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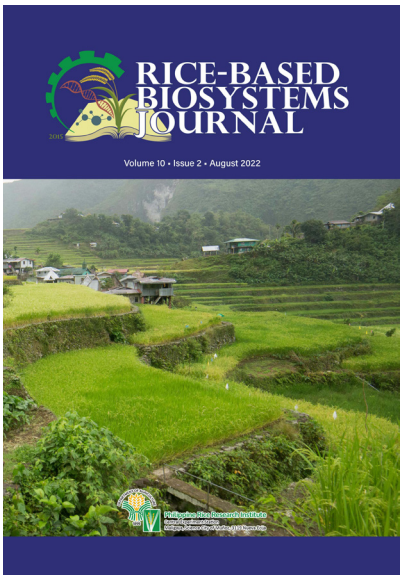

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This issue publishes studies on soil-related microorganisms' enrichment, direct-seeded rice ecosystem in irrigated and upland environments, abiotic stresses, grain quality characteristics that include health concerns, variety characteristics, and social issues on adaptive capacity of young people to climate change. As the journal progressed, we have covered a wider range of topics and studies in applied sciences, bringing new knowledge and technologies that can be used by researchers and farmers. We also continue to pursue basic research as a foundation of principles and application in rice science.

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FULL PAPER

PERFORMANCES, CHALLENGES, AND OPPORTUNITIES OF PHILRICE-DEVELOPED DIRECT-SEEDING MACHINES

Elmer G. Bautista*, Arnold S. Juliano, John Eric O. Abon, Alice B. Mataia, Manuel Jose C. Regalado, Aurora M. Corales, Jesusa C. Beltran, and Eduardo Jimmy P. Quilang

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Abstract

Crop establishment is one of the most labor-intensive activities in rice production, especially in manual transplanting practice. As labor becomes increasingly scarce and expensive, mechanized direct-seeding method offers an alternative production system that addresses challenges in agriculture. This method can enhance the farmers' profitability by reducing farm labor cost and addressing emerging labor shortage. The study assessed the performances, challenges, and opportunities of rice seeding machines developed by Philippine Rice Research Institute (PhilRice) such as manual drum seeder, hand tractor-drawn seeder, localized Korean riding-type seeder, hand tractor-drawn multi-purpose (MP) seeder, and tractor-drawn MP seeder. Results showed that MP seeder is the most efficient and economical for dry seeding while drum seeder for wet seeding. Although it is manually pulled, the drum seeder is the most cost-effective and readily available equipment for wet direct seeding using pre-germinated seeds. Mechanized direct seeding is highly recommended in irrigated lowland and rainfed areas to reduce risk of labor shortage during crop establishment. However, its adoption slowed down because of high initial investment, limited knowledge of farmers, and cultural practices. These machines must be widely promoted to attain the desired level of farmer's knowledge on direct seeding establishment in the country and reduce farm labor cost.

Keywords: *Crop Establishment, Direct Seeding, Multi-purpose Seeder, Dry Direct Seeding, Wet Direct Seeding*

Introduction

Rainfed lowlands in the Philippines totaling to 1.47 million hectares comprised about 31% of the total rice harvested area in the country (PSA, 2021; Visitacion et al., 2022). These areas are characterized by uncertain rainfall pattern, limited application of agricultural land reform, and the predominant presence of poor rice farmers mostly utilizing traditional practices. Accordingly, rice yield in rainfed lowlands is 3.22 t ha^{-1}, substantially lower than the harvests in irrigated areas averaging 4.48 t ha^{-1} (PSA, 2021). Cropping intensity in rainfed lowlands was observed at 1.3, while 1.6 in irrigated areas.

Manual transplanting is the most prevalent crop establishment method used by rice farmers in the Philippines. From the rice-based household survey conducted by PhilRice, transplanting was adopted by 64% of the farmers while 36% practiced direct seeding (Litonjua, 2019). Transplanted rice is advantageous in terms of crop establishment, weed management, and nutrient accessibility but it is a highly resource intensive practice in labor-use, water for irrigation, and other resources, which are becoming expensive (Kumar and Ladha, 2011).

Direct seeding of rice (DSR) offers an alternative production system because low input saves labor, water, and costs (Kaur and Singh, 2017). Studies have also shown that direct-seeding method in crop establishment, particularly on puddled soils, can significantly enhance farm efficiency (Alam et al., 2020). DSR used only around 3 person-day, which is substantially lower than transplanting that requires 30 person-day (RBFHS 2016-2017), resulting in less labor cost and improved farmer's productivity (Beltran, 2015).

Dry seeding is more popular in rainfed lowland, upland, and flood-prone areas, while wet seeding is commonly practiced in irrigated areas (Alam et al., 2020). This method is more appropriate in unfavorable rainfed areas due to water scarcity and erratic rainfall pattern (Sandhu et al., 2021). Comparative yields in DSR can be obtained by adopting cultural practices including selection of suitable cultivars, proper sowing time, optimum seed rate, and appropriate weed and water management. Moreover, DSR also offers the option to promote the adoption of crop rotation practices, which is highly in line with the directive of food system transformation for food diversification and improved food and nutrition security.

Direct seeding, however, has some adoption constraints (Affholder et al., 2010). The poor crop stand and high weed infestation are major concerns (Singh et al., 2005a). Xu et al. (2019) performed meta-analysis of grain yield data and found that overall, the yield of DSR was 12% lower than transplanted rice (TPR). However, the yield loss of DSR relative to TPR was highly variable depending on management practices, soil type, and climate conditions, which ranges from -2 to -42%. Weed and water management and climatic stress had the largest impact on yield performance, resulting in over 15% yield variation. According to Chaudary et al. (2022), there are several constraints associated to DSR such as the presence of more weeds, emergence of weedy rice, herbicides resistance, nitrous oxide emissions, nutrient disorders primarily N and micro-nutrients, increase in soil-borne pathogens, and lodging. In addition, traditional direct-seeding practice often uses a higher seed rate and requires further investment in weed management compared with TPR. Seeding rates for dry broadcast seeding are normally high at 150 - 250 kg ha^{-1}, anticipating the possible damages due to rats, birds, and snails (International Rice Research Institute [IRRI], 2016).

The paddy drum seeder is a manually pulled seeder composed of number drums with several holes on both ends, secured and rotated with a common shaft. Previous design was made of sheet metal but later improved into plastic drums to reduce machine weight. The latest design consisted of six drums with plastic wheel and tubular handle. Pre-germinated seeds are poured from the holes of these drums when rotated during pulling. Seeds are distributed in straight rows of 20-cm distance at pre-selected seed rates of 40, 60, and 80 kg ha^{-1}.

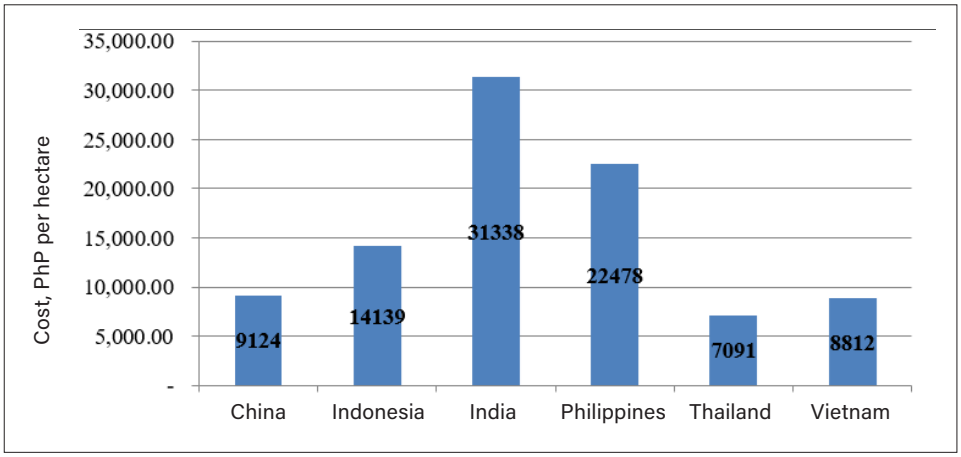
The handtractor-drawn drum seeder is a modified drum seeder attached to locally available power tiller. It was designed to replace the manual heavy work of pulling the drum seeder for easier operations and bigger capacity up to a double or thrice of manual seeding.

The localized riding-type seeder was adopted from the Korean precision seeder that precisely drops seeds into hills, resulting in significantly lower seed rate than local seeders. It is a self-propelled machine with riding operator, designed to use locally available materials and fabrication tool and practices.

The handtractor-drawn MP seeder is a newly developed dry seeding machine for rice, corn, and mungbean (Bautista et al., 2018). It was designed to suit locally available power tiller with riding operator for dry fields. The tractor-drawn MP seeder in the same case is a newly-developed machine for dry seeding of rice, corn, and mungbean intended for small tractors, which are popular to farmers nowadays.

Effects of direct seeding establishment on production cost

The costs of crop establishment activities in six major rice-producing countries are shown in Figure 1. Due to the prevalence of manual transplanting in India and Philippines, their costs of production respectively reached more than PhP30,000 and PhP20,000 per hectare. The countries that predominantly practice direct-seeding have lower production costs per hectare (Beltran et al., 2015). This is reflected in the annual incomes of farmers, in which Filipinos have the least net income per hectare after India. As manual crop establishment requires a substantial number of person-days per hectare (Devkota et al., 2020), it increases total production cost resulting in lower productivity (Pandey et al., 2000). Production costs in China, Thailand, and Vietnam are very low mainly due to their adoption of direct seeding and highly mechanized farming techniques.



(Source: Bordey et al., 2016)

Figure 1. Cost of rice crop establishment.

1. Labor requirement during crop establishment of rice

Total labor requirements are 69 and 42 ha^{-1} for transplanted and direct-seeded rice, respectively (Table 1). Transplanting needs the highest labor requirement among all operations. Direct-seeding substantially reduces labor for crop establishment to tenfold. The figure reflects that direct-seeding requires 65% less labor than transplanting, which improves farmer's productivity.

Table 1. Labor requirements in rice production, labor-days/ha

	Transplanted	Direct Seeded
Seedling establishment	3	0
Crop establishment	21	2
Harvesting and threshing	20	19
Land preparation	10	9
Crop care and maintenance	12	9
Post-harvest	2	2
Persons/ha	69	42
Cost/PhP	19,000	14,000

Source: Beltran, 2015

2. Direct-seeded areas in the Philippines

Rainfed areas have high chance of adopting the direct-seeding method due to water scarcity for irrigation. Annual data in Table 2 show that Western Visayas region has the highest percentage of adopting direct seeding (83.6%) followed by MIMAROPA (56.8%) and SOCCSKSARGEN (54.2%).

Table 2. Direct-seeded areas in the Philippines, 2013, % rice area.

Region	Average
CAR	14.5
Ilocos	2.4
Cagayan Valley	12.9
Central Luzon	29.5
CALABARZON	26.0
MIMAROPA	56.8
Bicol	27.1
Western Visayas	83.6
Central Visayas	0.8
Eastern Visayas	0.5
Zamboanga Peninsula	29.9
Northern Mindanao	20.4
Davao Region	28.5
SOCCSKSARGEN	54.2
CARAGA	22.5
ARMM	28.1

Source: RBFS, 2013

Other regions had lower percentage of direct-seeded areas probably due to water sufficiency or irrigation and perhaps, farmers are unwilling to take the risks associated with direct-seeding method such as pests (rodents, snails, weeds) and crop failure caused by natural phenomena (intense rain and flooding).

Generally, this study assessed the performance and identified emerging challenges associated with PhilRice-developed direct seeding machines. Specifically, it (a) analyzed the economic performance of mechanical direct seeding technologies vis-à-vis the manual direct seeding method in terms of yield, labor use, seeding rate, cost, and efficiency; and (b) determined the challenges based on literatures and field experiences corresponding the possible opportunities on the adoption of these direct seeding machines. This paper attempted to answer the questions: "Why were the machines not adopted?" and "What are the recommendations to enhance adoption of these machines?"

Materials and Methods

PhilRice's Rice Engineering and Mechanization Division developed five direct-seeding machines such as paddy drum seeder, handtractor-drawn drum seeder, localized Korean riding-type seeder for wet seeding, handtractor-drawn MP seeder, and tractor-drawn MP seeder for mechanized dry direct seeding. The economic performance of the direct seeding machines was assessed for mechanized direct seeding, relative to manual direct seeding method.

Source of data and analysis

Data were gathered from development stages to actual performances and end user's machine adoption, which included machine specifications, cost of crop establishment, labor use, seed rates, and farmer's adoption. Descriptive statistics such as averages and summations were used for comparisons of labor used and seeding rate. Partial budget analysis was also conducted to determine comparative efficiency of using the direct seeding machines compared with manual direct seeding method.

Results and Discussion

Wet direct-seeding technologies

Manual seeding

Manual direct seeding is done by manually placing dry seeds (Figure 2a) and broadcasting pre-germinated seeds on a well-prepared and leveled field (Figure 2b). Broadcasting requires more than two person-days ha^{-1} while placing dry seeds in furrows needs more than 20 persons ha^{-1}.

A practice in the manual direct seeding for straight row planted rice is called the dibbling method or *sud-sud* in Ilocos Norte. It requires around 26 person-days to plant a hectare. Seeding rates are high, which is up to 240 kg ha^{-1} and usually, results in decreased yield because of uneven crop stand during rice production. The dibbling method uses a piece of plastic pipe with a sharp end, inserted to the dry soil then put seed from the other end to plant and release it to leave seeds in the created hole.

Farmalite, a locally fabricated machine, is popularly used in Pangasinan and Tarlac for planting rice and corn. Similarly, it requires around 17 person-days to establish a hectare. It is a locally made device created from a pipe with funnel for easy placing of seeds. This is pointed down to the dry soil; seeds are placed in the funnel then released by pulling its trigger in the holes.

Another establishment method is creating furrows in a well-prepared field using a plow pulled by carabao or a handtractor. Seeds are directly broadcasted on the furrowed field then harrowed to cover the seeds with soil while digging them into the furrows.

These manual methods seem to be disadvantageous considering the labor requirement, mostly carried out by aging farmers. The disinterest of younger generation in farming also reduces farm labor pool. The high labor demand resulted in increasing prices of seeding per unit area.

Table 3 shows the typical cost comparison between mechanical and manual seeding for rice establishment. Land preparation is considered the same to all but crop establishments are comparing MP seeder with the manual dry-seeding. Manual furrow and broadcast seeding are more costly due to additional labor and greater number of seeds required. It was also found that higher yield was obtained with MP seeder, which resulted in higher harvesting cost including drying. It eventually resulted in higher net income per hectare of MP seeder when gross income is subtracted from the total cost.

Direct-seeding machines for wet land condition

Direct-seeding is the method of planting rice seeds directly in the field. It may be done in wet or dry seeding method based on the physical characteristics of the soil using dry or pre-germinated seeds.

Row seeding

Row seeding is mechanical seeding of dry or pre-germinated seeds mostly ending up in a straight direct-seeded rice crop. Seeds are evenly distributed in the field using a direct-seeding machine. With

Figure 2. Manual direct-seeding includes (a) placing dry seeds in furrows and (b) broadcasting pre-germinated seeds.

Table 3. Comparison between mechanical seeding vs manual seeding (Bautista et al., 2018)

	MP Seeder	Furrow Seeding	Broadcast Seeding	
		60 kg ha^{-1}	60 kg ha^{-1}	150 kg ha^{-1}
Land preparation	7,000.00	7,000.00	7,000.00	7,000.00
Crop establishment	3,030.00	5,975.00	4,475.00	7,625.00
Crop care	7,715.50	7,715.50	7,715.50	7,715.50
Harvesting and drying	7,166.40	7,140.00	5,860.80	6,280.80
Total production cost	24,911.90	27,830.50	25,051.30	8,621.30
Gross income	40,904.70	40,754.00	33,452.60	35,849.90
Net income	15,992.80	12,923.50	8,401.30	7,228.60

straight-seeded rice, humans and machines can easily enter fields to remove weeds and maintain the area and facilities.

Drum seeder

PhilRice has developed manually-pulled drum seeders, which places pre-germinated seeds in the soil surface with specified rows. It is composed of drums in a common shafting that holds and rotates drums when pulled. To improve the seeder (Figure 3), place seeds in shallow furrows to minimize pests and rainfall damage in manual and power tiller-drawn modes of operation.

Compared with the broadcast method, manually operated seeders require longer time to complete an operation in a given area. Drum seeder, which was made of metal drums, is now replaced with plastic consisting of 12 rows to lessen its weight and for increased capacity. A distinct advantage of the drum seeder is the straight rows to facilitate favorable access during crop maintenance and uniform plant spacing for better plant growth.

Figure 3. Drum seeder operation and seeded field.

Table 4. Specifications of the plastic drum seeder.

Seed capacity:	12 kg (2 kg/hopper)
Seeding capacity:	1 - 1.5 ha/day
Seeding rate:	40 - 80 kg ha^{-1}
Seed preparation:	24 hours soaking and 24 hours incubation
Number of rows	10 rows
Spacing	20 cm between rows
Machine weight	12 kg
Advantages	Output is straight in rows Light weight Affordable price
Disadvantage	Hard to pull especially in deep mud

Handtractor-drawn drum seeder

The handtractor-drawn drum seeder (Figure 4) was consequently developed to address farmer's complaint on heavy work of manually pulling a plastic drum seeder. It is based on the principle of drum seeder that drops rice seeds in small furrows created by furrows opener before seeds are being released. The power tiller-drawn drum seeder is not adopted by farmers due to the difficulty of its operation on small and irregular plots even though it has a bigger field capacity of 3 - 4 h ha^{-1}. It also needs significant power when turning at headlands, which resulted in uneven fields during operations.

Riding-type precision seeder

A local version of the Korean seeder made of locally available materials and through local manufacturers' fabrication techniques, was produced and field tested. The seeder (Figure 5) was introduced in the Philippines and demonstrated in areas where farmers commonly practiced direct seeding. The seeder can drop seeds from as low as 2 - 3 seeds per hill and high as 15 seeds per hill. Seeds are placed at 1-cm depth and slightly covered with mud. Field trials also showed that the seeder is functional during the rainy season without displacing the seeds in the rows.

Table 5. Specifications of the handtractor-drawn drum seeder.

Machine Type	**Attachment to Handtractor**
Total width	2.62 m
Length	1.36 m
Number of rows	12 (20 cm distance of planting)
Seeding rate	40 - 120 kg ha^{-1}
Field capacity	4 - 5 ha/day
Advantages	High seeding capacity One operator is needed Straight seed rows
Disadvantages	Difficult to turn at headland Paddy wheels usually destroy levelled fields

Figure 4. Handtractor-drawn drum seeder.

Figure 5. The riding-type (a) Korean precision seeder and (b) its localized version.

Table 6. Specifications of the localized riding-type Korean seeder

Capacity:	4 ha/day
Number of rows:	8
Row spacing:	25 and 30 cm
Seeding rate:	10 - 80 kg ha^{-1}
Fuel consumption:	8 L ha^{-1}
Advantages:	Seeds are covered right after sowing, making them less visible to birds and rats Seeds are not easily washed out by heavy rainfall after sowing Needs only one person a day to operate a hectare Capacity is 3 - 4 ha/day
Disadvantages:	Difficulty in maneuvering in deep muddy soils Wheels destroy levelled rice fields Locally fabricated machines are too heavy High initial investment cost High fuel consumption (approximately 15.7 L ha^{-1})

Machines for dry-seeding of rice and rice-based crops

Several dry direct-seeding machines have been locally developed. Promotion of these technologies could be hastened to increase farmers' awareness, which will eventually lead to adoption.

Multi-purpose seeder (MP)

The MP seeder (Figure 6) was developed through a Bureau of Agricultural Research -funded project, "Improving Crop Productivity in Drought-Prone Rainfed Lowlands in the Philippines with Mechanized Direct Seeding Technology" in collaboration with PhilRice, University of Los Baños, and International Rice Research Institute.

Tractor-attached multi-purpose (MP) tiller seeder

The tractor-attached MP tiller seeder was developed to be pulled by four-wheel tractors and is distributed by the government under the Rice Competitiveness Enhancement Fund (RCEF) program. This multi-crop seeder can plant rice, corn, and mungbean by replacing appropriate metering devices for each commodity.

Economic analysis of direct seeding technologies

Economics of these machines were tabulated and evaluated based on assumed value of utilization.

Figure 6. The MP seeder and established crops: (a) rice, (b) corn, and (c) mungbean.

Table 7. Specifications of the MP Seeder

Engine:	7 - 8 Hp
Prime mover:	local handtractor
No. of operator:	1
Capacity:	2.4 ha/day
Plant spacing:	20 cm rice, 60 cm corn, and 60 cm mungbean
Seeding rate:	60 kg ha^{-1} rice, 20 kg ha^{-1} corn, and 20 kg ha^{-1} mungbean
Advantages	No additional investment for farmers owning handtractor More precise seed placement than manual seeding Rice seeds in rows
Disadvantages	Uses fuel during operations Dry seeded rice is more prone to weeds than transplanted rice

Table 9 shows the economic analysis of direct seeding technologies. Investment cost of the riding-type Korean seeder is the highest followed by the 4W tractor-attached MP seeder, which is reflected in a payback period of 4.9 and 2.7 years, respectively. This showed that investing in four-wheel prime movers substantially increases the initial cost of the seeders. With regard to handtractor-drawn seeders, the MP handtractor-drawn seeder and handtrator-drawn drum seeder had almost the same price with a payback period of 0.2 and 0.4 years. The low-cost manually pulled drum seeder will be paid in just a month of seeding.

Comparison of Economics Among Machines

Table 10 shows that the total production cost using MP seeder (PhP24,912) is the lowest among the methods, followed by manual broadcast with 60 kg ha^{-1} seeds at PhP25,051 ha^{-1}, which resulted in the highest net income of PhP15,993 ha^{-1} among all methods. Production cost of using the MP seeder is lower than furrow seeding by 20% and broadcast seeding by 13%. Labor cost was also reduced by 60 and 37%, respectively.

The MP seeder helps to address problems in rainfed areas. Seeds can be evenly distributed using a rotary seed-metering plate that controls the amount of seeds released in soil. With this, crop establishment is done faster; thereby, saving cost. Overall, the MP seeder can help boost farmers' productivity and profitability in dry-seeded areas.

Figure 7. Tractor-attached MP tiller seeder.

Table 8. Specifications of the multi-crop reduced tiller seeder.

Prime mover	4-wheel tractor
Field capacity	2 - 3 ha^{-1} day
Labor	1
Number of rows	9
Seeding rate	20 - 60 kg ha^{-1} rice, 15-20 kg ha^{-1} corn
Advantages	High seeding capacity One operator needed Four-wheel tractors are popular among farmers
Disadvantages	High investment cost Size is too large for small plots

Table 9. Economics of direct seeding technologies (2018).

	Drum Seeder	Handtractor-drawn Drum Seeder	Riding-type Korean Seeder	4W Tractor-attached Multi-crop Tiller Seeder	Handtractor-attached MP Seeder
Cost of the machine (Php)	8,500	120,000	1,000,000	500,000	38,000
Utilization: days/year	30	60	60	60	60
Field capacity, ha/day	1.5	4	4.0	3.0	2.5
Fixed and variable costs, Php/yr:	11,188	72,334	394,156	265,406	34,453
Income generated, Php/ha:	2,251.4	2,198.6	857.7	1,025.5	770
Payback period, yr	0.1	0.2	4.9	2.7	0.3
Break-even hectares	3.8	54.6	1,165.9	487.6	49

Assumptions: Useful life is five years; salvage value, 10% investment; diesel PhP 40/L, labor rate, PhP 300 a day; custom rate of seeding PhP 2,500 ha^{-1}.

Table 10. Cost comparison between manual seedings (broadcast, furrow seeding) and MP seeder at different kg ha^{-1}.

	MP Seeder	Furrow Seeding 60 kg ha^{-1}	Broadcast Seeding 60 kg ha^{-1}	Broadcast Seeding 150 kg ha^{-1}
			PhP	
Land preparation	7,000	7,000	7,000	7,000
Seeding	3,030	5,975	4,475	7,625
Crop care	7,716	7,716	7,716	7,716
Harvesting	7,166	7,140	5,861	6,281
Total cost	24,912	27,831	25,051	28,621
Gross income	40,905	40,754	33,452	35,850
Net income	15,993	12,924	8,401	7,229
Yield (kg ha^{-1}) for paddy at PhP17/kg	2,406	2,396	1,973	2,108

Source: Bautista et al., 2017

Summary and Recommendations

The manual drum seeder is well-adapted to lowland conditions. Although it is manually pulled, it has distinct advantages over manual broadcasting such as reduction of required seeds from 80 - 250 kg ha^{-1} to 40 - 80 kg ha^{-1}, and straight-seeded crop that allows easier entry for cleaning and maintenance. It is the lowest-cost machine available for direct seeding pre-germinated seeds.

The handtractor drawn-seeder is designed to lessen the drudgery of manual pulling of the drum seeder during seeding. It is attached to the local handtractor to enhance the capability of the locally made power-tiller, allowing it to be more versatile and multi-purpose in usage. This resolves the time-consuming and burden of pulling the drum seeder in deep mud but adoption is limited due to farmers' complaints of hard turning at headland and disturbance to their well-leveled rice fields.

The Korean seeder was tested and worked effectively in lowland conditions. It is suited to local field conditions but the problem of high price was pointed out. A locally adapted unit similar to the Korean seeder was fabricated to reduce the cost of the imported machine. However, the local version is heavier, which makes it hard to operate in slightly deep mud soils.

The MP seeder is a promising technology as its benefits are not confined to rice production in rainfed lowlands. It promotes crop diversification and intensification as it is applicable to rice, corn, and mungbean. The convenience it offers can motivate farmers to expand their area of cultivation, which can result in a more abundant crop production; thereby, increasing income by 32 - 92%. Further tests and optimization studies or scaling out are recommended to establish the full potential of the machine in different locations in the country. Moreover, dry-seeding technologies should be extensively promoted for adoption; thereby, contributing to food security.

The multi-purpose reduced-till seeder pulled by small tractor was designed for seeding operation in rainfed areas. It was meant to direct-seed rice, corn, and mungbean on a dry-prepared field even at reduced tillage. It is expected to be popular when four-wheel tractor becomes the main power source in the farm.

Direct-seeding method of crop establishment was proven to incur lesser cost and labor requirements than manual transplanting. Mechanized direct-seeding is highly recommended for almost all ecosystems.

Table 11. Technical constraints and suggested strategies and experts' experience in adopting direct seeding in the Philippines.

Technical Constraints	Recommended Strategies
High initial investment cost (with the small land holdings of ordinary farmers, they cannot afford to own machines, which would eat up their earnings)	▪ Encourage farmers to form cooperatives for them to collectively own and optimize utilization of expensive machine ▪ Increase usage per season (optimize machine utilization among members, bolster capacity and benefit through planned operations) ▪ Subsidize acquisition of agricultural machines (lower production cost will result in lower cost of output; lower taxes to own a machine will motivate farmers to buy)
Adaptability of seeding machines to local field shapes and sizes; riding self-propelled seeder is not suited to wet land soil conditions of small plots	▪ Massively introduce and demonstrate direct-seeding machines (Lantin 1986) ▪ Introduce and demonstrate new agricultural machinery (In 1964, all local government units [LGU] were activated for machine promotion and demonstration throughout Japan. They conducted full-force demonstrations and training on the use of combine harvesters, transplanters, and other machines). ▪ Establish field service in strategic places. Fund research for machine adoption to local conditions.
Lack of education on direct-seeding machines (machines are still considered luxury, added hassle to common practices, more cost on production; farmers are intimidated by machine complexity)	▪ Conduct training on machinery operations to agricultural technicians in regional field offices, local government units, and farmers. In 1960, an agricultural mechanization training unit was created to elevate awareness of farmers and train farmer-beneficiaries of machines (Lantin, 1986). ▪ Educate farmers on the efficient and safe use of farm machines
4. Availability and capability of local manufacturers (unavailability of affordable machines; manufacturers are not ready to fabricate new machines; limited demand for machines limits fabrication; machines are produced with low quality; no after-sales services offered)	▪ Subsidize local manufacturers to encourage fabrication of not-so-popular local machines ▪ Support local manufacturers (subsidized provision of local machine designs from research institutions and technical assistance in the fabrication process)
5. Cultural problems (culture of people is difficult to change, inadequate knowledge on crop care and maintenance; labor displacement; machines are new things to them)	▪ Educate farmers on machine utilization ▪ Scale up promotion through actual demonstrations (to-see-is-to-believe mindset of farmers, reduce drudgery of field work) (Lantin 1986)
6. Weak promotional activities (lack of promotion in the field; farmers' mentality of to-see-is-to-believe limit adoption of direct-seeding machines; low appreciation of machine benefits)	▪ Extensively promote direct-seeding machines. During the enactment of the Agricultural improvement promotion law in 1984, Japan actively promoted locally fabricated and imported machines using almost 10,000 extension agents and 680 subject matter specialists

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IN SITU GROWTH OF THE CYANOBACTERIUM, *Nostoc commune* VAUCHER, IN THE UPLAND AND LOWLAND RICE FARMS IN NORTHERN LUZON, PHILIPPINES

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Abstract

Nostoc commune is an edible macroscopic cyanobacterium capable of fixing atmospheric nitrogen into the soil. This study reported its persistence in the upland and lowland rice farms and along the river of 11 municipalities in Northern Luzon. Water supply favors its persistence by affecting the surrounding climate. Generally, its population had significantly vanished except in a few paddies that were less disturbed by agricultural cultivation, specifically in areas that are shallowly tilled and applied with minimal chemicals. Human consumption and market demand are among the primary factors affecting the noticeable disappearance in the rice farms.

Despite some morphological variations, almost indistinguishable microscopic features were observed among the samples. The direct PCR sequencing of the 16S rRNA gene established the close genetic similarity between *N. commune* collected from different lowland rice farms in Ilocos Norte, which were also similar to sequences obtained from the GenBank database. On the other hand, the samples collected in the Ilocos Norte upland site were relatively and genetically distant from lowland samples.

Understanding the natural environmental conditions supporting the growth and development of the *N. commune* will help facilitate its restoration in the rice farms to provide supplemental nitrogen to crops. Results may also lead to the successful design of its clean and scalable cultivation for food. Apparently, the constant inoculation of *N. commune* in wet and less disturbed areas on the farm can possibly contribute to the establishment of its primary colonies and eventual proliferation. Its on-farm cultivation using natural farm resources can be promoted along with reduced-tillage technologies, especially in nutrient-poor areas.

Keywords: *16s rRNA Gene, Biofertilizer, Food, Heterocyst, Tabtaba*

Introduction

Nostoc species are nitrogen-fixing cyanobacteria formerly known as blue-green algae that belong to the family *Nostoc*aceae in the order *Nostoc*ales (Castenholz and Waterbury, 1989). These can be found in almost all terrestrial and aquatic habitats including the rice paddy field ecosystems wherein light, water, temperature, and nutrients provide a favorable environment for its growth (Roger, 1985). *N. commune* Vaucher is especially widespread globally (Potts, 2000; Wright et al., 2001; Novis and Smissen, 2006). Its persistence and success in terrestrial environments have been attributed to its ability to tolerate desiccation after an extended drought period and to rapidly rehydrate and recover metabolic activity once favorable conditions have been reestablished (Dodds et al., 1995; Potts, 2000; Fukuda et al., 2008).

It is consumed worldwide (Potts, 2000) and is known to be rich in proteins, amino acids, fatty acids, polysaccharides, flavonoids, vitamins, and various kinds of minerals (Diao et al., 2012; Li et al., 2003); hence, a remarkable healthy food. It was first consumed in early 2000, when the Chinese used *N. commune* as a famine food to survive during the Eastern Jin Dynasty in the third century A.D. (Han et al., 2004; Gao, 1998; Potts, 2000). Likewise, it exhibits antiviral and antitumor properties as well as anti-bacterial and anti-inflammatory effects (Kanekiyo et al., 2005; Tamaru et al., 2005; Dembitsky VM and Řezanka T, 2005). It is also known as traditional medicine with strong antifungal activities against *Phytophthora capsici* (Kim, 2006). It is a potential source of high-value products for biotechnological exploitation and can also be cultured on a large scale for the production of the desired bioactive molecules. Biotechnologically, some genomic projects have already embarked on understanding the

biosynthesis and physiological functions of Nostoc species (Skulberg et al., 2004). It is one of the major contributors to the sequestration of carbon dioxide in organic compounds, specifically in nitrogen-poor environments of India, Indonesia, and other Asian countries. Mixtures of viable cyanobacterial species, *Nostoc spp*. included, have long been used as biofertilizers of rice paddies (Santra, 1993).

In the Philippines, *N. commune* is locally called "tabtaba". In the 1980s to the early 2000s, it was predominantly observed co-existing with rice in large biomasses of Northern Luzon, particularly in the rice paddies and hilly areas of Ilocos and Cagayan Valley regions (Martinez, 1988) and in Albay, Bicol Region (Rodulfo, 1990). Its proliferation in the area has been associated with the richness of the soil but nowadays, the inhabited areas have significantly decreased. In this study, upland and lowland areas currently proliferated by *N. commune* were identified and the natural environmental conditions and factors that support its growth and development were determined. The results of this study can help facilitate the design of its scalable production for food or the establishment of its on-farm cultivation using natural farm resources in providing supplemental nitrogen to crops.

Materials and Methods

Collection of N. commune

The upland and lowland rice farm areas of Northern Luzon were surveyed from 2016 to 2017 to identify the presence of *N. commune*. These sites include Pangasinan, Ilocos Norte, Isabela, Nueva Vizcaya, Kalinga, Cagayan, and Mountain Province. The field technicians of the local government units, village officials, residents, and vendors in local markets were interviewed to locate the areas with *N. commune* bloom. Residents in Nueva Ecija, Negros Occidental, and Agusan Del Norte were also interviewed.

Morphological and molecular characterization of N. commune

The specimens were washed, rinsed, and stored in a dry condition at room temperature until use. Fresh or saturated biofilm specimens were hand-sliced using a razor blade homogenized and examined under the microscope. The species were identified morphologically by Dr. Milagros M. Goss, an *N. commune* expert, based on taxonomic keys provided by McGuire et al. (1984).

Representative samples were also characterized molecularly. The extraction of genomic DNA samples has been difficult. Among the various extraction procedures tested, the use of NucleoSpin® DNA extraction kit was the most effective. The 16s rRNA gene was amplified using universal primers 27F - AGAGTTTGATCMTGGCTCAG and 1492R - CGGTTACCTTGTTACGACTT (Lane, 1991). The PCR reaction was carried out in 6 uL volume, consisting of 1x PCR buffer, 2mM $MgCl_2$, 0.5 uM each of R and F primers, 0.2 mM dNTPs, 0.04U Taq, and 50 ng genomic DNA template. The PCR cycling was performed in Veriti™ 96-Well Fast Thermal Cycler) at 35 cycles as follows: 5 min 95°C, 30 sec 95°C, 40 sec 58.8°C (Turner et al., 1999), 40 sec 72°C, and 7 min 72°C.

The purified PCR products were sent to FirstBASE Laboratories Sdn Bhd, Selangor, Malaysia for sequencing. The DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) and compared with two available *N. commune* sequences in the NCBI GenBank database. The rRNA sequences were aligned using the multiple sequence alignment program CLUSTAL W (Larkin et al., 2007). The aligned sequences were checked for gaps manually, arranged in a block of 600 bp in each row (Ayyadurai et al., 2007), and used in the molecular evolutionary genetics analysis (MEGA) format in the software MEGA v6.0. The bootstrapped data sets were used directly for constructing the phylogenetic tree using the MEGA v6.0 program for calculating the multiple distance matrixes (Kumar et al., 2004). The multiple distance matrix obtained was used to construct phylogenetic trees through the neighbor-joining (NJ) method (Saitou and Nei 1987).

Maintenance of N. commune cultures

Dried *N. commune* specimens was soaked in sterile distilled water for an hour and washed twice with sterile water. Specimens were disinfected with 5% sodium hypochlorite and then washed with sterile water several times. Wet *N. commune* was ground in sterile petri plates and then inoculated in BG-11 agar slants. This medium is used successfully for most cyanobacteria. Culture tubes were then placed under four 20-watt fluorescent lamps until colonies appeared on the surface of agar. The purified cultures were maintained in test tubes with nutrient agar and kept in the freezer at PhilRice Central Experiment Station in the Science City of Muñoz, Nueva Ecija.

Chemical properties of soil and water samples and climatic conditions

The collected soil and floodwater samples from specific sites, both areas with and without *N. commune* bloom was collected for the *in situ* measurement of chemical properties. Stone, rubbish, trash, or grass on the surface of the land were brushed away and the surface or topsoil. Composite soil samples from 10 random spots in the area, with 5 - 10 cm deep layers, were collected using a sterile scalpel. Samples were temporarily kept in 50-mL Corning tubes that were

properly labeled with location, collection date, and other necessary details. Samples were sent to CAFS-ASI Analytical Services Laboratory in the University of the Philippines Los Baños, Laguna for analysis.

Results and Discussion

Survey areas for the presence of N. commune and site characterization

N. commune provides plants with an indigenous supply of nitrogen and can also be utilized as an alternative protein source for humans and farm animals. It is widely distributed in illuminated portions of the biosphere in aquatic and terrestrial habitats (Dodds et al., 1995). It forms colonies with mucilaginous layers on soil, stones, and mud in freshwater systems (Briones-Nagata, 2007). *Nostoc* species are known to be highly adapted to life in arid environments. They can tolerate high light intensities and UV radiation as these synthesize scytonemin, mycosporines, and other photo-protective pigments providing shade along with the surrounding microbial community (Castenholz and Garcia-Pichel, 2000).

In the Philippines, *N. commune* used to abundantly thrive in the provinces in Northern Luzon particularly Ilocos Norte, Cagayan, Isabela, La Union, Kalinga, Abra, Apayao, Nueva Vizcaya, and Pangasinan, based on the historical knowledge of the locals interviewed in this study and on information gathered from Dr. Milagros M. Goss, who led various projects in the Philippines on cyanobacteria including *N. commune*. The search was successful in Ilocos Norte and Nueva Vizcaya as only *N. commune*-like colonies were collected from other provinces.

In Ilocos Norte, locals claimed that the nitrogen-fixing cyanobacteria probably still exist in Adams, Dumalneg, Bangui, Pasuquin, Bacarra, Badoc, Currimao, Paoay, Laoag City, Piddig, Carasi, Solsona, Banna, Marcos, San Nicolas, Batac City, Burgos, Bacarra, Sarrat, Vintar, and Pagudpud. However, interviewees were unable to identify the exact areas in Adams, Pasuquin, Dumalneg, and Batac City as the populations had already declined significantly.

In 2016 wet season, the abundant proliferation of wet olive, green to dark green *N. commune* was documented in an upland rice farm at Sitio Ballit, Brgy. Santa Matilde, Pasuquin, Ilocos Norte (Figure 1). The dense brownish biomass of dried *N. commune* was found scattered on the paddy during the 2017 dry season. This is consistent with the findings of Pott (1997), which showed that some wet colonies with bluish-green color turn inconspicuous brownish mat when dry.

The interviewed farmer practices dry seeding; thus, the soil is not plowed deeply - a condition that probably favored the abundant growth of *N. commune* over the soil surface. The site is situated relatively at the most elevated part of the upland, with a creek situated underneath. However, the same clusters of *N. commune* hardly thrived in the nearby rice paddies.

In another upland rice paddy farm in Dilanis, Pasuquin, Ilocos Norte, the broad *N. commune* appeared like flat leaves stuck on the soil (Figures 2 and 4). Adjacent to the site is a man-made reservoir, where water from the mountains are stored for farm utilization. Meanwhile, in San Isidro, Dumalneg, Ilocos Norte, clusters of tiny raisin-like specimens were collected (Figure 2) from sandy soil near the Bolo River. Water from the hills flows down to the paddy site.

Abundant blooms of discoid and flat *N. commune* were documented in three rice paddy areas of Nueva Vizcaya (Figure 3): along the river in Purok 3 and Purok 4, in Magapuy, Bayombong, and Sto. Domingo, Bambang (Figure 4). After rice harvest, the abundant proliferation of *N. commune* on the soil surface became more visible. In 2015 wet season, a rice paddy in Purok 3, Magapuy was reported to be densely populated by *N. commune* and locals in the area obtained about two sacks of its fresh biomass for food after the rice harvest. The Magat River is the constant source of water in this area, which contributed to the dispersal of *N. commune* inoculum and supported its growth and proliferation. However, in 2017, the site was instantly disrupted by a road construction project

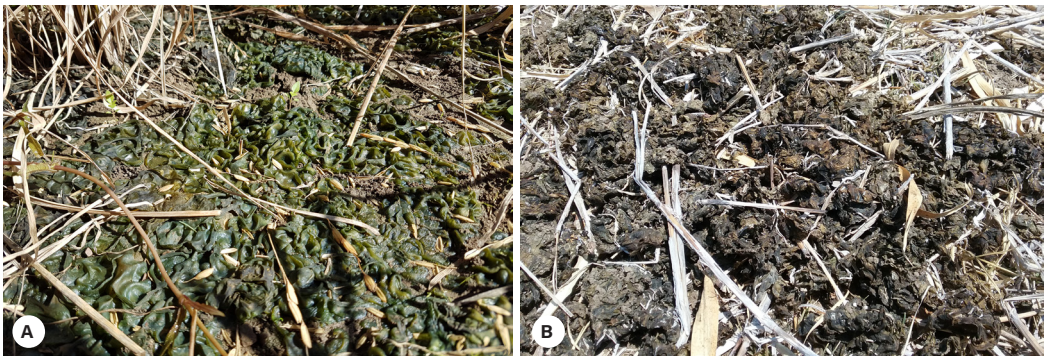

Figure 1. A site in Sitio Ballit, Brgy. Santa Matilde, Pasuquin, Ilocos Norte with an abundant proliferation of *N. commune* during the 2016 wet season (A) and 2017 dry season (B).

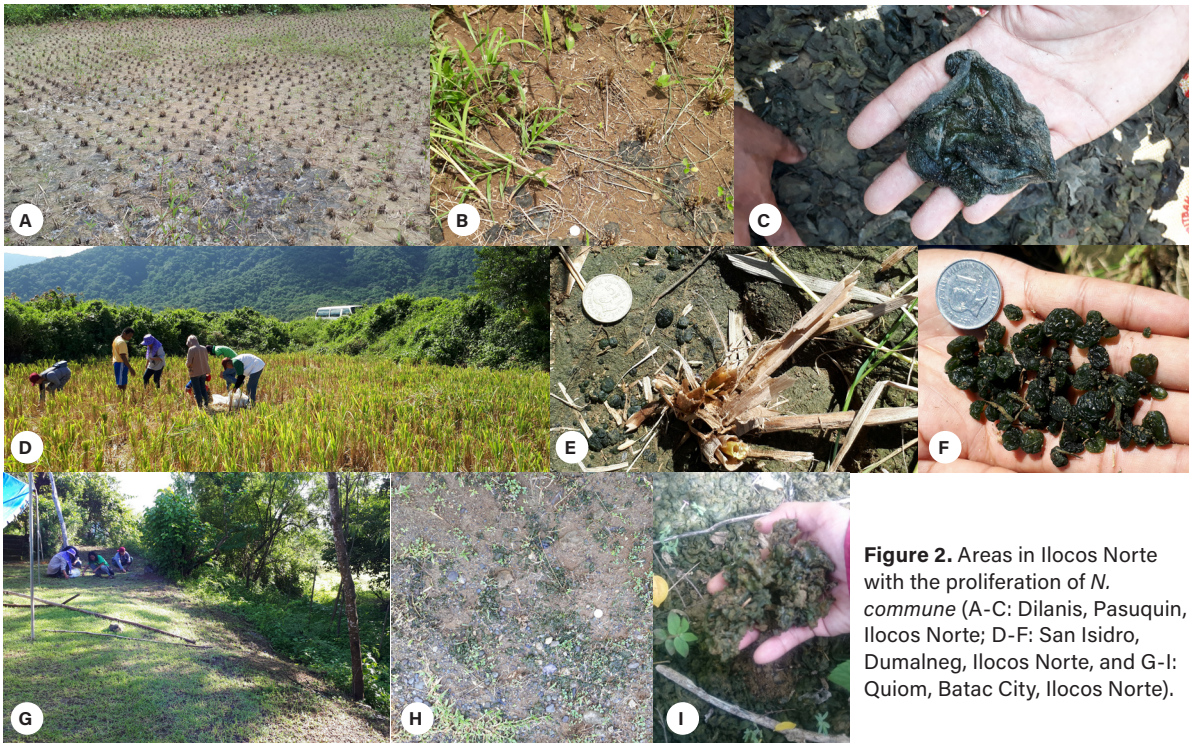

Figure 2. Areas in Ilocos Norte with the proliferation of *N. commune* (A-C: Dilanis, Pasuquin, Ilocos Norte; D-F: San Isidro, Dumalneg, Ilocos Norte, and G-I: Quiom, Batac City, Ilocos Norte).

(Figure 3). Locals also identified Quezon, Solano, and Bagabag for the proliferation of *N. commune* but presence of this species was not found.

Topography generally contributes to the persistence of *N. commune*. Being surrounded by mountains and constant rainfalls in the afternoon allows high moisture of soil, rendering a very conducive environment for abundant proliferation. Farmers with *N. commune* thriving in their rice fields also said that they apply fewer chemicals. However, the study of Tansay et al. (2021) concluded that commercial formulations of glyphosate and 2,4-D do not adversely affect the *Nostoc* species.

Fresh weight of *N. commune* collected in idle lands and hilly mountains collection sites in Batac, Ilocos Norte averaged 259 g/m^2 (of Biningan) and 1418.96 g/m^2 (Quiom). Meanwhile, 12666.67 g/m^2 in Sto Domingo, Bambang in Nueva Vizcaya was collected. The edible macroscopic cyanobacterium gathered in the rice farms and along the river of Magapuy, Bayombong weighed 280. 67 and 766.67 g/m^2, respectively. The comparison of average means showed no significant differences except at a 10% level of significance wherein large biomass collected at Quiom, Batac City, Ilocos Norte showed significant difference from the biomass collected from an upland site at Biningan, Batac City, Ilocos Norte and from a lowland rice farm at Magapuy, Bayombong, Nueva Vizcaya. However, it should be noted that the data presented were based on the disturbed populations of the *N. commune*, as only remnants of the frequent but irregular harvesting by the locals were gathered.

Utilization of N. commune

Most of the people in Ilocos and Cagayan Regions have known that *N. commune* proliferates mostly during wet seasons, especially when rainfall is enough to retain moisture in the soil. At such times, large biomasses are being harvested, consumed, and sold in local the market (Figure 5). As such, human consumption and market demand could have probably contributed to its significant decrease in population. In the market, fresh *N. commune* is sold at PhP10 – PhP20 per small noodle cup while dried forms are sold at PhP400 - 500 per 250 g. The dried form is more convenient for long-distance transport and sometimes sent by locals to relatives abroad. Aside from being consumed as green salad savored with salt or *bagoong* and tomatoes or calamansi, other people use it as garnish on mungbean or on *pinakbet,* an Ilukano dish.

According to reports, Bontoc folks 15 years ago add the spherical *N. commune* on *samalamig* and *halo-halo* as alternatives to *sago* and *gulaman*. In Cagayan, Abra, and Tabuk, locals also see this nitrogen-fixing cyanobacterium growing on rivers seasonally. In recent reports, however, some locals already doubt the safety of consuming *N. commune* due to fear of parasites or traces of chemical residues in its tissues. Instead of using as human food, *N. commune* in Tabuk, Kalinga is used as animal feed.

Figure 3. Areas along the Magat River in Magapuy, Bayombong, Nueva Vizcaya with an abundant proliferation of *N. commune* in 2016 (A-G at Purok 4; H-J at Purok 3), but ruined by a road construction project in 2017 (L-N).

Table 1. Fresh weight biomass of *N. commune* harvested in collection sites.

Areas	Quadrat 1	Quadrat 2	Quadrat 3	Mean
	N. commune Fresh Weight Biomass (g/m^2)			
Ilocos Norte				
Upland				
Biningan, Batac	177.64	341.63	-	259.67^{b}
Quiom, Batac	2250.82	579.65	1426.4	1418.67^{a}
Nueva Vizcaya				
Upland				
Sto. Domingo, Bambang	365.5	286	1129	593.67^{ab}
Lowland, rice farms				
Sto. Domingo, Bambang	1100	1000	1700	1266.67^{ab}
Magapuy, Bayombong	345	304	192.8	280.67^{b}
Along Magat river				
Magapuy, Bayombong	1000	400	900	766.67^{ab}

Means with the same letter are not significantly different at 10% level of significance

Figure 4. A rice paddy area in Sto. Domingo, Bambang, Nueva Vizcaya (along Magat river) with proliferation of *N. commune* before (A - B) and after (C - H) rice harvest.

Morphological and Molecular Characterization of N. commune

Despite having variations in the size (micro-colonies or macro-colonies), shape (discoid or flat), and texture (rough or smooth), the microscopic examinations (40x) of *N. commune* samples from upland and lowland rice farms revealed no significant morphological differences. Generally, long chains of vegetative cells and intercalating heterocysts were observed among specimens collected from the rice paddies of Bayombong and Bambang in Nueva Vizcaya and Adams, Pasuquin, and Dumalneg in Ilocos Norte (Figure 6). Heterocyst cells contain oxygen-sensitive enzyme nitrogenase and serve as sites for nitrogen fixation by converting N_2 to NH_3 (Dixon and Kahn, 2004). Its response to the amount of nitrogen varies: N_2 fixation is only stimulated when nitrogen in the surroundings becomes limited but with a non-limiting resource of N such as ammonium ($NH4^+$) and nitrate ($NO3^-$), these cells do not produce active heterocysts (Meeks et al., 2002; Campbell et al., 2007). Also, *Nostoc* species respond to nutrient limitations by producing relatively short, motile filaments called hormogonia, and differentiate spore-like structures called akinetes (Sarma et al. 2004).

The extraction of genomic materials from *N. commune* specimens has been challenging and high-quality DNA samples were only extracted from three samples: two from the geographically-distant lowland rice farms in Ilocos Norte (Adams and Pasuquin) and another from an upland area in Pasuquin, Ilocos Norte. The size of the 16S rRNA gene amplified was about 1.4 kb (Arima et al., 2012). Figure 7 shows that the nucleotide sequences of *N. commune* obtained from the lowland rice farms are identical to each other and also highly similar to the 16S rRNA gene sequences of *N. commune* strains AB088375.2 and HQ877827 obtained from the GenBank database. This also confirms a high level of genetic similarity indicating that these specimens are recognizable by

Figure 5. Locals of Nueva Vizcaya and Ilocos Norte collect *N. commune* from hills and rice farms and sell it in public markets for food (A - B harvesting by hand-picking; C - D selling fresh form in local markets; and E - H drying preparation).

