yielding irrigated rice. Field Crops Res. **47**:243-252

Peng S, Huang, J, Sheehy JE, Laza RC, Visperas RM, Zhong X, Centeno GS, Khush GS, Cassman KG (2014) Rice yields decline with higher night temperature from global warming. Proc. Natl. Acad. Sci. USA **101** (27): 9991-9975

Philippine Rice Research Institute (1991) Rice production technoguide. Philippine Rice Research Institute, Muñoz, Nueva Ecija, Philippines

Philippine Rice Research Institute (2008) "Leaf Color Chart." Rice Technology Bulletin No. 22: 12p., PhilRice, Maligaya, Science City of Muñoz, Nueva Ecija.

Sanico AL, Peng S, Laza RC, Visperas RM (1999) Differences in nitrogen use efficiency of rice varieties developed in the Philippines since 1966. Phil. J. Crop Sci. **24**: 1-94.

Schenk MK (2006) Nutrient efficiency of vegetable crops. Acta Hort. **700**:25–38

Shapiro CA, Wortmann CS (2006) Corn response to nitrogen rate, row spacing, and plant density in eastern Nebraska. Agron. J. **98**:529–535.

Shukla AK, Ladha JK, Singh VK, Dwivedi BS, Balasubramanian V, Gupta RK, Sharma S K, Singh Y, Pathak H, Pandey PS, Padre AT, Yadav RL (2004) Calibrating the Leaf Color Chart for Nitrogen Management in Different Genotypes of Rice and Wheat in a Systems Perspective. Agron. J. **96**:1606-1621.

STAR Version 2.0.1 (2014) Biometrics and Breeding Informatics, PBGB Division, International Rice Research Institute, Los Baños, Laguna, Philippines

Vergara BS, Lilis R, Tanaka A (1964) Relationship between length and growing period and yield of rice plants under a limited nitrogen supply. Soil Science and Plant Nutrition **10** (2):18-21

Weih M (2014) A calculation tool for analyzing nitrogen use efficiency in annual and perennial crops. Agronomy **4**: 470-477

Wu L, Yuan S, Huang L, Sun F, Zhu G Li G, Fahad S, Peng S, Wang F (2016) Physiological mechanisms underlying the high-grain yield and high-nitrogen use efficiency of elite rice varieties under a low rate of nitrogen application in China. Frontiers in Plant Science **7**:1-12

Yang W, Peng S, Laza RC, Sese MD (2008) Yield gap analysis between dry and wet season rice crop grown under high-yielding management conditions. Agronomy Journal **100**(5):1390-139

Yoshida S (1981) Fundamentals of Rice Crop Science. IRRI, Los Baños, Laguna, Philippines

Zhang A, Liu R, Gao J, Yang S, Chen Z (2014) Regulating N application for rice and sustainable eco-agro development in the upper reaches of Yellow River Basin, China. The Scientific World Journal **14**: 1-11. doi:10.1155/2014/239279

Zvomuya F, Rosen CJ (2002) Biomass partitioning and nitrogen use efficiency of 'Superior' potato following genetic transformation for resistance to Colorado Potato Beetle. J. Amer. Soc. Hort. Sci. **127**:703–709

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Research Note

Diazotrophic Isolates from Grasses can Improve Growth of Rice Seedlings

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Abstract Diazotrophs, like growth-promoting bacteria, can improve rice growth. However, more information is needed on the growth-promoting activities of diazotrophs, their common plant sources, and impact on growth of rice seedlings. This laboratory study isolated and screened potential growth-promoting diazotroph isolates from Bermuda grass (*Cynodon dactylon*) and *talahib* (*Saccharum spontaneum*) and assessed their influence on growth of rice seedlings (*Oryza sativa*, upland variety PSB Rc23). Four bacterial isolates obtained from Bermuda grass (i.e., CDL1, CDR1, CDR2, and CDRS) and three bacterial isolates from *talahib* (i.e., TRS1, TST1-1, and TST1-2) were screened using *in vitro* assays to assess their plant growth-promoting activities. CDL1, TST1-1, and TST1-2 fixed more nitrogen based on colorimetric method. Highest production of indole-3-acetic acid (IAA) was observed with TST1-1 while moderate levels of IAA were observed with CDL1, CDR2, and TRS1 based on colorimetric method. Bacterial ability to solubilize phosphorus was observed with CDR1, CDR2, TST1-1, and TST1-2 isolates based on Pikovskaya's Test. Siderophore production was observed with CDL1, TST1-1, and TST1-2 based on qualitative method. Inoculation was done by dipping rice seeds in suspension of bacterial isolates. Compared with the uninoculated Control and other bacterial isolates, CDL1 from Bermuda grass significantly increased shoot growth, root growth, and seedling vigor index of PSB Rc23 seven days after sowing. This was followed by TST1-1 from *talahib*.