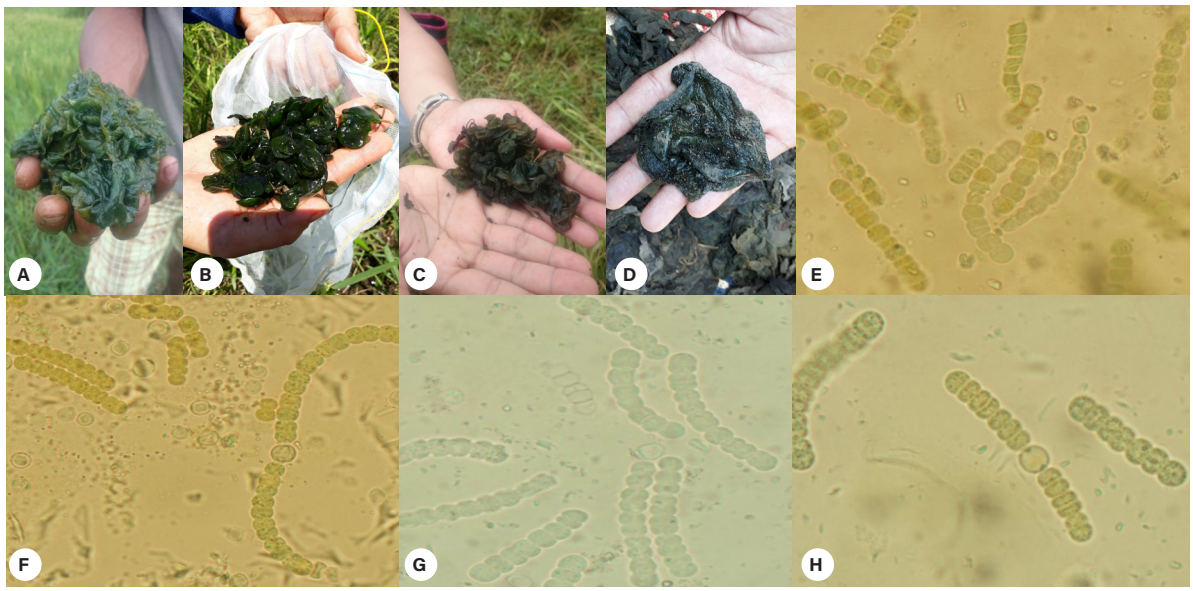

Figure 6. Fresh *N. commune* specimens collected from the rice paddies in Nueva Vizcaya (Bayombong [A]; Bambang [B]); and Ilocos Norte (Adams [C]; Pasuquin [D]) with corresponding microscopic examinations (E - H) based on heterocysts and vegetative cells.

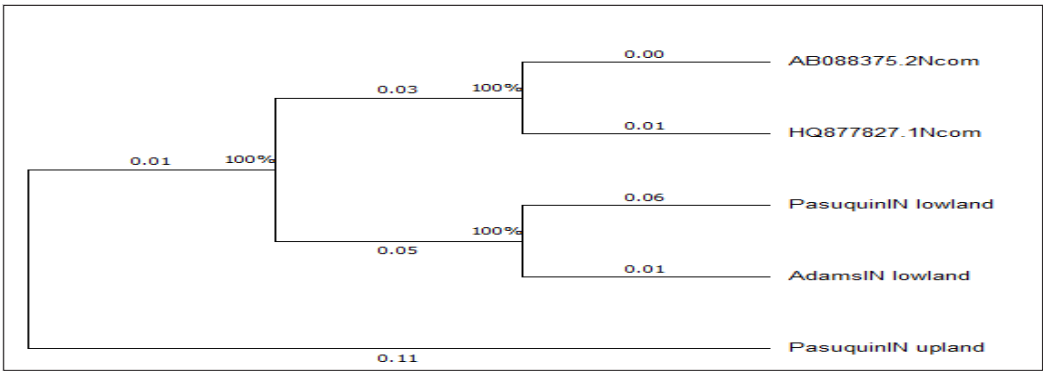

Figure 7. Molecular Phylogenetic analysis *N. commune* collected from upland and lowland farms in Ilocos Norte based on 27F-1492R primers by Maximum Likelihood method.

16S rRNA gene sequence. The specimens came from a common origin, but the type of environments that supported their persistence in the distant lowland rice paddies did not trigger genetic variations. However, a considerable genetic dissimilarity was observed in the upland specimen collected in Pasuquin, Ilocos Norte compared with those from the lowland rice farms. The estimated evolutionary divergence of *N. commune* from lowland farms of Adams and Pasuquin, Ilocos Norte is 0.07 (Table 2). The computed distance of *N. commune* from Pasuquin upland and Adams lowland sites were 0.17 and 0.23, respectively. The combinations of various factors such as the availability of water and nutrients, substrate conditions, and amount of UV radiation in these two lowland rice areas are among the major factors affecting the growth and development of both plants and cyanobacteria. In 2014, Borah et al. established the presence of higher amounts of total carotenoid content of *N. commune* isolated from a flat paddy field while the isolate from a terrace paddy field was richer in phycobiliproteins. The full-length 16S rRNA gene sequences of the collected samples should be examined in the succeeding phylogeny reconstruction to gain a deeper overview of their genetic diversity (Nübel et al., 1997).

The evolutionary history was inferred by using the Maximum Likelihood method that based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-2933.54) is shown (Figure 7). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances, estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node of the tree. The analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were 1,115 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al, 2016).

Table 2. Estimates of Evolutionary Divergence between *N. commune* sequences based on 16S rRNA gene sequences.

Specimen 1	Specimen 2	Distance
AB088375.2_N.com	HQ877827.1_N.com	0.01
AB088375.2_N.com	PasuquinIN_Lowland	0.14
HQ877827.1_N.com	PasuquinIN_Lowland	0.14
AB088375.2_N.com	AdamsIN_Lowland	0.09
HQ877827.1_N.com	AdamsIN_Lowland	0.10
PasuquinIN_Lowland	AdamsIN_Lowland	0.07
AB088375.2_N.com	PasuquinIN_Upland	0.15
HQ877827.1_N.com	PasuquinIN_Upland	0.16
PasuquinIN_Lowland	PasuquinIN_Upland	0.23
AdamsIN_Lowland	PasuquinIN_Upland	0.17

The number of base substitutions per site from between sequences are shown in Table 2. Analyses were conducted using the Maximum Composite Likelihood model (Tamura and Nei, 1993). The analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were 1,115 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Climatic conditions and chemical properties of soil and water samples

The chemical properties of soil samples collected from areas with and without the proliferation of *N. commune* (Pasuquin, Batac, San Nicolas, Bayombong, and Science City of Muñoz in October 2017) were determined (Table 3). However, no distinguishing properties of soil and floodwater samples were associated with *N. commune* proliferation. The pH of the samples from Santa Matilde in Pasuquin, Ilocos Norte falls within the range favorable to the growth of algae. The pH values are considered high for ionic activities of multivalent cations in the soil solution to remain favorable for absorption by plants. For instance, the activity of Zn^{+2} in solution will be depressed by the formation of Zn $(OH)_2$ when pH goes high (e.g. pH 8). Other metallic cations (Fe, Mn,

Table 3. Chemical Properties of soil and water samples collected in areas with- and without *N. commune*.

Place of Collection		Samples	pH	OM	N	P	Mg	K	Na	Ca	Mg	CEC	Fe	Zn	Cu	Mn	Cl
				%		ppm (Olsen)	ppm		me/100g*; ppm**			me/100g soil			ppm		
1	Nueva Viscaya with *Nostoc*	soil	7.7	0.48	0.08	11	172	0.15	0.2	10.37	1.63	12	-	-	-	-	-
		water	6.5	-	-	-	-	3	25	22	7	-	0.52	ND	0	0.22	85
2	Nueva Viscaya without *Nostoc*	soil	6.8	0.94	0.09	10	271	0.17	0.45	14.86	3.64	19	-	-	-	-	-
		water	7.2	-	-	-	-	0.4	8	9	10		0.08	ND	0.01	0.1	85
3	Adams, Ilocos Norte with *Nostoc*	soil	6.9	4.87	0.22	5	230	0.22	0.29	11.65	5.27	30	-	-	-	-	-
		water	6.9	-	-	-	-	7	11	15	5	-	0.05	ND	0.01	0.06	85
4	PhilRice-CES without *Nostoc*	soil	6.3	1.53	0.12	12	446	0.28	0.42	16.39	10.67	33	-	-	-	-	-
		water	7.5	-	-	-	-	6	43	29	21	-	0.04	ND	ND	ND	85
5	Tabug, Batac City, Ilocos Norte with *Nostoc*	soil	7.5	0.96	0.08	3	421	0.59	0.14	27.02	2.38	31.2	-	-	-	-	-
6	Quiling, Batac City, Ilocos Norte with *Nostoc*	water	7.5	-	-	-	-	0.4	18	27	8	-	0.03	ND	ND	ND	99
7	Tabug, Batac City, Ilocos Norte without *Nostoc*	soil	7.8	2.02	0.15	9	775	0.82	1.2	37.4	10.42	49.95	-	-	-	-	-
8	La Union with *Nostoc*	soil	6.1	1.14	0.11	55	208	0.1	0.42	18.06	2.51	25	-	-	-	-	-
9	La Union without *Nostoc*	soil	5.9	1.31	0.12	11*	247	0.08	0.36	17.03	3.01	22	-	-	-	-	-
		water	7.6	-	-	-	-	0.9	18	26	17	-	0.03	ND	ND	ND	114
10	Amulung,Cagayan with *Nostoc*	water	7.5	-	-	-	-	0.9	19	35	6	-	0.03	ND	ND	ND	99
11	PhilRice Batac without *Nostoc*	water	6.6	-	-	-	-	42	47	25	10	-	0.2	ND	ND	4	128
12	Mangatarem, Pangasinan with *Nostoc*	water	7.6	-	-	-	-	42	12	15	45	-	0.04	ND	0.01	ND	142
13	Mangatarem, Pangasinan without *Nostoc*	water	7.2	-	-	-	-	1	11	29	29	-	0.04	ND	0	ND	128

Cu) will suffer the same. Presumably, at these pH values, the concentration in soil and activities in the soil solution of Ca^{+2} and Mg^{+2} will be high. Among the samples collected and analyzed, one of the two samples from CES (sample #7 soil) exhibited a slightly acidic pH and this is the sample without *Nostoc*. The acidity of sample 7S also manifested high Fe and Mn, expectedly because of their high solubility within the acidic range of pH in soils. However, the levels are yet to be considered but site management must consider implications to soil fertility and vulnerability if further increase in acidity occurs.

The samples electrical conductivity (EC) or the amount of salts in soil samples ranged from 0.10 to 1.00 mS/cm. It is far below the range for seawater EC values that are considered detrimental to crops as reported in the Philippines and other countries with EC saline soils. Seawater has an average total soluble salt content of 35 g L^{-1}, corresponding to electrical conductivity of about 50 dSm^{-1}. Saline soils typically have EC values of >4 dSm^{-1}. High salt content could affect the growth and biomass production of sensitive crops including rice. The salts are due to any or all of the following constituents: Ca^{++}, Mg^{++}, Na^{+}, K^{+}, HCO_3^{-}, SO_4^{-}, and Cl^{-}. Saline soils affected by the intrusion of seawater have high content of Na and Cl. Samples from Batac City, Ilocos Norte did not exhibit salinity despite its proximity to the sea.

Organic matter content was low in all soil samples except the one from Science City of Munñoz in Nueva Ecija (sample #78) with organic matter content of 3.32%. The rest of the samples showed very low content of organic matter content considered for rice production (<2.5%). The high level of organic matter in this site can be associated with the history of cropping and crop management practices such as the incorporation of crop residues and application of organic fertilizer.

The soil samples exhibited high contents of plant-available phosphorus extracted using 0.5 M sodium bicarbonate (Olsen P). The highest was found in the same slightly acidic soil sample from CES (sample #7). High level of Olsen P indicates residual content from previous applications accumulated through time. Except for two samples (sample #5S and #8S), all soils possessed medium to high levels of potassium (0.4 - 0.6 me K/100g for medium and >0.6 me K/100g for high). The two samples also had the lowest exchangeable Ca. The sample from Bayombong, Nueva Vizcaya (Sample #5S) gave the lowest cation exchange capacity of 19.27 me/100g. In a study by PhilRice and University of the Philippine Los Baños, a comparison of 14 soil properties showed that Cu had significantly increased in places without blue-green algae bloom.

Conclusion and Recommendation

Results showed that the seasonal abundance of *N. commune* still persists in the upland and lowland rice farms of Ilocos Norte and Nueva Vizcaya. Factors enhancing its persistence were associated with fewer disturbances of its habitat and minimal use of chemical farm inputs. The presence of groundwater resources and afternoon precipitation even during the dry months ensure a conducive environment for the continued growth and proliferation of *N. commune*. Flowing water resources also significantly favor its distribution, as observed along the Magat river in Bayombong, Nueva Vizcaya. Its use as human food and the market demand had also reduced the populations of this undeniably slow-growing organism. While *N. commune* is popularly known to older locals of Ilocos Norte and Nueva Vizcaya, the younger generation showed unfamiliarity. Residents in Visayas and Mindanao are also unfamiliar with its presence.

The huge contribution of *N. commune* in nitrogen fixation has a potential help in supplementing nitrogen for rice crops. As there were no distinguishing soil and floodwater properties among samples collected from areas with and without *N. commune* proliferation, constant supplementation of rice paddies with inoculum until it establishes its population is seen as a promising approach to bringing it back into the rice paddies. Inoculation should initially concentrate on areas that are often wet and less disturbed.

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EFFECTS OF RED EARTHWORMS *Pontoscolex corethrurus* (MÜLLER) ON RICE SEEDLINGS AND REACTIONS TO PESTICIDES AND FERTILIZERS

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Abstract

This study investigated the infestations of earthworms (*Pontoscolex corethrurus)* on seedbeds and levees in the rice fields in Mt. Province, Kalinga, Pangasinan, and Nueva Ecija and determined the effects of commonly used synthetic pesticides and inorganic fertilizers under laboratory and screenhouse conditions to this pest.

Results showed that seedling emergence was reduced by 1.2 - 5.4%, 4.8 - 14.9%, and 18.1% when earthworm populations were at 5,000, 10,000, and 20,000 m^{-2}, respectively. Rice seedlings grown in soil with earthworms had shorter roots, lower shoots and dry weights than those cultivated in clean soil.

Laboratory bioassay showed that common synthetic pesticides used in rice fields had toxic effects on earthworms. Earthworms treated with niclosamide (molluscicide), butachlor (herbicide), pretilachlor (herbicide), and chlorpyrifos (insecticide) died after 24 h of exposure. Earthworm mortality was not observed when metaldehyde (molluscicide) and inorganic fertilizers (urea, ammonium phosphate, and complete) were applied. In screenhouse experiment, paddy soils applied with niclosamide (4.8) and carbofuran (5.0) had the least number of earthworms after 14 days of treatment. However, synthetic pesticides had no significant effects on *P. corethrurus* under screenhouse conditions. Soil applied with N fertilizer had 11.5 earthworms while giving P-K yielded 10.8 worms. In areas with high population of earthworms, it was recommended to use plastic trays or apply the *dapog* technique to avoid negative effects on rice seedlings.

Keywords: *Damage, Population, Mortality, Bioassay, Red Earthworm*

Introduction

Enhancing soil fertility is one of the positive effects of earthworms (Lee, 1985) as these maintain soil aeration, drainage, and porosity (Edwards, 2004) and facilitate soil macro-aggregation and nutrients availability (Brown et al., 1999; Ratsiatosika et al., 2021a). This endogeic and geophagous earthworm influences soil properties, plant-associated microbial communities, microbially-mediated processes, and plant biomass (Braga et al., 2016). In particular, *P. corethrurus* increases the concentration of soluble phosphorus (P) in the soil (Trap et al., 2021).

Earthworm burrows are usually lined with a protein-based mucus that helps stabilize these channels. Species with permanent burrows cast their feces around the lining of the burrows, with the cast material usually containing more plant nutrients in a readily available form than the surrounding soil. Earthworms move large amounts of soil from the deeper strata to the surface. The amounts moved in this way ranges from 2 to 250 t ha^{-1} every year, equivalent to bringing a layer of soil between 1-mm and 5-cm-thick to the soil surface, creating a stone-free layer. Some species make permanent burrows, whereas others move randomly through the soil, leaving cracks and crevices of different sizes (Edwards, 2004).

However, there were instances when earthworms were detrimental to plant growth. Biomass of legumes is sometimes negatively affected by earthworms (Brown et al., 2004). A small red earthworm, identified as *Dichogaster curgensis* Michaelsen that belongs to the family Occhtochaetidae was reported by Barrion and Litsinger (1997). In the Cordillera Region of the Philippines, the earthworm's burrowing activity caused leaks in rice levees while body setae mechanically injured roots resulting in plant stunting and seedling death.

Earthworm infestations were reported in rice farms in Mt. Province, Kalinga, Nueva Ecija, and Pangasinan in 2015. The earthworms covered emerging seedlings with thick mud castings, which eventually caused seedlings mortality. Earthworms collected in affected areas were identified as *Pontoscolex corethrurus* (Müller, 1857). Sandoval et al. (2022) asserted that flooding kills *P. corethrurus* that seek refuge in levees and other areas.

P. corethrurus is an invasive endogeic earthworm (Lavelle et al., 1987). It can colonize habitat and is well-adapted to human activity (Lavelle et al., 1987; Hendrix et al., 2008). A pantropical earthworm can tolerate a wide range of abiotic and biotic environmental conditions, which makes it a successful colonizer (Fragoso et al., 1999; Lavelle et al., 1987). Endogeic species increase soil macroporosity and water infiltration with some producing small-sized casts, which seal surfaces resulting in soil erosion (Blanchart et al., 1999). *P. corethrurus* can affect and compact the soil because of its feeding activity. As a result, it prevented plant growth and when drought occurs, the layer turns into a hard-compact thick crust (Alegre et al., 1996; Marichal et al., 2010). It may also modify biogeochemical processes as well as communities of plants, microbes, and native earthworms (Marichal et al., 2010). Earthworms decomposed faster during the early stage (between 0 and 3 days), as reflected by the higher rate of decomposition and increased accumulation of dissolved organic matter (DOM). This decomposition pattern was paralleled by bacterial community dynamics, in which bacterial richness and diversity were significantly higher during early decomposition ($p < 0.05$), with the relative abundances of many genera decreasing as decomposition progresses (Sun and Ge, 2021).

Rice farmers commonly use fertilizers and pesticides to improve crop growth and increase productivity. In the Philippines, more than 90% of the total area planted were applied with inorganic fertilizers (urea, complete, ammonium sulfate, and ammonium phosphate), from 2015 to 2018. Pesticide usage ranged from 89.1 to 93.2% of the total area. By classification, herbicide in liquid form had the highest application with 1.37 L ha^{-1}. Next were insecticide at 0.97 L ha^{-1} and molluscicide at 0.30 kg (PSA, 2019).

Earthworms are highly susceptible to pesticides (Edwards and Bohlen, 1992). Neonicotinoids, strobilurins, sulfonylureas, triazoles, carbamates, and organophosphates are families of pesticide that are harmful to earthworm (Pelosi et al., 2014). Effect of pesticides on earthworms depends on exposure pathways for each type of pesticide, information on earthworm habitat, and feeding preferences (Bertrand et al., 2015). It also affects earthworm mortality by directly distressing them or altering their physiology (Sabra and Mehana, 2015). However, combining effects of earthworms with fertilizers such as silicon reduced the severity of rice blast disease in a Ferralsol in Madagascar, considering with or without NPK fertilization. Dual treatment of earthworm inoculation and Si fertilization in a nutrient-poor tropical soil resulted in a higher tolerance of rainfed rice to *P. oryzae* than with treating with only Si. Dual treatment also provided the optimal agronomic balance in biomass and nutrition and reduced disease severity (Blanchart et al., 2020).

Materials and Methods

Effects on Rice Seedlings

Effects of *P. corethrurus* earthworms on rice seedlings (NSIC Rc 222) were studied in 2015 wet season. Seedling trays with 19.63 cm^2 hole area were used, filled with previously dried and pulverized paddy soil and moistened with tap water. Varying earthworm populations (T1: 0, T2: 5, T3: 10, T4: 15, and T5: 20) were placed in each hole to simulate population levels in a square-meter paddy: 2,500 (T2); 5,000 (T3); 7,500 (T4); and 10,000 (T5), ten seeds were sown per hole. The experiment was arranged in completely randomized design (CRD) with three replications.

In May 2016, the same experiment was conducted using size-4 clay pots in an area of 176.71 cm^2 each. Individual pot was filled with 1,000 g of dried and powdered soil, primarily sieved using mesh net. The substrate was watered and kept moist for 24 h, and then different numbers of earthworms were placed in each pot based on the treatment. The treatments were: T1: earthworm-free; T2: 45; T3: 90; T4: 180; and T5: 360 equivalent to population per square meter of 2,500 (T2), 5,000 (T3), 10,000 (T4), and 20,000 (T5) individual earthworms. Forty seeds of NSIC Rc 222 were sown per pot. The data gathered were percent seedling emergence, percent reduction in seedling emergence relative to percent emergence in the negative control, shoot and root lengths, and fresh and dry weights. The CRD experiment had four replications.

Reactions to pesticides

Laboratory bioassay

In June 2016, the toxicity of common synthetic pesticides and inorganic fertilizers was tested through a laboratory assay. Treatments were: T1: control (water); T2: Niclosamide (molluscicide); T3: Metaldehyde (molluscicide); T4: Butachlor (herbicide); T5: Pretilachlor (herbicide); T6: Chlorpyrifos (insecticide); T7: Urea (N-fertilizer); T8: Amonium phosphate (P-fertilizer); and T9: Complete (N-P-K fertilizer). The treatments were either dissolved or diluted in tap water, following the recommended rates for field application. Suspensions of one half and two times the recommended rates were likewise prepared as sub-treatments. The diluted treatments were applied to 6-well tissue culture plates containing 4 earthworms with 10 ml water per well. The percent mortality was gathered 24 h after treatment application. The CRD experiment had six replications.

Screenhouse experiment

Paddy soil with 6,000 ± m^{-2} earthworms was mixed thoroughly in a plastic basin and placed on size 4 clay pots (176.71 cm^{-2}). Each pot contained 10 ± 2 earthworms. Recommended rates for common synthetic farm inputs were applied on the surface of the potted paddy soil and watered based on the recommendation for each treatment. Treatments were: T1: control (water); T2: Niclosamide (molluscicide); T3: Butachlor (herbicide); T4: Pretilachlor (herbicide); T5: Chlorpyrifos (insecticide); T6: Carbofuran (insecticide/nematicide); T7: N-P-K (fertilizer); T8: N (fertilizer); T9: N-P (fertilizer); and T10: P-K (fertilizer). Treatments 2, 3, 7, 8, and 9 were flooded up to 2 cm water level, while the others were kept saturated after treatment application. Surviving earthworms and cocoons were counted 14 days later. The fresh and dry weights of recovered earthworms were also recorded. The CRD experiment had four replications.

Analysis

The data gathered were variance-analyzed (ANOVA) while the means were separated by standard deviation or Fisher's least significant difference test at a 5% level of significance using the SAS 9.1.3.

Results and Discussion

The earthworm reported by farmers in Mt Province, Kalinga, Pangasinan, and Nueva Ecija was identified as *Pontoscolex corethrurus* (Müller, 1857), which belongs to family Glossoscolecidae. This species is believed to have originated in South America (Righi, 1984). These earthworms are distinguished through their pink to almost transparent skin (Figure 1.A), zygolobic prostomium (B), saddle-type clitellum with (C), and 4 closely paired setae (hooked) per segment (D). Adult earthworms have 207 segments with average size of 74.7 mm (length) by 1.8 mm (width). Its 5 - 6 mm clitellum is located at the 9 - 11 segments 14^{th} - 24^{th} segment from the prostomium. The cocoon had either brown, dark green, or black color (Figure 1. D).

A screenhouse experiment on the effect of *P. corethrurus* on the destruction of levees was conducted by Sandoval et al. (2022). The result shows that after 14 days of flooding, levees with water level of 10 cm were degraded to 1.33 cm, levees flooded with 3 cm water had 7 cm height, and 8.33 cm on saturated soil condition. Flooding causes earthworm mortality. Earthworms flock in paddy levees and other elevated areas in the field to access to oxygen and survive the condition. Earthworm burrowing activity loosens the compactness of levees and terrace walls; collapsing the soil and releasing paddy water.

Effects on Rice Seedlings

Seed germination was reduced by 5.4 - 14.9% when the population of earthworms was at 5,000 - 10,000 m^{-2} (Table 1). This observation correlates with the study of Grant (1983) and Decaëns et al. (2001) which showed that several seeds of weed species had lower and slower germination rates in earthworm casts. Furthermore, Ratsiatosika et al. (2021b), reported that the ability of rice to exploit beneficial interactions among free-living soil organisms is influenced by its genetic background. However, the loss of earthworm-interactive abilities of rice crops are independent of the genetic distance among cultivars and breeders' agronomic criteria.

Moreover, 10-day-old seedlings grown in paddy soil with earthworms of more or less 7,500 - 10,000 m^{-2} had shorter roots (5.61 - 5.64 cm) than plants grown in earthworm-free soil, which averaged 6.9 cm (Table 1 and Figure 2). Roots of plants with approximately 5,000 m^{-2} earthworms were comparable with the plants in earthworm-free soil. Shoot lengths of plants grown in soil with and without earthworms were also comparable.

In clay pot experiment, mud castings by earthworms were observed to cover the emerging seedlings a day after sowing. Seeds were less visible due to mud castings as the number of earthworms increased per pot (Figure 3). The seeds sown in earthworm-free soil (T1) were undisturbed and visible on the surface. *P. corethrurus* is known

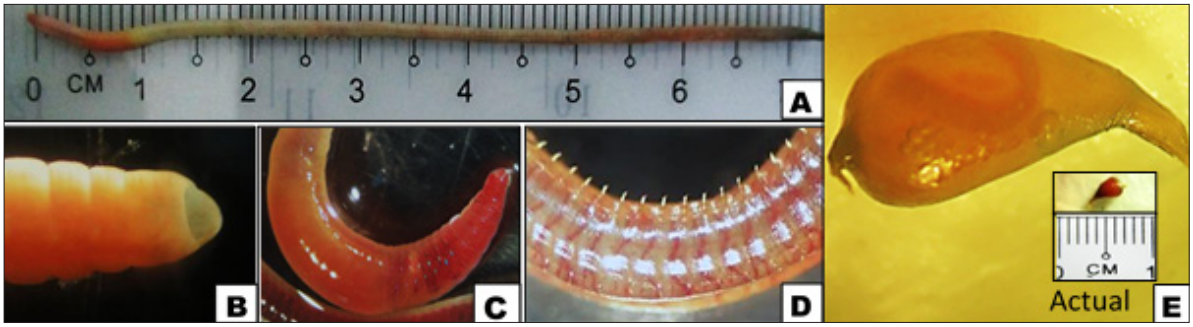

Figure 1. A 7.5 cm P. *corethrurus* earthworm with pink skin (A), zygolobic prostomium (B), saddle-type clitellum (C), setae in each segment (D) and 4.6 mm length and 1.9 mm width cocoon (E).

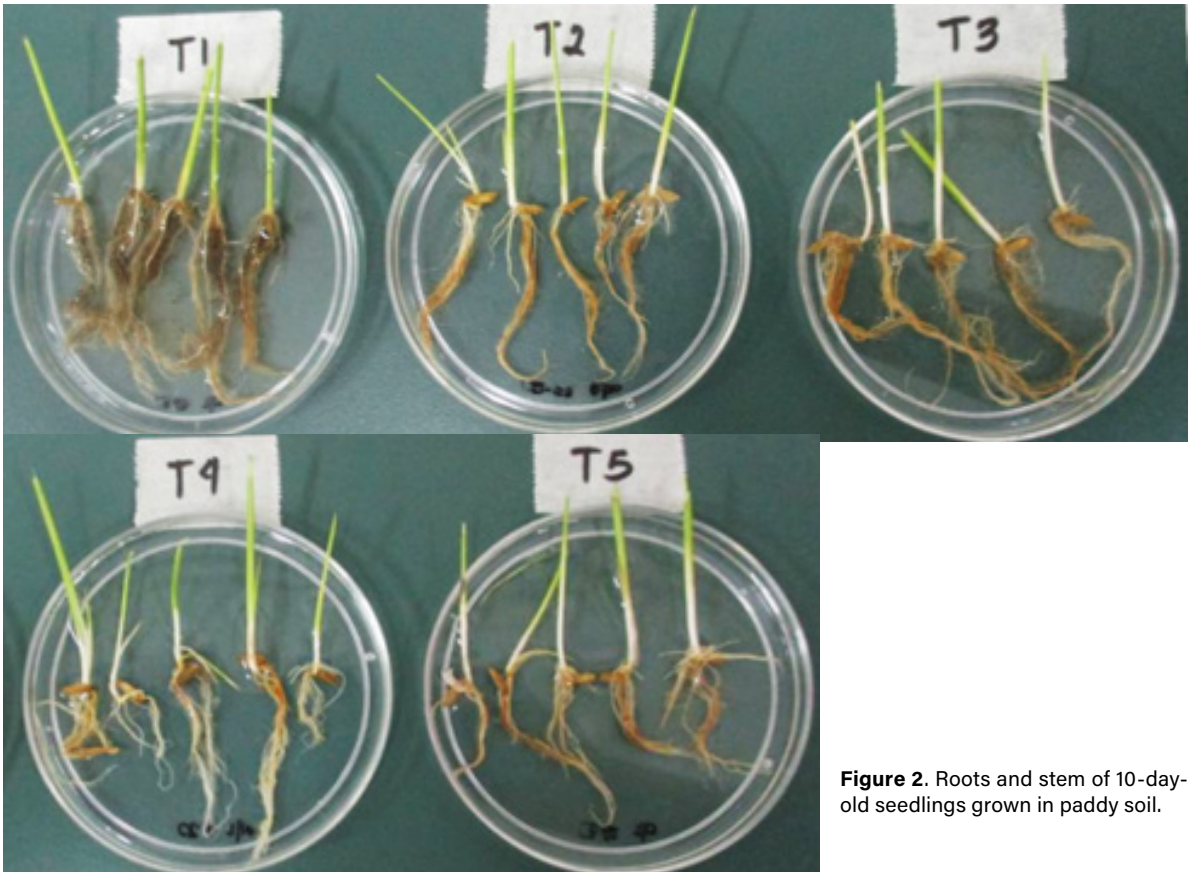

Figure 2. Roots and stem of 10-day-old seedlings grown in paddy soil.

Table 1. Effects of different numbers of earthworms on rice seedlings at 10 days after sowing (DAS).

No. of Earthworms in 19.63 cm^2 Hole	Seedling Emergence (%)	Reduction in Seedling Emergence (%)	Shoot Length (cm)	Root Length (cm)
0	82.2 ab	-	29.1 ab	6.9 a
5	87.8 ab	-6.8	31.7 a	7.2 a
10	77.8 ab	5.4	28.7 b	6.1 ab
15	71.1 b	13.5	28.3 b	5.6 b
20	70 b	14.9	28.4 b	5.6 b

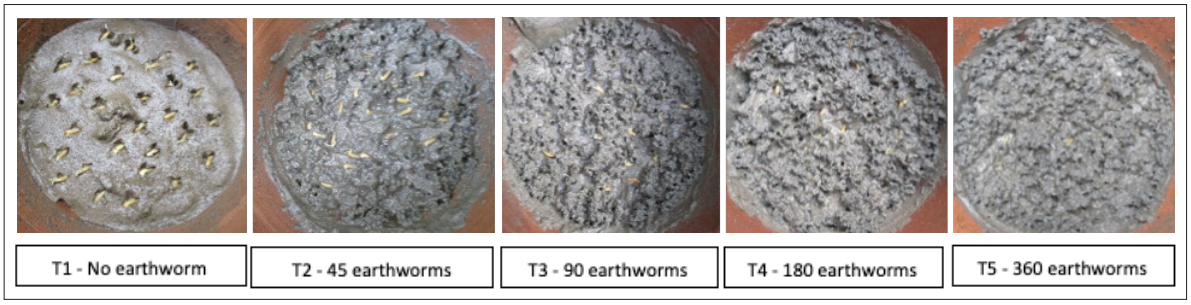

Figure 3. The emerging cultivated rice seeds in potted soil with and without earthworms at 1 DAS.

to compact soil. The accumulation of casts at the surface under moist soil conditions may result in the formation of a continuous muddy layer of earthworm casts (Alegre et al., 1996; Blanchart et al., 1997). The growth of plants is then prevented and when droughts occur, this layer turns into a compact thick crust. As a consequence, large patches of bare soil are generated, which is impermeable to water and air (Chauvel et al., 1999).

The reductions of seedlings emergence at 2.4, 1.2, and 4.8% were recorded in soils with 45, 90, and 180 earthworms, respectively. Seedling emergence in soil with 360 earthworms had 18.07% significant reduction. Root length was also reduced in 45, 90, 180, and 360 earthworms with 11.5, 11.7, 10.4 and 8.3 cm lengths, correspondingly. Seedling height did not vary when grown in soils with and without earthworms. However, seedlings grown in soil without earthworms had a significantly higher shoot dry weight of 124.7 mg compared with the 77.7 - 91.3 mg of those grown in soil with earthworms (Table 2). Seedlings grown in earthworm-free soil had longer roots and higher root dry weight than those grown in soil with earthworms (Figure 4). Seedlings with shorter roots due to earthworm infestation in the seedbed take longer time to recover when transplanted. Barrion and Litsinger (1994) reported that earthworm's burrowing activity resulted mechanical injury to the root system causing plants to stunt, wilt, and eventually die. Infested fields exhibited reduced plant stand and uneven growth. The highest percent damaged hills occurred in seedling stage (55%), followed by 30% at maximum tillering, with 0% in the reproductive stage.

Reactions to pesticides

Laboratory bioassay

A 100% mortality rate was observed on earthworms treated with niclosamide (molluscicide) after 15 min of application. Evident irritation was observed in the earthworms upon application of herbicides (butachlor and pretilachlor) and chlorpyrifos insecticide. Mortality of 70 - 100%

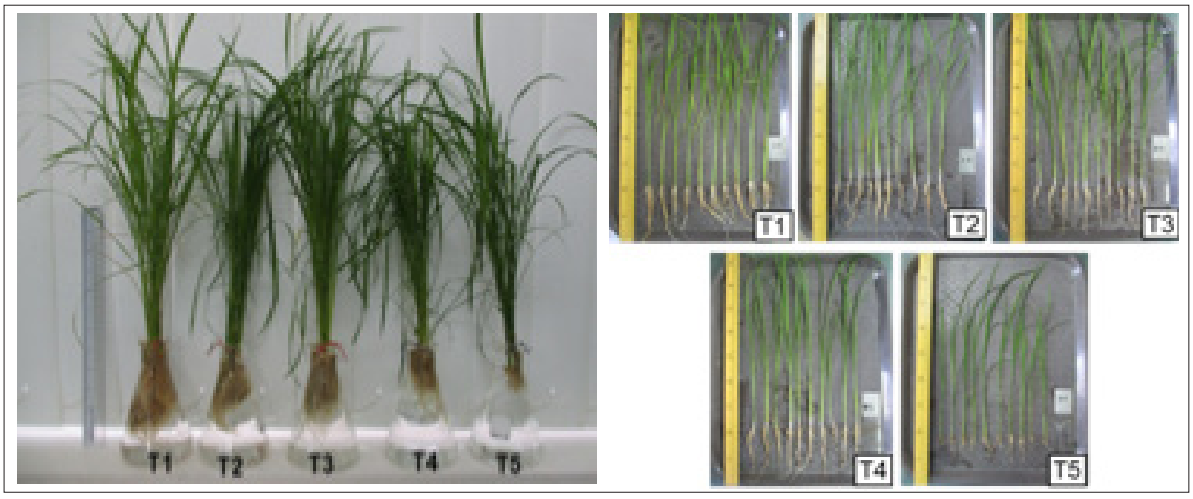

Figure 4. Roots and stems of 20-day-old seedlings grown in paddy soil.

Table 2. Effects of different numbers of earthworms on rice seedlings at 20 DAS.

No. of Earthworms in Clay Pot (176.71cm^2)	Seedling Emergence at 20 DAS (%)	Reduction in Seedling Emergence Relative to % Emergence in T1 (%)	Shoot Length (cm)	Root Length (cm)	Shoot DW (mg)	Root DW (mg)
0	92.2 a	-	43.2	16.4 a	124.7 a	39 a
45	90 a	2.4	43.8	11.5 b	91.3 b	21 b
90	91.1 a	1.2	45.1	11.7 b	77.7 b	21 b
180	87.8 a	4.8	42.2	10.4 cb	84.3 b	19.5 b
360	75.6 b	18.1	41.4	8.3 c	80 b	15.1 b

occurred in butachlor, pretilachlor, chlorpyrifos, and niclosamide after 24 h regardless of the rate applied (Figure 5 and Table 3). Their skin disintegrated starting from their tail (Figure 6). However, mortality was not observed on earthworms applied with metaldehyde (molluscicide) and inorganic fertilizers (urea, ammonium phosphate, and complete). Earthworms are highly susceptible to insecticides causing immobility and rigidity. Insecticides also cause significant biomass reduction and delay in growth and reproduction of earthworms by disrupting physiological activities (Miglani and Bisht, 2019). Different studies on the effects of pesticides on earthworms observed rapturing of cuticle, oozing out of coelomic fluid, and swelling and palling of body tissues (Solaimalai et al., 2004); damage to male reproductive system (Sorou and Larink, 2001); and coiling of tail (Espinoza-Navarro and Bustos-Obregon, 2004). Buch et al. (2013) reported that carbofuran and carbendazim had toxic effects on *P. corethrurus* and *Eisenia andrei.*

Screenhouse experiment

Paddy soil applied with niclosamide (molluscicide) and carbofuran (insecticide/nematicide) had the least number of earthworms, 14 days after treatment application with an average of only 4.7 and 5 earthworms per pot. The highest numbers of earthworms at 11.5 and 10.8 per pot were recovered from soils applied with N and P-K fertilizers (Table 4). Earthworms treated with fertilizers (N-P and P-K) and pesticides (butachlor, pretilachlor, and chlorpyrifos) had higher individual fresh weights of 0.132 - 0.184 g than the control with 0.084 g. Those applied with carbofuran had the least weight of 0.1 g.

Earthworms applied with fertilizer N-P had the heaviest dry weight, though comparable with those in other treatments. The numbers of cocoons recovered were comparable in most treatments. Treatments 3 (butachlor) and 4 (pretilachlor) had the most cocoons

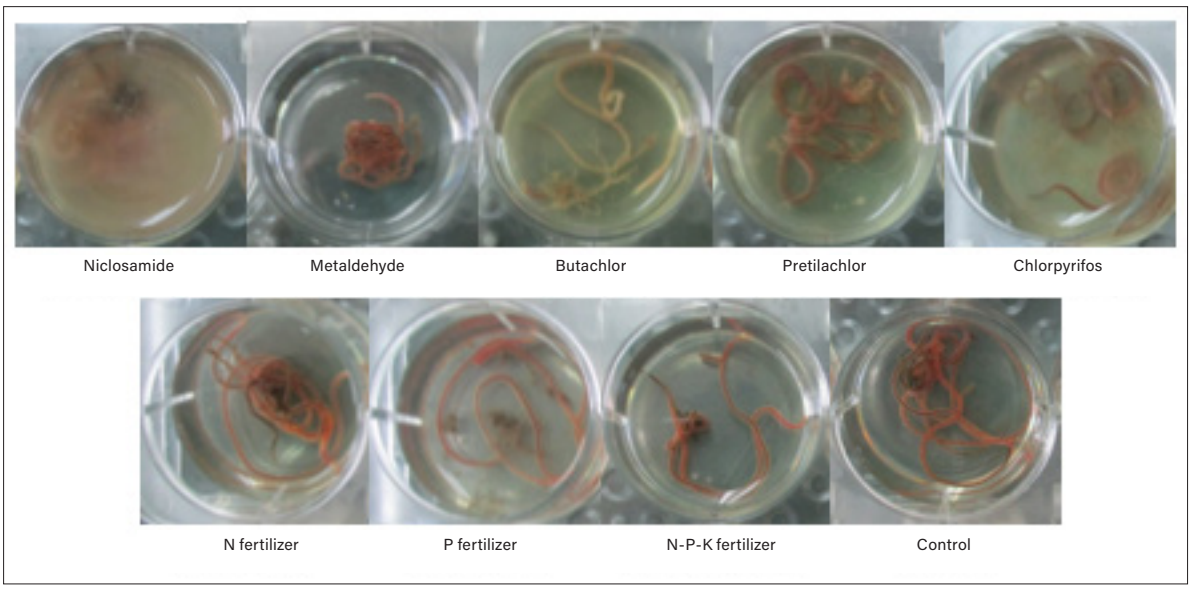

Figure 5. Effects of different farm inputs on *P. corethrurus* 24 h after application of treatment.

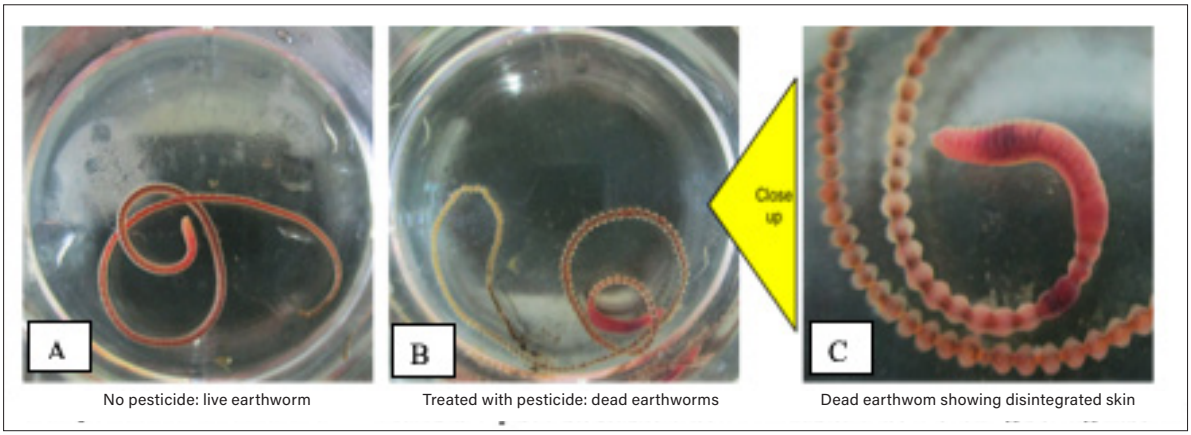

Figure 6. Healthy earthworm on untreated water (A), dead earthworm applied with pesticide (B), and a dead earthworm with disintegrated skin (C).

Table 3. Average percent mortality of earthworms exposed to common synthetic pesticides and inorganic fertilizers for 24 h.

Treatments	Mortality (%)		
	1/2 Recommended Rate	Recommended Rate	2X Recommended Rate
Control	0	0	0
Butachlor	87.5	100	95.8
Pretilachlor	100	100	100
Chlorpyrifos	70.8	79.2	95.8
Niclosamide	100	100	100
Metaldehyde	0	0	0
Urea (N)	0	0	0
Ammonium phosphate (P)	0	0	0
Complete fertilizer (NPK)	0	0	0

Table 4. Effects of synthetic farm inputs on earthworms when applied to potted paddy soil, 14 days after treatment application.

Treatments	Active Ingredient	No. of Earthworms	Fresh Weight	Dry Weight	No. of Cocoons
T1	Control	8.3 ab	0.084 bc	0.014 ab	1.8 ab
T2	Niclosamide	4.8 b	0.087 bc	0.017 ab	0.5 b
T3	Butachlor	8.3 ab	0.184 a	0.018 ab	2.8 a
T4	Pretilachlor	8 ab	0.150 a	0.014 ab	2.8 a
T5	Chlorpyrifos	7.8 ab	0.154 a	0.014 ab	0.5 b
T6	Carbofuran	5 b	0.070 c	0.011 b	2 ab
T7	N-P-K	7 ab	0.075 bc	0.011 b	0.8 b
T8	N	11.5 a	0.084 bc	0.014 ab	1 ab
T9	N-P	8.8 ab	0.132 ab	0.019 a	2 ab
T10	P-K	10.8 a	0.161 a	0.016 ab	1.8 ab

averaging at 2.8 per pot; treatments 2 (niclosamide), 5 (chlorpyrifos), and 7 (N-P-K) had less cocoons (Table 4).

Response of *P. corethrurus* to herbicides is variable. Glyphosate showed no toxic effects for *E. andrei* and *P. corethrurus* even at the highest concentration tested (47 mg a.i. kg^{-1}), although they displayed avoidance behavior at this concentration (Buch et al., 2013). Kale and Krishnamoorthy (1979) assessed the effects of the insecticide Sevin (i.e., 1-naphthyl-n-methylcarbamate), which was mixed with a clay loam in the laboratory, and found that lower concentrations (i.e., 37.5 - 75 ppm) have a stimulatory effect on earthworm growth and survival rather than inhibitory. However, the highest concentrations (i.e., >150 ppm) resulted in growth delays and reduced survival rates.

Synthetic pesticides had no significant effects on *P. corethrurus* when applied on soil under screenhouse condition. Moreover, inorganic fertilizer favors the *P. corethrurus* population.

Results explain why *P. corethrurus* became a problem in rice fields of Mt. Province, Kalinga, Pangasinan, and Nueva Ecija. Earthworm population build-up in these provinces was the consequence of continuous use of synthetic pesticides and application of inorganic fertilizers by the farmers. Management practices such as use of plastic trays or the dapog technique can be practiced in these areas to prevent damage and increase of earthworm population and avoid rice seedling damage.

Conclusion and Recommendations

P. corethrurus has infested rice seedbeds in Mt. Province, Kalinga, Pangasinan, and Nueva Ecija based on farmers' reports. The earthworms cover the emerging seedlings with thick mud castings resulting in seedlings with longer roots and stems that are succulent and hard to pull. In some instances, seedlings that were heavily covered with mud castings died. It was observed that an earthworm population of 50,000,000 - 100,000,000 ha^{-1} caused 5.4 to 14.9% reduction in seedling emergence. Seedlings emergence in soils with 45, 90, and 180 earthworms (equivalent to 25,000,000 - 100,000,000 ha^{-1}) was diminished by 2.4, 1.2, and 4.8%, respectively. Soil with 360 earthworms had 18.1% reduction in seedling emergence. Levees with 10 cm water level were degraded from 10 cm height to 1.33 cm after 14 days of flooding while levees flooded with 3 cm water had 7 cm height, and 8.33 cm high on saturated soil condition.

Common synthetic pesticides used in rice fields such as butachlor, preticlachlor, niclosamide, and chlorpyrifos had toxic effects on earthworms when tested through laboratory bioassay. However, when applied in the soil, these pesticides had no significant effect on earthworm population. Moreover, application of inorganic fertilizers favored the earthworm population. This is probably because *P. corethurus* are considered topsoil earthworms that live primarily in the upper 2 - 3 inch of the soil and do not build permanent burrows, but instead randomly burrow throughout the topsoil while ingesting residues and mineral, thereby, avoiding effects of pesticides that goes deeply in the soil.

Chlorpyrifos were previously reported to have effects on earthworms. However, result of this study shows that it has no significant effect on *P. corethrurus*.

Therefore, further study is recommended to determine how this earthworm ignores toxicity of some pesticides by studying their metabolism and behavior and to determine the effects of other cultural practices such as water management, fertilizer rates and application, frequency, and type of pesticide usage on earthworm population. It is also recommended to use plastic trays or the *dapog* technique in seedbed areas with high population of earthworms to avoid negative effects on rice seedlings considering that earthworm prefers to damage seedlings and seedbeds. Crop rotation can also be explored to further reduce the population of *P. corethurus*.

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FULL PAPER

EFFECTS OF STORAGE MATERIALS AND DURATION ON THE MILLING POTENTIALS, PHYSICOCHEMICAL PROPERTIES, AND PHENOLICS OF AROMATIC RICE

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Abstract

Storage conditions and time affect rice quality parameters in different ways. Philippine Rice Research Institute (PhilRice) in Batac City, Ilocos Norte has developed storage bins and their effects on the quality and phenolics of aromatic rice was investigated. Understanding probable consequences can contribute to the development of appropriate storage technologies that could help minimize losses and maintain the quality of aromatic rice. Such information will also help stakeholders, particularly farmers and processors, in producing aromatic rice with better quality that can satisfy consumer's preferences.

The study determined the (a) changes in milling potentials, physicochemical properties, and total phenolic content (TPC) of aromatic *Burdagol*-Laguna type rice and (b) best storage material and duration, which can give an excellent quality of aromatic rice with minimum losses. Brown rice and milled rice recoveries significantly decreased after 5 months while amylose content (AC) increased after 3 months regardless of the storage material. The TPC of the aromatic rice stored in both bins increased as storage duration progressed while those stored in sack increased during the 2nd - 4th month. However, TPC decreased again during the 5th month. The crude protein content (CPC) of the samples stored in the insulated bin remained constant throughout the 5-month duration and yielded the second highest TPC in *Burdagol*-Laguna type rice, followed by the uninsulated bin. It can be concluded that the insulated bin can maintain the CPC and is suitable in storing *Burdagol*-Laguna type for head rice recovery, CPC, and TPC. Hence, it can also be used as an alternative in storing paddy. Variations in the brown rice and milled rice recoveries, AC, CPC, and TPC of aromatic *Burdagol*-Laguna type were influenced by its storage material and duration.

Keywords: *Aromatic Rice, Milling Potentials, Physicochemical Properties, Storage Bin, Total Phenolic Content*

Introduction

Rice is the staple food of roughly 50% of the world population. As consumers become conscious of the quality of rice that they eat, the high quality specialty rice (aromatic or fragrant rice) have become popular and continuously commands higher price in local and international markets. Aromatic rice is preferred by consumers who are willing to pay for the value-added specialty rice because of its unique popcorn- or pandan-like scent (Juliano, 2003).

Rice quality such as milling potential, physical and physico-chemical properties, cooking parameters, and eating or sensory, largely determines its economic and market value as well as its acceptability to consumers (PhilRice, 2008). Quality rice is characterized by good nutritional and cooking quality, taste, aroma, and appearance. The market value of milled rice depends largely on its physical qualities such as percentage of head rice (Hafeel et al., 2008) and subsequently determines the income of farmers and rice processors.

Phytochemicals and antioxidant properties of rice recently gained interest from researchers. It was reported as a source of phytochemicals including phenolic compounds, which present potential health benefits due to their antioxidant activity (Walter and Marchesan, 2011). Hence, the nutritional quality of rice has received more attention (Boius et al., 2003) as cited by Thanajiruschaya et al. (2010).

Storage is one of the important stages in postharvest operations. Proper storage provides safer conditions for the grains; preventing losses caused by adverse weather, moisture, rodents, birds, insects, and microorganisms (IRRI, n.d.). Good storage conditions prolong the shelf-life and preserve the nutritional value of grains (GrainPro, 2019).