Keywords: Bermuda Grass, Diazotroph, Plant Growth-Promoting Activity, Rice, *Talahib*.

Introduction

Diazotrophs are microorganisms (i.e., bacteria and archaea) that convert atmospheric nitrogen (N) gas into a readily available form such as ammonia for the use of other organisms such as rice in paddy field (Ladha and Reddy, 2003; Raymond et al., 2004). They encode nitrogenase, the enzyme complex that catalyses the conversion of N_2 gas to ammonia (Santi et al., 2013). The interaction of N-fixing ability of diazotrophs with rice varieties is viewed as an alternative to replace part of the required N fertilizer of rice plant and to further study on other natural or added nutrients present in the soil (Baldani and Baldani, 2005; Araújo et al., 2013).

Diazotrophs exert their positive effects on plant growth directly and indirectly through various mechanisms. Direct effects include N fixation, production of phytohormones and enzymes, and mobilization of nutrients while indirect effects include increasing release of nutrients and fertilizer uptake efficiency, plant tolerance to stress, and production of pathogen-suppressing substances (Van Loon, 2007; Keyeo et al., 2011). Diazotrophs can affect plant growth directly by the synthesis of phytohormones such as IAA, solubilization of inorganic phosphate and zinc, mineralization of organic phosphate, and inhibition of plant ethylene synthesis (Peng et al., 2002; Dobbelaere et al., 2003; Govindarajan et al., 2008; Saravanan et al., 2008; Araújo et al., 2013).

IAA stimulates plant cell proliferation and cell elongation by loosening plant cell walls aiding root elongation and adventitious root formation (Glick, 2014). IAA and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, produced by growth-promoting bacteria, have synergistic interaction through induction of transcription of ACC synthase by IAA that catalyzes the formation of ACC. Okon and Labandera-Gonzales (1994) reported that diazotrophs like *Azospirillum* sp. produced IAA that stimulated increase in density and length of

root hairs, rate of appearance of lateral roots, and root surface area. Ferreira et al. (2015) inoculated *Azospirilium brasilense* AbV5 strain on several upland rice cultivars under field condition. Fifty percent of the rice cultivars had statistically significant increase in yield varying from 40 to 108% upon inoculation with *A. brasilense* AbV5 strain while 35% showed no significant change in yield. Results indicated a cultivar-dependent response of rice to inoculation with *A. brasilense* AbV5 strain.

Certain strains of diazotrophic bacteria were found to exhibit phosphorus (P)-solubilization (Lopez-Ortega et al., 2013; Chung et al., 2005). As majority of P is in insoluble forms (inorganic mineral), P-solubilization aids in converting P to soluble forms readily available for uptake of plants (Bhattacharyya and Jha, 2012). Solubilization of inorganic phosphorus occurs as a result of the action of low molecular weight organic acids such as gluconic and citric acids that are synthesized by bacteria (Rodriguez et al., 2004). The ability of these bacteria to solubilize P is considered as ecologically safe and economically reasonable option for supplying P as an alternative to phosphatic fertilizer application (Ahemad and Kibret, 2014).

Diazotrophs have been found to produce siderophore, an iron-chelating compound, which aids in iron absorption and influence plant uptake of other minerals like zinc and copper (Dimkpa et al., 2009; Gururani et al., 2012). The increased availability of minerals to the bacteria was reported to directly stimulate biosynthesis of antimicrobial compounds that suppress growth of pathogenic organisms important to crops such as *Rhizoctonia solani* (Joseph et al., 2007; Wahyudi et al., 2011). It also served as determinant of induced systemic resistance (Kirankumar et al., 2008) by competing with pathogenic fungi in terms of nutrient supply and synthesizing anti-fungal compounds and indirectly maintained plant growth through the reduction or prevention of the deleterious effects of one or more phytopathogenic organisms (Dobbelaere et al., 2003; Ji et al., 2014).

This study isolated the diazotrophic bacteria from *Cynodon* sp. and *Saccharum* sp. and assessed their plant growth-promoting activities and impact on rice seedling growth.

Materials and Methods

Isolation and Purification of Phyllospheric and Rhizospheric Bacteria

Fresh samples of leaf, stem, root, and rhizospheric soil from *Cynodon dactylon* and *Saccharum spontaneum* were used as sources of isolates. To isolate bacteria from the rhizosphere, the entire root system was collected and carefully tapped to remove soil adhering to the roots. The roots were placed in 100 mL diluent and blended thoroughly on a Wrist Action shaker. The soil suspension was diluted to make a series of four 10-fold dilutions. Similarly, 0.1 mL of each of the four dilutions were spread on duplicate Burk's nitrogen-free agar plates (20 g mannitol; 15 g agar; 5 g soil extract; 1 g dipotassium phosphate; 0.2 g magnesium sulphate; 0.2 g sodium chloride, trace amount of ferrous sulphate; 1000 mL distilled water). To obtain endophytic isolates, the procedure involved surface sterilization of root, stem and leaf with tap water, ethyl alcohol, chlorox, and sterile distilled water. Surface-sterilized root, stem, and leaf samples were macerated using sterilized mortar and pestle. Similarly, 0.1 mL of each sample extract was inoculated in nitrogen-free broth.

Colonies from both isolation processes were examined after incubation. Each viable microorganism present in the sample developed into a visible colony (Black et al., 1965). The visible colony was further purified by 2 successive quadrant streaks, a method to visually separate single pure colonies on agar plate. Pure isolates were then stored in agar slants incubated at room temperature (28-30°C).

Isolates were given codes based on the source plant and designated as CD from *Cynodon dactylon* and T from *Saccharum spontaneum*. The bacteria were isolated from stem, leaf, root, and rhizospheric soil. They were designated as ST for stem; L for leaf; R for root; and RS for rhizospheric soil. Isolates were grown in nitrogen-free broth.

In vitro Screening of Bacterial Isolates for Growth-Promoting Activities

Detection of nitrogen fixation. A loopful of each isolate from nitrogen-free broth was spread on a nitrogen-free malate agar plate containing bromo-

thymol blue (BTB). Change in color from green to blue or green to yellow indicated positive results for nitrogen fixation.

Indole-3-acetic acid (IAA) production. IAA production was measured by growing test culture in Burk's nitrogen-free broth media supplemented with tryptophan. After 7 days of incubation, the cultures were centrifuged and the IAA in the supernatant was detected qualitatively using Salkowski's reagent (1.0 mL 0.5M $FeCl_4$; 50 mL distilled water; 30 mL H_2SO_4). One mL of bacterial supernatant was reacted with 2 mL of the reagent. A color range from pink to red indicated positive reaction for IAA production.

Phosphate solubilization. The ability of isolates to solubilize P was evaluated using Pikovskaya's medium by Subba Rao (1999). The bacterial isolates were inoculated onto the surface of the agar. After 7 days, presence of clearing zone around the bacterial growth indicated phosphate solubilization.

Siderophore production. Procedure of Alexander and Zuberer (1991) was followed using Chrome Azurol S (CAS) agar media to detect siderophore production. The CAS plates were inoculated with the isolates using barbecue sticks. After 3-4 days of incubation, orange halos indicated siderophore production by the bacteria.

Effectiveness of Bacteria in Improving Seedling Growth and Vigor

Effects of bacterial isolates on seedling growth and vigor were assessed under laboratory conditions at the Philippine Rice Research Institute (PhilRice) in Nueva Ecija, Philippines. Twenty-five surface-sterilized PSB Rc23 seeds were inoculated by soaking in bacterial suspension for 30 min. Inoculated seeds were sown in sterile petri plate with moist filter paper for each treatment and grown at room temperature with three replications. The number of germinated seeds was counted 7 days after sowing. Seed was considered germinated when the radicle was ≥ 2 mm. Root and shoot lengths of randomly selected seedlings were measured.

Percent germination and seedling vigor index were calculated using the following formulas (Abdul Baki and Anderson, 1973).

$$\textit{Sporulation Index (SI in \%)} = \frac{\textit{Total number of plants bearing spores}}{\textit{Total number of plants collected}} \; X \; 100$$

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Results

In vitro Screening of Bacterial Isolates for Growth Promoting Activities

Nitrogen fixation. Three of the 7 bacterial isolates in the agar media (i.e., CDL1, TST1-2, and TST1-1) showed the highest intensity of color change from green to yellow, while the rest of the isolates (CDRS1, TRS1, CDR2, and CDR1) showed lesser intensity of color change (Figure 1). CDRS1 had almost the same green color as the Control.

Indole-3-Acetic Acid Production. Bacterial isolates were tested for IAA production following reaction with Salkowski reagent. Isolate TST1-1 exhibited the most intense pink color that indicated ample production of IAA (Figure 2). TST1-1 was followed by CDL1 and CDR2.