During storage, rice grains are prone to losses due to improper storage techniques and lack of proper

storage facilities (Fernando et al., 1985 as cited by Hafeel et al. (2008). In the Philippines, roughly 2 - 6.5% rice loss was reported during the storing (Jiatrakul, 2007). Improper storage also results in the deterioration of quality in the form of discoloration, off-flavor, and nutritional content (Baradi et al., 2016).

Many studies were conducted on the effect of various factors and conditions during storage of paddy. Ilieva et al. (2014) evaluated the effect of moisture content of paddy during harvest and storage time after harvest on the milling yield, percentage of head rice yield, and grain breakage of San Andrea rice variety. Result showed that milling yield and head rice yield were highest a month after the harvest with lowest grain breakage.

Tsado et al. (2005) also reported that the environment and duration of paddy storage have significant influence on the quality of milled rice. Storage period had a substantial effect on the fissured grains, broken grain, moisture content, CPC, crude fiber content, and crude fat content.

Hafeel et al. (2008) identified the effects of hermetic storage in the IRRI-super-bag and compared it with common poly-sack bag storage in terms of milling quality of different paddy varieties stored for 4.5 months. Results showed that an increase in the moisture content reduces the thousand grain mass with the use of common poly-sack bag. Specifically, storage duration of 4.5 months has significant effect on the physical and milling qualities of paddy.

Hull (1998) as cited by Roseline et al. (2009) reported that grains stored under normal conditions exhibit continous physico-chemical changes due to the physiological activities of the germ and endosperm, which affect the culinary properties and nutritive value.

Thanajiruschaya et al. (2010) studied the effect of storage time and temperature on the antioxidant components and properties of milled rice. Storage time caused decrease of extractable phenolic and antioxidant activities of milled rice. Similarly, milled rice stored at higher temperature (37ºC) also had higher phenolic contents and antioxidant activities than stored at 25ºC.

In the Philippines, farmers usually store their rice harvest in a conventional way using ordinary sacks, which do not provide adequate protection from pests and insects and quality deterioration. Rice produce must be secured from moisture accumulation brought about by temperature fluctuations. To address this concern, some farmers use wooden and metal bins for storage.

Many studies were conducted on the effects of storage conditions on ordinary rice but few on aromatic rice, which are preferred varieties because of its distinctive pleasant scent. Philippine aromatic rice varieties include NSIC Rc 72H (Mestizo 1), NSIC Rc 218, and *Burdagol*-Laguna Type. *Burdagol*-Laguna Type is an aromatic rice variety with long and intermediate grain from Laguna.

The grain quality and phytochemical content of aromatic rice is then hypothesized to be affected by the type of storage material and duration. By using insulated bin at ambient conditions, the quality and total phenolic content of the *Burdagol*-Laguna Type will not significantly change after 5 months of storage.

The study determined the changes in the quality (milling potentials, physico-chemical properties), and total phenolic content of aromatic rice with different storage materials (sack, insulated bin, uninsulated bin) and duration.

Materials and Methods

Storage Experiment

The effects of duration and storage materials (sack, insulated bin, and uninsulated bin) on the grain quality and phytochemical content of *Burdagol*-Laguna type rice were evaluated. The ordinary sack commonly utilized by farmers was used as the control. It is made of polypropylene (95 x 53 cm) with 50-kg capacity. On the other hand, the bins (insulated and unisulated) were made of plain GI sheet gauge #18. A ½-in thick polyethylene foam was used for the insulated bin. These materials are locally available and affordable to the farmers. Some farmers in San Nicolas, Ilocos Norte used uninsulated bins to store paddy and milled rice. Figure 1 shows the schematic diagram of the insulated and uninsulated bins. The study was conducted from November 2016 to April 2017 at the PhilRice station in MMSU Campus, Batac City, Ilocos Norte.

Burdagol-Laguna Type, which was harvested on October 2016 in a field experiment in San Nicolas, Ilocos Norte, was used in the study. Paddy samples with initial moisture content (MC) of 11.4 - 12.6% wet basis (wb) were stored in different materials (12 kg each) for five months at ambient condition in PhilRice Batac. Figure 2 shows the paddy samples and the storage setup. The recommended MC of dried grains should not be higher than 14% MC wb for long-term storage without significant deterioration in quality, under the average conditions in the Philippines with 85% relative humidity (% RH) and 25ºC temperature (Philippine Rice Postproduction Consortium, 2002). The temperature and % RH were monitored daily

at 8 a.m., 1 p.m., and 4 p.m. using a digital thermo-hygrometer.

Samples were gathered monthly for grain quality and phytochemical content evaluation at the PhilRice Rice Chemistry and Food Science Division. Paddy samples totaling 500 g were collected from each storage material per replication and placed in small paper bags (size 10), then packed in box during transport from PhilRice Batac to PhilRice Central Experiment Station (CES) in Science City of Muñoz, Nueva Ecija. The samples were processed and analyzed at the RCFSD laboratory, 3-4 weeks upon arrival.

Experiment was replicated thrice and the treatments were laid out in a two-way factorial on completely randomized design (CRD) presented in Table 1.

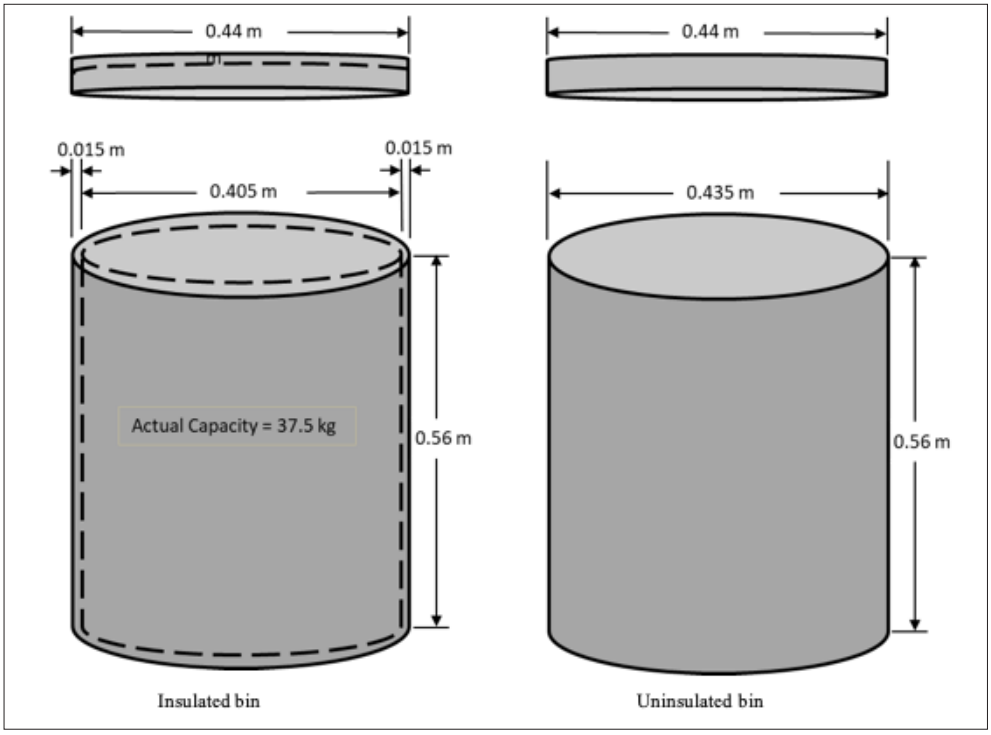

Figure 1. Schematic diagram of the insulated and uninsulated bins.

Table 1. Experimental lay-out and design of the study.

Storage Material (M)	Duration (D) Month	Replication (R)	Experimental Units
M1 - Sack	D1 – 0	R1	M1D1R1, M1D1R2, M1D1R3
M2 - Insulated bin	D2 – 1	R2	M2D1R1, M2D1R2, M2D1R3
M3- Uninsulated bin	D3 – 2	R3	M3D1R1, M3D1R2, M3D1R3
	D4 – 3		M1D2R1, M1D2R2, M1D2R3
	D5 – 4		M2D2R1, M2D2R2, M2D2R3
	D6 – 5		M3D2R1, M3D2R2, M3D2R3
			M1D3R1, M1D3R2, M1D3R3
			M2D3R1, M2D3R2, M2D3R3
			M3D3R1, M3D3R2, M3D3R3
			M1D4R1, M1D4R2, M1D4R3
			M2D4R1, M2D4R2, M2D4R3
			M3D4R1, M3D4R2, M3D4R3
			M1D5R1, M1D5R2, M1D5R3
			M2D5R1, M2D5R2, M2D5R3
			M3D5R1, M3D5R2, M3D5R3
			M1D6R1, M1D6R2, M1D6R3
			M2D6R1, M2D6R2, M2D6R3
			M3D6R1, M3D6R2, M3D6R3

Figure 2. *Burdagol*-Laguna type paddy samples during storage at ambient temperature.

Sample Processing

The samples were processed at the Rice Chemistry and Food Science Division, PhilRice CES in Science City of Muñoz, Nueva Ecija followed by the evaluation of grain quality, milling potentials, and physicochemical properties, and TPC.

Evaluation of Grain Quality Characteristics

Milling Potentials

The percent brown rice (BR), total milled rice (TMR), and head rice (HR) were evaluated as described in the National Cooperative Testing (NCT) manual of PhilRice (1997).

a. % Brown Rice

Rough rice seeds weighing 125 g were passed twice through a dehuller (SATAKE testing husker, Model 35A, Satake, Co., Hiroshima, Japan) to obtain BR grains. The average BR yield was determined based on three replications.

% Brown Rice = (weight of brown rice (g)/125 g) x 100

b. % Total Milled Rice

The BR grains obtained after dehulling (75% brown rice recovery) were placed in a small rice polisher (McGil No. 2 Mill, Seedburo Equipment Co., USA) for 30 sec to remove 10% of the bran and the embryos. The white milled rice was weighed while TMR yield was calculated based on the average of three samples.

$$\%\ \text{Total Milled Rice} = (\text{weight of total milled rice (g)}/125\ \text{g}) \times 100$$

c. % Head Rice

Milled rice grains were put into a grain separator (Satake testing width and length grader, Satake, Co., Hiroshima, Japan) to separate the HR from the broken grains. The head or unbroken grains were weighed while average HR yield was measured based on three replications.

$$\%\ \text{Head Rice} = (\text{weight of head rice (g)}/125\ \text{g}) \times 100$$

Physicochemical Properties

The amylose and crude protein contents were evaluated as described in the NCT Manual for Rice (PhilRice, 1997).

Amylose Content (AC)

The AC of rice sample was measured based on the iodine colorimetric method. An exact amount of 100 mg rice flour was weighed and transferred in a 100-mL

volumetric flask, added with 1 mL of 95% ethanol to wet the samples and disperse the clumps. The sample was soaked in 9 mL of 1N NaOH and incubated for about 18 - 24 h to gelatinize the starch fraction. A 5 mL of the sample was transferred in a 100-mL volumetric flask and diluted with approximately 50 mL distilled water. The solution was added with 1 mL of 0.9 M NH4Cl, swirled gently, then added with 2 mL of iodine reagent (0.15% iodine in 1.5% KI) then diluted to 100-mL mark with distilled water. Absorbance was measured at 620 nm within 20 - 60 min incubation using UV-Vis spectrophotometer (Shimadzu UV-1280, Japan). The AC was calculated based on the standard curve generated from potato amylose and milled rice undefatted checks.

Crude Protein Content (CPC)

The CPC of rice was analyzed using the Kjeldahl method. Rice flour (0.3 g) was weighed and transferred in a digestion tube, added with 1 Kjeltabs catalyst and 5 mL of concentrated sulfuric acid (H_2SO_4). The tubes containing the samples were placed in the digestion block and heated gently at 420°C until the solids became a clear solution. After cooling, the digested samples were added with approximately 25 mL of distilled water to dissolve the crystalline residue. The samples were distilled and titrated with 0.05N H2SO4 using a Kjeltec protein autoanalyzer (Velp DK-159, Italy) following the manufacturer's instructions. The CPC was computed using the formula:

$$CP\ (\%) = \frac{[Vol\ H_2SO_4\ (sample) - Vol\ H_2SO_4\ (blank)]\ x\ N\ H_2SO_4]}{Weight\ of\ sample\ (g)} \times 0.014 \times 5.95 \times 100$$

Evaluation of Phytochemical Content

Total Phenolic Content (TPC)

One-gram rice flour (milled rice) of each sample was weighed into a centrifuge tube added with 10 mL, 85% aqueous methanol. The mixture was shaken for 12 - 14 h and centrifuged at 3000 rpm for 15 min. The extract was transferred into a centrifuge tube and stored at 4°C until analysis. Polished or milled rice was used in determining the total phenolic content due to the fact that aromatic rice is commonly consumed as milled rice and not as brown rice although phenolics are concentrated in the bran.

The TPC was determined following the Folin-Ciocalteu method developed by Singleton et al. (1999) with minor modifications. Sample extract and standard solution (0.5 mL) were mixed with 2.5 mL freshly prepared Folin-Ciocalteu reagent (1:10 dilution). After 15 min of incubation, 2 mL of 7.5% sodium carbonate was added to the mixture. It was allowed to stand for 1 h for color formation. The absorbance of the blue color outcome was measured at 765 nm against a blank. Gallic acid (GA) was used as a standard while TPC was expressed as mg GA equivalent per gram sample. Modifications were done to fit the single spectrophotometric reading method using UV/Vis spectrometer.

Statistical Analysis

The Analysis of Variance (ANOVA) for two-way factorial in completely randomized design (CRD) was used to determine the main effects and interactions of storage material and duration on the grain quality and phytochemical content of rice.

The Least Significant Difference (LSD) test was also used for treatment mean comparison at 5% level while Statistical Tool for Agricultural Research (STAR) Software was used in for statistical analysis.

Results and Discussion

The aromatic *Burdagol-Laguna* type rice had grain length of 6.91 cm and grain shape (length/width) of 2.78 cm. Hence, it is classified as long and intermediate grain based on the NCT Standards.

Effects of Storage Materials and Duration on Grain Quality

Milling Potentials

a. % Brown Rice

The main effects of storage material and duration on the brown rice recovery were significant (Table 1). The brown rice recovery of *Burdagol*-Laguna Type significantly decreased after 5 months of storage regardless of the material used. This result counters the findings of Hafeel et al. (2008), which showed improvement in the brown rice recoveries of the paddy after 4.5 months of storage in both storage conditions (poly-sack bag and hermetic IRRI-super bag) due to decaying of hull. However, Donahaye et al. (2001) as cited by Hafeel et al. (2008) found that decaying rate can be significantly controlled by storing the paddy in a hermetic storage system.

The insulated bin gave lower brown rice recovery of *Burdagol*-Laguna Type than the uninsulated bin and ordinary sack (control). The higher percentage of brown rice recoveries stored in sack and uninsulated bin can be associated to the rapid decaying of hull. Donahaye et al. (2001) as cited by Hafeel et al. (2008) stated that decaying rate can be substantially managed by storing the paddy in a hermetic system. The use of insulated bin storage would minimize the effect of the fluctuating temperature and relative humidity of the surrounding air on the respiration metabolism of

grains, insects, and fungi present in the stored grain; and ultimately, the decaying or biological aging and grain damage.

% Total Milled Rice

The main effects of storage material and duration, as well as their interaction effects on the milled rice recovery were significant (Table 2). The milled rice recoveries of the paddy stored in insulated and uninsulated bins did not significantly change in four months. However, the milled rice recoveries in all three storage materials significantly decreased after five months of storage. Hafeel et al. (2008) found that after 4.5 months of storage, milled rice recovery increased by an average of 1.5% compared with the initial samples, both in poly-sack and IRRI-super bag. On the other hand, Ilieva et al. (2014) found that milling yield decreased after 2 and 3 months of storing paddy harvested at different moisture contents.

The milled rice recovery of paddy stored in sack fluctuated after the 4th month of storage, and then significantly decreased after the 5th month. This may be due to the unstable ambient temperature and % RH that could affect the grains consequently, the milled rice recovery. The ambient temperature and % RH of the storage area from November 2016 to April 2017 were 21.6 - 30.5^{o}C and 44.5 - 79 %, respectively.

The milled rice recoveries of the paddy samples in all the storage materials decreased after 5 months and were comparable with each variety (Table 3).

% Head Rice

The head rice recovery of *Burdagol*-Laguna Type was not significantly affected by the storage material and duration. Adikarinayake (2005) as cited by Hafeel et al., (2008) found that the head rice yields of Bg 94-1 and Bg 34-8 did not significantly change after 6 months of hermetic storage. Contrasting this study, Hafeel et al. (2008) found that the initial values of the head rice yield were significantly higher than the two storage methods: poly-sack bag and IRRI-super bag. Ilieva et al. (2014) reported that head rice recovery decreased after 2 and 3 months of storage of paddy harvested at different moisture contents.

Physicochemical Properties

Amylose Content (AC)

The AC, which is the major factor in determining cooked rice texture, rate, extent of starch digestion, and satiety index (Juliano, 2010), is inversely related to cohesiveness, tenderness, and glossines (Juliano, 1971 as cited by Zhou et al., 2001) of milled rice. Low AC affects the eating and cooking qualities of cooked rice (Zhou et al., 2002) as cited by Atapattu et al. (2018).

The main effect of storage duration was significant to the AC of *Burdagol*-Laguna Type (Table 4). It significantly increased after 3 months of storage regardless of the material used. The result indicates that rice texture becomes harder after 3 months of storage. Jiatrakul et al. (2007) reported that the hardness of the cooked rice of paddy stored at ambient temperature increased with storage time while the stickiness decreased.

Crude Protein Content (CPC)

The CPC is an important component of rice nutritional quality (Tang et al., 2004) as cited by Zhang et al. (2012). The nutritional value of milled rice improves with an increase of protein content (Juliano, 2010).

Table 1. Comparison of the brown rice recovery of *Burdagol*-Laguna Type stored at different durations at each level of storage material.

Month	% Brown Rice			
	Sack	Insulated Bin	Uninsulated Bin	Mean
0	77.63 ± 0.09	77.36 ± 0.30	76.63 ± 0.30	77.21 a
1	77.73 ± 0.50	77.66 ± 0.12	77.62 ± 0.06	77.67 a
2	77.91 ± 0.18	77.10 ± 0.33	78.14 ± 0.01	77.72 a
3	77.53 ± 0.20	76.93 ± 0.13	77.28 ± 0.19	77.59 a
4	77.82 ± 0.13	77.27 ± 0.21	77.67 ± 0.16	77.59 a
5	74.29 ± 0.23	74.01 ± 0.32	74.76 ± 0.18	74.35 b
Mean	77.15 a	76.72 b	77.02 a	
Significance				
Material (M)		*		
Duration (D)		***		
M x D		ns		

**** highly significant at 0.1% level, * significant at 5% level; ns-not significant*
Means followed by common letter(s) are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

Table 2. Comparison of the total milled rice recovery of *Burdagol*-Laguna Type stored at different durations and each level of storage material.

Month	% Total Milled Rice		
	Sack	Insulated Bin	Uninsulated Bin
0	70.47 ± 0.08a	69.58 ± 0.23a	69.35 ± 0.26a
1	68.95 ± 0.35b	69.10 ± 0.85a	69.88 ± 0.11a
2	69.92 ± 0.27ab	69.25 ± 0.39a	70.38 ± 0.29a
3	70.29 ± 0.12a	68.90 ± 0.04a	69.99 ± 0.20a
4	70.14 ± 0.13ab	68.77 ± 0.27a	69.65 ± 0.15a
5	66.45 ± 0.33c	66.33 ± 0.28b	67.19 ± 0.19b
Significance			
Material (M)		***	
Duration (D)		***	
M x D		*	

*** highly significant at 0.1% level, * significant at 5% level; ns-not significant
Means in a column with the same letter are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

Table 3. Comparison of the total milled rice recovery of *Burdagol*-Laguna Type stored using different materials at each level of storage duration.

Material	% Total Milled Rice					
	0	1	2	3	4	5
Sack	70.47 ± 0.08a	68.95 ±0.35b	69.92± 0.27ab	70.29± 0.12a	70.14 ± 0.13a	66.44± 0.33a
Insulated Bin	69.58 ± 0.23b	69.10 ± 0.85ab	69.24± 0.39b	68.90 ± 0.04b	68.77 ± 0.27b	66.33 ± 0.28a
Uninsulated Bin	69.35 ± 0.26b	69.88 ± 0.11a	70.38± 0.29a	69.99± 0.20a	69.65 ±0.15a	67.19 ± 0.19a
Significance						
Material (M)			***			
Duration (D)			***			
M x D			*			

**** highly significant at 0.1% level, * significant at 5% level; ns-not significant*
Means in a column with the same letter are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

Table 4. Comparison of the AC of *Burdagol*-Laguna Type stored at different durations.

Month	Amylose Content (%)			Mean
	Sack	Insulated Bin	Uninsulated Bin	
0	14.82 ± 0.18	14.92 ± 0.16	14.84 ±0.19	14.86 b
1	14.36 ± 0.12	14.84 ± 0.12	14.61 ±0.12	14.60 b
2	14.92 ± 0.21	14.97 ± 0.11	14.76 ± 0.04	14.88 b
3	16.72 ± 0.25	16.20 ± 0.12	16.17 ± 0.13	16.36 a
4	16.46 ±0.15	16.57 ±0.02	16.51 ± 0.01	16.39 a
5	16.50 ± 0.01	16.50± 0.05	16.52 ± 0.02	16.53 a
Significance				
Material (M)	ns			
Duration (D)	***			
M x D	ns			

*** highly significant at 0.01% level; ns - not significant
Means in a column with the same letter are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

The main effects of storage material and duration, as well as their interaction effects on CPC were significant (Table 5). The CPC of *Burdagol*-Laguna Type rice stored in the insulated bin did not significantly decreased throughout the 5-month storage duration. This implies that the insulated bin can maintain the CPC in the aromatic rice stored for 5 months. However, for the samples stored in sack and uninsulated bin, the CPC significantly decreased after the 1st and 2nd month. This may be due to the fact that the paddy samples stored in the sack and uninsulated bin were more easily affected by the temperature and humidity of the surrounding than the insulated bin, which may cause fungal or insect attack against the rice grains. Protein is a good source of nutrition for the growth and proliferation of insects (Oessoe et al., 2014).

Table 6 shows that the CPC of paddy samples stored in all three materials were comparable with each other, starting from the 3rd until 5th month. The highest decrease in the crude protein of paddy samples was observed from the uninsulated bin (22.98%), followed by grains stored in sack (9.31%), and least (5.88%) from the insulated bin.

Phytochemical Content

Total Phenolic Content

Phenolic compounds may provide antioxidant and radical scavenging activities (Thanajiruschaya et al., 2010). They have redox properties, which acts as antioxidants (Shorib and Shahid, 2015; Sobrattee et al., 2005) as cited by Johari and Khong 2019. In 2019, Kumar and Goel reported that phenolic content reduces disease risk and enhances human health.

The main effects of storage material and duration, as well as their interaction effects on the TPC were significant (Table 7). The TPC of samples increased as the storage duration progressed. This is in contrary with the result of Thanajiruschaya et al. (2010), which showed a consistent decrease in the phenolic acid content of milled rice following storage. This may be related to the form of rice being stored (milled or white rice), which is different from this study that used paddy or rough rice.

The samples stored in sack and insulated bin showed similar trend. TPC of the samples increased starting the 2nd month while TPC of the samples in the uninsulated bin started to increase in the 1st month.

The highest TPC of *Burdagol*-Laguna Type rice was observed during the 4th month using sack (0.14 mg/g GAE); the 4th and 5th months using insulated bin (0.15 mg/g GAE); and 5th month using uninsulated bin (0.16 mg/g GAE). Zhou et al. (2004) reported that phenolic acid particularly the ferulic acid of milled rice ranges from 61 to 84 mg/kg or 0.06 - 0.08 mg/g grain. Clifford (1999) and Scalbert and Williamson (2000) as cited by Kumar and Goel (2019) reported that an individual should have a daily intake of phenolic acids at around 200 mg/day or more, depending on his/her consumption of whole grains, fruits, and vegetables such as rice, corn, apples, citrus, and onions. Phenolic acids are key class of polyphenols and abundantly used in human diet (Kumar and Goel, 2019). Thus, results from this study indicate that *Burdagol*-Laguna Type aromatic rice can be a good source of phenolic acids to help meet daily nutrition requirement.

Thanajiruschaya et al. (2010) reported that TPC was observed to be higher in milled rice stored at higher temperature. In this study, the highest ambient temperatures were observed during the 4th and 5th month of storage (March and April 2017, which

Table 5. Comparison of the CPC of *Burdagol*-Laguna Type stored at different durations at each level of storage material.

Month	Crude Protein Content (%)		
	Sack	Insulated Bin	Uninsulated Bin
0	7.20 ± 0.21a	6.80 ± 0.12a	8.53 ± 0.03a
1	7.23 ± 0.32a	6.53 ± 0.23a	7.97 ± 0.13a
2	6.93 ± 0.09ab	6.60 ± 0.25a	8.03 ± 0.22a
3	6.47 ± 0.12b	6.23 ± 0.09a	6.27 ± 0.03b
4	6.70 ± 0.00ab	6.60 ± 0.06a	6.63 ± 0.07b
5	6.5 ± 0.03b	6.40 ± 0.00a	6.57 ± 0.03b
Significance			
Material (M)		***	
Duration (D)		***	
M x D		***	

*** highly significant at 0.01% level; ns - not significant
Means in a column with the same letter are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

Table 6. Comparison of the CPC of *Burdagol*-Laguna Type stored using different materials at each level of storage duration.

Material	% Crude Protein Content					
	0	1	2	3	4	5
Sack	7.20 ± 0.21b	7.23 ± 0.32b	6.93 ± 0.09b	6.47 ± 0.12a	6.70 ± 0.00a	6.53 ± 0.03a
Insulated bin	6.80 ± 0.12b	6.53 ± 0.23c	6.60 ± 0.25b	6.23 ± 0.09a	6.60 ± 0.06a	6.40 ± 0.00a
Uninsulated bin	8.53 ± 0.03a	7.97 ± 0.13a	8.03 ± 0.22a	6.27 ± 0.03a	6.63 ± 0.07a	6.57 ± 0.03a
Significance						
Material (M)	***					
Duration (D)	***					
M x D	***					

*** highly significant at 0.1% level, * significant at 5% level; ns-not significant
Means in a column with the same letter are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

Table 7. Comparison of the total phenolic content of *Burdagol*-Laguna Type stored at different durations at each level of storage material.

Month	Total Phenolic Content (mg g^{-1} GAE)		
	Sack	Insulated Bin	Uninsulated Bin
0	0.1100 ± c	0.1100 ± c	0.1100 ± d
1	0.1100 ± c	0.1133 ± c	0.1233 ±c
2	0.1300 ± b	0.1400 ± b	0.1500 ±b
3	0.1367 ±ab	0.1433 ±ab	0.1500 ± b
4	0.1400 ±a	0.1500 ± a	0.1567 ±ab
5	0.1330± ab	0.1500 ± a	0.1600 ± a
Significance			
Material (M)	***		
Duration (D)	***		
M x D	***		

*** highly significant at 0.01% level
Means in a column with the same letter are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

are considered the hottest months in the area). The result also confirmed the previous report of Baradi et al. (2016), which stated that the highest total phenolic contents (0.25 mg/g GAE) of the aromatic rice stored in paddy form were observed during the 3rd and 4th month. This could be due to the increase in temperature during the smoking or heating time in the storage hut, which enhanced the release of phenolics from the rice endosperm and bran/embryo fractions. Phenolic compounds have different forms (free, soluble-conjugated, and bound) found in the endosperm and bran/embryo fractions of the rice grain (Tyagi et al., 2022).

Contrasting the result, Lang et al. (2019) found a reduction of TPC in the black rice grains stored under normal-atmosphere (conventional), nitrogen-atmosphere, and vacuum-atmosphere for 12 months. Dar et al. (2016) stated that aside from the antioxidant activities of bran-enrich snacks (rice, wheat, oat), their TPC also decreased during storage at ambient temperature.

It can be observed that the TPC of samples stored in the bins (insulated and uninsulated) increased as the storage duration progressed. Meanwhile, the TPC of samples stored in sack increased during the 2nd - 4th month, but decreased again in the 5th month.

Among the three storage materials used, the paddy samples stored in the uninsulated bin had the highest TPC for *Burdagol*-Laguna Type rice, followed by the insulated bin (Table 8). The lowest TPC was observed from samples stored in sack.

Results show that storage material and duration had significant effects on the grain quality and phytochemical content of aromatic *Burdagol*-Laguna Type rice.

The brown rice recovery of *Burdagol*-Laguna Type significantly decreased after 5 months of storage, which contradicts the findings of Hafeel et al. (2008). The authors contended that increase in brown rice recoveries occurs after 4.5 months of storage in poly-sack bag due to hull decomposition.

Table 8. Comparison of the total phenolic contents *Burdagol*-Laguna Type stored using different materials at each level of storage duration.

Material	Total Phenolic Content (mg/g GAE)					
	0	1	2	3	4	5
Sack	0.11 ± 0.0004	0.11 ± 0.0002	0.13± 0.0013	0.14 ± 0.0006	0.14 ± 0.0006	0.13 ± 0.0003
Insulated bin	0.11 ± 0.0004	0.12 ± 0.0016	0.14 ± 0.0005	0.14 ± 0.0011	0.15 ± 0.0004	0.15 ± 0.0005
Uninsulated bin	0.11 ± 0.0005	0.12 ± 0.0014	0.15 ± 0.0030	0.15 ± 0.0010	0.16± 0.0003	0.16 ± 0.0007
Significance						
Material (M)	***					
Duration (D)	***					
M x D	***					

*** highly significant at 0.1% level, * significant at 5% level; ns-not significant
Means in a column with the same letter are not significantly different from each other at 5% level using LSD; 3 replicates per treatment

Higher brown rice recoveries of paddy samples were observed in grains stored in sack and uninsulated bin than those in the insulated bin. The insulated bin would have minimized the effect of the fluctuating temperature and relative humidity of the surrounding air, which slows down the respiration of the grains, insects, and fungi present in the stored grain; and ultimately, the decaying or biological aging and grain damage. Although the insulated bin had lower brown rice recovery than the sack and uninsulated bin, this storage material provided better protection against insect damage and deterioration of quality such as decrease in CPC and TPC.

The milled rice recoveries of *Burdagol*-Laguna Type also decreased. The milled rice recoveries of the samples stored in bins (insulated and uninsulated bin) remained constant until during the 4th month of storage while these fluctuate in the control storage. This can be associated to the changing ambient temperature and % RH that could affect the grains and consequently, the milled rice recovery.

The head rice recoveries of *Burdagol*-Laguna Type were not affected by storage material and duration. On the other hand, the AC increased after five months indicating that the rice becomes harder in texture regardless of storage material. This result supported the report of Tamaki et al. (1993).

Moreover, the CPC of *Burdagol*-Laguna Type rice stored in the insulated bin did not decrease throughout the 5-month duration implying that the insulated bin can maintain the CPC of the aromatic rice. This is contrary to the samples stored in sack and uninsulated bin where the CPC significantly decreased after the 1st month and 2nd month, respectively.

In addition, the TPC of the samples stored in bins (insulated and uninsulated) increased as the storage duration progressed. This may be due to enzymatic or nonenzymatic release of bound phenolics and highest ambient temperatures during the 4th and 5th month of storage (March and April 2017). Supporting the result, Zhou et al. (2004) found a significant increase of free phenolic acids in white rice during storage, which may be due to enzymatic or nonenzymatic release of bound phenolics while Thanajiruschaya et al. (2010) concluded that TPC was higher in milled rice stored. The result also confirmed the previous report of Baradi et al., in 2016. The TPC of paddy samples stored in sack increased during the 2nd - 4th month but decreased again during the 5th month.

Conclusion

Results show that 5-month storage is sufficient in determing the best material to be used in safekeeping aromatic rice. To minimize losses and deterioration of quality, insulated bin is a better alternative for storing *Burdagol*-Laguna Type paddy at ambient condition. Insulated bin can maintain the milled rice recovery and crude protein after 4 - 5 months of storage and gave the second highest TPC after 5 months of storage.

The best allowable storage duration in attaining the highest brown rice recovery, milled rice recovery, CPC, and total phenolic content is 4 months. However, the AC increased after 3 months indicating that rice texture becomes harder. The higher the brown rice and milling recovery, the greater amount of aromatic rice will be available. Rice is more nutritious with higher the CPC and TPC, which makes it more beneficial to human health. Thus, aromatic rice can be best stored for 4 months at ambient condition using insulated bin for better quality rice.

It is recommended that a similar study can be conducted using more samples of aromatic rice varieties to better understand the responses on various storage materials and durations.

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FULL PAPER

GRAIN QUALITY AND SAFETY ASSESSMENT OF LOCAL AND IMPORTED MILLED RICE IN THE PHILIPPINES

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Abstract

Despite the government's demand of importing rice to meet the country's declining buffer stock, the plight of every Filipino in accessing high-quality and safe rice remains as the top priority. This study determined the grain quality, pesticide residue, and heavy metal content of imported and local milled rice samples. There were 45 rice samples (38 imported and 7 local) collected from different warehouses of National Food Authority (NFA). Sixteen samples came from Thailand, 14 from Vietnam, 6 from Myanmar, and 1 each from Pakistan and India. Meanwhile, the 7 collected local rice samples from farmers of Bukidnon, Aklan, Albay, Batangas, Negros Occidental, Isabela, and Cagayan de Oro that were acquired through NFA, contained high amount of broken rice ranging from 19.8 to 60.9%. This result was based on the National Cooperative Testing (NCT) manual for rice. Seven samples from Thailand passed the preferred Grade 1 classification for chalky grains. Majority of the samples had long and slender grains, but only 10 samples, mostly from Vietnam and Thailand, exhibited both characteristics. Moisture content (MC) ranged 9.7 - 12.6% while most of the samples had high amylose content (AC) of 22.4 - 27.4%, including all rice samples from Thailand and Myanmar. Furthermore, rice imports from Thailand and Pakistan had low gelatinization temperature (GT), while samples from Vietnam, Myanmar, Philippines, and India had intermediate. Rice samples from Myanmar and majority from Thailand and Philippines had medium cooked rice texture, while most of the samples from Vietnam were under the soft classification. All samples had no off-odor both in raw and cooked forms. Rice imported from Thailand had separated; hard to slightly tender and rough cooked rice, which is a common characteristics of rice varieties with high AC – low GT property. Meanwhile, majority of Vietnam rice samples produced softer texture that could be attributed to their intermediate or high AC – intermediate GT property. In terms of pesticide residue, all rice samples are below the maximum residue limit (MRL) for organochlorine and some of the organophosphate pesticides (Fenthion and Chlorpyrifos). All samples contained safe level of arsenic and mercury. Unfortunately, 4 samples (2 from Myanmar, 1 each from Vietnam and Thailand) exceeded the MRL for lead (Pb) while two samples from Vietnam exceeded the MRL for Cadmium (Cd). In general, the collected NFA rice samples had good grain quality. However, the high Pb and Cd concentration of some imported rice may pose some serious health risks among Filipino consumers.

Keywords*: Grain Quality, Heavy Metals, Imported Rice, National Food Authority, Pesticide Residue, Safety*

Introduction

The continuous population growth in the country has been identified as one of the factors contributing to rice shortage. In December 2018, the Philippine Statistics Authority (PSA) reported a 4.59% (2,849.37 mt) decrease in the total rice stocks inventory (2,718.48 mt) from the previous year. The inevitable dependence of Filipinos to rice requires reliable and steady supply; thus, the government demands rice importation from neighboring countries like Thailand, Vietnam, and China to meet the country's declining buffer stock (Tobias, 2019). In 2019, the country has imported 3.12M mt of milled rice through the National Food Authority (NFA) (PSA, 2020). However, the quality of rice sold by NFA has sometimes become a subject of concern. Some consumers believe that most NFA rice in the market are old stocks and of poor quality. More importantly, reports involving the excessive application of pesticides in rice by top exporting countries, particularly Vietnam and Thailand, have caused panic among Filipino consumers. Huan et al. (2005) reported that Vietnam and Thailand were heavy users of pesticides. In fact, farmers in Vietnam applied more pesticides than necessary. These countries are now facing serious challenges with regard to the amount and toxicity of pesticides used. Bordey et al. (2016) stated that the Philippine government should enforce testing for pesticide residue of all imported rice to protect its citizens. Public concern on pesticide toxicity has increased over the years due to significant evidence of carcinogenic and mutagenic

effects in humans (Niaz et al., 2016). In 2016, the U.S. Food and Drug Administration (USFDA) rejected 95 shipping containers of imported rice from Vietnam due to the high levels of pesticide (Klein, 2016; Dao, 2016). In 2015, the European Food Safety Authority (EFSA) reported that the rice samples originated from India, Vietnam, and Pakistan have carbendazim; a fungicide ranging from 0.012 to 0.041 mg/kg, which is above the strict MRL of 0.01 mg/kg. Studies showed that continuous consumption of food even at low pesticide residue concentrations may eventually cause adverse effect in humans through bioaccumulation of pesticides in the body system. The largest proportion of human acute toxicity data is associated with pesticide intoxication (Onojoh et al., 2013). The huge potential risks include birth defects, hearing loss, cancer, and infertility. Additionally, there are symptoms related to acute intoxication such as vomiting, seizures, loss of appetite, and nosebleed. On their adverse and irreversible consequences on human health and environment, various government and international organizations have established MRL to regulate the concentration of the active ingredients found in pesticides applied in crops and food.

Meanwhile, the excessive input of potentially harmful heavy metals by different anthropogenic activities have created a contamination problem in soil, air, and water. Metals presents in the soils can be taken up directly by the standing crop. Dietary intake of heavy metals via soil-crop system has been considered as the predominant pathway of human exposure to heavy metals. One of the major routes is through rice consumption, especially in mining areas (Fan et al., 2017). Heavy metals such as arsenic (As), lead (Pb), cadmium (Cd), mercury (Hg), manganese (Mn), and antimony (Sb) are major concerns and become a worldwide environmental problem (Solidum et al., 2012; Li et al., 2013). In 2011, Ma et al., reported that rice samples collected from 40 countries were found to have high average As content of 0.129 mg/kg, which is enough concentration to potentially affect the health of local residents. In 2016, USFDA released an updated report on the inorganic As content of white rice samples. The inorganic As concentration was 23 - 196 ppb for white long grain, 39 - 174 ppb for white medium grain, and 52 - 102 ppb for white short grain (USFDA, 2016). Prolonged exposure to high As level can cause numerous health problems including skin lesions, cancer, developmental toxicity, neurotoxicity, cardiovascular diseases, and diabetes. Moreover, several studies also reported the high concentrations of Cd and Pb in rice in some countries (Zhuang et al., 2009; Orisakwe et al., 2012;). In 2014, a rice contaminated with Cd (termed as Cd rice) caused social panic in Hunan Province of China (Yu et al., 2017). High levels of Cd and Pb in polished rice, which exceeds the safety standard for milled rice were also found in China (Fu et al., 2008). Similarly in Australia, high levels of Cd and Pb were found in rice imported from India and Thailand (Rahman et al., 2014). Health effects associated with Cd bioaccumulation in the human body include diarrhea, bone fracture, reproductive organ and kidney failure, damage to central nervous system, and cancer (Sharma et al., 2015). In addition, prolonged exposure to low levels of Pb may cause loss of appetite and weight, depression, muscular weakness, joint stiffness, neuropsychological deficiencies, growth retardation, and cardiovascular abnormalities (Rosen, 1993; Gupta and Gupta, 1998). Reports on high levels of Hg from rice-producing countries also caused alarm among rice exporters and consumers. High concentrations of Hg (1,280 μg/kg) were found in rice grown of southwest China specifically in their Hg mining areas (Qiu et al., 2008). Milled rice and brown rice samples from major rice producing provinces of China contained Hg level of 3.4 - 4.9 μg/kg, respectively. Severe exposure to Hg can be harmful for the human brain, heart, kidneys, lungs, and immune system. It can also cause blindness, chromosome damage, paralysis, and birth defects (Venugopal and Luckey, 1978). Elevated levels of methyl mercury in the blood of fetus and young children can cause harmful effects on their developing nervous system, resulting in reduced learning ability.

Being one of the active members of the ASEAN, the Philippines is highly involved in the harmonization of food safety regulations including MRLs of pesticides. In 2014, the Philippine National Standards adopted the initial list of MRL for selected crops including rice. These limits aim to improve the trade and competitiveness of the local agricultural commodities and protect consumers against harmful effects of agrochemicals (BAFS, 2015). As the government continues to ensure sufficient rice supply to the country, the plight of Filipino rice consumers to access high-quality and safe rice must be a top priority. As a rice-consuming country, Filipinos are inevitably exposed to significant amounts of pesticide residues and heavy metals. This study assessed the quality and safety of local and imported milled rice for consumption. The information generated can be used as basis for crafting policies related to quality and safety of local and imported rice in the Philippines.

Materials and Methods

Collection of rice samples

The collection of milled rice samples followed the NFA's issued online track and status of rice import arrivals. To minimize duplication, the following factors were considered: importation scheme (government to government [G-to-G] and government to private [G-to-P]), country of origin, supplier, % broken rice, name of vessel, and point of disembarkation. The process was facilitated through close coordination with the Regional Directors, Provincial Managers, and Regional and Provincial Standards and Quality Assurance Officers of NFA offices.

About 5 kg of sample per source/type were collected at the warehouse following the sampling protocol of NFA (Figure 1). Briefly, samples were drawn from at least 10% of the bags within a given lot. Bags were randomly selected from various position in the entire pile. A grain probe was inserted in the rice bags for about 1/3 diagonally, lengthwise in another 1/3, and widthwise in remaining 1/3 to obtain samples. The collected samples were then mixed to constitute a homogenous composite or representative sample. The samples were then packed in a transparent plastic bag and labeled accordingly (NFA, 1998). Information such as supplier, crop year, date of milling, moisture content, % broken rice, and date of delivery to the warehouse were recorded. The warehouse practices and pest control measures such as fumigation, fogging, and spraying employed before and during the storage process were also noted. The collected rice samples were then kept in the refrigerator (4 - 8°C) prior to laboratory analyses. The rice samples were pulverized (0.5-mm mesh) using a cyclotec grinder for analyses that requires powder form samples.

Evaluation of grain quality

The rice grain quality characteristics were evaluated in accordance to the standard protocols of the NCT Manual for Rice (1997). It includes the determination of physical attributes, physicochemical properties, cooking parameters, and sensory characteristics.

Physical attributes

For % broken rice, 100 g of the collected rice samples was placed in a grain grader to separate broken rice from head rice. The latter was collected and weighed. Percent broken rice was computed as the difference of 100 g and the weight of head rice, divided by 100 g and multiplied by 100. Grain length and width were measured using a calibrated caliper while the shape was determined by dividing the grain's length to its width. The grain length and shape were calculated based on the average of ten grains. For % chalky grains, about 10 g of milled rice grains in triplicate were weighed and grains with 50% or more chalky area were considered as chalky.

Figure 1. The collection of NFA rice samples from NFA warehouses.

Physicochemical properties

Moisture content of the rice samples was determined using the oven-drying method. Approximately 1.0 g of powdered sample was placed in a tared pan, oven-dried at 130°C for 1 h, cooled at around 50°C, equilibrated inside a desiccator for 25 min, and weighed. Oven-drying was repeated until constant weight was obtained. The moisture content was calculated using the following formula:

$$\%\ \text{moisture content} = \frac{\text{weight of fresh sample} - \text{weight of dried sample}}{\text{weight of fresh sample}} \times 100$$

The AC of rice samples was determined using the iodine staining colorimetric method developed by Juliano et al. (2012). Rice flour was weighed and soaked overnight with reagents and percent amylose was computed based on the linear regression obtained from standard amylose curve. The gelatinization temperature (GT) was indexed based on the alkali spreading value (ASV) following a numerical scale described by Little et al. (1958) and Bhattacharya (1979). Six whole grains were spaced evenly in a small Petri dish added with sodium hydroxide (enough to submerge the grains in solution). The dish was covered and left undisturbed for 23 h at room temperature and visually evaluated using a seven-point numerical scale. Meanwhile, crude protein content was determined using Kjeldahl method, in which about 300 mg rice flour was weighed and transferred into a 250-mL Kjeldahl flask. One piece of Kjeltab (catalyst) and 5 mL of concentrated sulfuric acid were added to the samples then digested for 1.5 h until the sample became completely clear. After cooling, 30 mL of distilled water was added and the sample was homogenized using a vortex mixer. The sample was distilled and titrated using the Kjeltec autoanalyzer. Lastly, the texture of cooked grains was determined using an Instron autoanalyzer (Instron 3342, USA). Rice sample was cooked using a pre-determined rice-water ratio. The cooked rice was let cool for 45 min and subsequently weighed and transferred into the Instron cell. The force required to extrude the cooked grains into the cell was measured.

Cooking quality

The cooking quality evaluation started with the determination of the optimum cooking water, followed by the measurement of weight and height increases, and cooking time. The optimum amount of water for complete doneness (neither too dry nor too soft) was determined after preliminary trials.

Sensory characteristics

The sensory characteristics of raw and cooked rice samples were evaluated by a well-represented number of trained male and female panelists from the PhilRice Rice Chemistry and Food Science Division. Raw rice samples were assessed in terms of aroma, off-odor, color, glossiness, chalkiness, white belly, and hardness. On the other hand, cooked rice was evaluated for aroma, off-odor, color, glossiness, cohesiveness, tenderness, smoothness, taste, and off-taste. Samples with heavy metal and pesticide residues below the MRL were exclusively subjected to sensory evaluation.

Table 1 shows some of the grain quality parameters evaluated, their classification, and recommended or preferred values based on NCT (1997) rice manual.

Determination of pesticide residues and heavy metals

The pesticide residues and heavy metal content of the rice samples were determined by Jefcor Laboratories, Inc., located in Dasmarinas, Cavite while the organophosphate and organochlorine pesticides were identified using Gas Chromatography-Mass Spectrometry. Meanwhile, heavy metals specifically As, Cd, Pb, and Hg content of samples were analyzed using Inductively Coupled Plasma-Optical Emission Spectroscopy.

Data Analysis

All analyses were performed in twice, while descriptive analyses such as mean, range, and percentage distribution were computed using the Microsoft Office Microsoft Excel 2016.

Results and Discussion

Collected NFA rice samples

Thirty-eight local and seven imported rice samples were collected from NFA warehouses in the country (Table 2). All imported rice samples were harvested in 2018; 16 samples from Thailand, 14 from Vietnam, 6 from Myanmar, and 1 each from Pakistan and India (Figure 2). Almost half of the samples were imported by the Philippine government through G-to-G scheme and shipped via various vessels (Figure 3). Majority of the rice samples from Thailand were imported from DFT-Thailand. Vietnam rice were also acquired through G-to-G mode of procurement from either Vinafood I or Vinafood II, except for 1 sample procured from a private supplier (Olam International Ltd.). Moreover, rice imports from Myanmar, India, and Pakistan were supplied by private companies (G-to-P), mostly by Olam International Ltd. Meanwhile, the seven local rice samples were harvested from 2018 to 2019 and acquired by NFA from the local farmers of Bukidnon, Aklan, Albay, Batangas, Negros Occidental, Isabela, and Cagayan de Oro as enumerated based on collection date.

Table 1. Classification and recommended values for grain quality parameters.

Parameter	Classification		Recommended/ Preferred Value
Physical attributes			
Grain length (mm)	Extra long (EL)	≥ 7.5	6.6 - 7.4
	Long (L)	6.6 - 7.4	(Long)
	Medium (M)	5.5 - 6.5	
	Short (Sh)	≤ 5.4	
Grain shape (mm)	Slender (S)	> 3.0	> 3.0
	Intermediate (I)	2.1 - 3.0	(Slender)
	Bold (B)	≤ 2.0	
Chalky grains (%)	Premium (Pr)	< 2.1	≤5.0 (Grade 1 to Premium)
	Grade 1 (G1)	2.1 - 5.0	
	Grade 2 (G2)	5.1 - 10.0	
	Grade 3 (G3)	10.1 - 15.0	
	aa	≥ 15.1	
Physicochemical properties			
Amylose content (%)	Waxy/glutinous (W)	0.0 – 2.0	17.1 - 22.0
	Very low (VL)	2.1–10.0	(Intermediate)
	Low (L)	10.1 – 17.0	
	Intermediate (I)	17.1 – 22.0	
	High (H)	≥ 22.1%	
Gelatinization temperature (°C)	High (H)	1 – 2 (> 80.1)	4 - 5
	High-Intermediate (HI)	3 – (74.5 - 80.0)	(Intermediate)
	Intermediate (I)	4 – 5 (70.0 - 74.0)	
	Low (L)	6 – 7 (<70.0)	
Crude protein		6 - 9%	
Instron cooked rice hardness (kg/cm^2)	Hard	≥ 2.6	1.1 - 2.5
	Medium	1.9 - 2.5	(Soft to medium)
	Soft	1.1 - 1.8	
	Very soft	0.5 - 1.0	

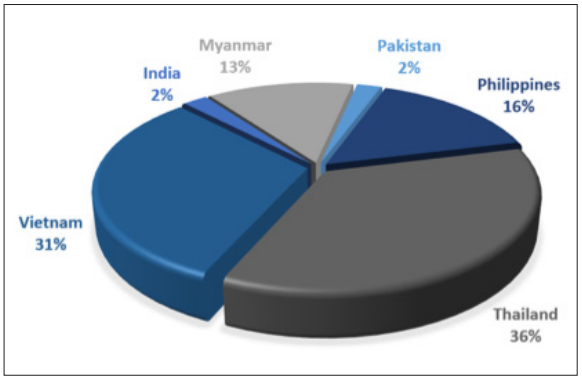

Figure 2. Percentage distribution of collected local and imported milled rice samples in terms of country of origin.

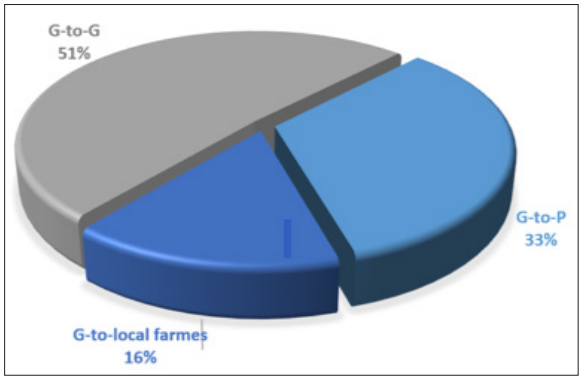

Figure 3. Percentage distribution of collected local and imported milled rice samples in terms of importation scheme.

Table 2. Detailed information of the collected NFA rice samples.

No.	Country of Origin	Supplier	Vessel	Crop Year	Date of Milling	Moisture Content	Broken Rice	NFA Warehouse**	Type	Scheme***
						(%) as declared				
1	Philippines	Local farmers of Bukidnon	N/A	2018	May 2018	-	31.3	Cagayan de Oro RO	Local	N/A
2	Vietnam	VINAFOOD I	N/A	2018	May 2018	14	25	Cagayan de Oro RO	Imported	G-to-G
3	Vietnam	VINAFOOD I	MV VINH HUNG	2018	May 2018	14	25	Agusan del Norte PO	Imported	G-to-G
4	Vietnam	VINAFOOD I	MV TAYSON 2	2018	May 2018	14	25	Tarlac City PO	Imported	G-to-G
5	Thailand	Ponglarp Co. Ltd.	MV VINH HUNG	2018	Jun 2018	14	25	Cebu City RO (1)	Imported	G-to-P
6	Vietnam	VINAFOOD I	VINASHIP GOLD	2018	May 2018	14	15	Cebu City RO (2)	Imported	G-to-G
7	Vietnam	VINAFOOD I	MV BMC ALPHA	2018	May 2018	14	25	Cebu City RO (3)	Imported	G-to-G
8	Thailand	DFT Thailand	MV DONG PHU	2018	May 2018	14	25	Iloilo City RO	Imported	G-to-G
9	Thailand	DFT Thailand	MV VTC DRAGON	2018	May 2018	14	25	General Santos City RO (1)	Imported	G-to-G
10	Vietnam	VINAFOOD I	N/A	2018	May 2018	14	25	General Santos City RO (2)	Imported	G-to-G
11	Vietnam	VINAFOOD I	N/A	2018	May 2018	14	15	General Santos City RO (3)	Imported	G-to-G
12	Thailand	DFT Thailand	N/A	2018	May 2018	14	25	Palawan PO	Imported	G-to-G
13	Myanmar	Olam International Limited	MV NMC GALAXY	2018	May 2018	14	25	La Union PO (1)	Imported	G-to-P
14	Thailand	Olam International Limited	MV OCEAN GLORY	2018	May 2018	14	25	La Union PO (2)	Imported	G-to-P
15	Thailand	DFT Thailand	MV GAZI	2018	May 2018	14	25	La Union PO (3)	Imported	G-to-G
16	Thailand	DFT Thailand	MV TAYSON 2	2018	May 2018	14	25	Leyte RO	Imported	G-to-G
17	India	Olam International Limited	MV Rising	2018	May 2018	14	25	Batangas RO (1)	Imported	G-to-P
18	Thailand	DFT Thailand	MV Tayson 2	2018	May 2018	14	25	Batangas RO (2)	Imported	G-to-G
19	Thailand	DFT Thailand	MV GAZI	2018	May 2018	14	25	Isabela RO	Imported	G-to-G
20	Thailand	Asia Golden Rice Co. Ltd	N/A	2018	Jun 2018	14	25	Kalibo 2018	Imported	G-to-P
21	Philippines	local farmers	N/A	2018	Dec2018	12-14	-	Kalibo 2018	Local	N/A
22	Vietnam	VINAFOOD I	MV PHUONG DUONG 5	2018	Dec 2018	14	25	Tarlac 2019 - 1	Imported	G-to-G
23	Vietnam	VINAFOOD I	MV VEGA STAR	2018	Dec 2018	14	25	Tarlac 2019 - 2	Imported	G-to-G
24	Vietnam	VINAFOOD II	MV BLUE STAR	2018	Dec 2018	14	25	Cabanatuan 2019 - 1	Imported	G-to-G
25	Vietnam	VINAFOOD II	MV PHUONG DUONG 5	2018	Dec 2018	14	25	Cabanatuan 2019 - 2	Imported	G-to-G
26	Thailand	DFT Thailand	MV CHARLENE	2018	Nov 2018	14	25	Cabanatuan 2019 - 3	Imported	G-to-G
27	Myanmar	Olam International Limited	M/V TAN BINH 127	2018	No 2018	14	25	La Union 2019 - 1	Imported	G-to-P
28	Thailand	DFT Thailand	M/V TRUONG MINH DRAGON	2018	Nov 2018	14	25	La Union 2019 - 2	Imported	G-to-G
29	Vietnam	VINAFOOD II	M/V VINASHIP PEARL	2018	Dec 2018	14	25	La Union 2019 - 3	Imported	G-to-G
30	Vietnam	VINAFOOD II	M/V ZIRCON	2018	Dec 2018	14	25	Iloilo City 2019 - 1	Imported	G-to-G

No.	Country of Origin	Supplier	Vessel	Crop Year	Date of Milling	Moisture Content	Broken Rice	NFA Warehouse**	Type	Scheme***
						(%) as declared				
31	Myanmar	Shwe Wah Yaung Agriculture Pro. Co. Ltd	M/VRAINBOW IVY	2018	Oct 2018	14	25	Iloilo City 2019 - 2	Imported	G-to-P
32	Myanmar	Shwe Wah Yaung Agriculture Pro. Co. Ltd	M/V RAINBOW FAIRY	2018	Oct 2018	14	25	Iloilo City 2019 - 3	Imported	G-to-P
33	Thailand	Olam International Limited	M/V DAI DUONG QUEEN	2018	Nov 2018	14	25	Batangas 2019 - 1	Imported	G-to-P
34	Vietnam	Olam International Limited	M/V DERYUONG SUNFLOWER	2018	Nov 2018	14	25	Batangas 2019 - 2	Imported	G-to-P
35	Myanmar	Olam International Limited	M/V VTC SUN	2018	Nov 2018	14	25	Batangas 2019 - 3	Imported	G-to-P
36	Thailand	Olam International Limited	M/V PHC MARITIME	2018	Nov 2018	14	25	Legazpi 2019 - 1	Imported	G-to-P
37	Myanmar	Olam International Limited	M/V HOANG PHUONG LUCKY	2018	Nov 2018	14	25	Legazpi 2019 - 2	Imported	G-to-P
38	Thailand	DFT Thailand	M/V BANGPAKAEW	2018	Nov 2018	14	25	Zamboanga 2019 - 1	Imported	G-to-G
39	Thailand	Asia Golden Rice Co. Ltd	M/V VINALINES MIGHTY	2018	Nov 2018	14	25	Zamboanga 2019 - 2	Imported	G-to-P
40	Philippines	Local farmers of Albay	N/A	2019	Dec 2019	14	N/A	Albay 2020 - 1	Local	N/A
41	Philippines	Local farmers of Batangas	N/A	2019	Sep 2019	12	N/A	Albay 2020 - 2	Local	N/A
42	Philippines	Local farmers of Negros	N/A	2019	Dec 2019	13	N/A	Negros Occidental 2020 - 1	Local	N/A
43	Philippines	Local farmers of Isabela	N/A	2019	Oct 2019	11.8	N/A	Isabela 2020 - 2	Local	N/A
44	Philippines	Local farmers of CDO	N/A	2018/2019	Nov 2019	12.4	N/A	Cagayan de Oro 2020 - 1	Local	N/A
45	Pakistan	Olam International Limited	M/V SEA MAGIC	2018	Dec 2018	14	25	Tarlac 2019 - 3	Imported	G-to-P

*DFT – Department of Foreign Trade
**RO - Regional Office; PO - Provincial Office
*** G-to-G - Government to Government; G-to-P - Government to Private Company

Grain quality characteristics of the collected rice samples

In terms of income and price, studies have shown that consumers tend to buy quality rice as income increases and that consumers shift to better quality rice when the prices decline. According to Abansi et al. (1990), rice grain quality is one of the most important factors affecting the consumers' preferences in urban or rural areas. Information on grain quality is also used by policy makers in formulating policies related to rice importation and commercialization.

Physical attributes

Physical attributes like broken rice, chalky grains, and grain dimensions affect the quality and market value of rice. According to Conway et al. (1991), the physical qualities of milled rice are strongly correlated with its transaction price. Broken rice are kernels that are less than 25% (1/4) of their normal length after milling. The amount of broken rice is highly affected by postharvest processes like drying and milling. The % broken rice of the NFA rice samples ranged from 19.82 to 60.87%. The five rice samples with highest % broken rice are from Thailand and Vietnam procured through a G-to-G scheme.

Chalk is an opaque area in the rice grain, which commonly occurs when grains are exposed to high temperatures during its development. The presence of chalky grains decreases the value of rice because of their undesirable appearance and eating quality. Chalky grains tend to rapidly absorb water during cooking and produce a slightly larger expansion volume than head rice. Lower AC of the chalky kernels causes more soluble solid and higher iodine index during cooking indicating a lower eating quality than the translucent kernels. Chalky kernels are also reported to produce harder and less adhesive (or sticky) texture of cooked rice, requiring more time for chewing than head kernels (Chun et al., 2009). The % chalky grain of the samples ranged from 3.2 to 50.1%. Majority of rice imports from Vietnam (n=13) contained significant amount of chalky grains (10.6 - 50.1%). Out of the 45 collections, only the seven samples from Thailand have passed the recommended classification of Grade 1.

Uniformity of grain dimensions (length and shape) is an important factor considered by the consumers whose preference varies from one group to another. Filipino consumers usually prefer long (6.6 - 7.4 mm) and slender shape (>3.0) grains. Figure 4 shows that majority of the collected samples were long (58%) and slender (53%), but only 10 samples, mostly from Vietnam and Thailand, possessed both characteristics. As per country of origin, majority of the rice imports from Thailand were extra long (69%) and slender (88%), while samples from Vietnam were long (93%) and either intermediate (50%) or slender (50%). Rice imports from Myanmar were medium (83%) and intermediate (83%). The lone sample from India and Pakistan had long and extra long grain, respectively, but with same slender shape. Meanwhile, all the seven local rice samples were classified as long and intermediate grains (Figures 5 and 6).

Physicochemical properties

Properties such as moisture, AC, GT, and protein are key indicators of cooking and eating qualities of rice. The collected samples had moisture content ranging 9.7 - 12.6%, which is within the ideal range of 10 - 12% for milled rice (Juliano, 2007). AC is the key determinant of eating quality that could either be waxy (0 - 2.0%), very low (2.1 - 10.0%), low (10.1 - 17.0%), intermediate (17.1 - 22.0%), or high (22.1% and above). Rice with high AC tends to cook hard and dry while low-AC rice has softer and stickier cooked grains. Waxy rice, which has little or no amylose, is also referred to as glutinous rice. Filipino consumers prefer low to intermediate amylose varieties because of their fluffy and soft cooked texture. Majority of the collected samples (76%) had high AC (22.4 - 27.4%) including all the imports from Thailand and Myanmar and six each from Vietnam and Philippines (Figures 7 and 8). The remaining 24% (n=11) were intermediate

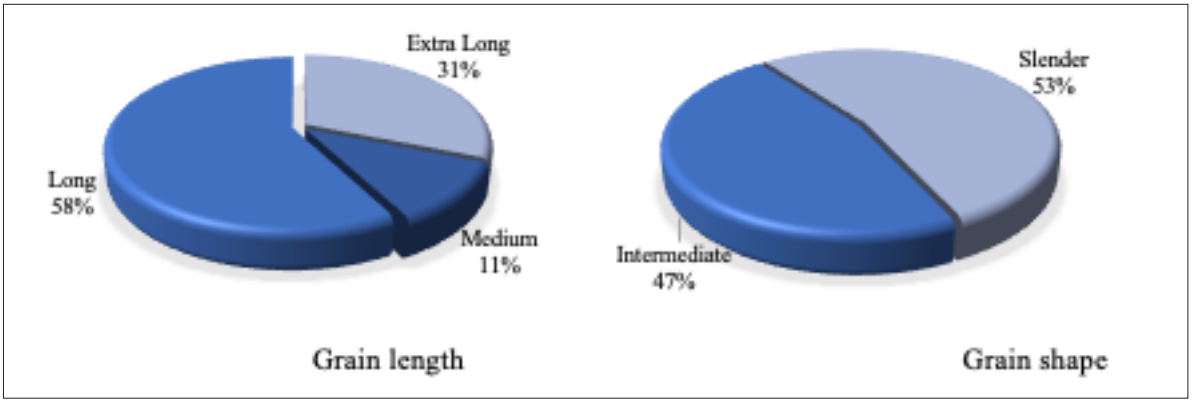

Figure 4. Percentage distribution of collected local and imported milled rice samples in terms of grain length and shape.

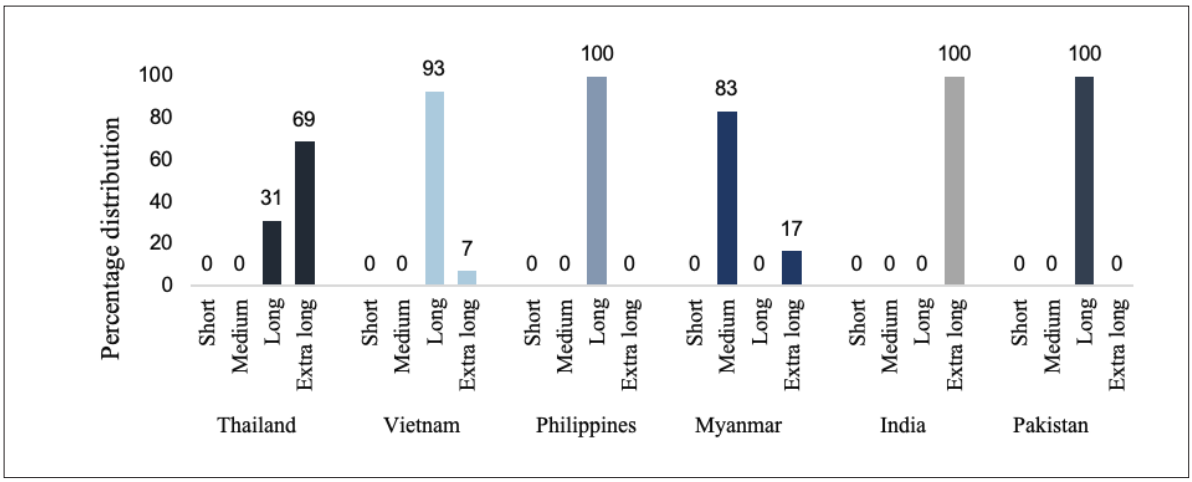

Figure 5. Percentage distribution of rice samples per country in terms grain length.

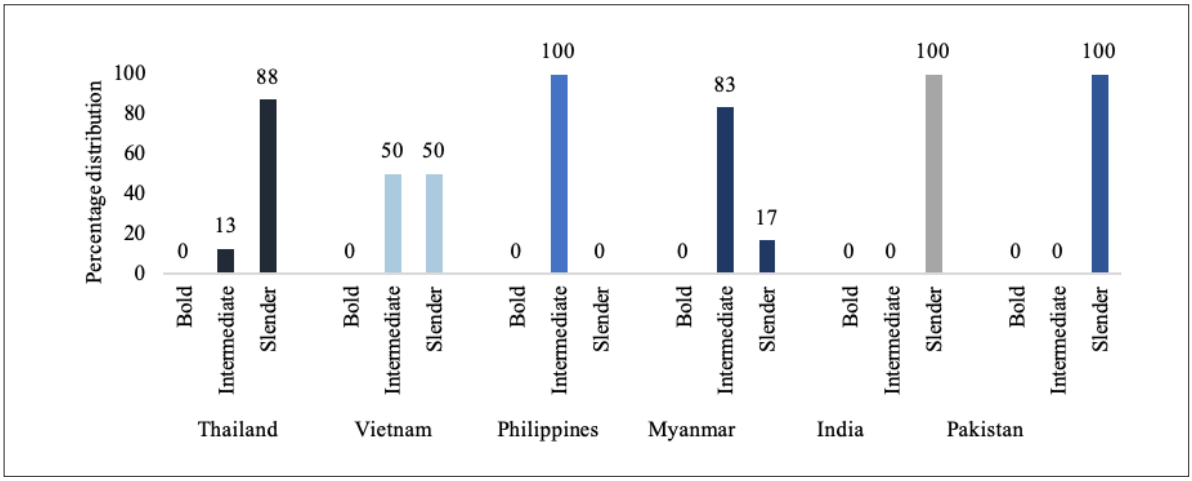

Figure 6. Percentage distribution of rice samples per country in terms of grain shape.

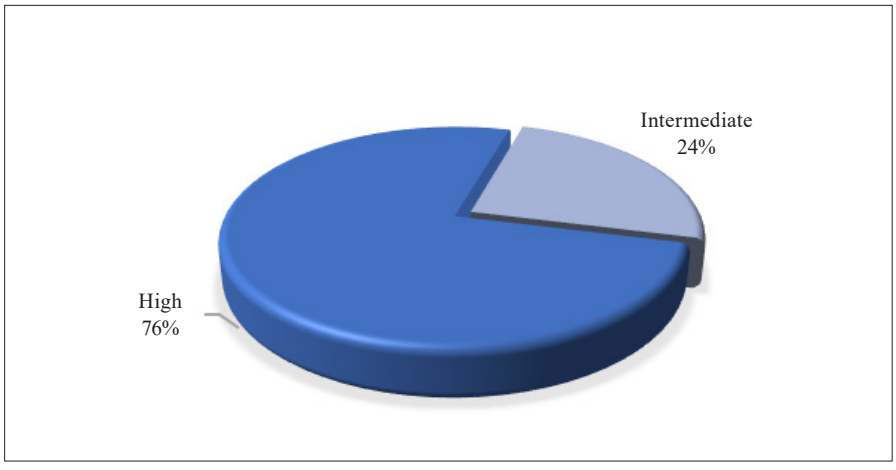

Figure 7. Percentage distribution of collected local and imported milled rice samples in terms of amylose content.

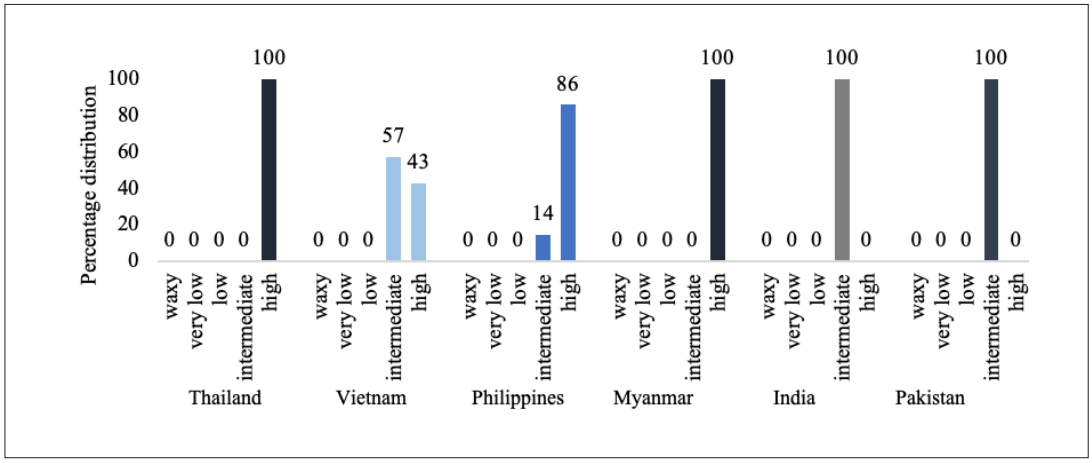

Figure 8. Percentage distribution of rice samples per country in terms of amylose content.

(18.8 - 21.7%), eight of which came from Vietnam, imported through G-to-G scheme. The other three samples were from India, Pakistan, and Philippines.

Another major factor affecting rice cooking quality is the GT. The starch granules GT is used to predict the cooking time of rice, which is indirectly measured using ASV. The GT of rice varieties usually ranges from 55 to 80°C grouped into: 6 - 7 low (< 70°C); 4 - 5 intermediate (70 - 74°C), 3 high-intermediate (74.5 - 79°C), and 1 - 2 high (>80°C) GT. Low GT rice cooks faster; high-GT rice becomes excessively soft when overcooked, elongates less, and requires more water and time for cooking compared with low or intermediate GT. Figure 9 shows that the rice samples had low to intermediate GT (3.7 - 6.8), indicating shorter cooking time at much lower cooking temperature. Rice imports from Thailand and Pakistan had low GT, while samples from Vietnam, Myanmar, Philippines, and India obtained intermediate GT classification.

The protein content of milled rice samples ranged from 5.9 to 8.3%, which is within the normal values of 6 - 9% . Aside from the nutritional benefit, protein contributes to the quality of cooked rice texture. Rice with high protein content tends to have a flakier and less sticky texture than with lower content of the same amylose category.

In terms of Instron cooked rice hardness, majority of the rice samples (96%) fell under the preferred soft to medium classification (1.38 - 2.50 kg/cm^2). All the rice imports from Myanmar and majority of the samples from Thailand and Philippines had medium cooked rice texture while most of the samples from Vietnam got soft classification (Figure 10). On the other hand, two samples from Thailand, both have high AC and low GT, obtained the hard classification.

Cooking quality

Samples were cooked using 1:1.6 rice to water ratio, which is 10 mL higher than the recommended ratio of 1:1.5. Higher ratio of water is often used for rice varieties with higher AC for complete doneness. For weight increase, the collected samples gained weight is 2 - 3 times their original weight (94.0 - 198.4%). Meanwhile, the height increase ranged from 150 to 316.7%. Five rice from Thailand were among the samples with the highest height increase. Furthermore, majority of the samples with the highest height increase were supplied by Olam International Ltd. The cooking time of samples ranged from 14 to 19 min. Among the samples with the longest cooking time (16 - 19 min) were five rice imports from Myanmar. On the other hand, majority of rice imports from Vietnam supplied by either Vinafood I or Vinafood II took shorter time (15 min) to cook.

Sensory properties

The sensory properties of rice samples are summarized in Table 3. All of the raw samples had no aroma and off-odor. Majority of rice samples from Thailand were dull and contained 21 - 40% chalky grains. The grains were hard, which corresponds to their high AC characteristics. Meanwhile, rice samples from Vietnam were slightly grayish, dull, and slightly hard. The samples were perceived to contain higher amount of chalky grains (41 - 60%), which is consistent with their actual value (10.6 - 50.1%). Rice samples from Myanmar were dull and slightly hard to hard grains with 21 - 40% chalkiness. Interestingly, rice imports from this country were among the few samples to have white grains. Sample from India and Pakistan both had dull, hard, and 21 - 40% chalky grains. Rice samples from the Philippines had slightly grayish, dull to slightly glossy, 21 - 40% chalky, and slightly hard grains.

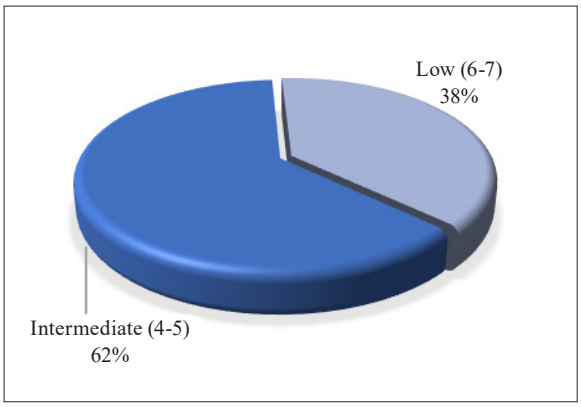

Figure 9. Percentage distribution of collected local and imported milled rice samples in terms of gelatinization temperature.

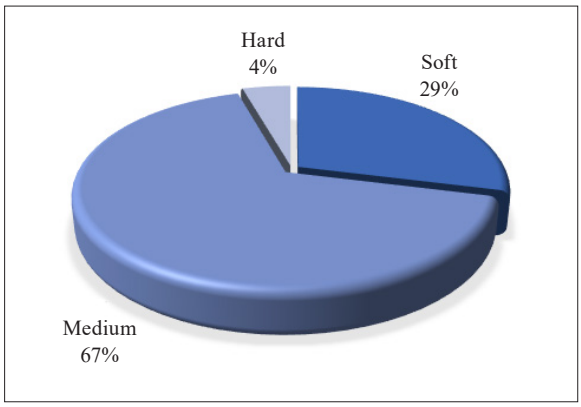

Figure 10. Percentage distribution of collected local and imported milled rice samples in terms of Instron cooked rice hardness.

Table 3. Sensory properties of the majority of samples per country of origin.

Origin Country	Raw Rice	Cooked Rice
Thailand	No aroma, no off-odor, slightly creamish/ slightly grayish, dull, 21 - 40% chalky, hard	No aroma, no off-odor, slightly creamish, slightly glossy, separated, hard to slightly tender, rough, bland, no off-taste
Vietnam	No aroma, no off-odor, slightly grayish, dull, 41 - 60% chalky, slightly hard	No aroma, no off-odor, slightly creamish, slightly glossy, slightly cohesive, slightly tender to tender, slightly smooth, bland, no off-taste
Philippines	No aroma, no off-odor, slightly grayish, dull to slightly glossy, 21 - 40% chalky, slightly hard	No aroma, no off-odor, slightly grayish/slightly creamish, slightly glossy, separated to slightly cohesive, slightly tender, slightly smooth, bland, no off-taste
Myanmar	No aroma, no off-odor, slightly white to white, dull, 21 - 40% chalky, slightly hard to hard	No aroma, no off-odor, creamish, slightly glossy, slightly cohesive, slightly tender, slightly smooth bland, no off-taste
India	No aroma, no off-odor, slightly grayish, dull, 21 - 40% chalky, hard	No aroma, no off-odor, slightly creamish, slightly glossy, slightly cohesive, slightly tender, slightly smooth, bland, no off-taste
Pakistan	No aroma, no off-odor, slightly creamish, dull, 21 - 40% chalky, hard	No aroma, no off-odor, slightly white, slightly glossy, slightly cohesive, slightly tender, slightly smooth, bland, no off-taste

For the cooked form, all the samples had no aroma, off odor, and off-taste, and were bland. Majority of the rice samples from Thailand were separated, hard to slightly tender, and rough. These characteristics are commonly observed among varieties with high AC and low GT. Meanwhile, majority of Vietnam rice samples had slightly creamish, slightly glossy, slightly cohesive, slightly tender to tender, and slightly smooth grains. Their softer cooked rice texture could be attributed to their intermediate or high AC and intermediate GT. Rice samples from Myanmar were slightly glossy, slightly cohesive, slightly tender, and slightly smooth. The color became creamish despite having white raw grains. Both samples from India and Pakistan had slightly glossy, slightly cohesive, slightly tender, and slightly smooth grains. Lastly, majority of the local rice samples were slightly grayish/slightly creamish, slightly glossy, separated to slightly cohesive, slightly tender, and slightly smooth. Rice samples collected from Aklan produced glossy, cohesive, tender, and smooth cooked rice.

Pesticide residue content of collected rice samples

Pesticide use helps reduce crop losses; however, continuous reliance on pesticides poses a serious threat to ecosystem and human health (Bordey et al., 2016). Results of the pesticide analysis showed that all the samples were below the MRL of organochlorine pesticides. Included here are the rice samples imported from Vietnam and Thailand wherein excessive pesticide application in rice production is reported. Additionally, the 45 rice samples were below the MRL of some organophosphate pesticides such as fenthion and chlorpyrifos. The fenthion content of the rice samples was <0.03 mg/kg, which is significantly lower than the 0.30 mg/kg limit. Similarly, the chlorpyrifos content of the samples (0.02 mg/kg) was below the 0.50 mg/kg limit. However, due to the detection limit of the equipment used, the actual concentrations of some organophosphate pesticides were still uncertain. It signifies that samples could

have higher or lower content than the MRLs of the compounds, example of this limitation is presented in Table 4.

Table 4. Organophosphate pesticide (Dichlorvos and Mevinphos) concentration of rice sample #1.

Compound	Content (mg/kg)	Limit of Detection (mg/kg)	MRL (mg/kg)*
Dichlorvos	<0.03	<0.03	0.01
Mevinphos	<0.03	<0.03	0.01

*EU Pesticide Database; Codex Alimentarius; Philippine National Standards

Heavy metal content of the collected rice samples

Rice is known to accumulate more metals than other cereals (Williams et al., 2007; Meharg et al., 2013; Khanam et al., 2020). The presence of heavy metals such as Pb, Cd, Hg, and As in rice has been reported in numerous studies including Fu et al. (2015), Jallad (2015), and Keshavarzi et al. (2015). In the Philippines, several studies on the heavy metal content of rice samples sold in the local market have been conducted. In 2012, Solidum et al., reported that rice samples (n=10) collected from the local markets in Metro Manila, including NFA rice, contained Cd ranging 0.0127 - 0.043 µg/g. Despite having concentrations below the safe limit of 0.2 µg/g, continuous consumption of these Cd-contaminated rice may still pose major health concerns for the population who relies on rice as their staple food. In a separate study, Solidum (2014) found that rice samples (n=10) sold in Metro Manila contained significantly high amount of Pb, which ranged from 0.2099 to 1.4795 ppm. The highest of which was detected in NFA rice.

Result of the heavy metal analysis showed that all of the rice samples were below the MRLs for As (0.20 mg/kg) and Hg (0.05 mg/kg). For Pb content, four imported rice samples supplied by Olam International Limited exceeded the MRL of 0.20 mg/kg (Table 5). Samples no. 35 and 37 from Myanmar had Pb content of 0.34 and 0.58 mg/kg, respectively. While sample no. 34 from Vietnam contained 0.48 mg/kg of Pb. The highest Pb concentration (0.64 mg/kg) among the samples was obtained in sample no. 36 from Thailand. In terms of Cd content, two samples from Vietnam exceeded the MRL of either 0.20 or 0.40 mg/kg. Sample no. 2 contained 0.54 mg/kg while sample no. 7 has 0.38 mg/kg of Cd (Table 6). Long-term consumption of these contaminated rice can pose potential health risks, especially to the vulnerable groups: pregnant women, children, elderly, and patients.

Table 5. Lead content of four samples from Vietnam (1), Myanmar (2), and Thailand.

Sample No.	Country of Origin	Supplier	Pb (mg/kg)	MRL (mg/kg)*
34	Vietnam	Olam International Limited	0.48	0.20
35	Myanmar		0.34	
36	Thailand		0.64	
37	Myanmar		0.58	

* Philippine National Standards

Table 6. Cadmium content of two samples from Vietnam.

Sample No.	Country of Origin	Supplier	Cd (mg/kg)	MRL (mg/kg)*
2	Vietnam	Vinafood I	0.54	0.20[a]; 0.40[b]
7			0.38	

[a] Commission Regulation (EC) No 1881/2006 (EU);
[b] Codex Alimentarius: General Standard for Contaminants and Toxins in Food and Feed (CXS 193-1995); Philippine National Standards

Conclusion

This study concludes that the grain quality characteristics of the NFA rice samples were associated with the country of origin, supplier, and procurement scheme. Based on the NCT method, collected samples generally possessed good grain quality characteristics and even passed some of the preferred characteristics of most Filipinos like grain dimension, Instron cooked rice hardness, AC, and GT. Majority of the samples also passed the standards for pesticide and heavy metal, except for the six imported samples, which exceeded the MRL for Pb and Cd. Thus, strict regulatory system must be implemented to protect the consumer from the potential health threats brought by contaminated rice samples. The information generated from this study can be used by policy makers in crafting relevant procedure on quality and safety of local and imported rice in the Philippines.

Acknowledgment

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IMPROVED SOMACLONES WITH MULTIPLE ABIOTIC TOLERANCE DERIVED FROM RICE LANDRACE *Y Dam Do*

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Abstract

Genetic variability enhancement is important in rice breeding to ensure selection of superior genotypes and improve specific undesirable characteristics of crops. *In vitro* culture (IVC) was utilized in 2008 dry season (DS) to induce genetic variation and improve the landrace '*Y Dam Do* in terms of phenotype. IVC resulted in generating somaclonal variants with improved phenotype. Tissue culture response showed that the genotype incurs a 2.9% callus formation and 8.3% plan regeneration efficiency. IVC_2 population showed variability in morphological traits during reproductive stage such as, flag leaf angle, panicle exsertion, culm angle and panicle branching. It also showed variability in major agronomic traits such as flowering, plant height, and tillering, revealing the phenotypic improvements induced by tissue culture that are acceptable to farmers. Evaluation for tolerance to abiotic stress specifically drought, salinity, and submergence at seedling stage identified somaclones with single or combined tolerance to the stresses. Further evaluation showed a 6 - 7 t ha^{-1} yield potential of the IVC lines under irrigated condition and 1 - 2 t ha^{-1} under managed drought. Check variety PSB Rc 14 only yielded 5 and 0.9 t ha^{-1} in irrigated and managed drought, respectively. Under submergence stress, yield potential of the lines was 1 - 2 t ha^{-1}, while the check variety IR64-Sub1 and PSB Rc68 yielded 3 and 2 t ha^{-1}, correspondingly. The evaluations led to the selection of three promising lines that are potential novel donors for combined abiotic tolerance in rice breeding. The output can also be nominated to the National Cooperative Test (NCT) as a potential variety for multiple stress-affected rice ecosystems.

Keywords: *In Vitro Culture, Somaclonal Variant, Multiple Tolerance, Drought, Saline, Submergence.*

Introduction

Changing climate has caused the occurrence of not just a single, but a combination of stresses in marginalized rice producing areas in the Philippines (Karl et al., 2009). Thus, the necessity to develop rice genotypes that can survive multiple abiotic stresses. Tissue culture has been used in rice breeding since its introduction by Haberlandt, a German scientist in the beginning of the 20th century (Thorpe, 2012), as one of the strategies in developing crops with improved abiotic stress tolerance. During culture process, uncontrolled and spontaneous variation known as somaclonal variation occurs. These variations are mainly due to novel mutations triggered by several factors such as wounding, exposure to mutagens, plant growth regulators, and other environmental factors (Krishna et al., 2016). Somaclonal variation results from base deletion or substitution, changes in chromosome number, chromosome rearrangements, or changes in epigenetic marks (Larkin and Scowcroft, 1981). Tissue culture-derived plants provide a genetic variability, which can be exploited for crop improvement and breeding through the creation of novel variants. Utilization of germplasm in rice breeding that may introduce high genetic variability in the field will lessen the vulnerability of rice crop to disease epidemics and insect infestation. Traditional rice varieties (TRVs), wild rices, and landraces are genetically and morphologically diverse germplasms, represent a gene pool for valuable traits such as abiotic stress tolerance.

The landrace *Y Dam Do* originated from Laos and was introduced to the Philippines in 1993. It is saline and drought-tolerant; however, it is low yielding and possesses poor phenotype. IVC was utilized to induce genetic variation to improve its phenotype such as flowering, plant height, plant type, tillering, panicle and grain traits, and yield ability, but with retained tolerance to abiotic stress. The identified improved mutant lines can be used as gene source for abiotic stress tolerance or can be nominated to NCT for possible release as a new variety for stress-prone rice environments.

Materials and Methods

The study evaluated somaclonal variants derived from seed culture of the rice landrace, *Y Dam Do* (Figure 1). The landrace was sourced out from the Philippine Rice Research Institute (PhilRice) Gene Bank. Researchers in this study characterized the landrace (Table 1) as secondary research produced inadequate information and literature about the subject. The landrace was selected because of its tolerance to multiple stresses and long-clustered panicles; however, it is late maturing, tall, and has low-tillering ability.

In vitro culture : Seed cleaning, sterilization, and callus induction

Rough rice grains were dehulled using Satake rice tester and were cleaned manually, removing broken and mix grains. Dehulled grains were sterilized with 50% (v/v) sodium hypochlorite with agitation at 200 rpm for 30 min, using Thermo Scientific MaxQ2000 orbital shaker and then rinsed with sterilized distilled water thrice. The procedure is repeated for another 30 min before blotting dry the grains in sterile petri plates, inside the laminar flow hood. The dried seeds were cultured in MS callus induction medium (Murashige and Skoog, 1962), hardened with 3 g L^{-1} agar (Pronadisa) and 2 g L^{-1} gelrite (Sigma) and autoclaved in TOMY SX-7000 autoclave at 115 psi for 15 min. The cultures were incubated in the dark at 27˚±2˚C for two weeks, resulting in callus tissues.

Table 1. Characteristics of *Y Dam Do*.

Morphological Trait	
Flag leaf angle	semi-erect
Culm angle	semi-erect
Panicle exsertion	well exserted
Panicle type	compact
Secondary branching	clustered
Leaf senescence:	early
Agronomic Trait	
Days to heading	92 days after seeding (DAS)
Culm length	108 cm
Plant height at maturity	135 cm
Panicle length	27 cm
Productive tiller	13 tillers
Phenotypic acceptability	7 (poor)
Grain Yield	2.007 tha^{-1}
Grain Trait	
Grain length	9.3 mm (extra-long)
Grain width	3.3 mm (broad)
Grain shape	2.8 mm (intermediate)
Endosperm color	white
Hull color	brown furrows in straw
Abiotic Stress Response	
Drought	Tolerant
Salinity	Tolerant
Submergence	Susceptible

Figure 1. Plant type and height (a), panicle (b), hulled (c) and dehulled grains (d) of *Y Dam Do*.

Characterization data show that *Y Dam Do* is tolerant to drought and salinity stress; however, it possesses poor phenotype (Cabusora et al., 2010; Valida et al., 2015; Balmeo et al., 2016; Buluran et al., 2018).

Plant regeneration, IVC_1 family, and IVC_2 seed generation

The calli generated were transferred into the regeneration medium containing similar components of the MS basal medium except for 1 mg ml^{-1} 6-BAP and 2,4-D. A different concentration of 0.5 mg ml^{-1} NAA and 2 mg ml^{-1} kinetin was used. The calli were incubated on lighted benches at 25±2˚C, at 16-day/8-night hour cycle until plantlets (IVC_1 generation) were fully regenerated with fully developed shoots and roots. Plantlets were hardened for 3 - 5 days under laboratory condition and for seven days in the head house before transplanting in the screenhouse. Plants were grown until maturity and IVC_2 generation seeds were harvested. IVC_2 plants were evaluated for variability in agro-morphological traits during vegetative and reproductive stages from which plants with improved phenotype were selected. These were further evaluated for another season to ensure the uniformity and stability of the IVC lines. Figure 2 shows the generation advance using *in vitro* culture breeding strategy.

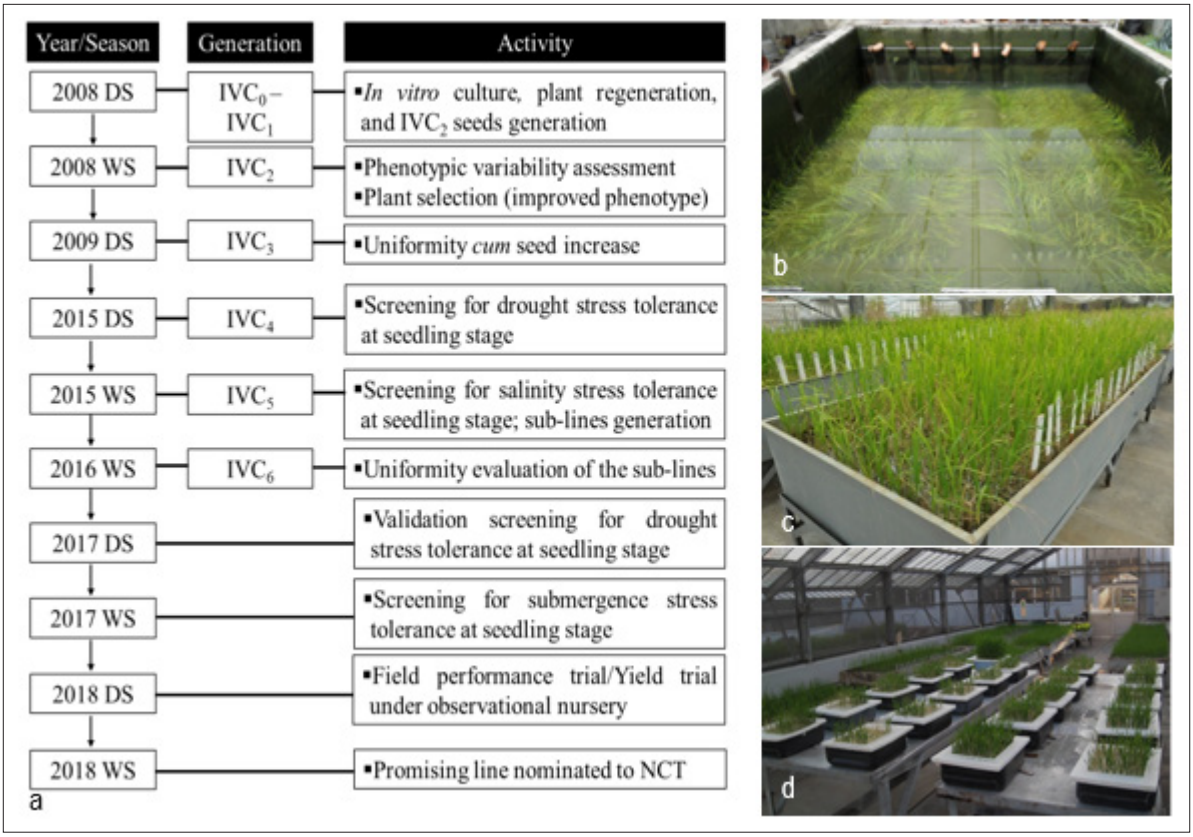

Figure 2. Generation advances using IVC technique from 2008 DS to 2018 WS (a); drought (b), salinity (c) and submergence (d) screening set-ups.

Evaluation for abiotic stress tolerance at seedling stage: Drought, salinity, and submergence

Drought tolerance was evaluated in a 5 m (length) x 1 m (width) x 0.4 m (depth) galvanized trays under greenhouse condition. The trays were filled with garden soil-sand mix at a ratio of 1:1 by weight. The checks, IR64 (susceptible), PSB Rc 14, and NSIC Rc 222 (tolerant), the wildtype, *Y Dam Do*, and the IVC lines were established randomly in unreplicated trays. Irrigation water was withdrawn 21 DAS and re-watered when IR64 reached leaf drying (LeD) score of 7. Leaf rolling (LeR), leaf drying (LeD), and drought recovery rate (DRR) were observed based on the Standard Evaluation System (SES) for Rice (IRRI, 2015). Salinity tolerance was screened through seedling floats with 100 holes, spaced at 10 cm x 10 cm and suspended in a 14-L capacity carboy trays. Checks FL478 (tolerant) and IR29 (susceptible) were established individually on the leftmost and rightmost of each seedling floats, respectively. Yoshida hydroponic solution was used as growing medium while table salt was used as source of salinity, with electrical conductivity (EC) adjusted from 6 to 16 dS m^{-1}. Salinization was done at 11 days after seeding while pH and EC were monitored daily. Tolerance score was observed based on visual leaf injury score (Gregorio et al., 1997) at 14 days after salinization.

Submergence tolerance was evaluated in seedling trays of 60 cm (length) x 31 cm (width) with 24 cm depth and submerged in a cemented tank with dimensions of 4 m (length), 3 m (width), and 1.1 m (depth). The FR13A (tolerant) and IR42 (susceptible) checks were established individually on the leftmost and rightmost of each seedling trays. Seedlings were submerged 14 DAS. Survival and tolerance were assessed 21 days from de-submergence based on SES for Rice (IRRI, 2015).

Field performance evaluation: Non-stress and stress condition

The selected IVC lines were evaluated for field performance under irrigated (IL), managed drought (MDR), and submerged conditions (SUB). The trials were established in a Randomized Complete Block design with three replications.For MDR, drought was imposed by withholding water once the test entries reached panicle initiation stage and re-watered when the susceptible check reached the LeD score of 5. Water table depth was monitored by installing three piezometers (1.5 m long) and a rain gauge. For submergence set-up, stress was imposed 21 days from transplanting that lasted for 8 days. Set-

up was drained when tissues decay and foul odor were observed in IR42 check. Water quality during submergence was monitored daily. Survival under complete submergence was observed 21 days after de-submergence.

The recommended fertilization rate of 120-60-60 during dry season was used for the IL and MDR conditions. Basal application of complete 14-14-14 at 240 g NPK/5.6 m^2 was also administered. The remaining N (46-0-0) at 73 g N/5.6 m^2 was top-dressed during panicle initiation. For SUB, fertilizer was applied only after the plants were fully recovered (14 days after submergence) and at panicle initiation. Urea at 12.5 kg ha^{-1} each was applied at 14 days after de-submergence and at panicle initiation.

Data Analysis

The variability and screening data were analyzed using descriptive statistics, histogram, and the Shanon Weaver Diversity Index (Hutcheson, 1970). Field performance data was analyzed by Analysis of Variance and Tukey's comparison of means using the Statistical Tool for Agricultural Research (STAR), Version 2.0.1 (IRRI, 2013). To determine the significance in yield reduction, estimate analysis was carried out using the SAS Portable Version 9.3.1 (SAS Institute, Inc., 2002).

Results and Discussion

In vitro culture response

The mature seeds of the landrace *Y Dam Do* were *in vitro* cultured in 2009 DS (Figure 3). There were 420 seeds cultured in callus induction medium, from which 12 (2.9%) seeds were induced to callus formation, 269 (64%) produced coleoptile, while 139 (33%) seeds did not respond. Plant regeneration was observed in 1(8.3%) callus only, from which 8 IVC_1 green plants were regenerated (Table 2). Regenerated plants were grown to maturity under screenhouse condition and IVC_2 seeds were harvested.

Genetic composition was identified as one of the factors influencing tissue culture responses (Niroula et al., 2005). It is known that some genotypes are recalcitrant to *in vitro* manipulation because of their poor callus production and regeneration ability (Benson, 2000). Studies of Abe and Fustsuhara (1986) showed that *japonica* varieties exhibited a higher rate of callus induction and regeneration compared with *indica*.

Variability evaluation in agro-morphological traits

One hundred-twenty IVC_2 plants were generated from the 8 IVC_1 plants regenerated *in vitro.* These were evaluated for variability in agro-morphological traits during vegetative and reproductive stages in 2008 WS. No variation was observed in leaf blade pubescence, leaf blade color, leaf sheath color, collar color, ligule shape and color, and auricle color during the vegetative stage. However, variations were observed in the reproductive stage. Flag leaf angle of the 78 (65%) plants were erect, 37 (30.8%) were intermediate, while the remaining 5 (4.2%) were horizontal (Figure 4a). Majority of the population (63%) had an erect culm angle while the rest (37%) had semi-erect (Figure 4b). Most of the plants had moderate panicle exsertion, while the rest were distributed to well exserted, just exserted, partly exserted, and enclosed (Figure 4c). Majority (59%) of the plants have heavy secondary panicle branching while no branching, light branching, and clustered

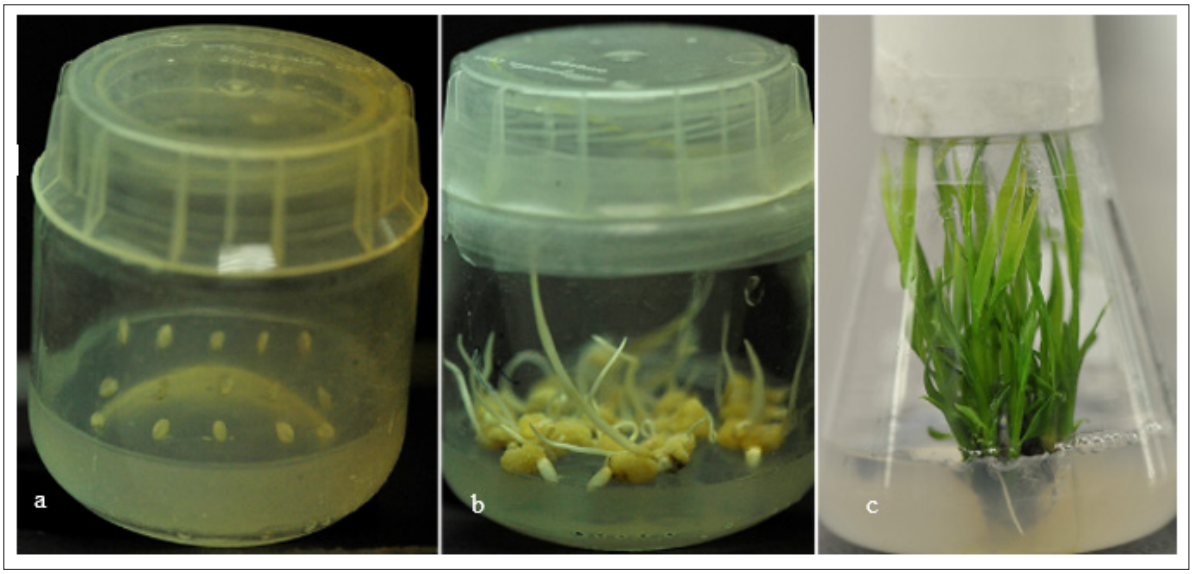

Figure 3. Cultured seeds of *Y Dam Do* in callus induction medium (a), callused seeds in 2-week culture (b), and callus with multiple regenerants in 4-week culture (c).

branching were observed in 9%, 13%, and 22% of the population, respectively (Figure 4d). Forty-four percent or majority of the plants have compact panicle type (Figure 4e) and also most of the plants (50%) have late leaf senescence (Figure 4f). The Shannon-Weaver Diversity Index (SWI) was computed to assess the phenotypic variation for each morphological trait. Most varied traits among the population have the highest SWI value. The SWI of panicle exsertion, secondary branching, panicle type, leaf senescence, flag leaf angle, and culm angle was 5.6, 2.9, 2.86, 2.58, 1.78, and 1.58, accordingly. Highest variability was observed in panicle exsertion but lowest in culm angle. This implies the possibility of a wider range selection in plant height and efficiency in pollen dehiscence.

Variation in agronomic traits was also observed among the generated IVC_2 plants as indicated in the population coefficient of variation (CV). CVs of the population for days to flowering, plant height, culm length, and panicle length were 10.2%, 19.8%, 15.4%, and 12.9%, correspondingly (Table 3). Days to first flowering of the 96 (80%) plants were 6 - 21 days earlier than the wild type with 92 DAS while 24 (20%) plants were comparable with this trait. Histogram of the trait indicated that most (53%) of the plants had earlier days of flowering in reference to the population mean of 77 DAS (Figure 5a). Early flowering, which is correlated to maturity, is important in selecting early maturing lines as it can contribute to the survival under drought stress condition (Shavrukov et al., 2017). Highest percentage or 96% of the population have culm lengths shorter than the wild type while the remaining 4% either have comparable or longer culm length. The plant height of the majority (96%) of the plants were 8.9% (123 cm) - 66.7% (45 cm) shorter than the wild type, standing at 135 cm. Taller height by 14.8% (20 cm) was observed in 2 (2%) plants while comparable height was also observed in 2 (2%) plants.