Phosphate Solubilization. *In vitro* assay for phosphorus solubilization revealed that CDR1, TST1-2, TST1-1, and CDR2 were able to solubilize P as observed from the formation of halo or clearing zone in the Pikovskaya medium.

Siderophore Production. With the use of the Chrome Azurol S agar media assay, formation of orange halo around the bacterial growth indicated siderophore production. Three of the 7 bacterial isolates (i.e., CDL1, TST1-2, and TST1-1) formed the largest orange halo diameter.

For Bermuda grass, CDL1 had ample degree N fixation while CDR1 and CDR2 had moderate degree of N fixation (Table 1). Except for P solubilization, CDL1 had higher degree of IAA production and siderophore production than CDR1, CDR2, and CDRS1.

For *talahib*, TST1-1 and TST1-2 had ample degree of N Fixation while TRS1 had moderate degree of N fixation (Table 1). Only TST1-1 and TST1-2 were positive for phosphate solubilization and siderophore production. TST1-1 had the highest degree or ample of IAA production.

Effectiveness of Bacteria in Enhancing Growth of Rice Seedlings

Among the 7 bacterial inoculants tested,

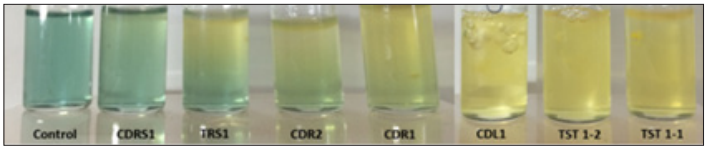

Figure 1. Nitrogen fixation on nitrogen-free agar medium by the 7 bacterial isolates. Change in color from green to yellow indicated N fixing activity.

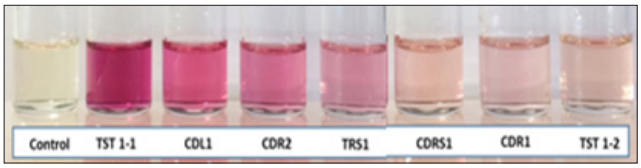

Figure 2. Colorimetric determination of indole-3-acetic acid (IAA) production by the 7 bacterial isolates. Color change from clear to pink or from clear to red color indicated IAA production.

inoculation with CDL1 resulted to the highest shoot length, root length, and seedling vigor index for the upland rice variety PSB Rc23 seven days after sowing (Table 2). CDL1 was followed by TST 1-1. However, when compared to the Control and the other bacterial inoculants, significant increases in shoot length, root length, and seedling vigor index were more consistent with CDL1 than with TST1-1.

Discussion

Based on shoot length, root length, and seedling vigor of the upland rice variety PSB Rc23 seven days after sowing, bacterial inoculant CDL1 from the leaf of Bermuda grass consistently ranked highest, followed by TST1-1 from the stem of *talahib*. Inoculants from the leaf or stem appeared to be more advantageous than inoculants from the roots and rhizospheric soil. Microbial differences between phyllosphere (above-ground plant portion) and rhizosphere (narrow soil region influenced by root secretions) communities were observed by Knief et al. (2012). In the phyllosphere, majority of proteins (60%) matched with members within the class *Alphaproteobacteria*, in particular with *Methylobacterium* and *Rhizobium*. However, *Bradyrhizobium*, *Azospirillum* and other Proteobacteria were dominantly present in the rhizosphere. Both bacteria have been extensively studied in the recent years and were revealed to have beneficial activities.

Certain strains of rhizobial diazotrophs promoted growth and yield of lowland rice through some changes in growth physiology or root morphology (Biswas et al., 2000). Isawa et al. (2010) showed that endophytic *Azospirillum* sp. strain B510 isolated from surface- sterilized stem of field-grown rice significantly increased tiller number and seed yield of paddy rice.

Araújo et al. (2013) evaluated the response of 10 traditional upland rice varieties of Marañao state, Northeast region of Brazil, to inoculation with 10 diazorophic strains, which were previously isolated from a regional soil and screened for their abilities to produce IAA. Results of their gnotobiotic experiment showed that the *Azospirillum amazonense* strain AR3122 increased the dry matter yield of the traditional rice varieties Cana Roxa and Cana Forte by 28 and 48%, respectively. Hence, based on our current results, more studies are needed to assess the impact of bacterial strains from the phyllosphere and rhizosphere of rice and non-rice plants on growth and dry matter yield of a particular rice variety. Studies will have to be conducted under a more controlled or gnotobiotic condition with a positive control prior to conducting inoculation studies in pot-soil condition.