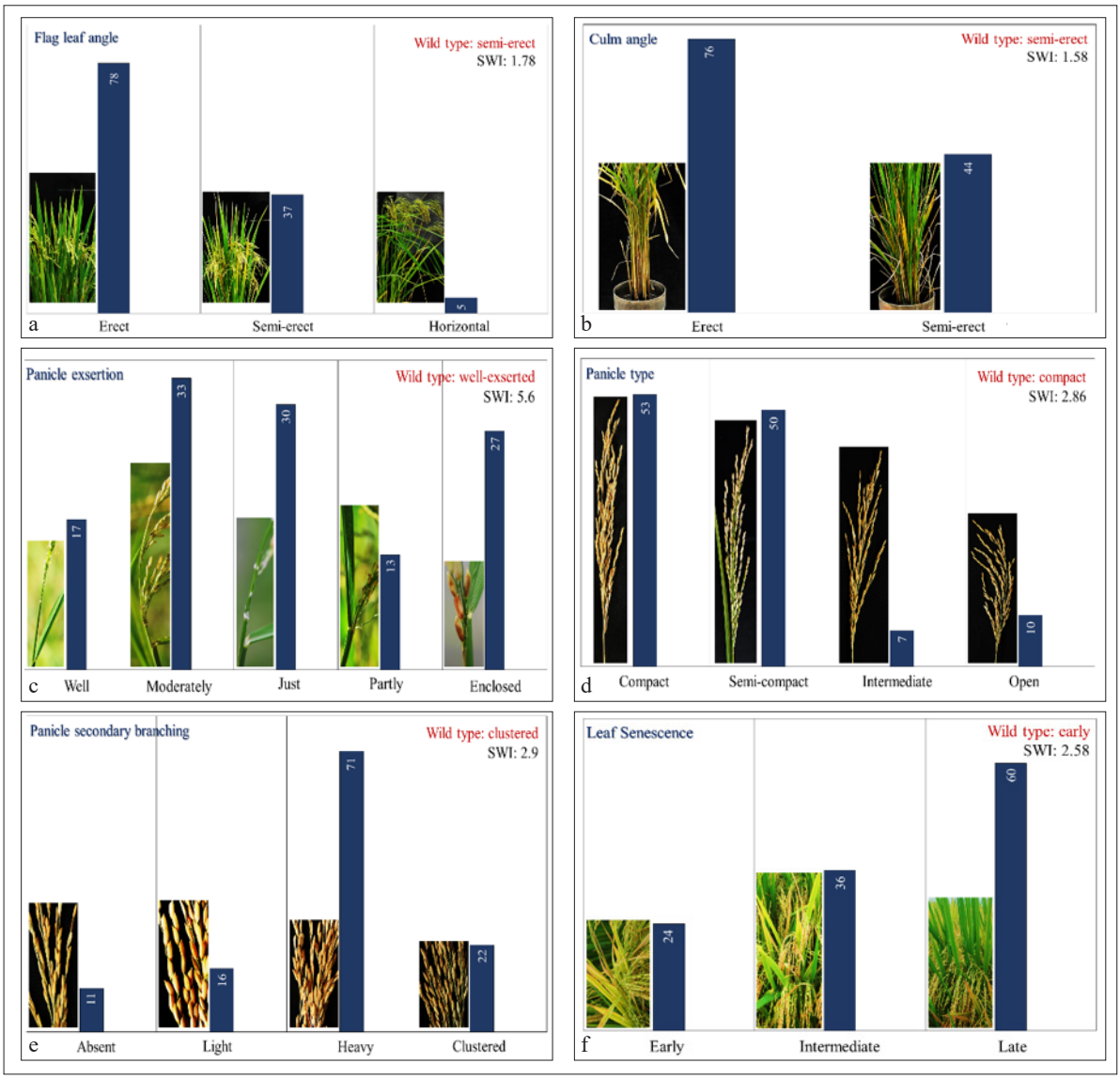

Figure 4. Variations in morphological traits of the IVC2 population observed at reproductive stage: flag leaf angle (a); culm angle (b); panicl,e exsertion (c); panicle type (d); panicle secondary branching (e); leaf senescence (f). PhilRice CES, 2008 WS.

Table 3. Population statistics for the four major agronomic traits, PhilRice CES, 2008 WS.

Descriptive Statistics	Number of Days to First Flowering (DAS)	Culm Length (cm)	Plant Height at Maturity (cm)	Panicle Length (cm)
Minimum	69	30	45	15
Maximum	97	127	155	33
Range	28	97	110	18
Population mean	76.6	66.0	89.9	24.0
Standard deviation	7.82	13.03	13.83	3.08
Population coefficient of variation	10.2	19.8	15.4	12.9

Positive Skewness for culm length (Figure 5b) and plant height (Figure 6a) indicates that there are more plants shorter in reference to the population mean (89.9 cm). Majority of the plants (53%) have comparable panicle length to the wildtype (27 cm), 2 (1.7%) plants have longer panicles while 54 (45%) have shorter panicles. Skewness of zero indicates varied panicle lengths among the population (Figure 6b).

At post-harvest stage, variations in grain morphology were observed including, hull color, endosperm color, grain length, grain width, and grain shape. Of the 120 plants, 90% had straw colored hull while 9.2% had brown furrows on straw identical to the wild type (Figure 4a). Majority (95%) of the population have brown kernel color and the rest (5%) have white glutinous-like kernel, same as the wild type (Figure 7b).

The grain length of 59 (49.2%) plants was shorter by 0.02 - 0.97 mm than the wild type with 9.3 mm. Meanwhile, 60 (50%) plants were longer by 0.5% (0.05 mm) to 9.2% (0.86 mm). Positive Skewness indicates that more plants have shorter grain length compared with the population mean of 9.2 mm (Figure 8a). Ninety-six percent of the plants had 10.3% (0.3 mm) to 32.7% (1.1 mm) increase in width, whereas 3.3% had reduction of 2.2% (0.1 mm) to 6% (0.2 mm), and 1 (0.8%) plant comparable with the wild type (3.3 mm). Skewness for grain width is almost zero which indicates a normal distribution (Figure 8b). Changes

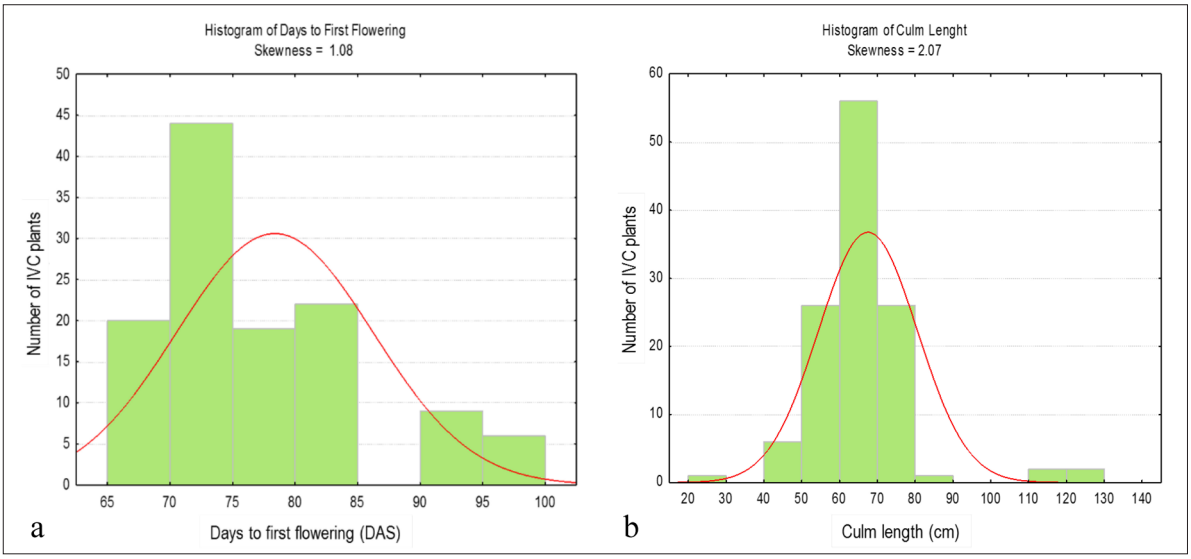

Figure 5. Variation and frequency distribution in days to flowering (a) and culm length (b) observed in the IVC2 population. PhilRice CES, 2008 WS.

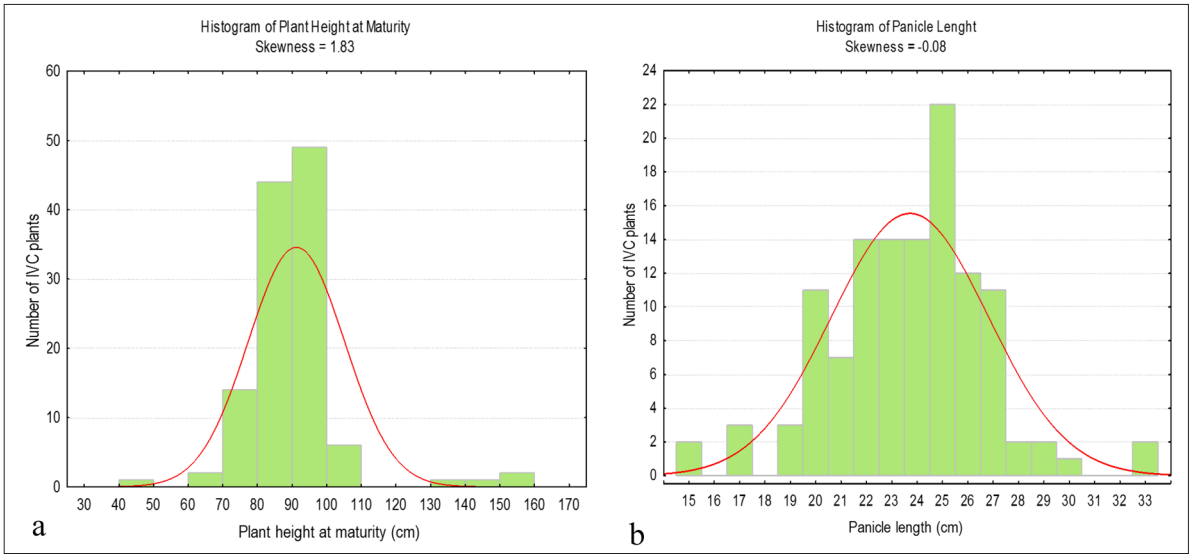

Figure 6. Variation and frequency distribution in plant height (a) and panicle length (b) observed in the IVC_2 population. PhilRice CES, 2008 WS.

Figure 7. Variation in hull (a) and endosperm (b) color observed among the IVC2 plants compared with the wildtype, *Y Dam Do*. PhilRice CES, 2008 WS.

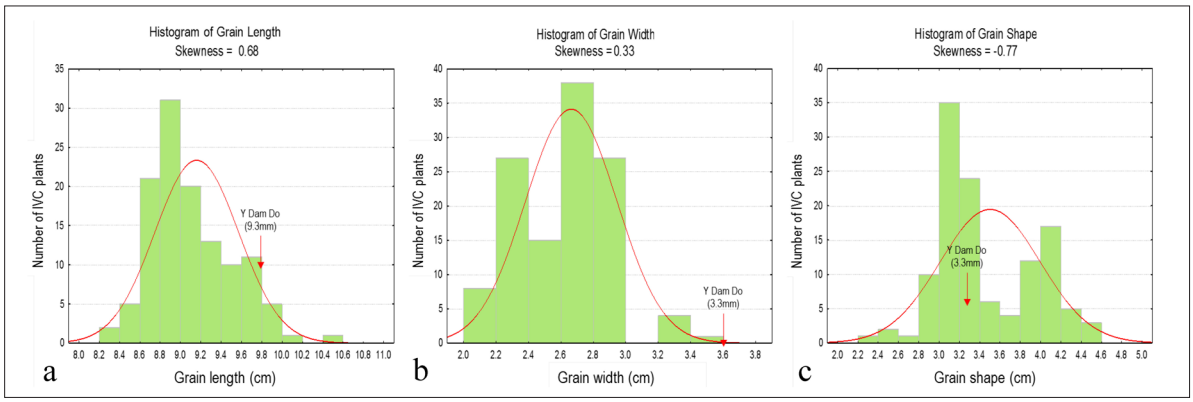

Figure 8. Variation and frequency distribution in grain morphology observed in the IVC_2 population: grain length (a), grain width (b), and grain shape (c). PhilRice CES, 2008 WS.

in grain length and width caused variations in grain shape, incurring reduction in 3.3% of the population by 5.3% (0.1 mm) - 16.2% (0.5 mm) and increased in 95% of the population by 5% (0.1 mm) - 59.9% (1.7 mm), in reference to the wild type with 2.8 mm grain shape (Figure 8c).

Tissue culture has been known to induce variation in phenotype and genotype of the regenerated plants (Orlowska et al., 2020; Fiuk et al., 2010). These are termed as somaclonal variations that can be inherited through a generative cycle (Morrison et al., 1988). The variations may be visible in the plant's morphological, biochemical, genetic, or epigenetic traits. The totipotency of the plants can be associated with genetic and epigenetic instabilities that are often translated into heritable phenotypic variations (Wang et al., 2015). Phillips and colleagues (1994) proposed that variations induced via tissue culture is a "self-imposed" mutagenesis that is largely attributable to the breakdown of normal cellular controls for genetic and epigenetic integrity. Recent revolutionary outbreaks in analytical tools such as the next generation sequencing (NGS) have established the molecular nature, mutation rate, and spectrum of somaclonal variation at the molecular level (Bednarek et al., 2021). The variability evaluation of the IVC_2 population selected 94 plants with improved plant type, and were advanced to further generations for seed multiplication, evaluation, and selection. Induced variability in agro-morphological traits is an advantage in breeding as it provides a wide platform for rice breeders in selecting superior genotypes with acceptable phenotypes (Roy and Shil, 2020).

Screening for seedling drought stress tolerance

The selected somaclones were screened for seedling drought tolerance in 2015 DS. Leaf rolling was observed 11 days after drought imposition (DI), an indication of moisture stress presence. Drought recovery rate (DRR) of the selected IVC lines ranged from 10.6% to 97.6% (Figure 9a), from which 64 IVC lines have 2 - 63% higher recovery than the wild type, with a DRR of 60% (Figure 9b). Eighty (85%) and 14 (15%) IVC lines were identified tolerant and susceptible to seedling drought stress, accordingly (Figure 9c). Soil moisture of 17 - 1.2% (Figure 9d) was recorded from DI to re-watering, when IR64 reached LeD Score of 7, 20 days from DI.

Screening for salinity stress tolerance at seedling stage

The 94-drought tolerant IVC lines were subjected to salinity tolerance at seedling stage in 2015 WS (Figure 10). The results identified 56 (60%) and 26 (28%) IVC lines that are tolerant and moderately tolerant, respectively (Table 4).

Table 4. Frequency distribution of the IVC lines for salinity tolerance at seedling stage, PhilRice CES, 2015 WS.

Tolerance Score	IVC Lines	
	Number	%
Highly tolerant	16	17.0
Tolerant	39	41.5
Moderately tolerant	25	26.6
Susceptible	10	10.6
Highly susceptible	4	4.3
Total		94

From the identified saline tolerant IVC lines, three plants were selected and transplanted, which created 246 sub-lines for each line. However, only 218 (87%) plants survived the maturity stage and produced seeds. These sub-lines were established panicle-to-a-row under field condition in 2016 WS for uniformity evaluation, in which 174 (80%) lines were similar in plant height and maturity.

Screening for submergence stress tolerance at seedling stage

The selected uniform lines were screened for submergence stress tolerance at seedling stage in 2017 WS. Water quality parameters such as pH, temperature, and dissolved oxygen (DO) were measured to describe the growing condition of the

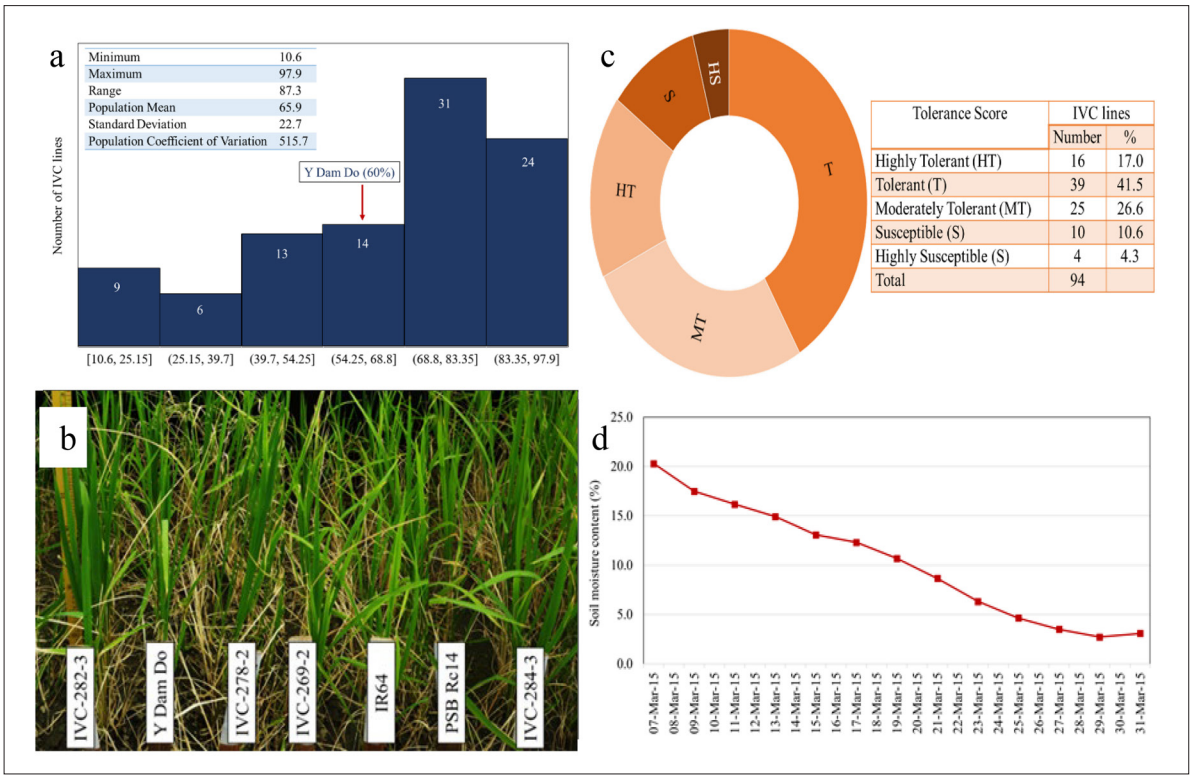

Figure 9. Drought stress tolerance response of the IVC lines: DRR (a), recovering plants, 14 days after rewatering (b), frequency of tolerant and susceptible lines (b) and soil moisture content (c). PhilRice CES, 2015 DS.

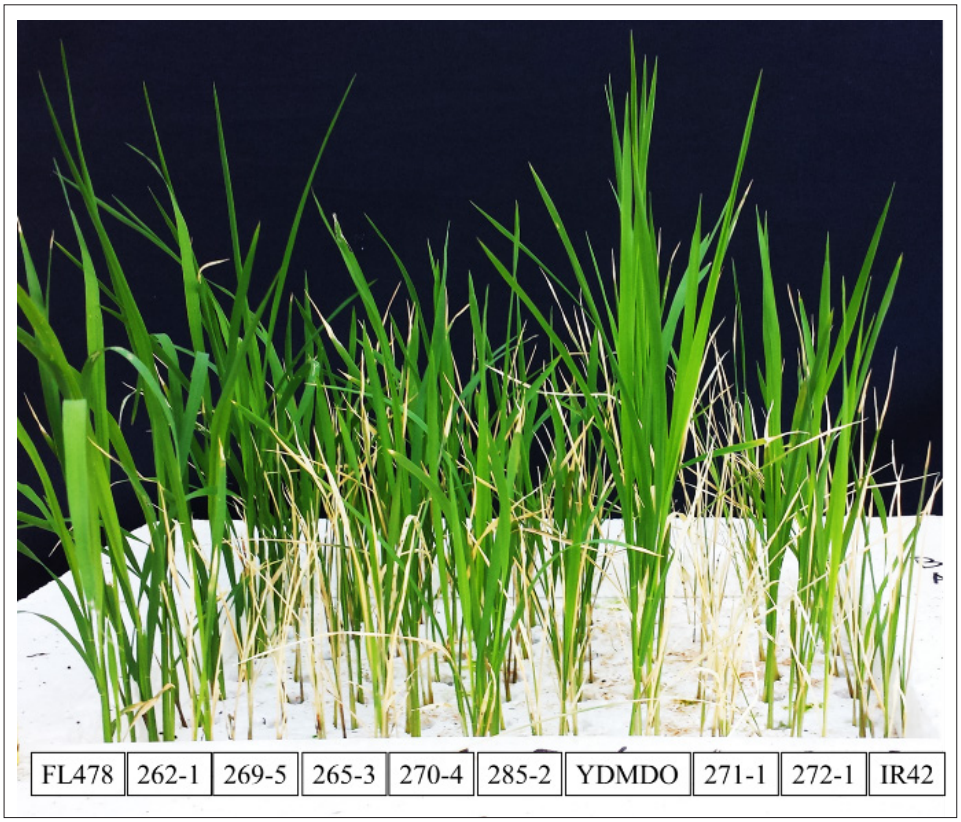

Figure 10. Response of the IVC lines to salinity stress at seedling stage. PhilRice CES, 2017 WS.

plants under submergence for 14 days (Figure 11a). The pH of the water from submergence imposition to de-submergence ranged from 6.1 to 7.8 while temperature and DO ranged from 26.9 to 30.5°C and 2.6 - 17.5%, respectively. The pH and temperature of the water seems to be at the optimum level; however, oxygen availability was low. Under submergence, low O_2 level results in hypoxic condition (<21% O_2) affecting respiration in plants and causes increase in the production of phytotoxic compound intensifying the fermentation processes; thereby, causing decomposition of plants (Fukao et al., 2019; Dennis et al., 2000; Sarkar et al., 1996; Setter et al., 1990). Survival of the IVC lines under complete submergence ranged from 0 to 80% (Figure 11b). The screening identified 3 IVC lines (2%): IVC-265-3, IVC-284-3, and IVC-285-1 tolerant to complete submergence (Figure 11c) and 171 IVC susceptible lines (98%).

Screening of the 218 IVC lines for drought, saline, and submergence tolerance at seedling stage identified 82 (38%), 113 (52%), and 3 (1%) lines with single tolerance to drought, salinity and submergence, respectively. The screening also identified 113 (52%) IVC lines with combined tolerance to salinity and drought, 3 (1.4%) IVC lines, each with combined tolerance to salinity and submergence, submergence and drought, and with tolerance to the three stresses (drought, saline, and submergence).

Field performance of the IVC lines under irrigated and managed drought stress condition

In 2018 DS, 18 IVC lines with combined abiotic stress tolerance were selected and evaluated in observational nursery for field performance under irrigated (IL) and managed drought (MDR) conditions (Figure 12a and 12b). Piezometer reading showed a 20 - 120 cm depth of water table indicating the progression of drought stress from the imposition at 37 days after transplanting (Figure 12c). Under IL, the average days to heading of the lines was 93 DAS while under MDR, the heading days were delayed by 7 days. Plant height under IL ranged from 85 to 118 cm, while height was reduced by 25 to 63 cm under MDR. Productive tillers in IL ranged from 12 to 16 tillers while 15 - 26 tillers were recorded under MDR (Table 5).

In rice plants, drought stress delays flowering to avoid reproductive growth under unfavorable condition (Bocco et al., 2012; Zhang et al., 2006). Recent transcriptomics studies revealed that drought condition causes reduced transcription of the Ehd1 florigen gene and accumulation of Hd3a and RFT1 genes, resulting in a delay in flowering (Galbiati et al., 2016). Furthermore, plant height is significantly reduced under drought stress as penalty to growth. Plant height is affected because rice plants use their

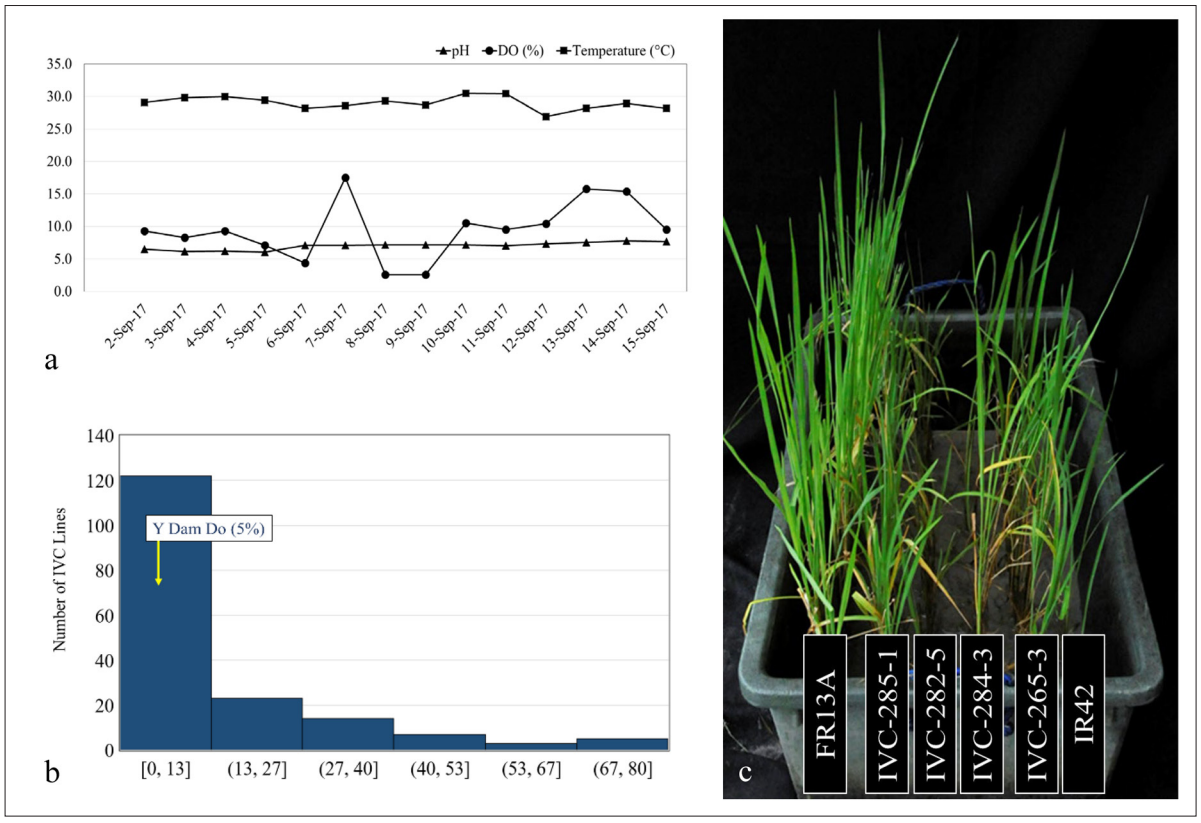

Figure 11. Quality of the water at 14 days of submergence (a), survival of the IVC lines under complete submergence, identified submergence tolerant IVC lines in comparison with FR13A and IR42. PhilRice CES, 2017 WS.

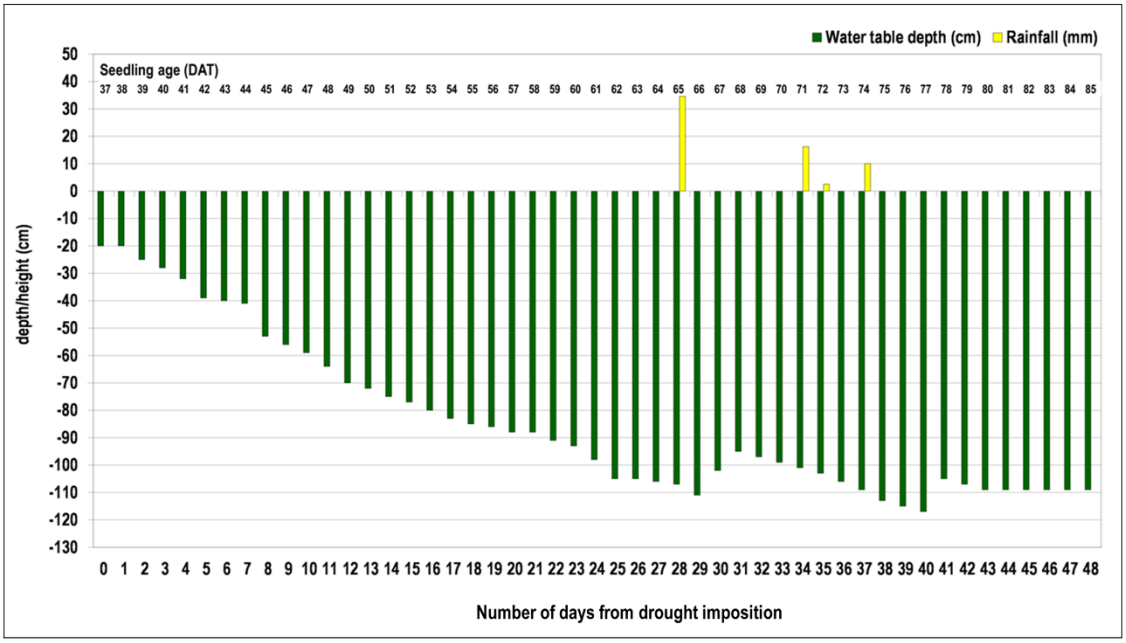

Figure 12. Field performance trial of the IVC lines under IL (a) and MDR (b) conditions at maturity; water table depth (cm) of the MDR set-up from drought imposition up to rewatering. PhilRice CES, 2018 DS.

Table 5. Agronomic traits of the IVC lines under irrigated (ILD) and managed drought stress (MDR) conditions, PhilRice, CES, 2018 DS.

Descriptive Statistics	Days to Heading (DAS)		Plant Height (cm)		Number of Productive Tiller	
	ILD	MDR	ILD	MDR	ILD	MDR
Minimum	80	78	85	60	12	15
Maximum	101	123	118	95	16	26
Mean	93	101	101	75	14	20
Range	21	45	33	35	4	11
Standard deviation	5.9	10.1	6.9	7.4	0.9	2.5
Standard error of mean	0.74	1.24	0.87	0.91	0.12	0.31

energy to produce more tiller for reproduction rather than stem elongation (Yang et al., 2019).

Under IL, grain yield of the IVC lines ranged from 5.074 to 7.013 t ha^{-1} from which no significant difference was observed compared with IR64, which is the highest yielding check with 6.612 t ha^{-1}. However, 1 line (IVC-284-3) incurred a 6% yield advantage over IR64. Under MDR, the grain yield range of IVC lines was 0.572 - 2.131 t ha^{-1} from which 14 (78%) lines were comparable with PSB Rc 14 yield of 0.938 t ha^{-1} (Table 6). These 14 lines have 3.7 - 127% yield advantage over PSB Rc 14.

Field performance of the IVC lines under non-stress and submergence stress condition

In 2019 DS, the three identified IVC lines with tolerance to submergence at seedling stage were evaluated for field performance under non-stress (NS) and complete submergence (SUB) stress conditions (Figure 13a-c). Water quality during the 7-day submergence period was monitored (Figure 13d). The water depth ranges from 79 to 91 cm, which kept the rice plants completely submerged. The pH of the water ranged from 9.02 to 9.22 showing an alkaline nature of the water. This condition may be attributed to the sulfate and carbonate residues from fertilization of the adjacent fields because the imposition of submergence coincided with the transplanting activities in the area (Zaman et al., 2018). The temperature of the water ranged from 26°C to 29°C creating a cool ambiance under water. However, cool temperature was found to hasten the decomposition of the plant tissues because of its high correlation to fermentation rate (Noguchi, 2007). Oxidation-reduction potential (ORP) reading of the water was 119 to 128 mV signifying a depletion of dissolved oxygen under water (Morard and Silvestre, 1996). The water quality data showed a non-favorable growing condition of the rice plants submerged under water.Survival of the IVC lines ranges from 32 to 47%, which is 35 - 41% relative to the tolerant check FR13A with a 92% survival (Figure 14a). Reductions in days to flowering and plant height were observed when submerging the IVC lines (Figure 14b). Under NS, days to flowering ranged from 93 to 94 DAS. A

Table 6. Grain yield of the IVC lines under IL and MDR conditions, PhilRice CES, 2018 DS.

No.	IVC Code	Grain Yield (t ha^{-1})				
		IL	Adv	MDR	Adv	Reduction
1	IR64	**6.612**		0.852^{b}		5.711*
2	PSB Rc 14	5.457		**0.938^{a}**		4.519*
3	Y Dam Do	2.130		0.844^{a}		1.286*
4	IVC-264-5	5.169	-21.8	0.973^{a}	3.7	4.197*
5	IVC-265-3	5.558	-15.9	0.850^{b}	-9.3	4.708*
6	IVC-265-4	5.094	-23.0	1.104^{a}	17.7	3.989*
7	IVC-265-5	5.784	-12.5	1.412^{a}	50.6	4.372*
8	IVC-266-3	5.803	-12.2	1.120^{a}	19.4	4.683*
9	IVC-267-4	6.574	-0.6	1.234^{a}	31.5	**5.340***
10	IVC-267-5	5.588	-15.5	1.498^{a}	59.7	4.090*
11	IVC-267-1	5.427	-17.9	1.517^{a}	61.8	3.910*
12	IVC-282-1	5.825	-11.9	1.349^{a}	43.9	4.476*
13	IVC-282-2	5.320	-19.5	0.823^{b}	-12.2	4.497*
14	IVC-282-3	5.500	-16.8	1.226^{a}	30.7	4.274*
15	IVC-282-4	5.477	-17.2	1.047^{a}	11.6	4.430*
16	IVC-282-5	5.074	-23.3	0.609^{b}	-35.1	4.466*
17	IVC-283-2	6.279	-5.0	0.981^{a}	4.6	5.298*
18	IVC-283-4	5.374	-18.7	1.710^{a}	82.3	3.664*
19	IVC-284-3	**7.013**	**6.1**	**2.131^{a}**	**127.1**	**4.882***
20	IVC-285-1	5.219	-21.1	1.129^{a}	20.3	4.091*
21	IVC-285-2	6.150	-7.0	0.572^{b}	-39.0	5.578*
Minimum		5.074	-23.3	0.572	-39.0	3.664
Maximum		7.013	6.1	2.131	127.1	5.578
Range		1.938	29.3	1.558	166.1	1.914
Mean		5.679	-14.1	1.183	26.1	4.497
Standard deviation		0.530	8.0	0.386	41.1	0.517
Coefficient of variation		9.326	-56.8	32.626	157.8	11.488
Pr>F		0.1526		0.0482		
Alpha		0.05		0.05		

Means with the same letter are not significantly different by Tukey's comparison of means at Alpha=0.05, STAR Version 2.0.1; *Significant reduction by Estimate Analysis, SAS Portable, Version 9.3.1

No. - number

Adv – advantage over the highest yielding check, IR64 (IL) and PSB Rc 14 (MDR)

delay of 7 days in days to flowering was observed under SUB condition. Reduction of plant height from 110 to 116 cm under NS and 92 to 103 cm was observed when lines were submerged. Plant height reduction is the result of the quiescence mechanism of plants under complete submergence. When submergence subsides, rice plant conserves its energy under water by halting all biochemical processes and later on channeling the conserved energy to rejuvenation of stems (Pucciariello, 2015). This also explains the increase of tiller number observed among the lines under SUB condition.

Under NS, the grain yield of IVC lines ranged from 5.013 to 6.852 t ha^{-1}, significantly higher than the tolerant check, FR13A with 1.147 t ha^{-1}. Lines IVC-284-3 and IVC-285-1 had yield comparable with PSB Rc 68 (5.260 tha^{-1}) and IR64-Sub1 (6.071 t ha$^{-1)}$. Under SUB, grain yield was 1.840 - 2.167 t ha^{-1}, which were significantly higher than FR13A (0.710 t ha^{-1}). These grain yields were also comparable to the yield of the check varieties PSB Rc 68 (3.228 t ha^{-1}) IR64-Sub1 (2.208 t ha^{-1}) (Figure 15).

Significant reduction of grain yield was observed due to submergence stress (Yadav et al., 2018; Rahman et al., 2018; Reddy et al., 1985). Photosynthesis system is damaged when plants are submerged; thus, production of assimilates is affected resulting in yield reduction (Mauchamp and Methy, 2004; Pedersen et al., 2013).

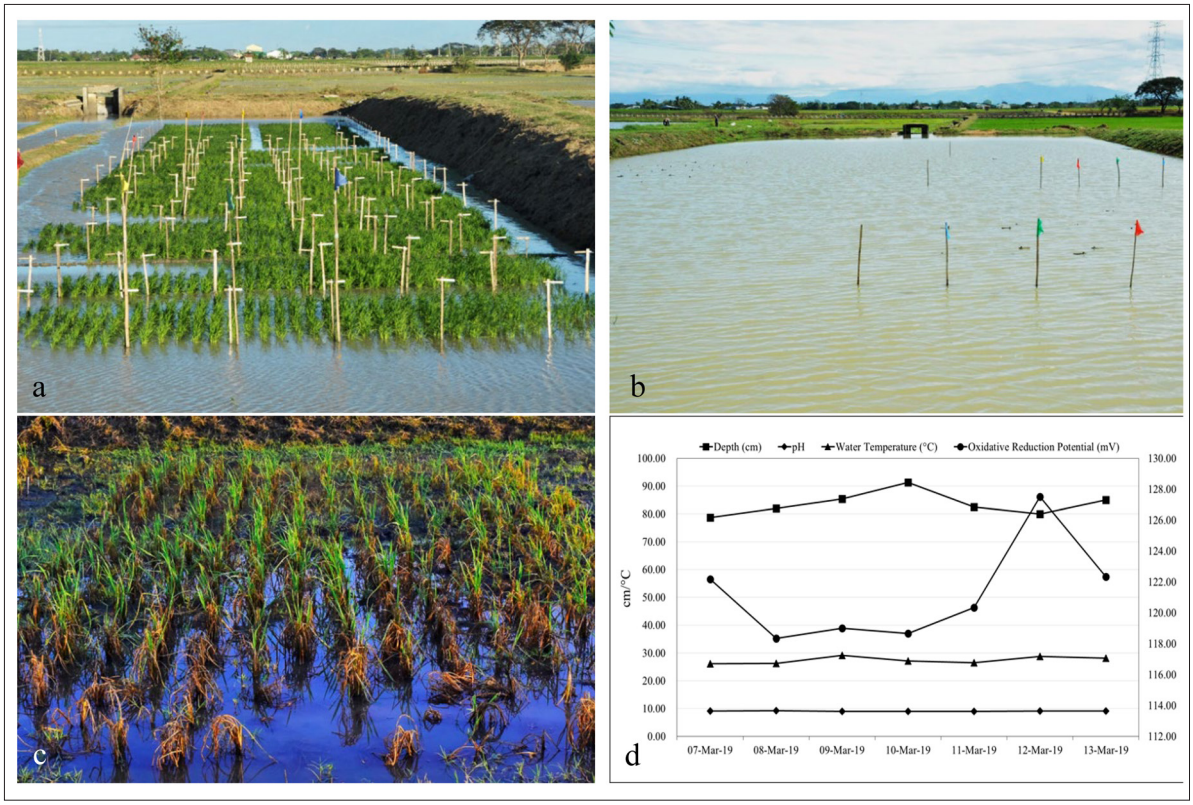

Figure 13. IVC lines at 14 days from transplanting before submergence imposition (a); submergence, 5 days from imposition (b); recovering plants, 14 days from de-submergence (c); quality parameters of water during submergence period of 8 days. PhilRice CES, 2019 DS.

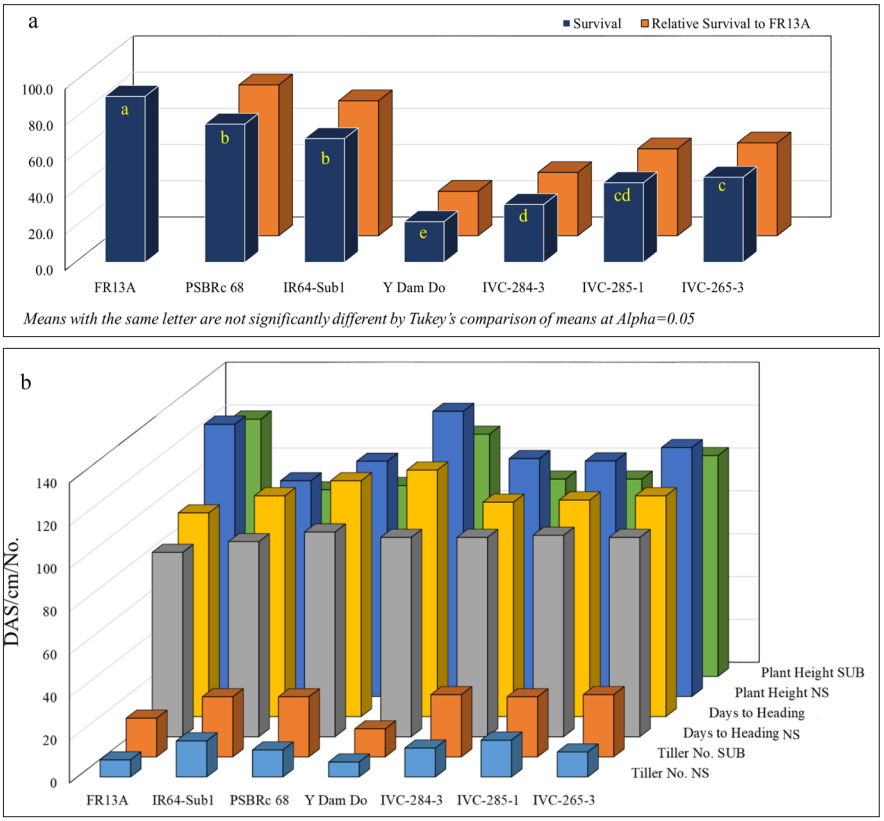

Figure 14. Survival (a) of the IVC lines after 21 days from de-submergence; agronomic traits (b) of the IVC lines under NS and SUB growing conditions. PhilRice CES, 2019 DS.

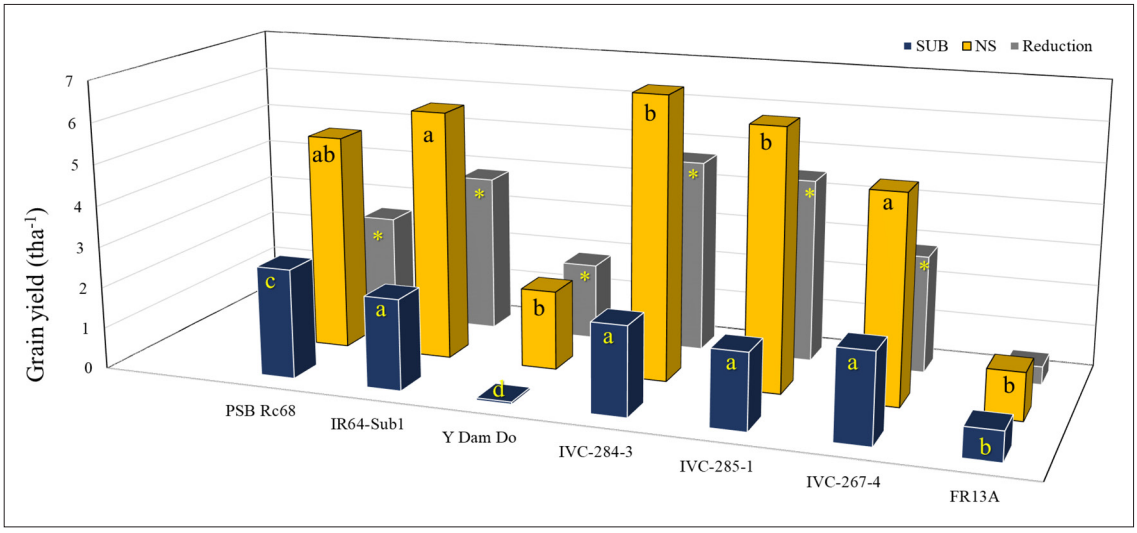

Figure 15. Comparison of grain yield under NS and SUB growing conditions of the IVC lines. PhilRice CES, 2019 DS.

Correlation and regression analysis of survival and grain yield under submergence

Pearson's correlation analysis of survival and grain yield (Figure 16a) under complete submergence showed a strong and significant negative correlation (r=-0.7004). Linear regression analysis (Figure 16b) provides an inverse relationship between the two parameters, which means that genotypes with high survival under complete submergence tend to produce lower yield. Similar observation was also obtained from a previous study evaluating NSIC Rc 222 mutants under submergence (Cabusora et al., 2022).

A study conducted by Nugahara et al. (2012) claimed that severely stressed plants lose biomass, leaves, and tillers; eventually recover longer before the resumption of new organ development. These effects influence the production of assimilate that will be translocated to the sink. Furthermore, the study revealed that submerged plants are depleted in above ground dry matter weight resulting in lower harvest index. This means that the plants need to produce more biomass by sacrificing grain development and filling.

From these evaluations, IVC-284-3, IVC-285-1, and IVC-265-3 were identified as lines possessing tolerance to combined abiotic stresses that produced acceptable yield under non-stress, managed drought, and submerged growing conditions.

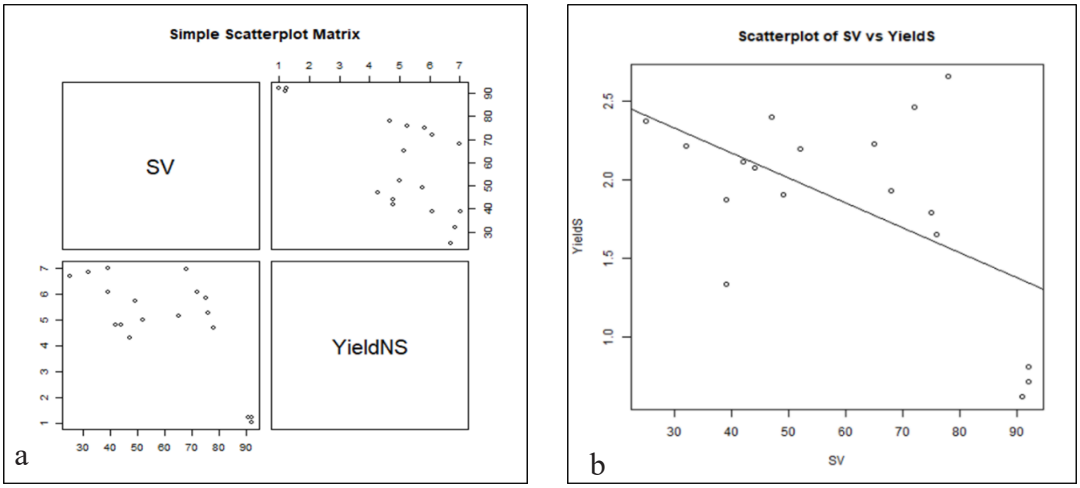

Figure 16. Correlation (a) and regression (b) analysis of plant survival and grain yield under complete submergence. PhilRice CES, 2019 DS.

Table 7. Selected promising IVC-derived Y Dam Do lines, PhilRice CES.

No.	IVC Code	Abiotic Tolerance at Seedling Stage						Grain Yield (t ha^{-1})				SV (%)*
		Drought		Salinity		Sub		IL	MDR	NSTR	SUB	
		Rec (%)	Score	LII	Score	SV (%)	Score					
1	IVC-265-3	84	T	3	T	75	T	6.574	1.234	5.013	2.167	72
2	IVC-284-3	87	T	1	HT	76	MT	7.013	2.131	6.852	2.154	75
3	IVC-285-1	73	T	1	HT	80	MT	5.219	1.129	6.304	1.840	76

Rec- Recovery, LII – Leaf Injury Index, SV-survival *survival at vegetative stage submergence, IL-irrigated lowland, MDR – Managed Drought Stress, NSTR-Non-Stress, SUB-Submergence

Summary and Conclusion

In vitro culture of the rice landrace, *Y Dam Do,* generated somaclonal variants with improved plant type, plant height, days to flowering, and yield compared with the wild type. These somaclonal variants with improved phenotypes and retained abiotic tolerance are good breeding materials in developing rice lines with multiple abiotic stress tolerance and acceptable morpho-agronomic traits. Assessment of the generated somaclones for abiotic stress tolerance identified IVC lines with combined tolerance to drought and salinity, drought and submergence, salinity and submergence, and combination of the three. Field performance of the lines under irrigated, managed drought, and submerged conditions, resulted in the selection of promising lines with multiple abiotic stress tolerance, acceptable agronomic traits, and with comparable grain yield to the check varieties (Table 7).

The identified promising lines can be used as novel sources of genes for multiple abiotic stress tolerance, or can be nominated to the National Cooperative Test for single or multiple abiotic stress ecosystems.

Acknowledgment

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FULL PAPER

A NUTRIENT MEDIUM FOR OUTDOOR CULTURE OF *NOSTOC commune* VOUCHER USING MALIGAYA SOIL WATER EXTRACT WITH MINIMAL PHOSPHORUS ENHANCEMENT

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Abstract

The *Nostoc commune* is a nitrogen-fixing cyanobacterium with huge potential as sustainable alternative to the expensive inorganic fertilizers. Its indoor culture using BG-11 has been optimized; however, indoor mass production will consume high electricity. Hence, outdoor culture is being utilized to take advantage of the solar radiation and naturally-available farm resources.

A 10% soil-water extract (SWE) made from Maligaya heavy clay soil with a minimal amount of phosphorus (P) for growth enhancement was tested as an outdoor culture medium for *N. commune*. The results showed a significant increase in weight and size of *N. commune* supplemented with 2.6 ppm P, distinctly in the fifth week. It also produced dark green sheaths with rough texture due to the formation of tiny buds. Meanwhile, the number of heterocysts was not significantly affected by the different media formulations. Generally, the SWE-based media were shown better than BG-11 for outdoor use. While the enhancement with 2.6 ppm P generally promoted outdoor growth and development of *N. commune* and relevantly did not induce toxic algal bloom. The formulated culture medium is easy-to-prepare and necessary materials are accessible. This can lead to the production of starter *N. commune* colonies for consequent on-farm co-cultivation, which can help increase rice productivity, especially in the nutrient enrichment-poor areas.

Keywords: *Biofertilizer, Cyanobacteria, Food, Phosphorus, Rice, Soil-water Extract*

Introduction

Recent tremendous increase in the price of imported inorganic fertilizers limits farmers' capacity to apply the recommended rates in rice production. Data from the Fertilizer and Pesticide Authority (FPA) show that the Philippines primarily import fertilizer from China (40.66%), Indonesia (16.70%), and Malaysia (12.20%) from 2018 to 2021. Inorganic nitrogen (N) fertilizer is the most popular type in the Philippines (Briones, 2017). Producing inorganic fertilizers is quite energy-intensive with natural gas is its main energy source. Thus, the price of fertilizer depends on the supply and price of natural gas. A modern ammonia production plant requires net energy consumption of approximately 29.7 million BTUs per ton of N, and upgrading ammonia to other N fertilizers requires even more energy – 35.9 million and 31.4 million BTUs per ton for urea and urea/ammonium nitrate manufacture, respectively (Kongshaug, 1998; Jenssen and Kongshaug, 2003). N-fixing organisms that can augment N sources and help in biological nutrient cycling is one of the main reasons for the increasing interest in sustainable biofertilizers in agriculture. Rice production in tropical countries mainly depends on biological N_2 fixation by cyanobacteria, which are natural components of paddy fields (Vaishampayan et al., 2001). Annually, in the cultivated agriculture systems, 32 Tg of N is fixed by biological N fixers (Singh et al., 2016), and cyanobacteria add about 20 - 30 kg fixed N/ha along with an organic matter to the paddy fields (Subramanian and Sundaram, 1986; Issa et al., 2014). The most effective N-fixing biological systems in the rice fields are certain cyanobacteria contributing about 25 - 30 kg N/ha every season (Venkataraman, 1979). Under the symbiotic association, cyanobacteria including the *N. commune* have an enormous biological N-fixation ability of 30 - 100 kg N/ha; hence, has been considered a valuable source of N for rice crop (Ito and Watanabe, 1985; Singh and Singh, 1987; Begum et al., 2011; Roy et al., 2016; Rai, 2019). These directly or indirectly help improve Nitrogen (N), Phosphorus (P), Iron (Fe), and other mineral content in the soils, which promote plant growth (Kumar, 2015). Cynobacteria are known to secrete growth-promoting substances (Gupta, 1966) and can compete with native flora and fauna (Singh et al., 2016). In many Asian countries, *Nostoc*

species have historically been utilized as healthy food and a traditional medicine. Comprehensive details of nutritional characteristics of *N. commune* and derived extracts such as antioxidative, anti-inflammatory, anti-carcinogenic, and immune regulation properties were also identified (Li and Guo, 2018).