The higher plant growth-promoting activities of CDL-1 and TST1-1 can be associated with N fixation, production of IAA, and production of siderophore, all of which were assessed qualitatively. According to Keyeo et al. (2011), the first step in screening possible beneficial bacteria

Table 1. Plant growth-promoting activities 4 bacterial isolates from Bermuda grass and 3 isolates from *talahib*. CD for *Cynodon dactylon*, T for *Talahib*, L for Leaf, R for Root, RS for Rhizospheric Soil, and ST for Stem. (+) trace, (+ +) moderate, and (+ + +) ample.

		Plant Growth-Promoting Activity			
Source	**Isolate**	**Nitrogen Fixation**	**IAA Production**	**Phosphate Solubilization**	**Siderophore Production**
Bermuda grass *(Cynodon dactylon)*	CDL1	+++	++	-	++
	CDR1	++	+	+	-
	CDR2	++	+ +	+	-
	CDRS1	+	+	-	-
Talahib (Saccharum spontaneum)	TRS1	++	+ +	-	-
	TST1-1	+++	+ + +	+	+
	TST1-2	+++	+	+	+

Table 2. Effect of bacteria inoculation on shoot length, root length and seedling vigor index of the upland rice variety PSB Rc23 seven days after sowing. Means followed by a common letter are not significantly different at 5% level by Tukey's post hoc test.

Treatment	Shoot Length (mm)	Root Length (mm)	Seedling Vigor Index
Control	35.3 ^{c}	44.0 ^{b}	250.2 ^{c}
CDL1	58.2 ^{a}	78.8 ^{a}	490.9 ^{a}
TST1-1	46.9 ^{ab}	47.2 ^{b}	340.1 ^{b}
CDRS1	40.8 ^{bc}	50.7 ^{b}	302.8 ^{bc}
CDR1	39.8 ^{bc}	48.3 ^{b}	300.3 ^{bc}
TRS1	40.3 ^{bc}	52.3 ^{b}	305.5 ^{bc}
CDR2	33.7 ^{bc}	42.0 ^{b}	233.8 ^{bc}
TST1-2	40.7 ^{bc}	49.7 ^{b}	303.1 ^{bc}

that have the ability to transfer fixed nitrogen to the host plant is to assess nitrogen fixation in free-living condition. Preliminary screening through quantitative assessment of acetylene reduction activity (ARA) of diazotrophs is usually used.

Keyeo et al. (2011) utilized ARA and tested the inoculation effects of isolated diazotrophic rhizobacteria on MR220 rice variety and reported that promotion of growth by IAA should be optimal as the excess concentration supplied by diazotrophs (e.g., 0.12 μg mL^{-1}) would suppress plant growth. Mohite (2013) reported that L-Tryptophan is a precursor of IAA production of bacteria by testing culture media with and without tryptophan. Maximum IAA production was observed on 0.05%, 0.1%, and 1.5% L-Tryptophan for wheat, banana, and maize rhizosphere isolates, respectively. IAA was not produced on L-Tryptophan free medium.

Siderophore production activity is one way of determining the ability of different microorganisms to improve plant development. Siderophores can be defined as small peptide molecules containing side chains and functional groups that can provide high-affinity set of ligands to coordinate ferric ions (Crosa and Walsh, 2002). Ghosh et al. (2015) quantitatively estimated siderosphore production of bacterial isolates using Chrome Azurol S (CAS) – Shuttle assays wherein the absorbance of the mixture of culture supernatant and CAS reagent was measured at 360 nm against a control reference (containing mixture of uninoculated broth and CAS reagent) by spectrophotometry. Under iron-limiting condition, siderophore chelates unavailable iron and make it all available to plants and co-habiting microorganisms; thus, depriving pathogens (Compant et al., 2005). In addition to biocontrol, siderophores are known to play multiple roles in diazotrophic bacterial species as they require both iron and molybdenum for the activity of nitrogenase.

In the present laboratory study that utilized agar and broth media, CDL1 was positive for N fixation, IAA production, and siderophore production, but was negative for P solubilization.

However, TST1-1 was positive for the aforementioned four growth-promoting activities. Results showed that CDR1, CDR2, TST1-2, and TST1-1 were able to solubilize P. Costa et al. (2014) simultaneously analyzed plant growth-promoting bacteria from studies that had similar methodology for bioprospection and phenotype screening using the Categorical Principal Component Analysis Model. The model classifies soil richness according to isolate's indolic compound (IC) production, siderophore production, phosphate solubilization ability, and bacterial genera composition. The result of the analysis through the model suggested that in rich soils, production of indolic compounds was high while phosphate solubilization and siderophore production were low. The model indicated that growth hormone producers had favorable plant interaction under rich nutrient soil. Under poor soil conditions, nutrient solubilizers were favored. Hence, it will be relevant to assess the performance of CDL1, TST1-1, and the other bacterial isolates under nutrient-rich and nutrient-deficient soil conditions.