The application of local strains of *N. commune* as biofertilizers shows potential in increasing the nitrogen use efficiency on rice (Pereira et al., 2009). The *N. commune* is locally known as a healthy food; hence, human consumption and market demand are among the major factors that led to significantly reduced population. To support its widespread utilization, especially in areas where it is not naturally existing, optimizing its scalable outdoor culture is essential in producing suitable biomass for inoculating rice farms.

In 1976, Pantastico and Gonzales reported a medium formulation for *N. commune* under laboratory conditions. However, its indoor production protocol is expensive because it requires high electricity consumption. As a result, they optimized an outdoor pond culture with SWE reinforced with minimal concentrations of superphosphate. There are related studies that implied a possible association of P in the distribution and productivity of *Nostoc* species in the field such as the usual proliferation of colonies around the rice roots where enhanced amounts of P are made available by inhabiting microorganisms (He et al., 2005). It is usually thought to be a major limiting factor for the growth of phytoplankton in aquatic ecosystems and in natural content of available P rarely exceeds at 10 uM (Bieleski, 1973). The variable availability of P in the medium used in culturing another *Nostoc* species was shown to influence the production of new cells and extracellular polysaccharides (Gao et al., 2012). The nutrient composition and properties of soil in different places vary and the total amount of P should be kept in enough concentration in order not to cause toxic algal bloom.

In this study, the heavy clay Maligaya soil series and naturally available tap water at the Central Experiment Station (CES) of Philippine Rice Research Institute (PhilRice) in Science City of Muñoz, Nueva Ecija were used as primary components in formulating a nutrient medium for outdoor growth and development of *N. commune*. The media formulations were reinforced with minimal P using the common Solophos fertilizer (0-18-0) applied in rice framing to enhance growth and development. The increase in *N. commune* fresh weight, growth size, shape, color, and the number of heterocysts were evaluated weekly for five consecutive weeks. The results of this study provide a baseline information for the optimization of low-cost and sustainable culture medium for outdoor production of *N. commune* in the locality. It also demonstrates the potential of SWE-based culture medium formulation with minimal P enhancement in establishing a reliable source of accessible *N. commune* inoculum. This eventually helps initiate its wider on-farm cultivation and help in improving productivity, especially in nutrient-poor areas.

Materials and Methods

Biological material

The *N. commune* samples used in the experiment were collected from a flat paddy field in Adams, Ilocos Norte in 2016 through a project implemented in the former Applied Biology Center for the Rice Environment (ABCRE). Samples were kept in the dry form for almost a year but easily regenerated into fresh form by 48-h soaking in tap water before establishing the experiment.

Medium preparation

Heavy clay Maligaya soil series and tap water collected at PhilRice CES were used as the primary components of preparing the SWE nutrient medium by mixing 50 g of soil per 200 ml of water for 10 min. The soil used was collected in a site that has not been proliferated by the *N. commune*. The mixture was allowed to stand for two days and autoclaved at 121°C at 15 psi for 20 min. From this sterile stock, three 3-liter of 10% SWE solutions were prepared for the three experimental treatments that were respectively added with 1.3 ppm (T3) and 2.6 ppm (T4) P using commercial Solophos fertilizer (Atlas 0-18-0). The P content of Solophos fertilizer is usually written as P_2O5 or phosphorus pentoxide but chemically in the soil solution, it works in the ionic phosphate (PO_4^{-3}) form. There is 43.7% P in P_2O_5; hence, the computed 3 and 6 ppm Solophos equate to 1.3 and 2.6 ppm elemental P, in the respective treatments. A control treatment without P enhancement (T2) was also included. To compare the efficiency of the test media formulations in supporting outdoor growth and development of *N. commune*, the established indoor *N. commune* culture medium, BG-11 (Yang et al., 2014) was also included as another control treatment (T1).

Outdoor culture set-up

The rectangular glass containers, measuring 28 cm x 18 cm x 18 cm, were filled with culture medium (not more than 15 cm deep) representing different experimental treatments. Each container was inoculated with 4 g (about 10 pieces) of fresh *N. commune.* The volume of the culture medium was maintained at 3 L, added with tap water if needed, and stirred daily for about 10 min. The experiment was carried out in 2018 wet season (WS) at PhilRice CES outdoors, in a completely randomized design

with three replications. The containers were covered with a thick and clear polyethylene sheet cover on sunny days or when it rains.

Sheath fresh weight and growth size (diameter), texture, and color of N. commune

The growth of *N. commune* was evaluated based on the fresh sheath weight (FW) and growth size (GS), which was measured using a digital weighing balance and a ruler, respectively. The formula used in computing the increase in FW) and GS are as follows:

Increase in FW = Actual FW - Initial FW

Increase in GS = Actual GS - Initial GS

The sheath texture was rated as "smooth" if there were very few buds on the surface or "rough" if otherwise. Moreover, the sheath color was rated as follows: 1 - yellowish to light green; 2 - moderate green; and 3 - dark green. Data were gathered weekly for 5 weeks. Heterocysts of the sheath of *N. commune* were examined and counted weekly using a compound microscope in a high-power field in three different areas of the prepared specimens in the glass slide at 7 DAI. Means were compared using the Star-IRRIStat program with Tukey's HSD.

Results and Discussion

Formulation of N. commune culture medium

The SWE contains nutritional substances that can naturally sustain the growth and development of a number of microorganisms but the composition of the various soil types may vary. In this study, the Maligaya clay soil was used as the primary component of an outdoor culture medium for *N. commune*. The use of readily-available on-farm resources can make the medium formulation affordable and sustainable. The Maligaya clay soil is a lowland suited for irrigated and rainfed paddy rice. It has a fine loamy textured with high clay content (35 - 60%) and isohyperthermic temperature regime (>22°C) with pH ranging from 6.8 to 7.2 (Collado et al., 2008). It is a Vertisol (-ert), dominated by shrink-swell clays that cause deep wide cracks and slickensides; very sticky when wet and compact when dry. When repeatedly saturated with water, it is manifested by a grayish color with or without mottles. Among the soil series in Nueva Ecija, it has the highest inherent and potential productivity ratings of 0.83 and 0.93, respectively. The inherent productivity index is the natural capacity of the soil to produce a given yield while potential refers to the capability of the soil to produce yield after correctible soil constraints had been remedied. This also equates to high inherent fertility, which could be one of the primary factors that prepared the soil-water, a promising nutrient medium for outdoor growth and development of *N. commune*. Heavy clay contains more humus and organic matters and other essential minerals than other kinds of soil such as silt, loamy, and sandy.

The SWE medium was supplemented with varying concentrations of P using the non-traditional Solophos (0-18-0). In crops, Solophos fertilizer promotes vigor, optimum root development for maximum absorption of nutrients, and resistance to lodging (www.atlasfertilizer.com). The dissociation of elemental P is highly dependent on the pH. With *Nostoc* species, P has been associated with its distribution and productivity (He et al., 2005) and in the production of new cells and extracellular polysaccharides (Gao et al., 2012). It is also thought to be a major limiting factor for the growth of phytoplanktons (Bieleski, 1973). The application of fertilizer like P has been demonstrated to have a beneficial effect on the establishment and growth of N-fixing cyanobacteria (Jha et al., 1965). However, high levels of nutrients caused harmful algal blooms (Giles et al., 2015), and algal toxins can be harmful to human and animal health. In the BG-11 indoor culture medium used by Diao and Yang (2014), the amount of P released from KH_2PO_4 is about 17 ppm. Collado et al. (2008) reported that the Maligaya clay soil contains high P, which is relatively higher than the soils in Nueva Ecija; however, its N and K levels are low.

The *N. commune* used was a paddy rice area in Adams, Ilocos Norte. Samples have been desiccated for almost a year and kept at room temperature but regenerated by soaking for about 48 h in water. The mechanisms involved in its desiccation-tolerance reflect both simple and complex interactions at the structural, physiological, and molecular levels (Potts, 1999). Under terrestrial and aquatic habitats, it can remain dry for months or years and can fully recover its metabolic activity within hours to days after rehydration with water (Dodds et al., 1995). It is important that the most common storage products of cyanobacteria are polyphosphate as a P storage compound, cyanophycin or phycobilin protein pigment as an N storage product, and glycogen as a storage product of both carbon and energy (Krompkamp, 1987). The fitness of *N. commune* to persist despite environmental stresses is advantageous but further improvement may still be necessary to enhance its survival, especially in areas where they are not naturally occurring.

Results showed that enhancement of the Maligaya clay soil-derived culture medium of up to 2.3 ppm did not produce algal blooms. Prasanna et al. (2012) also added that cyanobacteria enhanced the decomposition and mineralization of P_2O_5 and transformed it into available soluble organic phosphates/orthophosphates

through extracellular phosphatases and the excretion of organic acids for inorganic P_2O_5. This process is important for the growth and development of cyanobacteria. Most cyanobacteria have the ability to solubilize insoluble phosphates such as $(Ca)_3(PO_4)_2$ (tricalcium diphosphate), $FePO_4$ (ferric orthophosphate), $AlPO_4$ (aluminum phosphate), and $Ca_5(PO_4)_3(OH)$ (hydroxylapatite), which can be found in soils, sediments, or in pure cultures.

In 2014, Diao and Yan reported the culture of *N. commune* in China, specifically the formation of macro-colonies and discoid forms using a sterile BG-11 nutrient medium consisting of macronutrients. Under an artificial condition, their culture was incubated at 25°C with continuous rocking and 16 h of illumination using white fluorescent lights afterwards, in continuous darkness for 8 h every day. The BG-11 was renewed once every seven days. While this method is effective, this cannot be used for large-scale production as it is seen as costly as it requires high electricity consumption and a sterile environment.

Biomass growth of N. commune under outdoor conditions

The biomass growth of *N. commune* in different nutrient media formulations was assessed and measured by the increase of fresh weight biomass from first to fifth week (Figure 1 and Table 1). Data shows a consistent increase in *N. commune* fresh weight. The lowest increase in weight was recorded with BG-11. Apparently, BG-11 does not perform well in supporting the outdoor growth and development of *N. commune* despite of being the established indoor culture media for *N. commune*. Meanwhile, from Week 1 - 2, no significant increase was observed in fresh weight samples grown in SWE alone (896.67 and 1850 mg, respectively) compared with the weights in SWE + 1.3 ppm P (676.67 and 1410 mg, respectively), and SWE + 2.6 ppm P (943.33 and 1990 mg, respectively). Noticeably, from Weeks 3 - 5, the biomass weights in SWE + 1.3ppm P (1980, 2260, and 2440 mg, respectively) appeared as not significantly different from BG-11 with 1500, 1860, and 2336.67 mg. Though the biomass weights of *N. commune* in

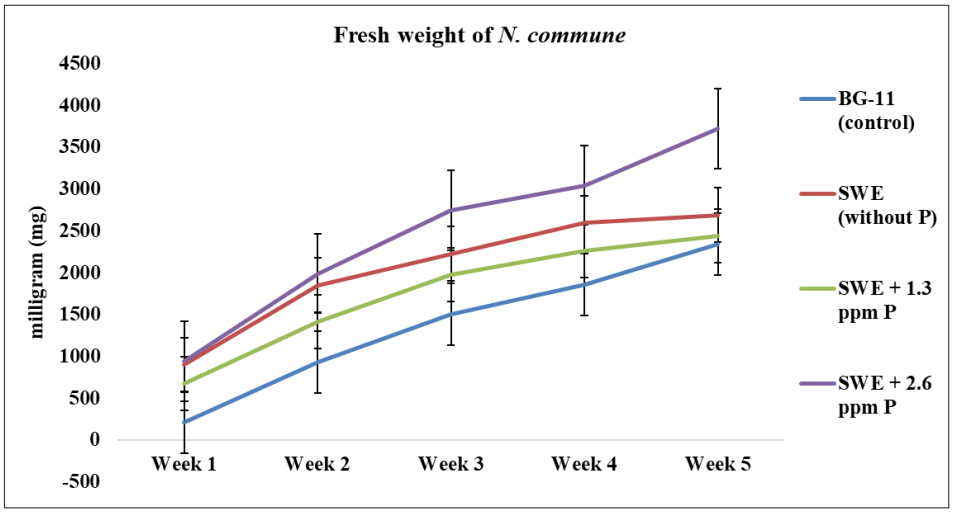

Figure 1. Biomass increase of *N. commune* in different formulations of nutrient media under outdoor culture conditions. (Weight increase = Actual weight of *N. commune* - initial weight).

Table 1. Statistical analysis for the increase in biomass of *N. commune* in different formulations of nutrient media under outdoor culture conditions.

Treatment		Fresh Weight (mg)				
		Week				
		1	2	3	4	5
T1	BG-11 (control)	213.33b	933.33c	1500.00c	1860.00c	2336.67b
T2	SWE	896.67a	1850.00ab	2226.67ab	2593.33ab	2686.67b
T3	SWE + 1.3 ppm P	676.67a	1410.00b	1980.00bc	2260.00bc	2440.00b
T4	SWE + 2.6 ppm P	943.33a	1990.00a	2746.67a	3043.33a	3720.00a
CV		22.53	14.86	13.43	10.18	16.85
SE Treatment		125.53	187.62	231.66	202.79	384.57
F Value		14.13	12.94	10.04	12.26	5.42
Pr (> F)		0.004	0.005	0.0094	0.0057	0.0382

Means with the same letter in a column are not significantly different at 5% level by Tukey's HSD (Weight increase = Actual weight of *N. commune* - initial weight).

SWE + 2.6 ppm P are relatively higher with SWE alone throughout the duration of the experiment, there is no significant difference in the increase in weights except by Week 5 when the significant increase in the *N. commune* fresh weight in SWE + 2.6 ppm P was emphasized. Different species of *Nostoc* had a flexible ability to utilize P, which play an important role in its growth and development as well as its widespread distribution in the environment (Dong et al., 2019). The P_2O_5 enrichment of cyanobacteria was also shown to enhance the growth rates and reproductivity of various cyanobacteria including *Nostoc* species (Kuffner and Paul, 2001). Apparently, among all the formulations tested, SWE + 2.6 ppm P is the most promising nutrient medium for supporting the outdoor growth and development of *N. commune,* yielding a biomass fresh weight of 3,720 mg. Under laboratory conditions, Pantastico and Gonzales (1976) reported a maximum yield of 15,800 mg fresh *Nostoc* per week or an average increase of 214% per day with the indoor nutrient medium they had formulated.

The biomass growth of *N. commune* in different nutrient media formulations was likewise assessed in terms of size increase. Figure 2 and Table 2 showed the increase in measurement of the widest diameter of each *N. commune* ball. Consistently, the size of *N. commun*e in BG-11 was the smallest that was significantly different from the size of *N. commune* grown in SWE-based media from Week 1 to 2. The results on fresh weights agree with the data presented. However, from Week 3 to 5, the *N. commune* cultured in BG-11 consistently remained the smallest with no significant increase in size despite the observed increase in weight. On the other hand, there was no significant difference in the sizes of *N. commune* cultured in the different SWE formulations except towards the last 2 weeks when SWE + 2.6 ppm P significantly recorded the biggest sizes at Week 4 (3.27 mm). In the 5th week, the size in SWE + 2.6 ppm P (3.1 mm) was relatively higher than in SWE + 1.3 ppm P (2.53 mm); however, these sizes were not significantly different. Results showed that generally,

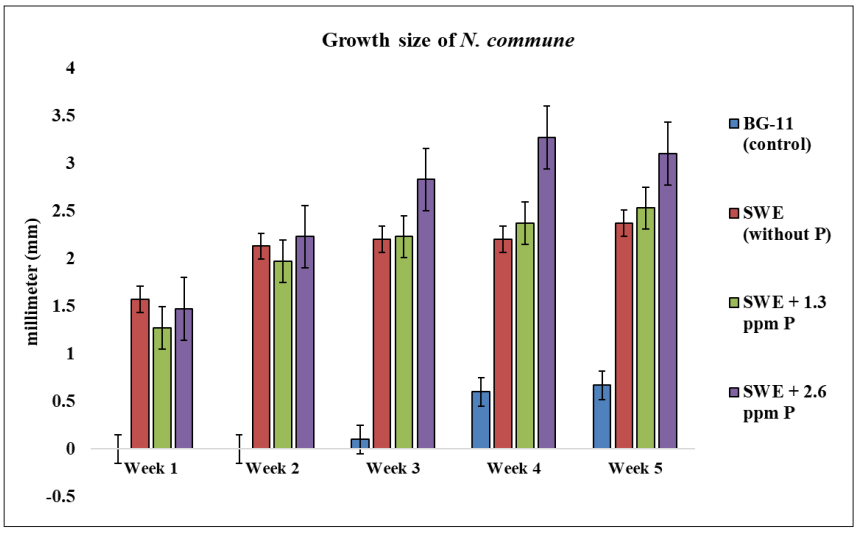

Figure 2. Growth of *N. commune* in different culture media. (Growth size increase = Actual growth size of *N. commune* - initial growth size).

Table 2. Statistical analysis data of the growth of *N. commune* in different culture media.

Treatment		Growth (mm)				
		Week				
		1	2	3	4	5
T1	BG-11 (control)	0.00b	0.00b	0.10b	0.60c	0.67c
T2	SWE	1.57a	2.13a	2.20a	2.20b	2.37b
T3	SWE + 1.3 ppm P	1.27a	1.97a	2.23a	2.37b	2.53ab
T4	SWE + 2.6 ppm P	1.47a	2.23a	2.83a	3.27a	3.1a
CV		18.01	19.52	20.22	18.79	14.02
SE Treatment		0.1581	0.2524	0.304	0.3235	0.248
F Value		42.33	35.36	31.01	23.52	35.73
Pr(> F)		0.0002	0.0003	0.0005	0.0010	0.0003

Means with the same letter in a column are not significantly different at 5% level by Tukey's HSD. (Growth size increase = Actual growth size of *N. commune* - initial growth size).

the SWE-based nutrient media were more efficient in supporting the size development of *N. commune* under outdoor culture conditions.

Color and texture of N. commune under outdoor conditions

In terms of effects on color and texture, no significant changes were observed among all treatments until the second week. However, in Week 3, notable darker green *N. commune* balls with and rough surface texture (Figure 3) appeared in SWE with 1.3 and 2.6 ppm P enhancement (Table 3), which was retained and became more intense until Week 5. Among the treatments, only SWE retained the initial moderate color of the *N. commune*, along with its smooth texture. Meanwhile, with BG-11 the *N. commune* turned yellowish with some green color but relatively lighter than the original color. Under the indoor culture of *N. commune,* BG-11 was replaced weekly; however, in this outdoor experiment, there has been no change and its volume was maintained by replacing with tap water. Therefore, it is interesting to investigate how the nutrients in the BG-11 had changed over the five-week experiment duration, and if this has something to do with the change in color or yellowing of *N. commune* balls. Moreover, with SWE alone, the smooth surface texture was retained all throughout the experiment.

Based on previous studies, the availability of P in culture media influences the activities of symbiotic and non-symbiotic N-fixing organisms in producing chlorophyll or carotenoids. It absorbs solar radiation and rough textures for their buds or new sheaths (balls) of cyanobacteria specifically *N. commune* (Smith, 1992; Couradeau et al., 2016; Roncero-Ramos et al., 2019). The enhancement of SWE medium made from Maligaya soil series with 1.3 - 2.6 ppm P increased the chlorophyll production in the *N. commune* samples, producing the dark green pigments. Moreover, available nutrients in SWE with P enhancement may have been a demonstration of triggering essential hydrological drivers on the surface of cyanobacteria for their reproduction (Rodrigo-Caballero et al., 2012).

Figure 3. The formation of tiny buds on the surfaces of *N. commune* balls culture in SWE with P enhancement made its texture appear rough. Initial moderate green color of the samples became darker, which became obvious on Day 3 and more intense toward the end of the experiment.

Table 3. Evaluated colors and textures of the *N. commune* in different culture media.

Treatment		Color						Texture					
		Week											
		0	1	2	3	4	5	0	1	2	3	4	5
T1	BG-11	2	2	2	2	1	1	S	S	S	S	S	S
T2	SWE	2	2	2	2	2	2	S	S	S	S	S	S
T3	SWE + 1.3 ppm P	2	2	2	3	3	3	S	S	S	R	R	R
T4	SWE + 2.6 ppm P	2	2	2	3	3	3	S	S	S	R	R	R

Color: 1 - yellowish to light green; 2 - moderate green; and 3 - dark green.
Texture: S (smooth) - no or very few buds on the surface; R (rough) - there are lots of buds on the surface of the ball.

Heterocyst count under microscope

Number of heterocyst in N. commune under outdoor conditions

The heterocyst (Figure 5) is specialized cells of *N. commune* that serve as the site of N-fixation, which is about 5 - 10% of cells in a filament. It occurs in the middle or at the end of the filaments, which are about 7 uM in diameter and are bigger than the vegetative cells (Honghun, 2006). The changes in the number of treatments were compared to determine possible effects on N-fixing ability. Böhme (1998) confirmed that generally, the observed heterocyst number of *N. commune* among the various culture media did not show significant differences until Week 5, wherein a significantly low number of heterocysts (11) was recorded (Figure 4 and Table 4). Hence, this observation has to be verified because an abrupt declined in the count was also recorded. In Week 5, the number of heterocysts in BG-11, SWE alone, and SWE + 2.6 ppm of P ranged from 19 to 22. This shows that the number of heterocysts is generally not affected in any nutrient media formulations tested.

Heterocysts are terminally differentiated cells, which interior becomes anaerobic, allowing the O_2-sensitive process of N-fixation to continue mainly as a consequence of respiration. Heterocysts are spaced at semi-regular intervals along filaments with approximately 7% of the cells differentiating into heterocysts in free-living *Nostoc* species. The fertilization of P has also been reported to stimulate the N-fixation activity of cyanobacteria (Liengen, 1999). Moreover, it protects nitrogenase from O_2 that is produced by photosystem II in adjacent vegetative cells and from matters entering the cell. The differentiation of heterocysts from vegetative cells in the filament is a complex, regulated process that involves changes in structure and function (Wolk et al., 1994). Furthermore, López-Igual et al. (2010) explained that heterocysts help in fixing atmospheric N compounds. In *N. commune*, heterocysts function as resting pores, its protoplasm becomes functional and germinates to form a new filament. Additionally, its abundant number or presence in the sheath ball of *N. commune* indicates that P is utilized efficiently. Heterocysts are responsible for the photosynthetic activities of the *N. commune* and are observed in the darker green sheath ball of the *N. commune*.

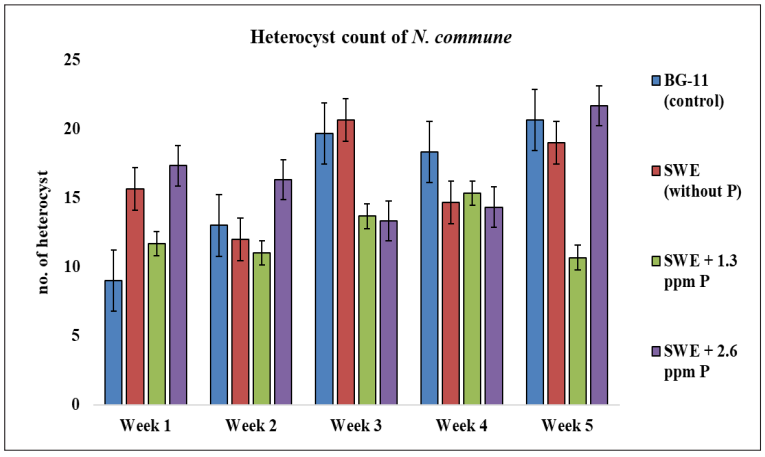

Figure 4. Heterocyst count of *N. commune* in different culture media.

Table 4. Statistical analysis for heterocyst count of *N. commune* in different culture media.

Treatment		Heterocyst Count				
		Week				
		1	2	3	4	5
T1	BG-11 (control)	9a	13a	20a	18a	21a
T2	SWE	16a	12a	21a	15a	19a
T3	SWE + 1.3 ppm P	12a	11a	14a	15a	11b
T4	SWE + 2.6 ppm P	17a	16a	13a	14a	22a
CV		27.44	44.13	35.31	29.65	15.96
SE Treatment		3.01	4.71	4.85	3.79	2.35
F Value		3.17	0.48	1.27	0.46	9.13
Pr(> F)		0.11	0.71	0.37	0.72	0.01

Means with the same letter in a column is not significantly different at the 5% level by Tukey's HSD.

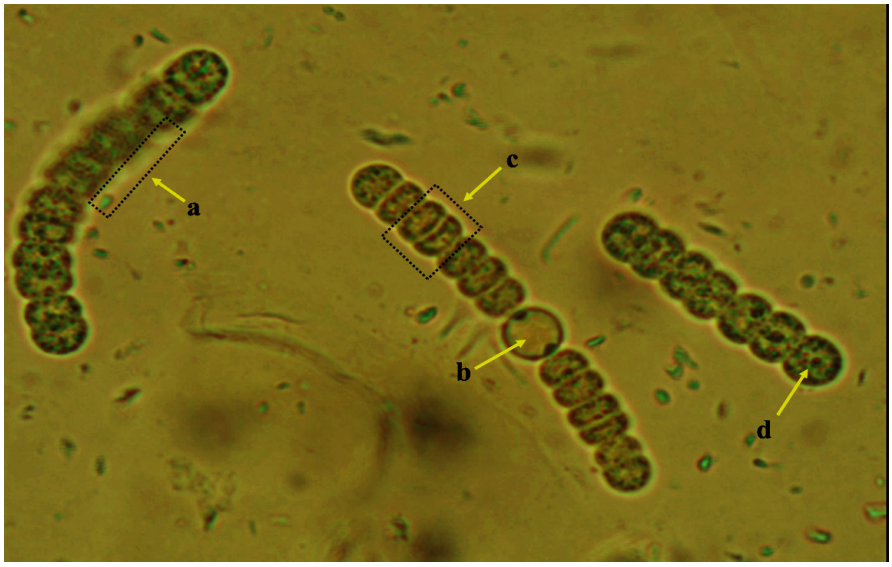

Figure 5. The gelatinous extracellular matrix and development of *N. commune* observed under microscope with high power field (HPF): (a) mucilage sheath; (b) heterocyst; (c) dividing cell; and (d) vegetative cell.

Summary and Conclusion

The formulation of a promising, cheap, and sustainable culture medium for the outdoor culture of *N. commune* was initiated through an SWE-based nutrient medium, prepared from Maligaya clay soil and tap water available at PhilRice CES. Its minimal enhancement with 2.6 ppm phosphorus showed a relatively highest increase in fresh weight and size of cultured *N. commune,* particularly towards Week 5. The enhancement of SWE-based nutrient media with 1.3 and 2.6 ppm P also produced a darker green color and rough buds on the surfaces of *N. commune*. Apparently, the *N. commune* colonies collected from the lowland rice farm in Adams, Ilocos Norte is potentially fit for outdoor culture with the formulated nutrient medium. Meanwhile, there has been no significant difference in the number of heterocysts among all the SWE formulations compared with BG-11. While BG-11 is an established indoor culture media for *N. commune*, results showed that it is not efficient for outdoor use. Despite the supplemental 2.6 ppm, the total amount of P in the formulated SWE nutrient medium showed an efficient in support in *N. commune* growth and development but it is too high to induce a disadvantageous algal bloom. It can propagate a suitable amount of biomass that can be used as inoculum in establishing a large-scale cultivation specifically, on-farm cultivation along with rice. This technology can be promoted especially in nutrient enrichment-poor areas to help increase productivity and less dependence on inorganic nitrogen fertilizers.

However, the effects of a much higher P supplementation (>2.6 ppm) in the formulated SWE medium derived from Maligaya clay soil needs further investigation. Subsequent optimization should also include the quality and quantity of solar radiation, temperature, humidity, pH, and available nutrients. The adaptability of *N. commune* in the new environment will also matter; hence, starter biomass can be taken either from upland or lowland rice environments. There is still a huge potential to restore these populations; thus, human intervention may be necessary especially in the consistent provision of starter colonies until such time that it can proliferate itself. This will particularly benefit the nutrient-poor soils that need fertility enhancement, wherein *N. commune* can help provide supplemental N and nutrients to the soil and crops.

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FULL PAPER

TRAITS IMPROVEMENT OF GAL-ONG, A TRADITIONAL RICE VARIETY, THROUGH INDUCED MUTATION

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Abstract

Gal-ong is a traditional rice variety with good eating quality and intense aroma; however, it has low-yielding potential that can be attributed to some inferior agro-morphological traits. In this study, radiation-induced mutagenesis was used to improve the variety. During the early generations, exposure of the putative mutants to natural biotic stresses under field conditions allowed the selection of mutants with apparent resistance against pests and diseases. Among the surviving mutants, at least 16 had about 180 - 373% yield advantage over the wild-type Gal-ong (WTGO) and had yields comparable with NSIC Rc 222. The days to maturity were also reduced by 22 - 40. The altered physical and physicochemical attributes of mutant rice resulted in a combination of good to excellent grain quality traits. The intensity of the aroma ranged from aromatic to none. Other interesting morphological variations observed were grain size, shape, color, and amylose content. The level of genetic variation among representative mutants was relatively high at 50%. The two high-yielding white-pericarp mutant lines that both lost aromas appeared to be 93% similar, while the two mutants with most of the WTGO traits and apparent blast resistance were 90% similar. The white-pericarp mutant with apparent stem borer and bacterial leaf blight resistance appeared genetically closest to the WTGO. These Gal-ong mutant rice lines can serve as valuable resources for broadening the genetic base of rice germplasm and interesting materials for functional genomics.

Keywords: *Aroma, Bacterial Leaf Blight, Blast, BPH, Gamma Irradiation, Rice*

Introduction

Traditional rice varieties are produced, commercialized, and exported because of their known good eating quality and aroma. In the Philippines, the nutritional and health benefits of these kinds of rice are promoted to create general public awareness. These varieties contain flavonoids and anthocyanin and the highest radical scavenging activity by polyphenols, which are favored by health-conscious consumers (Bhat and Riar, 2015). Gal-ong is among the traditional rice varieties, which is non-glutinous, red-pericarp rice with good eating quality, and intense aroma. Like most of the low-yielding pigmented rice grown for local markets (Wickert et al., 2014; Mau et al., 2017), Gal-ong has poor traits such as tall stature that makes it more prone to lodging, long maturity, and susceptibility to insect pests and diseases.

Mutations induce genetic changes in organisms through radiation or chemical agents. The use of gamma-ray irradiation is the most common (Ahloowalia and Maluszynski, 2001). This non-conventional method is one of the potential breeding tools used to generate genetic variability of traits in many crop plants, including rice (Maluszynski et al., 2000). Since the 1950s, over 2,000 crop varieties have been developed through induced mutation to randomly alter genetic traits and select improved types among progeny. In the Philippines, several rice mutants had been improved through mutation breeding. The Philippine Nuclear Research Institute (PNRI) developed the atomic rice PARC-2 released in 1973, an improvement of the high-yielding IRS-283-8, with better grain quality and improved tungro virus resistance. Other rice mutants developed by PNRI were improved from Milagrosa (1973), Azmil (1976), Bengawan (1981), Sigadis Milagrosa in collaboration with the Philippine Rice Research Institute (PhilRice) in 1999, Denorado, Perurutung NBB, and Malagkit Sungsong. The partnership between PNRI and PhilRice continued with the development of NSIC Rc 222-derived mutants with putative complete submergence tolerance (Cabusora et al., 2022). Based on IAEA data on commercial mutant varieties (Ulukapi and Nasircilar, 2018), the percentage of mutant varieties by mutation breeding constitutes 49.5% cereal, 21.9% ornamental plants and flowers, 15% legume, 2.4% fruit nuts, 2.4% vegetable crops, 2.3% fiber crop, 2.1% oil crops, 1.2% forage crops, 0.6% root-tuber crops, 0.4% herbs, 0.2% medicinal plants, and 2% other crops (Joint FAO/

IAEA Division of Nuclear Techniques in Food and Agriculture, 2018). Mutations had synergistic effects resulting in increasing the yield and quality of the crop and improving agronomic inputs, crop rotation, and consumer acceptance (Ahloowalia et al., 2003).

In this study, the mutation has been employed as an innovative technology to improve the morpho-agronomic traits of Gal-ong. The materials generated are useful in the pre-breeding, which can be used as donors for variety improvement and molecular application leading to gene discovery and marker development.

Materials and Methods

Plant materials

Wild-type Gal-ong (WTGO) was acquired from the germplasm resources of PhilRice, Batac City in Ilocos Norte, which was multiplied and characterized in the 2015 wet season based on the standard evaluation system for rice (SES) (IRRI, 2014). From the harvest, 250 g of healthy seeds were sent to PNRI for gamma irradiation at 250 gy. From this pool of irradiated seeds, the M1-M7 populations of putative mutants were generated in three batches under field conditions: Nos. 1-199 (1st batch), 200-series (2nd batch), and 300-series (3rd batch).

Crop establishment and maintenance

Seedlings at 21 days after sowing (DAS) were transplanted individually at 20 cm x 20 cm spacing and subjected to standard cultural management at PhilRice. Basal fertilizer of 14-14-14 (complete) was applied on plants 10 days after transplanting (DAT), and top dressed with the recommended rate of 46-0-0 (urea) at 35 - 45 DAT. The plants were maintained with proper water requirements and control of weeds, insect pests, and diseases.

Plant selection, characterization, and evaluation

At maturity, one panicle from each M_1 plant was collected, threshed, and bulked to constitute the mutant seed pool. The M_2 population comprised 5,000 individual plants. From the early stage of M_2, putative mutants for interesting traits such as reduced plant height, tiller count, reduced number of days to maturity, longer panicle length, and grain trait improvement were visually examined. Each of the selected plants was tagged accordingly, designated with a number, and advanced to verify the consistency of the improved traits. To purify the mutant rice, five panicles of individual plants were sown and transplanted in a single row with 15 hills per panicle (5 rows per selection) with a distance of 20 cm x 20 cm. The sampling area for yield was taken from the 15 rows of 21 hills and yield was computed using the grain moisture content, hills per plot, plot area (m^2), and grain yield (g). The reaction of mutant rice lines to blast, bacterial leaf blight, brown planthopper, and stem borer under natural field conditions was evaluated based on the Standard Evaluation Systems for Rice (SES, IRRI, 2014).

Molecular characterization

Selected promising putative M_4 and M_5 rice lines were subjected to molecular analysis using SSR markers to establish the degree of genetic similarity with the WTGO. High-quality DNA was extracted from leaf samples using the CTAB method with modifications (Doyle and Doyle, 1990). The quality and quantity of the DNA samples were evaluated using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA) and loading aliquot on 0.8% agarose gel prepared in a 1X TBE buffer. The amplification of PCR fragments was carried out in a 7.5 µL reaction containing 50 ng of DNA, 1X PCR buffer, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 0.5 nM of each primer, 2 mM MgCl2 and 0.5 U of Taq DNA polymerase in a programmable Thermal Cycler PTC 100TM (MJ Research Inc., Watertown, MA, USA) with a cycle consisting of an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 50 - 58°C for 30 sec, and 72°C for 30 sec; and then a final elongation of 72°C for 5 min. The PCR-amplified products were analyzed in 8% denaturing polyacrylamide gel in 1X TBE buffer at 120 v for an hour or in 2% agarose gel in 1x TBE buffer at 150 v. The electrophoresis gels were stained with Gel Red (TM) staining solution and documented using a Molecular Imager® Gel Doc™ XR+ System (BioRad, USA). For gel scoring, the allele size of PCR-amplified WTGO fragments was compared with the improved mutant lines.

Yield and grain quality evaluation

Gal-ong mutant rice lines were selected and characterized in three consecutive cropping seasons at PhilRice Batac City, Ilocos Norte (2017 dry season [DS] and 2017 wet season [WS]) and PhilRice Central Experiment Station, Maligaya, Science City of Muñoz, Nueva Ecija (2018 DS). The rice lines were established in a randomized complete block design with three replications. Stable mutant lines in M_4-M_6 generations and WTGO were mass-produced and deposited in the PhilRice Genetic Resources Division.

The weight of grain from each plot was recorded for yield assessment. The moisture content (MC) of grains of each plot was determined by the moisture meter and the final grain yield was adjusted at 14% by

dividing the actual MC by 86. The grain weight was expressed in tons/ha:

Yield kg/ha = MF x YF x yield per plot (g)
MF = (100-MC)/86
YF = 10/harvest area (10 to adjust for 1 ha)

In 2019 DS, 58 pre-NCT Gal-ong mutant rice lines were profiled for grain quality (GQ). The Rice Chemistry and Food Science Division (RCFSD) of PhilRice CES facilitated the sensory evaluation of rice samples based on the NCT Manual (1997). A maximum of four cooked and corresponding raw rice samples were evaluated per session using a 5-point hedonic scale scorecard. Trained panelists, composed of seven PhilRice staff, served as evaluators. Cooked non-aromatic rice samples were evaluated for aroma, color, glossiness, cohesiveness, tenderness, smoothness, and taste while non-glutinous aromatic samples were evaluated for aroma, color, tenderness, and taste. The raw rice samples were evaluated for aroma, color, glossiness, translucency, and hardness for the non-aromatic samples and for the non-glutinous aroma samples; color, translucency, and hardness.

Results and Discussion

In improving rice productivity and quality traits, genetic alteration through induced mutation created rapid progress in improving several traits in many crops. The change in the genetic material of an organism relies on the effectiveness of the dose used and its efficiency in relation to creating undesirable changes like sterility, lethality, and injury caused by the mutagenic agents (Girija and Dhanavel, 2009). The mutation alters base pairs in a DNA sequence and the genetic variation can be passed from generation to generation.

Morphological and agronomic characteristics of wild type Gal-ong

Special traditional rice varieties such as Gal-ong are sought after in the local and international market due to their good eating quality, nutritional significance, and aroma. Table 1 shows the yield and agronomic traits of the WTGO grown and purified in the field in 2015 WS at PhilRice Batac City, Ilocos Norte. It has an average of five productive tillers and the number of days to maturity was 156 days. The average grain yield was only 3.4 t/ha. The plants are very tall, reaching an average height of 150 cm. The low yield potential of WTGO was attributed to its very tall stature, low tillering ability, and late maturity. Gal-ong is produced and utilized for commercial and export, but satisfying its demands was unsuccessful because its average yield per hectare is low. Table 2 shows the detailed morphological descriptions of the WTGO.

Mutation-induced genetic variability

Improved pest and disease resistance

Three batches of Gal-ong putative mutants were generated from 2015 DS to 2016 DS (mutant series 100, 200, and 300). The most advanced lines were at M_5, M_6, and M_7 generation cultivated in 2018 DS. In earlier generations, the field set-ups were naturally infested with insect pests and diseases; hence, several lines were lost. In 2016 WS at PhilRice Batac City, Ilocos Norte, mutant plants with putative resistance to rice blast (Figure 1A) and brown plant hopper (BPH;

Table 1. Yield and agronomic traits of the wild type Gal-ong in 2015 WS at PhilRice Batac.

Agronomic Traits	Remarks
Plant height (cm)	150
Ave. number of productive tillers	5
Days to 50% heading	96
Days to maturity	156
Average number of seeds per panicle	148
Panicle exertion	Well-exerted
Spikelet fertility (%)	87
1,000 grain weight (g)	34.6
Average grain yield (t/ha)	3.4

** based on the Standard Evaluation Systems for Rice (SES, IRRI, 2014)*

Table 2. Morphological characteristics of Gal-ong wild type under field conditions at maturity in 2015 WS at PhilRice Batac.

Morphological Characteristics	Remarks
Leaf length (LL) (cm)	53.3
Leaf width (LW) (cm)	1.8
Leaf blade pubescence (LBP)	2 (intermediate)
Leaf blade color (LBC)	2 (green)
Basal leaf sheath color (BLSC)	1 (green)
Leaf angle (LA)	1 (erect)
Flag leaf angle (FLA)	5 (horizontal)
Ligule color (LC)	1 (white)
Ligule shape (LS)	2 (cleft)
Collar color (CC)	1 (light green)
Culm length (CmL) (cm)	117.4
Culm angle (CmA)	1 (erect < 30°)
Culm internode color (ApC)	1 (green)
Apiculus color (ApC)	6 (purple)
Panicle type (PnT)	2 (intermediate
Panicle axis (PnA)	2 (droopy)
Panicle length (PnL) (cm)	32.6
18. Stigma color (SgC)	1 (white)
Sterile lemma color (SLmC)	4 (purple)
Awning (An)	0 (absent)

* based on the Standard Evaluation Systems for Rice (SES, IRRI, 2014)

Figure 1B and Table 3) were selected. GXB-209 was outstanding as it manifested putative resistance against both rice blast and BPH. In 2016 DS, about 3000 M_1 plants (300 series) grown at PhilRice CES were naturally infested with stem borer (SB) (Figure 1c - d and Figure 2a - b) and BLB (Figure 1e). From this batch, only 14 M_1 plants survived (Figure 2c), which were advanced to generate the putative SB-resistant M_2 population (designated as "SB" lines). Stem borer feeding causes severe dead heart or drying of the central tiller during the vegetative stage and causes whiteheads during the rice reproductive stage. In 2017 WS, several M_5 and M_6 mutant lines with moderate resistance (MR) to resistant (R) reaction to BLB were also selected from PhilRice CES field trial, in which GXB-209 manifested moderate resistance. The BLB infestation at the maximum tillering stage of a rice crop can cause a significant yield reduction of about 20 - 30% to as high as 80% (Mew et al., 1992). The WTGO was consistently observed with severe susceptibility to rice blast, BLB, BPH, and SB.

Mutants with improved yield and other desirable trait variations

Among the selected advanced M_5-M_7 Gal-ong mutants, the top three highest-yielding lines at PhilRice CES in 2018 DS were the GXB-229 (9.7 t/ha), followed by the GXB-204 (8.5 t/ha) and SB-3 (8.2 t/ha). The yield exceeded the average yield of the check variety NSIC Rc 222 (7.5 t/ha) and was 300 - 400% improved over WTGO (2.6 t/ha). Despite the natural insect pest and disease that affected the experiment, which severely damaged WTGO, a relatively good harvest was achieved among several improved mutants. The GXB-229 appeared to have a consistently high potential yield, in wet and dry seasons. GXB-204 and some other mutants were observed with

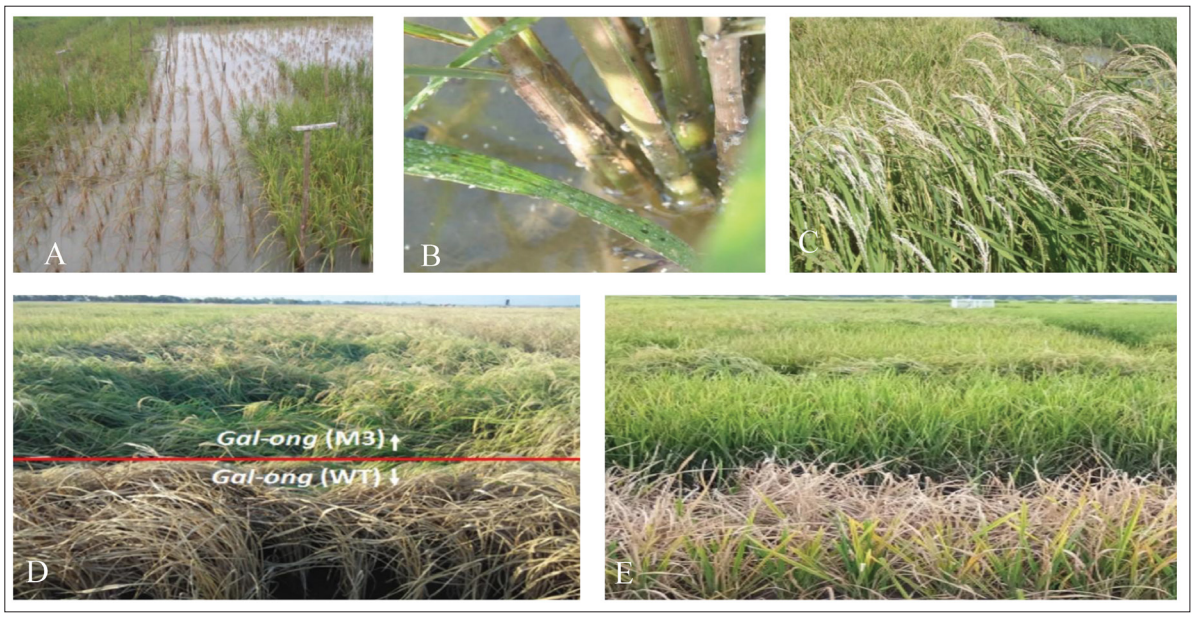

Figure 1. The Gal-ong wild type was shown susceptible to various insect pests and diseases under natural field conditions: (A) rice blast, (B) brown plant hopper, (C-D) stemborer (white heads and dead heart), and bacterial leaf blight (E).

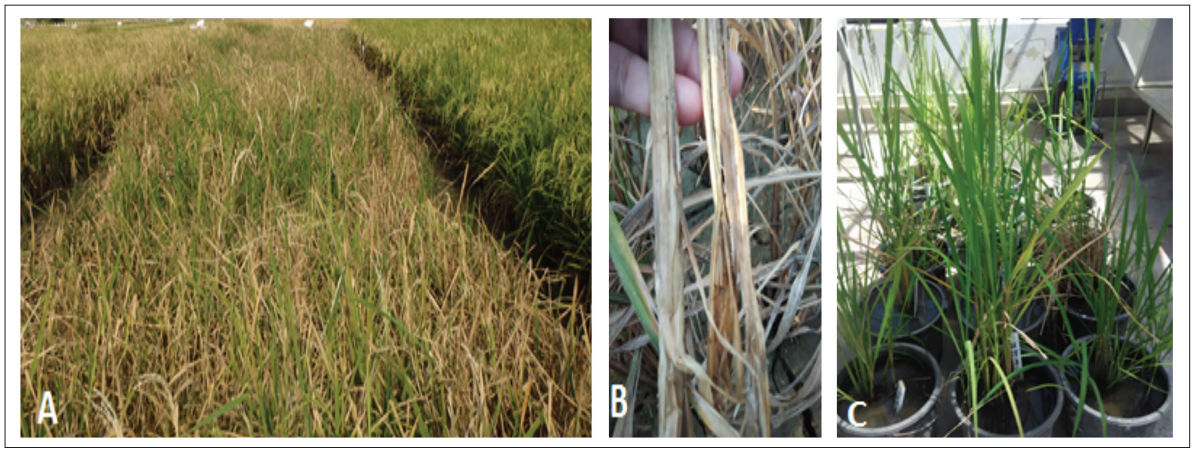

Figure 2. Severe stem borer incidence nearly destroyed all the putative Gal-ong mutants in the field in PhilRice CES (A-B), leading to the selection of M_1 plants with apparent SB resistance (C).

Table 3. Plant height, maturity, yield, and reaction to insect pest and diseases of Gal-ong and selected mutant lines.

Mutant Lines/ Rice Variety	Generation 2018 DS	Plant Height (cm)	Days to Maturity	Yield (tons/ha)	Plant Reaction to Pest and Diseases 2017 WS**		
					Blast	BLB	BPH
Non-glutinous aromatic							
GXB-310-2	M5	138	122	8.1	S	R	NP
GXB-311-7	M5	150	119	4.7	R	MS	NP
SB-1	M7	126	125	7.9	S	R	NP
SB-3	M7	124	125	8.2	S	R	NP
GXB-1	M7	146	121	7.4	NI	MR	MR
GXB-2	M7	142	119	7	NI	MR	MR
Glutinous							
GXB-309	M5	-	-	5.3	R	S	NP
GXB-317	M5	-	-	5.8	R	MS	NP
GXB-210-1	M6	147	134	5.5	MR	MR	S
GXB-209	M6	148	116	6.2	MR	MR	MR
High-yielding							
GXB-204	M6	108	121	8.5	MR	MR	NI
GXB-205	M6	113	122	7.8	MR	MR	NI
GXB-207	M6	118	120	7.7	MR	MR	S
GXB-208	M6	124	119	7.3	MR	MR	S
GXB-229	M6	124	117	9.7	S	R	NP
SB-5	M7	131	119	7.8	S	R	NP
Gal-ong	Wild type	157	156	2.6	S	S	S
NSIC Rc222	Check variety	107	119	7.5	-	-	-

** evaluated at PhilRice Batac City, Ilocos Norte using the Standard Evaluation Systems for Rice (SES, IRRI, 2014) Legend: S-Susceptible, MS-Moderately Susceptible, MR-Moderately Resistant, R-Resistant (Note: NI - No infestation/with apparent Resistance, NP- No Pressure)

high yields in the dry season but relatively reduced during the wet season due to adverse environmental conditions. The other two SB breeding lines, SB1 and SB5, which survived the severe stem borer infestation in the early generation, were also improved with at least 200% yield increase relative to the WTGO. In terms of maturity, the WTGO was 156 days while a 14 - 24% reduction in the average number of days was observed among the shortlisted mutant lines GXB-209, GXB-229, GXB-311, GXB-2, and SB-5 (Table 3). In terms of plant height, a 25 - 30% reduction was recorded in GXB-204, GXB-205, and GXB-207. A high frequency of mutants with altered height was widely reported in rice (Shadakshari et al., 2001). Semi-dwarf and early maturity are the traits most frequently desired in mutant rice (Shadakshari et al., 2001; Domingo et al., 2007; Bughio et al., 2007).

Figure 3 shows the other desirable traits observed among the mutant lines with an emphasis on a wide range of variations in grain shape (from long-slender to long-semi bold grains), endosperm color (from translucent to opaque), and pericarp color (white to pigmented). Figure 4 shows more altered traits in Gal-ong ranging from early to medium maturity, increased tiller count, long and dense panicles, and reduced plant height. There were also reports on the successful development of profuse leaves and large grain mutants in rice after mutagenic treatments (Shadakshari et al., 2001; Singh et al., 2004; Patnaik et al., 2006, Satish et al., 2012). Mutagenesis was proven very effective in generating desirable trait improvements that help in developing superior breeding lines (Balotch et al., 2003; Chakravarty, 2010).

Grain quality of Gal-ong mutant rice

Table 4 shows the milling potentials, physical, and physicochemical attributes of selected pre-NCT Gal-ong rice mutants along with recommended values. Table 5 shows the physical and physicochemical attributes of each promising mutant line. The majority, 56 entries, passed the requirements for percent brown rice recovery rated as fair (F). For milled rice recovery, 50 lines were rated as G, and six were Pr including GXB-229, GXB-311, and GXB-319. For head rice, 33 lines were rated as G1, and six including SB-1, SB-3, and GXB-207 qualified as Pr. On physical attributes, eight of the selected mutant lines composed of SB-1, SB-5, GXB-207, GXB-210-1, GXB-209, GXB-201-1, GXB-309, and GXB-317 qualified as G1 based on percent chalky grains. All the entries were rated G to Pr based on the percent immature grains. On grain length, 33 were rated L (long) while 28 were S (short). For the physicochemical properties, 28 were intermediate (I) amylose content and 6 were waxy (W) or glutinous.

Figure 3. Induced mutation created a range of phenotypic variations in grain shape (bold to long and slender) and pericarp color (red, white, brown, and purple/ black) among Gal-ong mutants (A. WTGO; B-E and bottom are mutants) 2017 DS.

Figure 4. Agronomic important trait variations among putative mutant lines: (A) early to medium maturity; (B) yield; and (C) reduced plant height.

Table 4. Milling potentials, physical, and physicochemical attributes of selected pre-NCT Gal-ong mutant lines with the recommended values.

Grain Quality	Classification		(n = 58)
Milling Potentials			
% Brown rice	Good (G)*	80% and above	1
	Fair (F)*	75 - 79.9%	55
	Poor (P)	below 75.0%	2
% Milled rice	Premium (Pr)*	70% and above	6
	Grade 1 (G1)*	65.1 - 70%	50
	Grade 2 (G2)	60.1 - 65%	2
% Head rice	Premium (Pr)*	57% and above	6
	Grade 1 (G1)*	48 - 56.9%	33
	Grade 2 (G2)	39 - 47.9%	11
	Grade 3 (G3)	30 - 38.9%	4
Physical Attributes			
Chalky grains	Premium (Pr)*	<2%	2
	Grade 1 (G1)*	2 - 5%	11
	Grade 2 (G2)	5.1 - 10%	14
	Grade 3 (G3)	10.1 - 15%	10
Immature grains	Premium (Pr)*	<2%	45
	Grade 1 (G1)	2 - 5%	13
Grain length	Extra long (EL)	7.5 and above	-
	Long* (L)	6.6 - 7.4	33
	Medium (M)	5.5 - 6.5	22
	Short (S)	5.4 and below	3
Grain shape	Slender* (S)	>3.0 mm	28
	Intermediate (I)	2-3 mm	29
	Bold (B)	<2 mm	1
Physicochemical Properties			
Amylose content	Waxy/glutinous (W)	0 - 2%	6
	Very low (VL)	2.1 - 10.0%	4
	Low (L)	10.1 - 20.0%	18
Gelatinization temperature	Intermediate (I)*	20.1 - 25.0%	28
	High (H)	>25%	2
	High (H)	74.5 - 80.0°C	2
	Intermediate (I)*	70 - 74oC	19
	Low (L)	<70 - 80oC	17

* Recommended values based on National Cooperative Testing (NCT) Manual for Rice. 1997. Rice Technical Working Group, National Seed Industry Council, Department of Agriculture.

The mutation resulted in the shifting of the rice starch quality among mutant rice lines; hence, the rice samples were categorized as non-glutinous aromatic or glutinous. Another group of the potentially high-yielding Gal-ong mutant was also included. For the glutinous rice samples, improved Malagkit Sungsong (MS) was used as a check. When cooked, it is slightly white, aromatic, smooth, and tasty. Among the cooked glutinous Gal-ong mutant rice, GXB-209 and GXB-210-1 were the most improved in terms of aroma, smoothness, and taste. When cooked, the slightly purple raw rice color of mutants GXB-209 and GXB-201-1 appeared slightly black but the others retained their raw rice color despite cooking. These are even much smoother and tastier than the check MS rice. These are slightly purple when raw and becomes slightly black when cooked. Generally, when cooked, the other mutant rice lines evaluated under this category were slightly aromatic, smooth to very smooth, and slightly tasty to tasty. While the MS rice has soft raw rice grains, the grains of raw mutant lines were relatively harder except for GXB-210-1, which was slightly hard. The raw rice form of all other entries, including the MS, was non-aromatic, dull, and 81 - 100% chalky in terms of translucence. The raw rice grain pigment of the mutants varied from slightly red, slightly purple, to slightly black. Compared with white rice, rice variants with pigmented grains are known to have improved nutritional value, as these accumulate higher levels of nutrition within the endosperm. Figure 3 shows that the **i**nduced mutation created a range of phenotypic variations in grain shape (bold to long and slender), and pericarp color (red, white, brown, and purple/ black). The black and purple colors of rice pericarps are due to flavonoid anthocyanin accumulation while the red color is due to proanthocyanidins (Gunaratne et al., 2013; Samyor et al., 2017). Pigmented rice varieties also tend to have a higher protein content with a well-balanced amino acid composition, a better glycemic index, and higher levels of fats, fiber, and vitamin E (Gunaratne et al., 2013; Hedge et al., 2013; Kushwaha, 2016). They also exhibit strong antioxidant and free radical scavenging capacity due to high levels of phenolic compounds such as anthocyanin, proanthocyanidin, and phenolic acids (Samyor et al., 2017).

Among the non-glutinous Gal-ong mutants, only the GXB-310-2 retained a slight aroma of cooked rice, which is relatively less aromatic than the check varieties WTGO, MS, and Burdagol. Its raw rice was also translucent. The third group of mutant rice is composed of potentially high-yielding lines. The aroma was lost in the WTGO except for the white-grained, smooth, and slightly tasty GXB-310-2.

Molecular characterization of promising Gal-ong mutants

Figure 5 shows the genetic variation among the 10 representative putative Gal-ong mutants with various combinations of phenotypic variations, constructed using NTSYS, and grouped by UPGMA. This analysis is partial because only 10 polymorphic SSR markers were examined. The phylogenetic tree showed three distinct clusters with GXB-310-2 being the most genetically distant with only about 50% relatedness to the rest of the samples. The second cluster, the largest one, had the 8 mutants grouped together. Interestingly, the two promising mutant lines that ranked 1st and 2nd in the yield trial

Table 5. Physical and physicochemical attributes of selected promising Gal-ong mutant lines.

Designation/ Code	Generation	%Mc	%, Grain Class		Grain Length (Mm, Class)	Grain Width (Mm)	Grain Shape (L/W, Class)	Amylose (%, Class)	Gel Temp (Asv**, Class)	Raw Rice (Good Eating)		Cooked Rice (Good Eating)				
			Chalky	Immature						Color	Translucency	Aroma	Color	Tenderness	Smoothness	Taste
Non-glutinous aromatic																
GXB-310-2	M5	11.9	G2	G1	L	2.3	I	L	L	Slightly creamish	21-40% chalky	Aromatic	White	Tender	Smooth	Slightly tasty
GXB-311-3	M5	13.1	G2	Pr	M	2.2	I	I	I/L	Very red	translucent	No aroma	Red	Slightly tender	Rough	Bland
SB-1	M7	12.6	G2	Pr	M	2.3	I	L	I/HI	Slightly red	81-100% chalky	Slightly aromatic	Slightly red	Tender	Smooth	Slightly tasty
SB-3	M7	11.7	G3	Pr	M	2.3	I	L	I	Creamish	21-40% chalky	No aroma	Slightly creamish	Tender	Smooth	Bland
GXB-1	M7	11.1	G3	Pr	L	2.2	S	I	I/HI	Slightly grayish	21-40% chalky	No aroma	Slightly white	Slightly tender		Bland
GXB-2	M7	12.0	aa	G1	L	2.4	I	I	I	Slightly grayish	21-40% chalky	No aroma	Slightly grayish	Slightly tender		Bland
Burdagol (control)	-	-	-	-	-	-	-	-	-	White	21-40% white belly	Aromatic	White	Tender		Slightly tasty
Glutinous																
GXB-309	M5	8.0	G1	G1	Sh	2.7	I	W	I/HI	Slightly red	81-100% chalky	Slightly aromatic	Slightly red	Very tender	Very smooth	Tasty
GXB-317	M5	8.8	G1	G1	Sh	2.6	I	W	L	Slightly red	81-100% chalky	Slightly aromatic	Slightly red	Very tender	Very smooth	Tasty
GXB-210-1	M6	12.5	G1	Pr	M	1.9	S	W	L	Slightly purple	81-100% chalky	Aromatic	Slightly black	Very tender	Very smooth	Tasty
GXB-209	M6	10.8	G1	G1	M	1.9	S	W	L	Slightly purple	81-100% chalky	Aromatic	Slightly black	Very tender	Very smooth	Tasty
IMS (control)	-	-	-	-	-	-	-	-	-	White	81-100% chalky	Aromatic	Slightly white	Very tender	Smooth	Tasty
High-yielding																
GXB-204	M6	12.9	aa	Pr	L	2.1	S	L	H/I/HI	Slightly grayish	81-100% chalky	No aroma	Slightly creamish	Slightly tender	Slightly smooth	Bland
GXB-205	M6	10.8	G2	G1	L	2.0	S	VL	H	Slightly grayish	61-80% chalky	No aroma	Slightly grayish	Slightly tender	Slightly smooth	Bland
GXB-207	M6	12.7	G1	G1	L	2.1	S	VL	H	Slightly grayish	21-40% chalky	No aroma	Slightly grayish	Slightly tender	Slightly smooth	Bland
GXB-208	M6	12.3	aa	G1	L	2.2	I	L	H/I	Creamish	translucent	No aroma	Slightly creamish	Slightly tender	Slightly smooth	Bland
GXB-229	M6	10.6	G3	Pr	L	2.2	S	L	HI/I	Slightly grayish	41-60% white belly	No aroma	Slightly creamish	Tender	Slightly smooth	Bland
SB-5	M7	11.4	G1	Pr	M	2.3	I	L	I	Slightly creamish	21-40% chalky	No aroma	White	Slightly tender	Slightly smooth	Bland
IR 64 (Control)	-	-	-	-	-	-	-	-	-	Slightly creamish	21-40% chalky	No aroma	Slightly white	Tender	Slightly smooth	Bland
Gal-ong	Wild Type	10.0	Pr	Pr	M	2.7	I	L	L	Slightly red	61-80% chalky	Aromatic	Red	Slightly hard	Slightly smooth	Slightly tasty

Aroma: Extremely aromatic - perceived/smelled when the container is opened; slightly aromatic - perceived/smelled when the sample is sniffed several times; none - not perceived; smells like an ordinary rice; "-" data not available

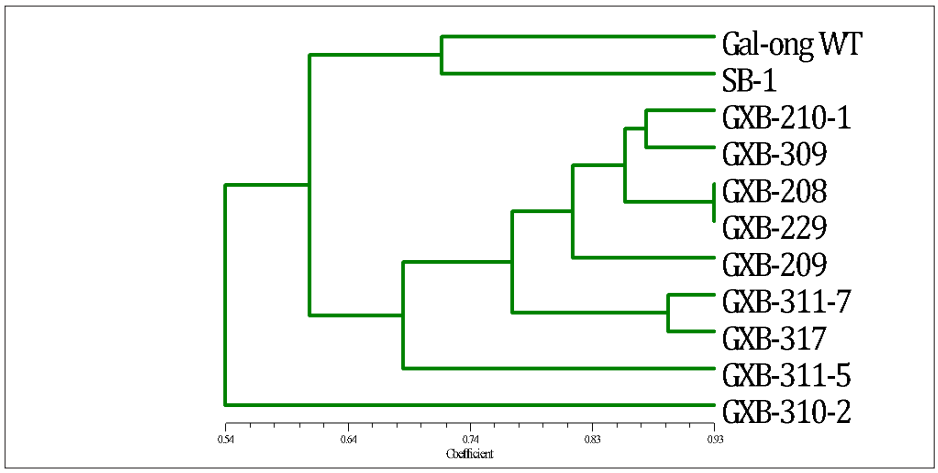

Figure 5. Genetic analysis on the similarity of putative Gal-ong mutants with the WTGO, constructed using NTSYS, and grouped by UPGMA.

conducted in 2017 WS, the GXB-208, and GXB-229, were 93% genetically similar at this point. This is consistent with their observed phenotypic variations as both were white-pericarp, non-glutinous, with good kernels, and excellent eating quality, but without aroma. Likewise, GXB-311-7 and GXB-311-5 were almost 90% genetically similar. These two are red-pericarp and non-glutinous, with the good kernel, excellent eating quality, and extremely aromatic. Notably, SB-1 appeared closest to the genotype of the WTGO. Comparing the phenotypic variations, a significant difference in the kernel and eating quality was observed, with the WTGO being relatively inferior. At this point, the level of genetic variation in the mutant population is somehow high as the degree of similarity can be measured from 50 to 60%. Though this stillneeds further genetic characterization and analysis, partial data show that the representative Gal-ong mutants can serve as valuable resources for enlarging the genetic base in the breeding programs (Nachimuthu et al., 2015). Though numerous kinds of molecular markers have been used in analyzing genetic dissimilarity in rice (Ni et al., 2002), the use of SSR markers is particularly prominent in evaluating genetic diversity as this can detect a remarkably great level of polymorphism among closely related rice cultivars (Ram et al., 2007; Akagi et al., 1997). It is also interesting to use these Gal-ong mutants in further research involving mapping techniques and next-generation sequencing to explore the function of key genes affected by a mutation, which resulted in a certain desirable phenotypic variation and a boost in functional genomics discoveries.

Conclusion and Recommendations

Mutation breeding generated M_5-M_7 Gal-ong rice mutants with increased yield and other combinations of improved traits. This study demonstrated the efficiency of gamma irradiation at 250 gy as a tool for inducing a wide range of genetic variations. At least 16 promising M_4-M_6 Gal-ong mutant rice lines were generated with about 200 - 300% yield advantage over the WTGO. Varying grain color from slightly red to slightly black or purple was also observed among the selected mutant lines; hence, interesting to determine the variations in anthocyanin contents. The aroma and taste of WTGO were generally lost in mutant lines classified with high yield improvement, except for white-grained and putatively BLB-resistant line GXB-310. GXB- 209, which is slightly black-pigmented and glutinous, is another interesting mutant line. Its grain quality was relatively better than the three check varieties, specifically in terms of smoothness and taste. Among the significant trait improvements in the WTGO that may help improve its yield are the apparent long-dense panicles, reduced the number of days to maturity, short stature, and insect pest and disease resistance of the promising lines. The mutants generated in this study are promising sources of agronomically important genetic variations useful in rice breeding, and good materials for gene discovery, and molecular marker development. The outputs of this study will eventually help satisfy the demands of high-grain quality Gal-ong rice.

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FULL PAPER

PURITY AND STABILITY EVALUATION OF PHILRICE-BRED LINE PR35742A AND PR35742B

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Abstract

Genetic purity of parental lines is a corner stone of three-line hybrid development and yield performance of the F1 hybrids. In this study, purity and stability of cytoplasmic male sterile (CMS) and maintainer (B line) of PR35742 bred by Philippine Rice Research Institute (PhilRice) were evaluated using the following processes: (1) seed source purity evaluation; (2) selection of true genotype by paircrossing; (3) classification of true genotype in identification nursery; (4) evaluation of true genotype by pair re-backcrossing; and (5) confirmation and validation based on pollen sterility and stability. Results showed that purity of the starting material of PR35742 A and B was <80% and did not pass the NSIC criteria for seed classification for parental lines. This was further confirmed when the selected A line plants from phenotypically evaluated uniform plants showed various degrees of pollen sterility. Only 55% of them were completely sterile. The recovered genotype selected in the series of re-backcrossing showed sterility fluctuation after four generation of re-backcrossing. Moreover, selected A plants from advanced generation line showed changes in pollen sterility and partial seed set when ratooned. This indicates that the original genotypes of PR35742A and PR35742B were already lost or altered, which could be due to biological and mechanical admixture. It can also be attributed to the natural variation or the maternal origin of PR35742A, which is the CMS line 28A carrying CMS Boro II-Taichung 65 (BT), type cytosterility source. This type of cytosterility source is easily influenced by the environmental condition that may result to sterility fluctuation and instability. Understanding the parentage information of developed CMS and maintainer lines is vital because it provides fundamental facts that can clarify their genetic and behavioral characteristics. Furthermore, established data can facilitate genetic basis particularly when interpreting sterility and stability research.

Keywords: *CMS, Maintainer, Pollen Sterility, Stability*

Introduction

Cytoplasmic male sterile (CMS) or A line, maintainer (B line), and restorer lines (R line) are needed in the development of three-line hybrids. The seeds purity of these parental lines play important role for expressing yield potential of developed F_1 hybrids. The A and B lines have similar morphology except that A line has cytoplasmic sterile gene with nuclear recessive gene (*S, rf*) while maintainer has cytoplasmic fertile gene with nuclear recessive gene (*N, rf*) (Pacada et al., 2021). Genetic purity of A and B can be determined by their genetic and phenotypic expression, further validated by their A x B progeny. CMS lines with wild abortive (WA) type has irregular or withered pollen, some are triangular and shuttle-shape, and remain unstained by IKI (Iodine Potassium Iodide) solution. By visual inspection, anther is empty, slender and thin, with whitish color. The WA type pollen abortion occurs during single nucleus stage or in uninucleate stage; thus, withered shape pollen morphology is observed (Yuan and Fu, 1995; Rao, 1988). The physiological and phenotypic characters of CMS derived from WA types are more stable in pollen sterility despite of varying environmental condition compared with other CMS types specifically in Boro II-Taichung 65 (BT) and Tian 1 type (Li et al., 2007; Joshi et al., 2001; Yuan and Fu, 1995; Pradhan et al., 1990). Thus, the most commonly used cytosterility sources in developing F_1 hybrids in local and abroad are WA (Li et al., 2007; Virmani et al., 1997). However, not all the cytosterility sources have the same interaction to various fertility restoring (*rf*) gene, particularly if the cytosterility source originated from other countries.

PhilRice-bred lines PR35742A and PR35742B developed in 2002 were the product of crossing IRRI-bred maintainer line IR72079B and PR35732A. CMS line PR35732A was developed by utilizing CMS line 28A as source of cytosterility for breeding CMS line and their maintainers at PhilRice. The CMS line, however, was observed to be unstable as pollen morphology of A line changed from unstained (infertile) to stained (fertile). To evaluate and further examined its useability for three-line hybrid

development, pollen sterility and stability were investigated in this study.

Materials and Methods

Plant materials

Two seed sources of parent lines from three-line breeding were used, namely A line from Foundation seed production and B line from purification nursery produced during 2019 dry season (DS).

Experimental set up establishment and layouting

Six set ups were established. The first three set ups were established in the field (2019 wet season [WS], 2020 DS, 2020 WS), while the other three set ups were established in a PhilRice screenhouse (October 2020 - January 2021, March - June 2021, and July - November 2021). Illustration of purity and stability evaluation process is shown in Figure 1. All the seeds were pre-germinated and planted after 21 days. One seedling/hill of A and B lines of PR35742 were transplanted using 20 cm x 20 cm spacing in the field and screenhouse. For the 2nd to 6th set up, the A line (depending on the availability of A seedlings) was planted in 1 - 5 rows with 22 hills/row, and two rows of 22 hills for the B line.

Purity evaluation

Classification of degree of purity of PR35742A and PR35742B seed source was based on the classification by National Seed Industry Council (NSIC) for parental seeds of hybrid rice. A and B were observed for their purity from maximum tillering until physiological maturity. The true type features were based on the agro-morphological traits of PR35742A and PR35742B. Identification of % mixture or off types were determined by ratio of off types to total plants planted x 100. PR35742A purity classification was based on Virmani et al. (1997), whereby plants exhibiting the true type and with 100% pollen sterility were selected.

Selection of true genotype for testcrossing

Generation of pair crosses (PCs), were carried out by selecting A plants with complete sterility (CS) and unstained pollen, while the B plants were selected from the population exhibiting true type features based on agronomic and morphological characterization.

Spikelet collection and pollen evaluation of a plants

Spikelet was collected when 30% of the panicle has emerged from the plants. Two approaches were

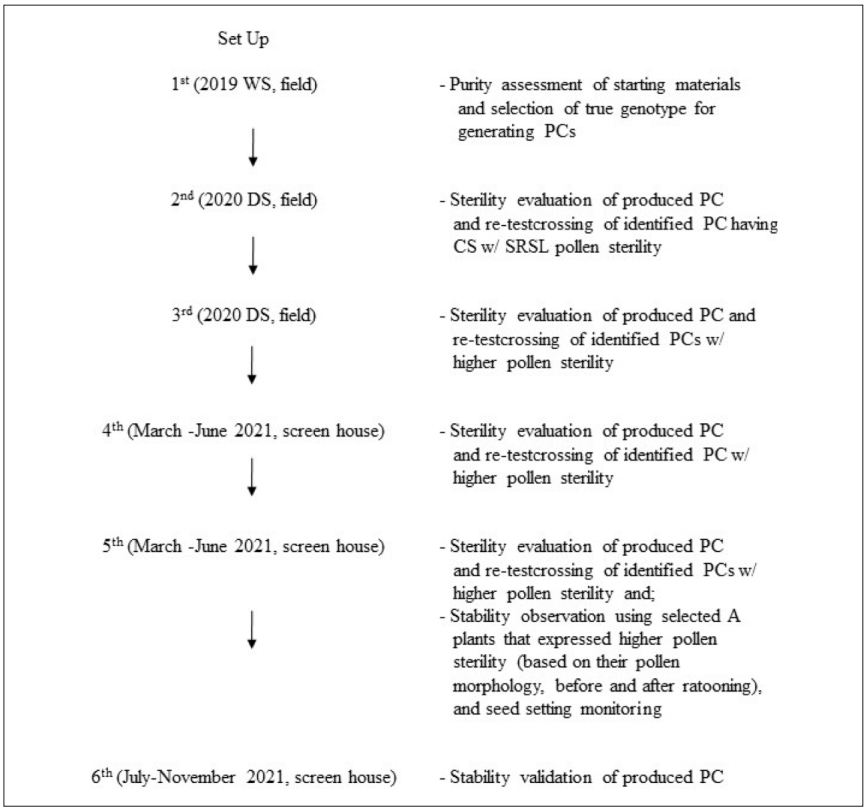


Figure 1. Purity and stability process of A and B line of PR35742. PC - pair crosses; CS -completer sterile; SRSL - stained round sterile light.

used: collection of main shoots with emerged panicle and direct collection of five unopened and matured spikelets from three areas (upper, middle, and bottom), within the emerged panicle. After tagging, the collected main shoot was placed immediately in pail with water while the collected spikelets were placed in 2 mL tube with 70% alcohol.

Anthers from three unopened and matured spikelets from top, middle, and bottom were placed and squashed on glass slides with 1% IKI solution. The same procedure was followed in spikelet, in which anthers were directly collected from the emerged panicle. Anthers were then pressed gently using a micro spatula to release pollen grains. All the debris or any foreign materials were removed. Three random microscopic fields were observed to examine the pollen.

Classification of true genotype

Selected genotype was evaluated by establishing the generated PCs in the identification nursery. The same process was followed for purity evaluation; however, a detailed classification for pollen sterility was carried out. In addition, pollen morphology of each A x B progeny was evaluated and categorized based on unstained withered (UWS) and unstained spherical sterile (USS), stained round sterile (SRS), and stained round fertile (Table 1). True genotype possessed unstained pollen in all evaluated A x B progenies. Backcrossing in pairs was executed when no unstained pollen was identified among the evaluated A x B progenies. The first produced PC was used for the source of re-backcrossing for extracting the true genotype. It was done by selecting plants within the evaluated PCs with no fertile pollen or less stained pollen. Pollen sterility inheritance of CMS line PR35742A was assessed in this process and pollen morphology information were classified as completely sterile, not completely sterile, and fertile (Table 1).

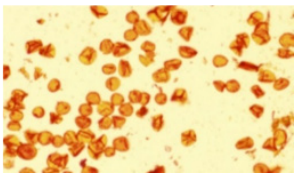
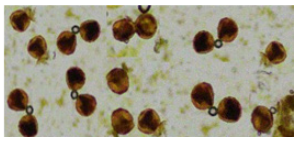
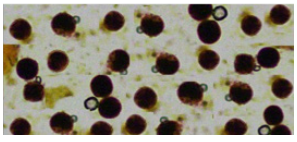
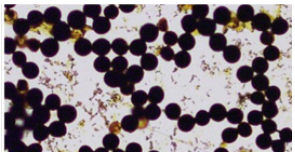
Evaluation of selected A and B line genotype

Generated paircross was evaluated in the identification nursery set up. The pollen sterility from evaluated PCs with unstained pollen or 100% CS and unstained pollen + stained round sterile-light were monitored. The two classifications were also used for pollen sterility stability evaluation.

Confirmation and validation of sterility and stability

The nature of pollen morphology was classified and concluded by observing the pollen morphology of all evaluated A x B progenies. In addition, selected A plants were ratooned to validate the pollen sterility stability by observing the percentage of unstained

Table 1. Key guide for selection for A plants. Pollen magnified using 10X electron microscope.

Pollen Category	Pollen Morphology Appearance	Classification	Remarks
Unstained withered sterile (UWS) Unstained spherical sterile (USS)		Complete Sterile (CS)	selected for PC
Stained round sterile-light (SRS-L)		not Complete Sterile (nCS)	not selected for PC
Stained round sterile-dark (SRS-D)		not Complete Sterile (nCS)	not selected for PC
Stained round fertile (SRF)		Fertile	not selected for PC

pollen from un-ratooned and ratooned plants. All ratooned plants were isolated to prevent pollen contamination while all tillers of the ratooned plants were observed for pollen sterility and seed setting. Percentage of seed setting was evaluated using tillers from ratooned A plants. Below is the formula for % seed set: number of unfilled grains were divided by the total number of filled and unfilled grains.

$$\%\ Seed\ set = \frac{\text{Number of unfilled grains}}{\text{Total number of filled and unfilled grains}} \times 100$$

Results and Discussion

Selection of best pair that produced typical, uniform, and stable progeny

There were 480 A line plants and 210 B line plants evaluated during the first set up in 2019 WS. Based on the phenotypic observation for uniformity and true type of PR35742, an estimated of 30% and 20% off types were observed among B and A plants population, respectively (Figure 2). From the observed true type, % distribution of pollen morphology from the selected 30 A plants showed that only 45% have 100% CS pollen, combination of withered sterile (UWS), and unstained round pollen (USS). Meanwhile, 13% of plants were observed to have dark stained round sterile pollen, 16% have sterile pollen, and 26% has fertile pollen (Figure 3). Based on 2005 National Seed Industry Council (NSIC) guideline, the maximum number of off-types for A and B breeder and foundation seeds are 0.02 and 0.05%, correspondingly. The observed off types were too high compared with the set guidelines of NSIC; thus, did not pass the NSIC criteria. This indicates that the degree of purity for foundation seed specifically for the CMS line was significantly low. Moreover, A and B plants expressing phenotypic uniformity in the field did not guarantee their genetic purity, which was confirmed by the percentage pollen sterility observed from the 30 A plants with uniform growth stand, 26% of them exhibited fertile pollen (Figure 3). The off types in the B line population were expected because the seed source was from the purification nursery and the desired genotypes for B (*N, rf*) was not yet achieved or fixed. To assure the genetic purity of parentals used in three-line breeding, genetic purity should be re-evaluated particularly for breeder and foundation seed stocks. Performance of hybrids depends of the purity of parental lines. Based on the research every 1% of decrease in purity of hybrid seeds is equal to a yield loss of 100 kg ha^{-1} (Virmani et al., 1997). This may also be applied for developing experimental F_1 hybrids, in which expected yield potential will be masked due to poor seed quality of parent lines; thus, limiting the selection of heterotic combination.

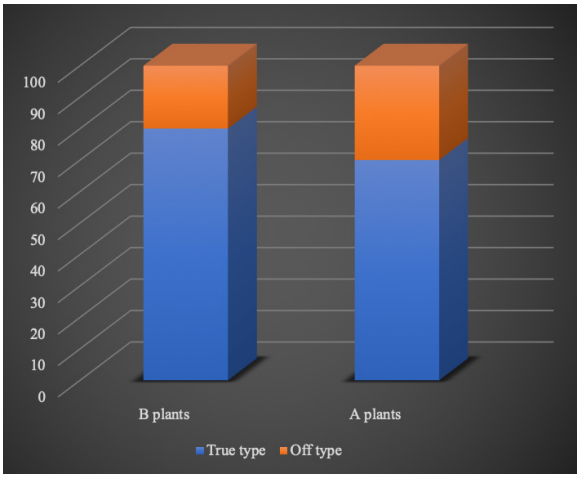


Figure 2. Purity evaluation of PR35742A and PR35742B based on grow-out uniformity expression during 2019 WS, 1st set up.

Confirmation and validation of sterility and stability

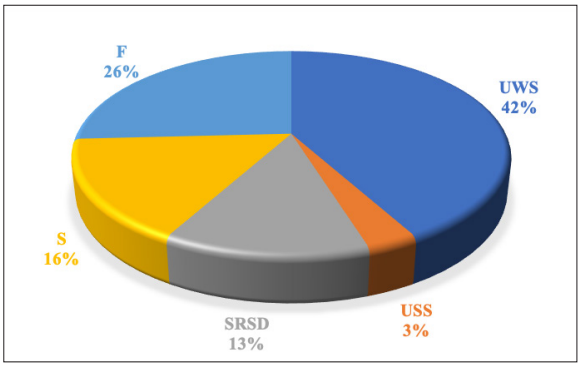


Figure 3. Purity evaluation of 30 A plants of PR35742 based on the degree of pollen sterility plants during 2019 WS. UWS - unstained withered sterile; USS - unstained spherical sterile; SRS-D - stained round sterile-dark; S - sterile; F - fertile.

From the selected genotype, seven PCs were established in the identification nursery during the second set up in 2020 DS. All plants from each PCs were uniform during the overall growth stage; however, no PCs showed 100% unstained pollen among the evaluated A x B progeny. Expression of stained and fertile pollen were also detected (Table 2). Six PC was used for re-backcrossing from the seven produced PC. Twenty-one PCs were produced and evaluated during third set up in 2020 WS. True genotypes of A and B were identified from PC3.6, PC3.7, PC3.9, and PC3.14. The A x B progenies of these PCs exhibited unstained pollen or 100% CS (Table 3). However, during the validation and confirmation of A and B genotype of PC3.6 and PC3.7 during the fourth set up last October 2020 to January 2021, stained pollen (light and dark) was expressed (Table 4). If PC 3.6 and PC3.7 contain cytoplasmic sterile gene with nuclear recessive gene (*S, rf*), and cytoplasmic fertile gene with nuclear recessive gene

Table 2. Pollen sterility evaluation of A x B progeny from selected A and B genotype during 2020 DS (2nd set up). CS - complete terile/unstained pollen; UWS - unstained withered sterile; USS - unstained spherical sterile; SRS-D - stained round sterile-dark; S - sterile; F - fertile.

2020 DS Code (2nd Set Up)	Pollen Morphology of Evaluated Plants					Total No. of A x B Progeny Evaluated	A x B Progeny Pollen Sterility	
	UWS	USS	SRSL	SRSD	F		% CS	% CS + SRSL
2.1	16	0	2	0	1	19	0	-
2.2	10	0	1	0	1	12	0	-
2.3	10	0	1	0	0	11	0	100
2.4	11	0	1	1	0	13	0	-
2.5	17	0	2	2	0	21	0	-
2.6	9	0	2	1	2	14	0	-
2.7	13	0	1	0	0	14	0	100

Table 3. Pollen sterility evaluation of recovered genotype during 2020 WS (3rd set up). CS -complete sterile/unstained pollen; UWS - unstained withered sterile; USS - unstained spherical sterile; SRS-D - stained round sterile-dark; S - sterile; F - fertile.

2020 WS Code (3rd Set Up)	Source Code	Pollen Morphology of Evaluated Plants					Total No. of Evaluated A x B Progeny	A x B Progeny Pollen Sterility	
		UWS	USS	SRSL	SRSD	F		% CS	% CS + SRSL
3.1	2.1	26	0	0	2	1	29	0	-
3.2	2.1	26	24	0	4	2	56	0	-
3.3	2.1	15	3	1	2	1	22	0	-
3.4	2.1	6	6	0	4	0	16	0	-
3.5	2.1	1	2	0	2	0	5	0	-
3.6	2.1	6	2	0	0	0	8	100	
3.7	2.1	26	15	0	0	0	41	100	
3.8	2.2	38	24	6	0	0	68	0	100
3.9	2.2	3	4	0	0	0	7	100	
3.10	2.2	18	10	0	2	0	30	0	-
3.11	2.2	10	11	0	2	0	23	0	-
3.12	2.2	8	3	0	0	3	14	0	-
3.13	2.3	4	1	0	3	1	9	0	-
3.14	2.3	2	4	0	0	0	6	100	
3.15	2.3	4	4	0	1	0	9	0	-
3.16	2.3	2	0	2	0	1	5	0	-
3.17	2.4	16	15	0	2	0	33	0	-
3.18	2.5	21	12	0	2	0	35	0	-
3.19	2.7	9	17	1	2	1	30	0	-
3.20	2.7	17	13	0	0	1	31	0	-
3.21	2.7	9	0	2	2	1	14	0	-

(*N, rf*), all the produced progeny (PC4.1 to 4.13) should have unstained pollen or CS and there will be no pollen sterility fluctuation observed among the A X B progeny evaluated.

These assumptions can be further examined in PC3.7 progeny, in which the 148 evaluated plants (PC 4.3 - PC4.13), a13 plants or only 9% exhibited 100% CS and the rest have stained sterile pollen. Pollen sterility variation among the evaluated among progeny suggests that the recovered B and A genotype carry the incorrect pair of gene (cytoplasmic sterile gene and cytoplasmic fertile gene) that lead to intercept or block the expression of complete sterile plants among the evaluated progenies. Moreover, this theory can be validated in the pollen sterility expression of A x B progeny of PC5.2 - 5.12 where no plants with complete sterility (100% CS) was identified. In addition, out of 547 A x B progenies evaluated, only 78% were unstained, and category of the produced A x B progeny was only % CS + SRSL. Instability was further confirmed from the established re-backcrosses from eight PC combination. Data showed that unstained pollen was reduced from 78%

(Table 5) to 59% (Table 6). Moreover, the number of dark stained round sterile was increase in evaluated progeny of PC5.1 (PC 6.1), PC5.5 (PC6.7), and other PC except for PC6.9 - PC 6.1 where % CS + SRSL category was maintained in the evaluated A x B progeny. Schematic diagram of purity and stability evaluation used in this study is illustrated in Figure 4.

It is concluded that PR35742A and PR35742B were unstable in the selected 23 A plants from the 5th ratooned set up. Only seven retained the unstained pollen four showed light stained pollen, while the remaining 12 A plants displayed dark stained pollen. From the stained pollen, some of the A plants showed seed settings (Table 7), suggesting that PR35742 true genotype had been lost due to biological and mechanical admixture. This instability may also be attributed to the CMS 28A line, PR35742A maternal origin, which carry BT cytosterility. The introduced CMS 28A line from India was also used in breeding PR35732A, a CMS line used for the development of PR35742A and for CMS conversion of PSB Rc12 (PR35732B) and IR72079B (PR35742B).

Based on the report of Yuan and Fu (1995), anther and pollen morphology of these BT cytosterility source, 28A anthers are slender, milky yellow but indehiscent. The pollen grains are round and lightly stained by IKI. The BT cytosterility pollen abortion happened at the latter stage of microspore

Table 4. Pollen sterility re-evaluation of A x B progeny with 100% CS during October 2020 - January 2021 (4th set up). CS - complete sterile/unstained pollen; UWS - unstained withered sterile; USS - unstained spherical sterile; SRS-D - stained round sterile-dark; S - sterile; F - fertile.

Oct. 2020 – Jan. 2021 (4th Set Up)	Source Code	Pollen Morphology of Evaluated Plants					Total No. of Evaluated A x B Progeny	A x B Progeny Pollen Sterility	
		UWS	USS	SRSL	SRSD	F		% CS	% CS + SRSL
4.1	3.6	7	0	0	1	0	8	0	-
4.2	3.6	17	0	3	3	0	23	0	-
4.3	3.7	3	0	1	0	0	4	0	-
4.4	3.7	6	0	1	2	0	9	0	-
4.5	3.7	21	0	5	1	0	27	0	-
4.6	3.7	9	0	0	2	0	11	0	-
4.7	3.7	16	0	2	0	0	18	0	-
4.8	3.7	27	0	1	5	0	33	0	-
4.9	3.7	12	0	2	2	0	16	0	-
4.10	3.7	1	0	0	0	0	1	100	
4.11	3.7	0	0	2	0	0	2	0	-
4.12	3.7	12	0	0	0	0	12	100	
4.13	3.7	13	0	0	2	0	15	0	-

Table 5. Confirmation and validation of A x B progeny of recovered B genotype during March - June 2021 (5th set up). CS - complete sterile/unstained pollen; UWS - unstained withered sterile; USS - unstained spherical sterile; SRS-D - stained round sterile-dark; S - sterile; F - fertile.

March - June 2021(5th set up)	Source Code	Pollen Morphology of Evaluated Plants					Total No. of A x B Progeny Evaluated	A x B Progeny Pollen Sterility	
		UWS	USS	SRSL	SRSD	F		% CS	% CS + SRSL
5.1	4.10	22	5	17	2	0	46	0	-
5.2	4.12	35	21	14	0	0	70	0	100
5.3	4.12	13	16	8	0	0	37	0	100
5.4	4.12	15	8	10	0	0	33	0	100
5.5	4.12	17	15	9	1	0	42	0	-
5.6	4.12	22	4	4	0	0	30	0	100
5.7	4.12	17	20	8	0	0	45	0	100
5.8	4.12	15	4	1	0	0	20	0	100
5.9	4.12	15	7	6	0	0	28	0	100
5.10	4.12	29	21	7	0	0	57	0	100
5.11	4.12	25	16	9	0	0	50	0	100
5.12	4.12	41	22	26	0	0	89	0	100

development; thus, presence of starch is detected when stained by IKI solution. (Virmani et al., 1997; Yuan and Fu, 1995). However, when this CMS line used in CMS conversion of IR72079B (PR35742B), the pollen morphology of developed CMS line PR35742A showed WA type but with some light and partial stained pollen. This interaction between the genetic factors in the cytoplasm and the nucleus of IR72079B and CMS line PR35732A may result in different pollen abortion within the plants, some abort during uninucleate, binucleate, and some in trinucleate stage. This can be seen in the pollen morphology of evaluated plants from first to sixth set up, in which the expression of UWS (uninucleate stage), USS (binucleate stage), and SRS (trinucleate stage), were observed in generated A x B progeny. This pollen characteristics of CMS line when established at higher temperature and lower moisture conditions, favored anther dehiscense in the CMS line resulting in selfing (Li et al., 2007; Yuan and Fu, 1995). Virmani et al. (1997) indicated that useable CMS line for developing commercial hybrids should have stable and complete pollen sterility across environments and easily maintained. Similarly, Joshi et al. (2001) found that stability of pollen sterility of a CMS line is an important characteristic for hybrid rice to be commercially useful with the established pollen sterility and stability. As such, the use of CMS line, PR35742A, is not recommended in developing three-line hybrids.

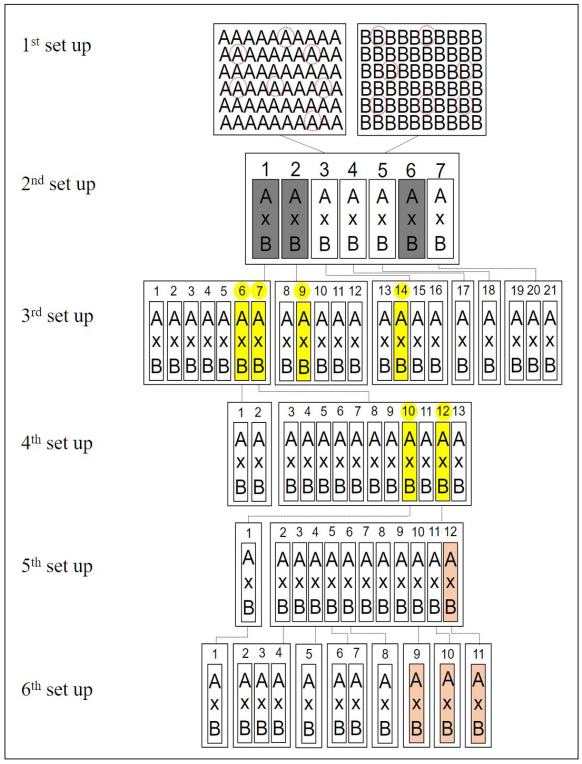


Figure 4. Schematic diagram of purification of PR35742A and PR35742B. Yellow shade denotes complete sterile (CS) A x B progeny, black indicates the presence of fertile pollen in the evaluated A x B progeny, and light orange represents the combination of complete sterile and light stained round sterile (SRSL) pollen among the evaluated progeny. 1st set up, 2021 WS; 2nd set up, 2020 DS; 3rd set up, 2020 WS; 4th set up, Oct. - 2020 – Jan. 2021 DS; 5th set up, March - June 2021; 6th set up, July - Nov. 2021.

Table 6. Pollen sterility evaluation of generated 11 pair crosses during July - November 2021 (6th set up). CS - complete sterile/ unstained pollen; UWS - unstained withered sterile; USS -unstained spherical sterile; SRS-D - stained round sterile-dark; S - sterile; F - fertile.

July – Nov. 2021 (6th Set Up)	Source Code	Pollen Morphology of Evaluated Plants					Total No. of A x B Progeny Evaluated	A x B Progeny Pollen Sterility	
		UWS	USS	SRSL	SRSD	F		% CS	% CS + SRSL
6.1	5.1	13	5	11	4	0	33	0	-
6.2	5.6	2	1	4	3	0	10	0	-
6.3	5.11	21	4	9	4	0	38	0	-
6.4	5.2	4	4	6	1	0	15	0	-
6.5	5.2	22	6	12	2	0	42	0	-
6.6	5.2	8	5	7	2	0	22	0	-
6.7	5.5	15	8	14	3	0	40	0	-
6.8	5.5	10	2	3	2	0	17	0	-
6.9	5.12	9	0	5	0	0	14	0	100
6.10	5.4	11	2	11	0	0	24	0	100
6.11	5.10	1	2	5	0	0	8	0	100

Table 7. Evaluation pollen sterility fluctuation and self-fertilization among the selected complete sterile plants from the 5th set up. UWS - unstained withered sterile; USS - unstained spherical sterile; SRS-D - stained round sterile-dark.

Source of Selected Plant	A x B Progeny Pollen Sterility		% Seed Set (Bagged Panicle, Before Ratoon)	Pollen Sterility (After Ratoon)	No. of Panicle Evaluated	No. of Panicle with Seed Set	% Seed Set/ Panicle
	% CS	% CS + SRSL					
5.1.1	0	-	-	SRSD	13	0	0
5.2.1	0	100	-	SRSD	5	0	0
5.2.2	0	100	-	UWS	9	0	0
5.2.3	0	100	0	SRSL	12	0	0
5.2.4	0	100	-	SRSL	8	0	0
5.2.5	0	100	-	SRSL	2	1	2.0
5.2.6	0	100	-	SRSL	7	0	0
5.3.1	0	100	-	SRSD	18	0	0
5.4.1	0	100	0	UWS	5	0	0
5.4.2	0	100	0	SRSD	4	1	1.1
5.4.3	0	100	-	USS	2	0	0
5.5.1	0	-	-	SRSD	20	0	0
5.5.2	0	100	0	USS	4	0	0
5.6.1	0	100	-	SRSD	6	0	0
5.10.1	0	100	-	SRSD	4	0	0
5.10.2	0	100	-	SRSD	5	0	0
5.11.1	0	100	-	USS	4	0	0
5.11.2	0	100	-	USS	4	0	0
5.12.1	0	100	-	SRSD	9	0	0
5.12.2	0	100	-	SRSD	14	3	3.3/5.3/3.3
5.12.3	0	100	0	USS	2	0	0
5.12.4	0	100	-	SRSD	7	0	0
5.12.5	0	100	0	SRSD	20	1	1.0

Conclusion

Parentage information of developed parent lines is vital specifically in the development of CMS and maintainer lines. These data support the unexplained pollen stability/sterility of CMS and maintainer lines like PR35742A and PR35742B.

Deterioration of purity of CMS or A and B line can be expressed basically by segregation in plant type, growth duration, and other traits. In WA type CMS line, degeneration of degree of sterility started when stained (light and dark) pollen appeared followed by expression of fertile pollen, and the occurrence of partial-self-fertilization. Maintaining the purity of parent lines used in developing three-line hybrids is a challenge to a hybrid rice breeder due to factors influencing purity degradation such as biological mixture, mechanical admixture, and natural variation. Adequate breeder seeds stock prevents the degradation of seed purity that may be associated in biological and mechanical admixture. Furthermore, establishment of quality assurance procedures in purifying CMS and its maintainer are important to ensure the quality of genetic material produced. It is recommended to refine the existing techniques that are currently used for parental purification.

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IDENTIFICATION AND CHARACTERIZATION OF RHIZOBACTERIAL DIVERSITY IN THE IRRIGATED RICE ECOSYSTEM IN CENTRAL LUZON USING 16S RIBOSOMAL RNA GENE SEQUENCING

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Abstract

Rhizobacteria live around the plant roots, producing compounds or performing activities that improve soil and plant health. Its diversity determines the capacity of the soil to sustain maximum microbial activity, hence also an indicator of soil fertility. In this study, 90 rhizobacterial isolates collected from the roots and rhizosphere of irrigated lowland rice from four provinces in Central Luzon were molecularly identified and characterized based on their 16S rRNA gene sequences. The rhizobacteria were categorized under 19 genera and 41 species. The phylogenetic tree analysis grouped the rhizobacteria into two significant phyla: Proteobacteria (60%) & Firmicutes (40%). The rice roots were found predominated by Enterobacter (28%), while Bacillus (48%) primarily dominated the rhizosphere. This study confirms the intimate association between potential PGPR and rice plants in the irrigated rice ecosystem. The identification of bacterial populations naturally existing in this type of ecosystem will provide the baseline information for selecting bacterial strains that can be used in the formulation of agricultural products to help improve soil fertility, reduce farm inputs, and increase productivity toward sustainable and safe agriculture in the irrigated lowlands.

Keywords: *PGPR, Roots, Rhizosphere, Soil, Agricultural Product*

Introduction

Soil microorganisms have a profound influence on plant and soil health (Bhattacharyya and Jha, 2012; Saharan and Nehra, 2011; Barea et al., 2005). The primary functions of soils depend on the processes regulated by microbial community structure. The structure and function of such microbial communities are directly regulated by the carbon flux and release of root exudates by the plant which in return, these plant-associated microorganisms influence plant health and growth (Dakora and Phillips, 2002). The rhizosphere is a habitat of diverse soil microorganisms and zone of intense microbial activity resulting in a pool of confined nutrients essential for plant development (Tilak et al., 2005; Burdman et al., 2000). Within the communities, three major groups of root-inhabiting bacteria are present: growth-promoting, growth-retarding, and growth-neutral. Beneficial symbiotic and free-living rhizobacteria have been termed plant growth-promoting rhizobacteria (PGPR) (Gray and Smith, 2005; Kloepper et al., 1980) and yield-increasing bacteria (Tang, 1994). PGPR significantly affects plant growth through a wide variety of mechanisms such as stress tolerance, nutrient fixation for easy uptake by the plant, plant growth regulation, production of siderophores, volatile organic compounds (VOCs), and protection enzymes such as chitinase, glucanase, and ACC-deaminase (García-Fraile et al., 2015; Choudhary et al., 2011). Bacterial species from diverse genera including *Pseudomonas, Klebsiella, Enterobacter,* and *Bacillus* have been reported to improve plant growth (Kloepper et al., 1989; Glick, 1995; Gururani et al., 2012). It is undisputed that rhizobacteria play a critical role in preserving soil fertility and improvement of plant growth and development (Saharan and Nehra, 2011); thus, its diversity should be conserved for maximum activity.

Taxonomists have studied different approaches to characterize soil microorganisms and understand their diversity. The traditional phenotypic and morphological way of characterization has many limitations in examining the diversity of soil microbes. This paved the way for the development of advanced molecular tools and techniques that provide the greatest and maximum information in analyzing the microbial diversity useful in establishing phylogeny. Phylogenetic analyses are important in identifying similarities among organisms, which leads to understanding the physiology and ecology of both culturable and non-culturable species (Kumar et al., 2011). Researchers rationalize the use of molecular techniques and bioinformatics tools to facilitate fast identification and characterization of soil microorganisms (Aquilanti et al., 2004; Becking, 2006). Molecular tools such as DNA sequence

analysis provide accurate information on the genetic diversity of soil microbes (Yang et al., 2007). The 16S rRNA gene of bacteria has been routinely used as a reliable molecular marker for phylogeny identification wherein it contains a conserved region and a unique array of sequences that are relative among species or different species (Woese, 1987).

This study was conducted to analyze, identify, and characterize isolated rhizobacteria from the irrigated lowland rice ecosystem in Central Luzon.

Materials and Methods

Sample collection

Samples were randomly collected from four irrigated lowland rice fields across Central Luzon (Nueva Ecija, Bulacan, Aurora, and Tarlac). About 45-day-old rice plant samples with soil adhering to the roots were pulled out from the ground. Soil and root samples were separately collected, placed in polyethylene bags, and kept cool in an ice box during transport to the laboratory.

Bacterial culture and purification

One gram of soil sample was serially diluted in Luria-Bertani broth (LB) up to 10^{-6} and mixed by shaking at 150 rpm for 30 min. Meanwhile, rice root samples were first washed with tap water to remove adhering soil. Root samples were washed thoroughly in sterile distilled water, disinfected with commercial bleach for 5 min, then with 75% ethyl alcohol solution for 3 min, rinsed with sterile distilled water six times, treated with 0.5% chloramine-T for 3 min, and finally rinsed with sterile distilled water. Air-dried root tissues were homogenized using a sterile mortar and pestle and suspended in 10 mL of sterile LB broth.

Bacterial genomic DNA isolation

The purified bacterial isolates were cultured for 48 h in 10 mL nutrient broth (NB) at 28°C±1, with shaking at 150 rpm. A 1.5 mL sample aliquot was centrifuged at 10,000 rpm for 2 min and the bacterial pellet was used in the DNA extraction following the modified CTAB protocol. The pellet was washed with 1 mL 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA) and centrifuged at 10,000 rpm for 2 min. The washed pellet was resuspended in 700 µL lysis buffer (1.4 M NaCl, 100 mM Tris pH 8, 20 mM EDTA pH 8, 2% CTAB) and 100 µL 10% sodium dodecyl sulfate (SDS) and incubated at 65°C for 30 min. The suspension was added with chloroform: isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 15 min. The upper aqueous phase was collected and added with 100 µL absolute isopropanol to precipitate the DNA. The precipitation was extended for 30 min at -20°C. The solution was centrifuged at 12,000 rpm for 15 min and the resulting DNA pellet was washed twice with 500 µL of 70% ethanol before resuspension in 50-100 µL 1X TE buffer, depending on the DNA pellet size. An equal amount of each DNA sample was added with 1X loading dye and electrophoresed on 1% agarose gel in TBE buffer (w/v) gel at 150 V cm-1 for 90 min. The gel was stained and documented using the Alpha Imager UV transilluminator gel documentation system. The extracted DNA samples were kept at -20°C until use.