P-solubilizing activity is determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe et al., 1998). Furthermore, Wani et al. (2007) revealed that microbes with the ability to solubilize P not only improved the availability of soluble P to plants, but also improved the yields of important crops through other mechanisms.

Quantification of phosphatase enzyme at pH 5, 7, and 8 from isolated phosphate-solubilizing diazotrophs was conducted by Lopez et al. (2013). They used a culture broth without P as culture media of inoculum and measured concentration of enzyme by spectrophotometry, in which genus *Azotobacter* resulted in the highest values of phosphatase activity compared to *Azosporillum*, *Rhizobium*, and *Klesiella*. Hence, such quantitative approach to assess the P-solubilizing activity will be useful to validate the P-solubilizing activities of CDL1, TST1-1, and the other bacterial isolates assessed qualitatively in the present study.

Conclusion

In association with the growth-promoting activities of diazotrophic bacteria (e.g., N fixation, production IAA, phosphate solubilization, and production of siderophore), the present laboratory study showed that among the 7 bacterial isolates, CDL1 from leaves of Bermuda grass and TST1-1 from stems of *talahib* were most effective in enhancing seedling growth of rice. However, significant increases in shoot length, root length, and seedling vigor index were more consistent with CDL1 than with TST1-1. Quantitative methods such as acetylene reduction assay and spectrophotometry will have to be used to validate the present results on growth-promoting activities of diazotrophic bacteria assessed by qualitative methods. The differential importance of phyllospheric and rhizospheric bacterial strains from rice and non-rice plants in terms of growth-promoting activities and impact on rice seedling growth and dry matter yield needs assessment. Such studies have to be conducted under gnotobiotic or more controlled condition with positive control.

Literature Cited

Abd-Alla MH, Issa AA, Ohyama T (2014) Impact of harsh environmental conditions on nodule formation and dinitrogen fixation of legumes. In Ohyama T, ed, Advances in Biology and Ecology of Nitrogen Fixation, pp 978-953

Abdul Baki AA, Anderson JD (1973) Vigor determination in soybean seed by multiple criteria. Crop Sci. **13**: 630–633

Ahemad M, Kibret M (2014) Mechanisms and applications of plant growth promoting rhizobzcteria: Current perspective. Journ. of King Saud Univ. – Sci. **26**: 1-20

Alexander DB, Zuberer (1991) Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol Fert Soils **12**(1): 39-45. doi: 10.1007/BF00369386

Araújo AES, Baldani VLD, Galisa PS, Pereira JA, Baldani JI (2013) Response of traditional upland rice varieties to inoculation with diazotrophic bacteria isolated from rice cropped at the Northeast region of Brazil. Applied Soil Ecology **64**: 49-55.

Baldani JI, Baldani VLD (2005) History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. An. Acad. Bras. Cienc. **77**: 549–579

Baldani J, Baldani VL, Dobereiner J (2000). Biological dinitrogen fixation in gramineae and palm trees. Critical Reviews in Plant Sciences **19**(3): 227-247. doi:10.1080/07352680091139213

Baldani J, Caruso L, Baldani VL, Goi SR, Döbereiner J (1997) Recent advances in BNF with non-legume plants. Soil Biology and Biochemistry **29**(5-6): 911-922. doi:10.1016/s0038-0717(96)00218-0

Baldani J, Oliveira I, Guimarães AL, Baldani SL, Reis VL, Silva FB, Döbereiner J (2000) Biological Nitrogen Fixation (BNF) in Non-Leguminous Plants: the Role of Endophytic Diazotrophs. Nitrogen Fixation: from Molecules to Crop Productivity, 397-400. doi:10.1007/0-306-47615-0_216

Bhattacharyya PN, Jha DK (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. **28**: 1327–1350.

Biswas JC, Ladha JK, Dazzo FB (2000) Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Sci. Soc. Am. J. 64: 1644-1650

Black CA, Evans DD, Dinauer RC (1965) Methods of Soil Analysis. Part II. Chemical and Microbiological Properties. Amer. Soc. Agron.,Inc., Madison, Wisconsin, **9**: 653-708

Boddey RM, Döbereiner J (1995). Nitrogen fixation associated with grasses and cereals: Recent progress and perspectives for the future. Nitrogen Economy in Tropical Soils, 241-250. doi:10.1007/978-94-009-1706-4_24

Chung H, Park M, Madhaiyan M, Seshadri S, Song J, Cho H, Sa T (2005). Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biology and Biochemistry **37**: 1970-1974

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol **71**:4951–4959. doi: 10.1128/AEM.71.9.4951-4959.2005

Costa DPB, Granada CE, Ambrosini A, Moreira F, de Souza R, dos Passos JFM, Arruda L, Passaglia LMP (2014) A Model to Explain Plant Growth Promotion Traits: A Multivariate Analysis of 2,211 Bacterial Isolates. PLoS ONE **9**(12): 1-25

Crosa JH, Walsh CT (2002) Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev **66**:223-249

Dimkpa C, Merten D, Svatos A, Bu¨chel G, Kothe E (2009) Siderophores mediate reduced and increased uptake of cadmium by Streptomyces tendae F4 and sunflower (*Helianthus annuus*), respectively. J. Appl. Microbiol. **107**:1687–1696

Dobbelaere S, Vanderleyden J, Okon Y (2003) Plant Growth-Promoting Effects of Diazotrophs in the Rhizosphere. Critical Reviews in Plant Sciences **22** (2): 107-149

Engelhard M, Hurek T, Reinhold-Hurek B (2000) Preferential occurrence of diazotrophic endophytes, Azoarcus spp., in wild rice species and landraces of *Oryza sativa* in comparison with modern races. Environmental Microbiology **2**: 131–141

Eskew DL, Focht DD, Ting IP (1977) Nitrogen fixation, denitrification, and pleomorphic growth in a highly pigmented *Spirillum lipoferum*. Appl. Environ. Microbiol. **34**(5): 582-585

Ezawa T, Smith SE, Smith FA (2002) P metabolism and transport in AM fungi. Plant and Soil **244**: 221-230

Ferreira EPB, Castro AP, Martin-Didonet CCG, Fageria NK (2015) Agronomical performance of upland rice cultivars inoculated with *Azospirillum brasilense* depends on the plant genotype. Communications in Soil Science and Plant Analysis **46**:14,1751-1762. DOI: 10.1080/00103624.2015.1043456

Ghosh SK, Pal S, Chakraborty N (2015) The qualitative and quantitative assay of siderophore production by some microorganisms and effect of different media on its production. Int. Journ. Chem. Sci. **13**(4): 1621-1629

Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiological Research **169**(1): 30-39. doi:10.1016/j.micres.2013.09.009

Govindarajan, M, Balandreau J, Kwon S-W, Weon H-Y, Lakshminarasimhan C (2008) Effects of the inoculation of *Burkholderia vietnamensis* and related endophytic diazotrophic bacteria on grain yield of rice. Microb. Ecol. **55**: 21–37

G**ururani MA, Upadhyaya CP, Baskar V, Venkatesh J, Nookaraju A, Park SW** (2012) Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. J. Plant Growth Regul. **32**: 245-259

Hassan W, Hussain M, Bashir S, Shah A, Bano R, David J (2015). ACC-deaminase and/or nitrogen fixing rhizobacteria and growth of wheat (*Triticum aestivum* L.). Journal of Soil Science and Plant Nutrition **15**(1): 232-248. 0-0. doi:10.4067/s0718-95162015005000019

Isawa T, Yasuda M, Awazaki H, Minimisawa K, Shinozaki S, Nakashita H (2010) *Azospirillum* sp. Strain B510 Enhances Rice Growth and Yield. Microbes and Environments **25** (1): 58-61

James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PPM, Olivars FL, Ladha JK (2002) Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z 67. Mol. Plant. Microbiol. Interact. **15**: 894–906

Ji SH, Gururani MA, Chun S-C (2014) Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol. Reasearch **169**: 83-98

Joseph B, Patra RR, Lawrence R (2007) Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). Int. J. Plant Prod. **2**:141–152

Karthikeyan B, Joe MM, Islam MR, Sa T (2012). ACC deaminase containing diazotrophic endophytic bacteria ameliorate salt stress in *Catharanthus roseus* through reduced ethylene levels and induction of antioxidative defense systems. Symbiosis **56**(2): 77-86. doi:10.1007/s13199-012-0162-6

Keyeo F, Ai`shah ON, Amir H (2011) The Effects of nitrogen fixation activity and phytohormone production of diazotroph in promoting growth of rice seedlings. Biotechnology (Faisalabad) **10**(3): 267-273

Kirankumar R, Jagadeesh KS, Krishnaraj PU, Patil MS (2008) Enhanced growth promotion of toma to and nutrient uptake by plant growth promoting rhizobacterial isolates in presence of tobacco mosaic virus pathogen *Karnataka*. J. Agric. Sci. **21**:309–311

Kloepper JW (1993) Plant growth-promoting rhizobacteria as biological control agents. In: Metting B (ed) Soil microbial technologies. Marcel Dekker, New York, USA, pp 255–274

Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, von Mering C, Vorholt JA (2012) Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. International Society for Microbial Ecology **6**: 1378-1390

Kraepiel AML, Bellenge JP, Wichard T, Morel FMM (2009) Multiple roles of siderophores in free-living nitrogen-fixing bacteria. Biometals **22**:573–581. doi:10.1007/s10534-009-9222-7

Ladha JK, Reddy PM (2003) Nitrogen fixation in rice systems: state of knowledge and future prospects. Plant and Soil **252**: 151– 167

López-Ortega MD, Criollo CPJ, Gómez VRM, Camelo RM, Estrada BG, Garrido RMF, Bonilla BR (2013) Characterization of diazotrophic phosphate solubilizing bacteria as growth promoters of maize plants. Revista Colombiana de Biotecnología **15**(2): 115-123. doi:10.15446/rev.colomb.biote.v15n2.36303

Mohite B (2013) Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. Journ. Of Soil Sci. and Plant Nutri. **13**(3): 638-649

Muthukumarasamy R, Cleenwerck I, Revathi G (2005) Natural association of *Gluconacetobacter diazotrophicus* and diazotrophic *Acetobacter peroxydans* with wetland rice. Systematic and Applied Microbiology **28**: 277–286

Okon Y, Labandera-Gonzales CA (1994) Agronomic applications of *Azospirillum*: An evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem **26**: 1591-1601.

Okon Y, Labandera-Gonzales C, Lage M, Lage P (2015) Agronomic applications of *Azospirillum* and other PGPR. Biological Nitrogen Fixation 1: 925-936 doi:10.1002/9781119053095.ch90

Peng S, Biswas JC, Ladha JK (2002) Influence of rhizobial inoculation on photosynthesis and grain yield of rice. Agron. J. **94**: 925–929

Raymond J, Siefert JL, Staples CR, Blankenship RE (2004) The natural history of nitrogen fixation. Molecular Biology and Evolution **21**: 541-554

Reinhold-Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K, De Ley J (1993) *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass [*Leptochloa fusca* (L.) Kunth], and description of two species *Azoarcusindigens* sp. nov.and *Azoarcus communis* sp. nov.. Int. J. Syst. Bacteriol.**43**: 574–584

Rodriguez H, Gonzalez T, Goire I, Bashan Y (2004). Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. Naturwissenschaften **91**(11): 552-555. doi:10.1007/s00114-004-0566-0

Sagoe CI, Ando T, Kouno K, Nagaoka T (1998) Relative importance of protons and solution calcium concentration in phosphate rock dissolution by organic acids. Soil Science Plant Nutrition **44**:617-625

Santi C, Bogusz D, Franche C (2013) Biological nitrogen fixation in non-legume plants. Annals of Botany **111**(5): 743-767. doi:10.1093/aob/mct048

Saravanan V, Madhaiyan M, Osborn EJ, Thangaraju M, As T (2008) Ecological occurrence of *Gluconacetobacter diazotrophicus* and nitrogen-fixing *Acetobacteraceae* members their possible role in plant growth promotion. Microb. Ecol. **55**: 130–140

Subba Rao NS (1999) Soil Microorganisms and Plant Growth. Oxford and IBH Pub. Co., New Delhi, India

Ramamoorthy V, Viswanathan R, Raguchander T, VPrakasam V, Samiyappan R (2001) Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and disease. Crop Protect. **20**: 1–11

Tran VV, Berge O, Ke SN, Balandreau J, Heulin T (2000) Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. Plant and Soil **218**: 273–284

Van Loon LC (2007) Plant responses to plant growth-promoting rhizobacteria. Eur. J. Plant Pathol. **119**: 243-254

Wahyudi AT, Astuti RP, Widyawati A, Meryandini AA, Nawangsih AA (2011) Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. J. Microbiol. Antimicrob. **3**: 34–40

Wani PA, Khan MS, Zaidi A (2007) Synergistic effects of the inoculation with nitrogen fixing and phosphate-solubilizing rhizobacteria on the performance of field grown chickpea. J. Plant Nutr. Soil Sci.**170**: 283-287

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