PCR amplification

The 16S rRNA gene region of the bacterial genome was amplified by PCR using a pair of universal primer 27F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R, 5'-TACGG(C/T)TACCTTGTTACGAC TT-3' (Polz and Cavanaugh, 1998). The optimum annealing temperature and PCR amplification conditions for each primer pair were determined. The 50 µL PCR contained 125-200 ng DNA, 1 U Taq polymerase, 1X PCR buffer, 2 mM MgCl2, 0.2 mM each dNTPs, 0.2 mM each of the primers.For the PCR cycling, initial denaturation was carried out for 3 min at 94°C. This was followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The final elongation was carried out at 72°C for 5 min. The amplified PCR products were separated on 1.5% agarose (w/v) gel electrophoresis at 150 V cm-1 for 90 min in 1X TBE buffer and stained and documented as mentioned above. The 100 bp DNA ladder was used as a size marker.

The PCR-amplified products were purified using Vivantis Technologies Sdn Bhd, Malaysia DNA purification kit, and sent to 1st BASE Laboratory Sdn Bhd (Selangor, Malaysia) for DNA sequencing.

16S rRNA gene sequence analysis

BioEdit Sequence Alignment Editor v7.0.5.3 and ClustalX 2.1 were used to initially align and manually adjust the nucleotide sequences. Species identity and percent homology were confirmed using the BLASTn program (www.ncbi.nlm. nih.gov). The 16S rRNA gene sequences have been deposited in the GenBank nucleotide sequence database under Accession Nos. MK791688-791707, MK796024-796036, MK834677-834731, MK691597, and MK691689. The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei, 2021) with the MEGA (Version 11.0.13) software packages.

Results and Discussion

The key life-supporting functions of soils primarily depend on the processes mediated by microbial community structures, which include diverse populations of Rhizobacteria that aggressively colonize plant roots to promote plant productivity and immunity. In this study, 90 bacterial isolates from the rice rhizosphere and roots were purified and identified molecularly using 16S rRNA gene sequencing. These bacterial isolates from the irrigated lowland rice ecosystem in Central Luzon showed excellent viability and culturability. The full-length 16s rRNA gene region of the bacterial DNA measuring about 1500 bp was amplified using primer 27F-1492R. On the basis of 16S rRNA gene sequencing, the rhizobacterial community in rice ecology was generally classified into two phyla, 19 genera, and 41 species. Figure 1 illustrates that about 60% of the live, culturable isolates belong to the phylum proteobacteria indicating that most of them are gram-negative bacteria. Meanwhile, the remaining 40% were from the phylum *Firmicutes*, which is a group of gram-positive bacteria.

The existence of a diverse population of rhizobacteria belonging to genera of *Citrobacter, Acinetobacter, Klebsiella, Leclercia, Pseudomonas, Enterobacter, Bacillus, Paenibacillus, Enterococcus, Serratia, Aeromonas, Staphylococcus, Brevibacillus, Phytobacter, Dickeya, Burkholderia, Paraburkholderia, Atlantibacter,* and *Pantoea* was established and identified based on their 16s rRNA gene sequences as shown in Table 1. In the National Center for Biotechnology Information (NCBI) BLAST (basic local alignment search tool) alignment of gene sequences, the e-value describing the number of hits expected to see by chance was all zero, which implies a very significant sequence match (https://blast.ncbi.nlm.nih.gov/).

Table 1 presents the respective unique accession numbers given by the NCBI upon registration in the NCBI GenBank database. Because of the presence of root exudates and rhizodeposits, rhizospheric soil is considered a hot spot of extreme microbial interaction and activity (Compant et al., 2010); therefore, a habitat for a diverse group of soil microorganisms including growth-promoting bacteria. Our findings show that isolates belonging to the genus *Bacillus* were the most dominant, abundant, and ubiquitous in irrigated rice ecosystems comprising 32% of the total population as shown in Figure 2 and Table 2. *Enterobacter,* on the other hand, ranked second recording roughly 26% of the total population.

All other genera of rhizobacteria had a very low abundance ranging from about 1 to 6% of the total population. As illustrated in Figures 3 and 4, the roots were dominated by the species belonging to the genera of *Enterobacter* with 28% of the population while the rhizosphere was dominated by *Bacillus* species with 48%, respectively. Our findings showed that the rice roots and rhizosphere were colonized by different rhizobacteria reported with high physiological traits and a diverse group of bacterial populations exists in the rhizosphere and roots of rice plants in the irrigated lowland rice ecosystem.

The current findings agreed with the results of the study on the rhizobacterial diversity in rice fields as it showed *Bacillus* as the most dominant genera in

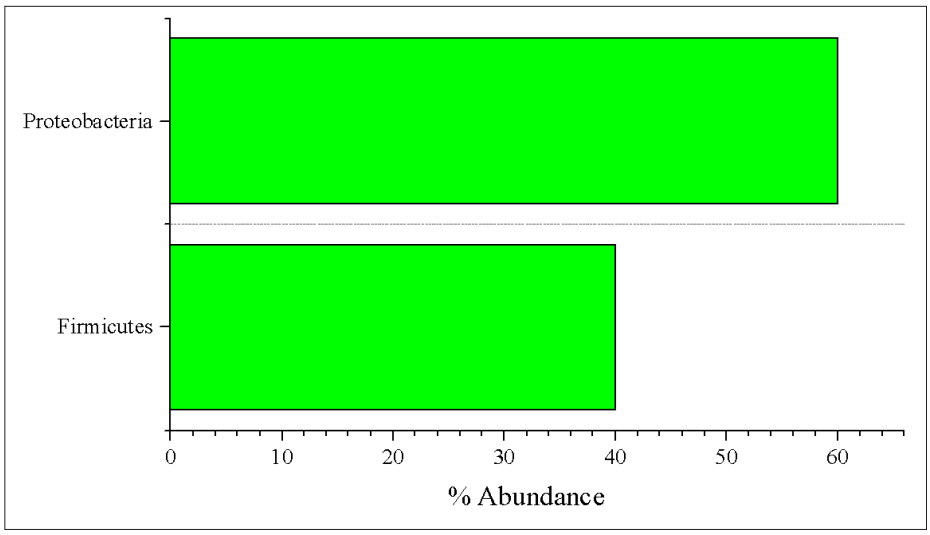

Figure 1. The abundance of bacterial isolates by phylum from rice roots and rhizosphere across four provinces in Central Luzon.

Table 1. The genetic identity of 90 rhizobacteria isolates in irrigated rice locations in four provinces in Central Luzon with their respective accession numbers deposited in the NCBI GenBank database.

Isolate No.	Rhizobacteria Registered Species Name	Accession No.	Source of Isolation	Location	BLAST Identified Species Name	Query Cover (%)	Similarity (%)	Accession Number
1	*Citrobacter* sp. FBMAX1	MK791688	Rice Roots	Nueva Ecija	*Citrobacter sp. XT-7*	100	99.64	KR063538.1
2	*Citrobacter* sp. FBMAX2	MK791689	Rice Roots	Nueva Ecija	*Citrobacter sp. XT-7*	100	99.50	KR063538.1
3	*Acinetobacter* sp. FBMAX3	MK791690	Rice Roots	Nueva Ecija	*Acinetobacter sp.*	98	96.71	KU530224.1
4	*Klebsiella pneumoniae* strain FBMAX4	MK791691	Rice Roots	Nueva Ecija	*Klebsiella pneumoniae strain CCFM8369*	100	99.35	KJ803926.1
5	*Klebsiella pneumoniae* strain FBMAX5	MK791692	Rice Roots	Nueva Ecija	*Klebsiella pneumoniae strain JPR9*	100	97.93	KM083804.1
6	*Leclercia adecarboxylata* strain FBMAX6	MK791693	Rice Roots	Nueva Ecija	*Leclercia adecarboxylata strain DC1-1-2*	100	99.50	MF716711.1
7	*Acinetobacter vivianii* strain FBMAX7	MK791694	Rice Roots	Nueva Ecija	*Acinetobacter vivianii strain AVi9*	100	97.65	MG202002.1
8	*Pseudomonas* sp. FBMAX8	MK791695	Rice Roots	Nueva Ecija	*Pseudomonas sp. t6(2014)*	100	98.31	KF898098.1
9	*Enterobacter cloacae* strain FBMAX9	MK791696	Rice Roots	Nueva Ecija	*Enterobacter cloacae strain IAE204*	100	99.78	MK414906.1
10	*Klebsiella* sp. FBMAX10	MK791697	Rice Roots	Nueva Ecija	*Klebsiella sp. G-40*	100	100.00	KM434224.1
11	*Bacillus amyloliquefaciens* strain FBMAX11	MK791698	Rhizosphere	Aurora	*Bacillus amyloliquefaciens strain L55*	98	84.87	KU922494.1
12	*Bacillus subtilis* strain FBMAX12	MK791699	Rhizosphere	Nueva Ecija	*Bacillus subtilis strain isolate L31*	100	99.93	KY652944.1
13	*Pseudomonas* sp. FBMAX13	MK791700	Rhizosphere	Nueva Ecija	*Pseudomonas sp. C9B1*	100	99.71	DQ184514.1
14	*Bacillus subtilis* strain FBMAX14	MK791701	Rhizosphere	Tarlac	*Bacillus subtilis strain ABS2*	99	97.01	KU533850.1
15	*Pseudomonas putida* strain FBMAX15	MK791702	Rice Roots	Aurora	*Pseudomonas putida strain M77*	100	92.05	HM008955.1
16	*Acinetobacter* sp. FBMAX16	MK791703	Rice Roots	Aurora	*Acinetobacter sp. SE63*	98	91.26	KU353552.1
17	*Enterobacter asburiae* strain FBMAX17	MK791704	Rice Roots	Aurora	*Enterobacter asburiae strain FC18569*	100	99.08	MK577384.1
18	*Bacillus megaterium* strain FBMAX18	MK791705	Rice Roots	Nueva Ecija	*Bacillus megaterium strain MER_31*	100	99.93	KT719611.1
19	*Paenibacillus polymyxa* strain FBMAX19	MK791706	Rice Roots	Nueva Ecija	*Paenibacillus polymyxa strain E8*	100	99.93	MG963206.1
20	*Enterobacter* sp. FBMAX20	MK791707	Rice Roots	Nueva Ecija	*Enterobacter sp. KN/03/24*	100	99.93	HQ235641.1
21	*Enterococcus gallinarum* strain SBMAX21	MK796024	Rice Roots	Nueva Ecija	*Enterococcus gallinarum strain HCD7-6*	100	99.86	MH111475.1
22	*Serratia marcescens* strain SBMAX22	MK796025	Rice Roots	Nueva Ecija	*Serratia marcescens strain JF1*	100	99.07	KC171984.1
23	*Serratia marcescens subsp. sakuensis* strain SBMAX23	MK796026	Rice Roots	Nueva Ecija	*Serratia marcescens subsp. sakuensis strain WTB52*	100	99.79	MK241850.1
24	*Enterococcus casseliflavus* strain SBMAX24	MK796027	Rice Roots	Nueva Ecija	*Enterococcus casseliflavus strain JFL12*	100	100.00	KT343156.1
25	*Bacillus cereus* strain SBMAX25	MK796028	Rice Roots	Nueva Ecija	*Bacillus cereus strain MER_TA_49*	100	99.93	KT719454.1
26	*Serratia marcescens* strain SBMAX26	MK796029	Rice Roots	Nueva Ecija	*Serratia marcescens strain SYJ1-9*	100	99.93	KR262852.1
27	*Bacillus cereus* strain SBMAX27	MK691597	Rice Roots	Nueva Ecija	*Bacillus cereus strain ANY*	99	99.02	GQ375259.1
28	*Bacillus cereus* strain SBMAX28	MK796030	Rice Roots	Nueva Ecija	*Bacillus cereus strain D26*	100	99.72	KC441765.1
29	*Bacillus tequilensis* strain SBMAX29	MK796031	Rice Roots	Nueva Ecija	*Bacillus tequilensis strain IAUK2309*	99	99.51	MH819732.1
30	*Bacillus cereus* strain SBMAX30	MK796032	Rice Roots	Nueva Ecija	*Bacillus cereus strain RTR*	100	98.72	MK014289.1
31	*Serratia* sp. SBMAX31	MK796033	Rice Roots	Aurora	*Serratia sp. NT3*	100	99.64	GU458275.1
32	*Acinetobacter calcoaceticus* strain SBMAX32	MK796034	Rice Roots	Aurora	*Acinetobacter calcoaceticus strain 5*	98	90.71	MH910109.1
33	*Serratia marcescens* strain SBMAX33	MK796035	Rice Roots	Nueva Ecija	*Serratia marcescens strain XJ-01*	100	99.22	FJ530951.1
34	*Enterobacter cloacae* strain SBMAX34	MK796036	Rice Roots	Nueva Ecija	*Enterobacter cloacae strain Bio103*	100	99.29	JX495602.1

Isolate No.	Rhizobacteria Registered Species Name	Accession No.	Source of Isolation	Location	BLAST Identified Species Name	Query Cover (%)	Similarity (%)	Accession Number
35	*Bacillus altitudinis* strain TBMAX35	MK834677	Rhizosphere	Aurora	*Bacillus altitudinis strain FC24763*	100	97.63	MK577401.1
36	*Bacillus* sp. TBMAX36	MK834678	Rhizosphere	Aurora	*Bacillus sp. BCK-7*	100	98.89	KP992120.1
37	*Bacillus pumilus* strain TBMAX37	MK834679	Rhizosphere	Aurora	*Bacillus pumilus strain IP10*	100	99.93	KY621526.1
38	*Bacillus pumilus* strain TBMAX38	MK834680	Rhizosphere	Aurora	*Bacillus pumilus strain HTI 3*	100	100.00	MK521055.1
39	*Bacillus drentensis* strain TBMAX39	MK834681	Rhizosphere	Aurora	*Bacillus drentensis strain BF-R7*	99	100.00	KY292423.1
40	*Bacillus altitudinis* strain TBMAX41	MK834682	Rhizosphere	Aurora	*Bacillus altitudinis strain WTB89*	100	99.93	MK241863.1
41	*Klebsiella* sp. strain TBMAX42	MK691689	Rhizosphere	Tarlac	*Klebsiella sp. strain B12*	99	99.72	MH578616.1
42	*Bacillus subtilis* strain TBMAX43	MK834683	Rhizosphere	Tarlac	*Bacillus subtilis strain ZGL14*	100	99.86	MH362700.1
43	*Aeromonas dhakensis* strain TBMAX44	MK834684	Rhizosphere	Tarlac	*Aeromonas aquariorum strain RD5*	99	99.57	KF307776.1
44	*Acinetobacter variabilis* strain TBMAX45	MK834685	Rhizosphere	Tarlac	*Acinetobacter variabilis strain L23*	99	99.93	KX832725.1
45	*Bacillus pumilus* strain TBMAX47	MK834686	Rhizosphere	Tarlac	*Bacillus pumilus strain HTI 3*	100	99.86	MK521055.1
46	*Bacillus thuringiensis* strain TBMAX48	MK834687	Rhizosphere	Tarlac	*Bacillus thuringiensis strain BPR162*	100	99.72	KU161299.1
47	*Pseudomonas* sp. TBMAX49	MK834688	Rhizosphere	Tarlac	*Pseudomonas sp. NTUIOB TPH6*	99	99.79	AB696793.1
48	*Bacillus subtilis* strain TBMAX50	MK834689	Rhizosphere	Tarlac	*Bacillus subtilis strain BX-13*	100	100.00	MK120493.1
49	*Bacillus cereus* strain TBMAX51	MK834690	Rhizosphere	Tarlac	*Bacillus cereus strain SML_M123*	100	99.93	MG937670.1
50	*Staphylococcus hominis* strain TBMAX52	MK834691	Rhizosphere	Aurora	*Staphylococcus hominis strain PbT3*	99	98.48	KT717627.1
51	*Bacillus subtilis* strain TBMAX53	MK834692	Rhizosphere	Aurora	*Bacillus subtilis strain CICC 10023*	100	99.86	GU980947.1
52	*Bacillus pseudomycoides* strain TBMAX54	MK834693	Rhizosphere	Aurora	*Bacillus pseudomycoides strain AB-CSL9*	100	99.65	MG780243.1
53	*Bacillus subtilis* strain TBMAX55	MK834694	Rhizosphere	Aurora	*Bacillus subtilis strain CICC 10023*	100	100.00	GU980947.1
54	*Brevibacillus* sp. strain TBMAX56	MK834695	Rhizosphere	Nueva Ecija	*Brevibacillus sp. strain W22*	100	99.93	MH773164.1
55	*Enterobacter cloacae strain TBMAX57*	MK834696	Rhizosphere	Nueva Ecija	*Enterobacter cloacae strain Bio103*	99	99.37	JX495602.1
56	*Bacillus cereus* TBMAX58	MK834697	Rhizosphere	Nueva Ecija	*Bacillus cereus BI-C3*	98	93.98	LT548959.1
57	*Enterobacter cloacae* strain TBMAX59	MK834698	Rhizosphere	Bulacan	*Enterobacter cloacae strain Bio103*	99	99.86	JX495602.1
58	*Enterobacter cloacae* strain TBMAX60	MK834699	Rhizosphere	Bulacan	*Enterobacter cloacae strain RJ30*	99	99.93	KC990813.1
59	*Enterobacter cloacae* strain TBMAX61	MK834700	Rhizosphere	Bulacan	*Enterobacter cloacae strain R6-355*	100	99.86	JQ659814.1
60	*Enterobacter cloacae* strain TBMAX62	MK834701	Rhizosphere	Bulacan	*Enterobacter cloacae strain Bio103*	99	99.86	JX495602.1
61	*Pseudomonas* sp. TBMAX63	MK834702	Rhizosphere	Bulacan	*Pseudomonas sp. P7(2009b)*	100	99.51	GU113077.1
62	*Enterobacter* sp. TBMAX64	MK834703	Rhizosphere	Bulacan	*Enterobacter sp. 1384*	99	99.65	KJ499995.1
63	*Brevibacillus* sp. TBMAX65	MK834704	Rhizosphere	Bulacan	*Brevibacillus sp. B13*	100	99.86	KT316416.1
64	*Burkholderia cepacia* TBMAX66	MK834705	Rhizosphere	Nueva Ecija	*Burkholderia cepacia isolate MSMB10*	100	99.86	EF114400.1
65	*Brevibacillus* sp. TBMAX67	MK834706	Rice Roots	Aurora	*Brevibacillus sp. B13*	100	99.79	KT316416.1
66	*Brevibacillus* sp. TBMAX69	MK834707	Rice Roots	Aurora	*Brevibacillus sp. B13*	100	99.51	KT316416.1
67	*Enterobacter cloacae* strain TBMAX70	MK834708	Rice Roots	Aurora	*Enterobacter cloacae strain DF3*	99	99.30	MG774409.1
68	*Phytobacter massiliensis* TBMAX71	MK834709	Rice Roots	Aurora	*Metakosakonia massiliensis JC163*	99	98.59	NR_125600.1
69	*Dickeya zeae* strain TBMAX72	MK834710	Rice Roots	Aurora	*Dickeya zeae strain DZ-E4-6*	99	98.94	KJ438953.1

Isolate No.	Rhizobacteria Registered Species Name	Accession No.	Source of Isolation	Location	BLAST Identified Species Name	Query Cover (%)	Similarity (%)	Accession Number
70	*Bacillus amyloliquefaciens* strain TBMAX73	MK834711	Rice Roots	Aurora	*Bacillus amyloliquefaciens strain PP19*	100	100.00	MH719375.1
71	*Enterobacter* sp. strain TBMAX74	MK834712	Rice Roots	Tarlac	*Enterobacter sp. strain DMKU-RP206*	99	99.72	MF125281.1
72	*Paraburkholderia kururiensis* strain TBMAX75	MK834713	Rice Roots	Tarlac	*Burkholderia kururiensis strain PR1*	99	96.67	JX083379.1
73	*Bacillus pumilus* strain TBMAX76	MK834714	Rice Roots	Tarlac	*Bacillus pumilus strain HTI 3*	100	99.93	MK521055.1
74	*Enterobacter* sp. TBMAX77	MK834715	Rice Roots	Tarlac	*Enterobacter sp. B16(2013)*	99	99.65	KF010363.1
75	*Enterobacter* sp. strain TBMAX78	MK834716	Rice Roots	Tarlac	*Enterobacter sp. strain PQ02*	99	99.93	KY570322.1
76	*Enterobacter* sp. TBMAX79	MK834717	Rice Roots	Tarlac	*Enterobacter sp. CC-30P5*	99	99.79	KR067593.1
77	*Bacillus pumilus* strain TBMAX80	MK834718	Rice Roots	Tarlac	*Bacillus pumilus strain HTI 3*	100	100.00	MK521055.1
78	*Atlantibacter hermannii* strain TBMAX81	MK834719	Rice Roots	Tarlac	*Escherichia hermannii strain T88*	99	99.86	HQ407263.1
79	*Klebsiella sp.* strain TBMAX82	MK834720	Rhizosphere	Tarlac	*Klebsiella sp. strain L1*	100	99.65	MH891613.1
80	*Bacillus megaterium* strain TBMAX83	MK834721	Rhizosphere	Tarlac	*Bacillus megaterium strain RTM*	100	100.00	MK014286.1
81	*Klebsiella pneumoniae* strain TBMAX84	MK834722	Rhizosphere	Tarlac	*Klebsiella pneumoniae strain UEF04*	99	99.65	MK696418.1
82	*Aeromonas caviae* strain TBMAX85	MK834723	Rhizosphere	Tarlac	*Aeromonas punctata strain 4LNC309*	99	99.65	FJ940796.1
83	*Aeromonas aquatica* strain TBMAX86	MK834724	Rhizosphere	Tarlac	*Aeromonas aquatica strain M_89*	100	97.33	MG428980.1
84	*Enterobacter mori* strain TBMAX87	MK834725	Rhizosphere	Tarlac	*Enterobacter tabaci strain PSMK*	99	99.86	MK641315.1
85	*Enterobacter cloacae* strain TBMAX88	MK834726	Rice Roots	Tarlac	*Enterobacter cloacae strain Bio103*	100	99.72	JX495602.1
86	*Enterobacter cloacae* strain TBMAX89	MK834727	Rice Roots	Tarlac	*Enterobacter cloacae strain Bio103*	100	96.54	JX495602.1
87	*Enterobacter mori* strain TBMAX90	MK834728	Rice Roots	Nueva Ecija	*Enterobacter tabaci strain PSMK*	99	99.79	MK641315.1
88	*Enterobacter* sp. strain TBMAX91	MK834729	Rice Roots	Nueva Ecija	*Enterobacter sp. strain TC165*	100	99.16	MK459533.1
89	*Enterobacter mori* strain TBMAX92	MK834730	Rice Roots	Aurora	*Enterobacter tabaci strain PSMK*	99	99.86	MK641315.1
90	*Pantoea* sp. TBMAX93	MK834731	Rice Roots	Nueva Ecija	*Pantoea sp. XJ3*	99	99.79	GU140078.1

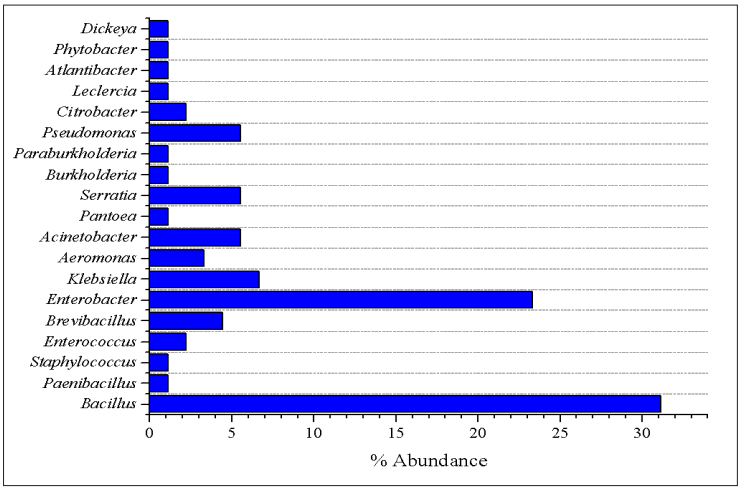

Figure 2. The abundance of bacterial isolates by genus from rice roots and rhizosphere across four provinces in Central Luzon.

Table 2. Affiliation of rhizobacteria isolates from rice roots and rhizosphere. Numbers in parentheses represent the number of isolates that were classified as members in each genus and species.

Phylum	Genus	Species	Rice Ecological Niche	
			Roots	Rhizosphere
Firmicutes	*Bacillus* (28)	*B. subtilis* (6)	0	6
		B. megaterium (2)	1	1
		B. tequilensis (1)	1	0
		B. thuringiensis (1)	0	1
		B. amyloliquefaciens (2)	1	1
		B. cereus (6)	4	2
		B. altitudinis (2)	0	2
		B. pseudomycoides (1)	0	1
		B. drentensis (1)	0	1
		B. sp (1)	0	1
		B. pumilus (5)	2	3
	Paenibacillus (1)	*P. polymyxa* (1)	1	0
	Staphylococcus (1)	*S. hominis* (1)	0	1
	Enterococcus (2)	*E. gallinarum* (1)	1	0
		E. casseiflavus (1)	1	0
	Brevibacillus (4)	*B. sp* (4)	2	2
Proteobacteria	*Enterobacter* (21)	*E. cloacae* (10)	5	5
		E. asburiae (1)	1	0
		E. mori (3)	2	1
		E. sp (7)	6	1
	Klebsiella (6)	*K. pneumonia* (3)	2	1
		K. sp. (3)	1	2
	Aeromonas (3)	*A. aquatica* (1)	0	1
		A. dhakensis (1)	0	1
		A. caviae (1)	0	1
	Acinetobacter (5)	*A. viviani* (1)	1	0
		A. calcoaceticus (1)	1	0
		Acinetobacter sp. (2)	2	0
		A. variabilis (1)	0	1
	Pantoea (1)	*P. sp* (1)	1	0
	Serratia (5)	*S. marcescens* (4)	4	0
		S. sp (1)	1	0
	Paraburkholderia (1)	*P. kururiensis* (1)	1	0
	Burkholderia (1)	*B. cepacia* (1)	0	1
	Pseudomonas (5)	*P. putida* (1)	1	0
		Pseudomonas sp. (4)	1	3
	Citrobacter (2)	*Citrobacter sp.* (2)	2	0
	Leclercia (1)	*L. adecarboxylata* (1)	1	0
	Atlantibacter (1)	*A. hermannii* (1)	1	0
	Phytobacter (1)	*P. massiliensis* (1)	1	0
	Dickeya (1)	*D. zeae* (1)	1	0

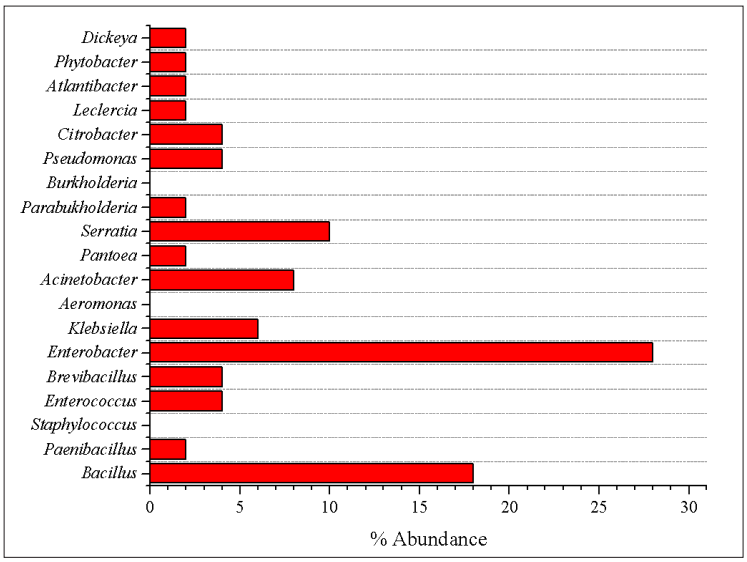

Figure 3. The abundance of bacterial isolates by genus from rice roots across four provinces in Central Luzon.

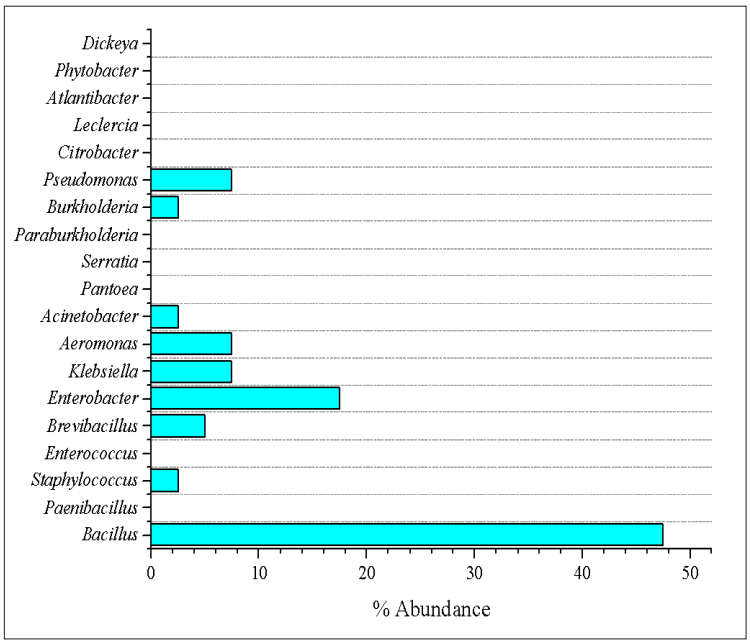

Figure 4. The abundance of bacterial isolates by genus from rice rhizosphere across four provinces in Central Luzon.

irrigated rice rhizosphere(Joshi et al., 2011). Similarly, studies showed that the rhizosphere of wheat and *Elaeagnus angusti folia* L. were also dominated by *Bacillus* (Joshi and Bhatt, 2011; Ramos et al., 1998). The ability of *Bacillus* to efficiently use nutrients provided by the plant through exudates has been the main reason and evidence for its predominance. Furthermore, many *Bacilli* have the ability to inhibit the growth of other strains because of their potential to produce growth-inhibiting substances against other microorganisms (Lunares et al., 1993). Our result is consistent with the findings of the metagenomic analysis conducted in Brazil that the predominant root colonizers in rice belonged to the phylum *Proteobacteria* comprising mostly *Enterobacter*-related endophytes (Sessitsch et al., 2012). In another study in Thailand, it was reported that the rice roots were dominated by *Bacillus* species (Raweekul et al., 2016). This discrepancy in the dominance of isolates in rice roots was partly due to several factors such as differences in environmental conditions because rice plants were grown in geographically different countries.

The phylogenetic tree constructed based on 16S rRNA gene sequences (Figure 5) shows two major groups: one comprised of the *Bacillus*, *Paenibacillus*, *Brevibacillus,* and *Enterococcus* species; and another group one comprised of *Enterobacter*, *Phytobacter*, *Seratia*, *Acinetobacter*, *Citrobacter*, *Pseudomonas*, *Burkholderia*, *Aerogenosa*, *Dickeya*, *Lectercia*, *Klebsiella*, and *Pantoea*. The bacterial population is very diverse and the 16s rRNA gene sequencing-based phylogenetic tree analysis delivered a better understanding in the assessment of the diversity of rhizobacteria isolated from the same and different ecological niches (Kumar et al., 2011).

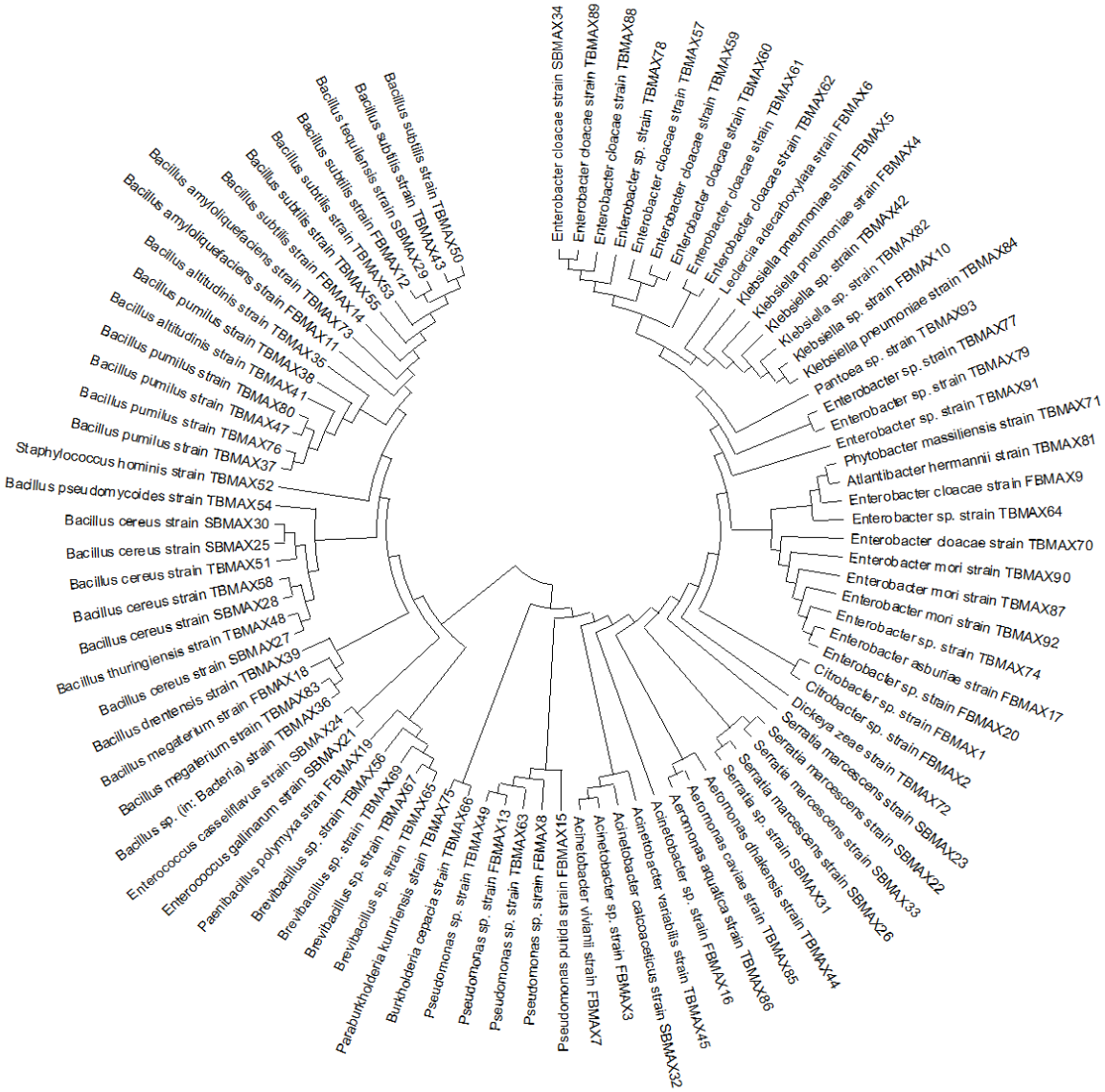

Figure 5. Phylogenetic tree of bacterial isolates based on 16s rRNA gene sequences constructed with MEGA version 11.0.13 software and grouped by Maximum Likelihood Tree Method (Tamura et al., 2021).

This result indicates that rice ecosystem has a rich biodiversity of rhizobacteria with reported high physiological traits and can be a good pool of potential rhizobacteria with growth-promoting abilities. Among the desired traits are nitrogen-fixing and capable of solubilizing insoluble phosphate, potassium, and zinc *in vitro.* These traits are vital for rhizobacteria to help plants uptake nutrients into bio-available forms. These are essential considerations in choosing bacterial strains that can be used as biofertilizers to help meet the ongoing need of the world to improve soil fertility, increase yields, and agricultural productivity toward sustainable and safe agriculture. Zhu et al. (2021) verified that *B. subtilis* can produce amylase, protease, chitinase, and lipase; showed by the clear zones around colonies on organic phosphorus and inorganic phosphorus agar medium. It can also produce IAA plant growth hormone suggesting that it promotes rice plant growth even though glucanase activity, HCN, and siderophore production were not detected. It likewise reduces the damages caused by rice blast fungus *Magnaporthe oryzae.* Among others, *Bacillus* and *Pseudomonas* were among the most dominant and most commonly found in various plant studies (Mahwish et al., 2015). *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Serratia sp.* are also beneficial in IAA production, phosphate dissolution, and N_2 fixation and were also used for crop production as bioinoculants (Hayat et al., 2012).

Conclusion

A rich diversity of bacteria was identified colonizing the rhizosphere of rice growing in an irrigated lowland rice ecosystem in Central Luzon. Among the identified species are very important as these were also very well known for their promising potential in plant growth promotion. The collection of rhizobacteria was molecularly characterized and can serve as a good pool of potential materials for formulating agricultural products helpful in improving soil fertility, increasing yields, and creating more sustainable and safer agriculture.

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RESEARCH NOTE

THE ROLE OF YOUNG PEOPLE IN ENHANCING FARMERS' ADAPTIVE CAPACITY TO CLIMATE CHANGE IMPACTS

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Abstract

Agriculture is among the worst-impacted sectors of climate change. In many developing countries, farmers suffer massive livelihood losses due to weather extremes such as typhoons and floods. Given this, the need to adapt towards climate change impacts is paramount. Focusing on farmers' adaptation towards climate change impacts is having enhanced adaptive capacity. This paper inquires how young people can help enhance the adaptive capacity of rice farmers to climate change impacts. Youth engagement in relation to enhancing farmers' adaptive capacity is an unexplored territory in the climate change adaptation literature. It also presents a model to explain how young people can be mobilized in climate change adaptation efforts. The model combines insights from *Community Youth Development Theory*, *Model of Proactive Private Adaptation to Climate Change*, and from the literature on youth engagement in rural communities. It can be concluded that there are entry points for young people engagement with respect to climate change adaptation efforts, with serving as infomediaries or information mediators for their farmer-parents as among the key roles that they may perform.

Keywords: *Adaptive Capacity, Climate Change Adaptation, Community Youth Development Theory, Infomediary, Youth and Climate Change*

Introduction

Weather extremes such as flood and drought are expected to be more frequent given the climate change trends (Cai et al., 2018). Agriculture is said to be among the worst-impacted sectors of climate change (Zougmoré et al., 2019). The world has seen the devastating impacts of climate change in terms of massive yield losses among farmers. In some areas, drought had resulted in a complete crop failure and millions of income losses.

With this, it goes without saying that farmers suffer the brunt of climate change impacts. Farmers remain poor in the Philippines rice sector and in many rice-growing countries. Most of the farmers are solely relying on rice production as their source of income; hence, it is imperative to enhance their adaptive capacity towards climate change impacts. Adaptive capacity is the potential or capability of a system to adapt to climatic stimuli (Smit and Wandel, 2006).

Farmers' adaptive capacity to climate change impacts is complex as it is influenced by a myriad of factors such as technological, social, and access to resources (Asfaw et al., 2016). Thus, adaptive capacity is context-specific. This is consistent with the idea that impacts of climate change are socially differentiated (McNeeley and Lazrus, 2014); therefore, it will be experienced differently in diverse contexts.

Hence, an important question that must be answered is: How to enhance the farmers' adaptive capacity to the impacts of climate change? This question is not a novel one, and this has been addressed using a number of lenses or strategies. Among these lenses is the technological lens, i.e., developing technologies that are climate-resilient such as water management technologies or varieties that could withstand challenging climatic conditions (Orge et al., 2019). In terms of strategies, the roll out of crop insurance to farmers may also be considered to lessen the impacts of climate change (Fonta et al., 2018). There is also a group of scholarship that tackles the importance of social capital in enhancing adaptive capacity. It has been argued that farmers' social network will help farmers cope with the impacts of weather extremes (Kansanga, 2017). Farmers usually borrow from their co-farmers or relatives to finance their operations whenever they encounter losses after severe weather conditions (Manalo et al., 2020).

One area that remains inadequately explored is youth engagement in relation to the enhancement of farmers' adaptive capacity. With more than 1.2 billion people within the age group of 15 - 24, young people can be a force to reckon with enhancing adaptive capabilities of farmers. Thus, this paper seeks to offer a framework on how to mobilize young people in enhancing farmers' adaptive capacity with regards to climate change impacts. This paper discusses the justification on the need to engage young people in

climate change adaptation efforts. It also presents the necessity to discuss model to enhance farmers' adaptive capacity with young people at its helm.

Young people: challenges, strengths, and why should they be engaged?

There is a significant documentation that young people no longer want to pursue agriculture-based livelihood (Asis, 2019). Youth outmigration from agricultural communities is a common literature theme. There are several reasons for this, which suggest that young people do not sit well with laborious activities in the agricultural areas (Manalo and van de Fliert, 2013). Another common reason is the fact that young people are more educated compared to the previous generations; thus, the desire to migrate is significantly high. Asis (2019) concludes that young people search for more opportunities abroad while others noted that lack of opportunities in rural communities (WB-IFAD, 2017).

Despite these challenges and issues, there are also several strengths that young Filipinos possess. They have high self-esteem, are resilient, see themselves as individuals with unique strengths and strong belief in God (Puyat, 2005). This paper anchors on capitalizing these strengths.

I argue that there is wisdom in mobilizing young people in relation to enhancing farmers' adaptive capacity to climate change impacts. There are three reasons for believing that this discernment will work. First, it is incorrect to argue that all young people do not want to be involved in agriculture. In the study of Rietveld et al. (2020), it was found that young men and women in Uganda remain engaged in agriculture at some way, irrespective of their gender or residence. A study among young people in Thailand proved that young people are willing to continue their agriculture ventures if given an opportunity (Salvago et al., 2019). According to Gultiano and Xenos (2004), young people may be classified into: leavers, returners, and those who-will-stay-no-matter-what. Leavers are the ones who show strong disdain to rural and agricultural life in general while returners are those who may leave but may return after some time, and those who-will-stay-no-matter-what are the ones who show strong likeness to live in a rural community and pursue agriculture-based livelihoods. The study of Gultiano and Xenos (2004) argues that sweeping statements saying that young people are no longer interested in farming must be avoided because it might possibly put a complete stop on youth engagement in agriculture discourse.

Second, there is a need to re-read the youth outmigration data (Manalo and van de Fliert, 2014). Usually, reading youth outmigration data moves towards its negative dimension, i.e., young people refused to be involved in agriculture. Although there are indeed young people who show disdain in pursuing agriculture-based livelihood, it is important to pay attention to other aspects of the data. Hence, a good question would be: Is it the case that young people do not want to farm or they just want to do farming differently? The question seeks to signpost the need to think of creative ways to engage the youth in the agriculture.

The third reason is the solid evidence of youth involvement in the climate change discourse. Young people are instrumental in shaping climate change governance and policymaking processes (Han and Ahn, 2020). Youth activism on climate change has succeeded in bringing the agenda to the fore, resulting in increased awareness of this issue (Luthfia and Alkhajar, 2018; O'Brien et al., 2018). Aside from climate awareness, there is also evidence of young people who actively participate in the climate resilience planning, all geared towards enhancing adaptive capacities of their respective communities (MacDonald et al., 2015). Scholars who have worked in the area of youth and climate change utilized various creative methods such as participatory video and digital technologies (MacDonald et al., 2015). These methods are effective in capturing stories and insights of young people regarding climate change. Despite their focused activities and deeply serious involvement, young people are not always recognized or at the very least taken seriously (Narksompong and Limjirakan, 2015). They barely receive the support needed to amplify the impact of their hard work (Salvago et al., 2019). Scholars emphasized the need for governments and other entities to extend full support to young people (Narksompong and Limjirakan, 2015). In general, youth involvement in the climate change discourse gravitates around mitigation efforts and/or climate awareness. However, youth involvement in climate change adaptation discourse in the agriculture sector is not very common.

Evidence of successful youth mobilization in agriculture

The Philippine Rice Research Institute's (PhilRice) Infomediary Campaign has shown successful cases of high school students acting as information mediators (infomediaries) in their respective rice-farming communities (Manalo et al., 2016). In this project, instances of adopting various rice production technologies have been reported. The project also claimed improved information-seeking behaviour of farmers brought about by the Infomediary Campaign activity in the school whereby students were mobilized to serve as infomediaries (Manalo et al., 2015). There

are also reports of students serving as climate-smart agriculture infomediaries in 2016 (Manalo et al.). In the publications of the Infomediary Campaign, high school students used various available platforms such as print publications, the PhilRice Text Center, and the Pinoy Rice Knowledge Bank - a website that contains most of the relevant information on rice cultivation in the Philippines.

Aside from the Infomediary Campaign, there are number of organizations with success stories to tell on engaging young people in agriculture. Among them is the 4-H club, which is modeled from the 4-H movement in the US, and overseen in the Philippines by the Agricultural Training Institute, and the Young Professionals for Agriculture Development-Philippine Chapter, which provides an international discussion platform for agriculture development (Asis, 2019).

There are also a number of initiatives that utilize online tools such as the *Millennial Bukid Girl* and *The Agrillenial*, which aim to serve as infomediaries between technical experts and the target audiences (Pamplona, 2019). Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) is also active in engaging youth in agriculture with its initiative *'Young Forces for Agricultural Innovation'* (#Y4AGRI). It promotes participation of youth towards agriculture and rural development with one of its recent projects called the *'Youth COVIDeo Contest'*, aimed at tapping the Southeast Asian youth's creativity through video production, showcasing their local food production practices (Pasiona et al., 2021). SEARCA also promoted gardening with its *'School - Plus- Home Gardens Project (S+HGP)'* to elementary school students. It serves as a participatory and inclusive model for sustainable development that promotes vegetable production and consumption towards better nutrition (Calub et al., 2019). Collectively, these initiatives show potential and, in some cases, impact of engaging young people in agriculture.

Rice farming context in the Philippines

In Philippines, the average age of farmers is 56. This figure was derived from the *5th round of the regular monitoring of rice-based farm households in the Philippines* conducted by PhilRice Socioeconomics Division (2019). While Filipino farmers can boast of more than three decades of rice farming experience, most of them spent eight years of formal education. Most (89%) of the rice farmers in the Philippines rely on rice cultivation as their main source of livelihood. The average landholding is relatively small at 1.5 ha^{-1}. Filipino farmers deal with a myriad of issues in relation to their farming venture. Among these issues are cutthroat arrangements with their landowners, lack of access to capital, unreliable access to water and other important resources, and low price for their produce (Palis et al., 2015). Poor access to extension services has been a perennial issue in the Philippines, which is particularly true among farmers in remote communities given that ageing agricultural extension workers is also a concern (Manalo, 2013). In the literature, poor access to information is an indicator of low adaptive capacity because it implies that farmers have inadequate access to information that could help them better adapt to climate change impacts (Khanal et al., 2018). In the Philippines, millions of farmers remain poor (Philippine Statistics Authority [PSA], 2017). The average annual income for irrigated and rainfed ecosystems is USD 6,338.78 at 1 US$= PhP 51. For rainfed farm, the situation is far worse with an average annual income of USD 3,797.50 or USD 316.46 monthly. The combination of these factors makes farmers more vulnerable to the impacts of climate change. In recent studies, it is known that poorer communities are generally more vulnerable to the impacts of climate change, and therefore have lower adaptive capacity.

This paper discusses about the rice farming context in the Philippines; however, the model that is being proposed should not be limited in its application to Philippine setting. It is drawn from literature review on climate change adaptation and youth development studies. The below discussion only provides a reference in terms of context to better appreciate the dynamics of rice-farming communities, with the Philippine context as an example.

A model on youth involvement in enhancing farmers' adaptive capacity to climate change impacts

In developing the model of youth engagement in enhancing adaptive capacity of farmers to climate change impacts, I turn to insights from Community Youth Development Theory and Model of Private Proactive Adaptation to Climate Change (Figure 1). The two phases in Figure 1 is from MPPACC while I have conceptualized the rest.

The main premise of *Community Youth Development Theory* is that young people are assets that can be mobilized, not problems that must be fixed (Brennan and Barnett, 2009). This premise is the overall guiding idea of the current model; the necessity to see young people as productive members of society. There is a need to pay attention in harnessing the possible immense contribution of young people, which in the case of this paper relates to enhancing adaptive capacity of farmers to climate change impacts.

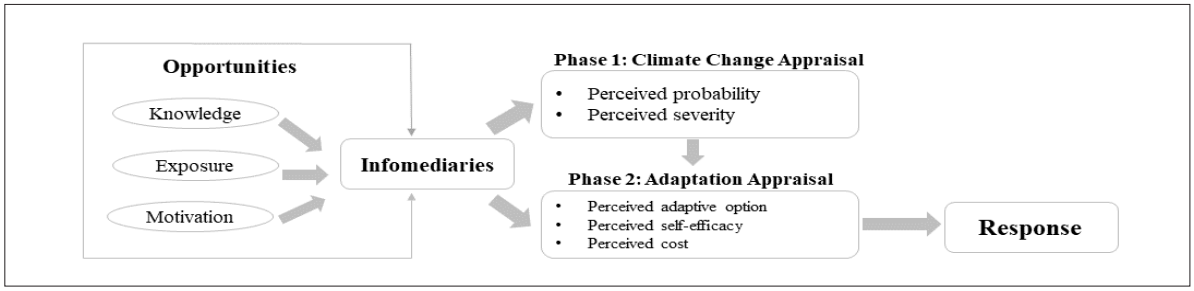

Figure 1. Model of youth engagement in agriculture to enhance farmers' adaptive capacity to climate change impacts.

The MPPACC, on the other hand, is a theory that explains the process of individual adaptation towards climate change (Grothmann and Patt, 2005). Aspects of MPPACC are being used in this current model to understand how farmers make adaptive decisions. It is important to look at adaptive decisions as pursuing (or not) an adaptive option since it may indicate adaptive capacity. It has also been used and proven applicable in different studies regarding the understanding of farmers' capability to make adaptive decision on impacts of climate change (Frank et al., 2011).

I adapt two phases in the decision-making process described in MPPACC (also see Manalo et al. 2019). The first of which is called Climate Change Appraisal phase. In the case of farmers, this is the phase where they assess if a weather extreme is likely to occur (perceived probability). If a farmer is convinced that a weather extreme is likely to occur, s/he will then assess the damage that the phenomenon is likely to cause him/her (perceived severity). If a farmer is convinced that a weather extreme is likely to occur and that s/he is likely to be affected, then risk perception is formed. That is, the farmer moves to preparing to adapt, leading to the Adaptation Appraisal, which is the second phase. In the Adaptation Appraisal phase, a farmer asks if there are adaptive options (perceived adaptive option), if s/he can implement the adaptive option (perceived self-efficacy), and if the adaptive option is affordable (perceived adaptation cost). The outcome of adaptation Appraisal phase is adaptive capacity.

How can young people enhance farmers' adaptive capacity to climate change impacts? In Figure 1, young people may have roles to play both in the climate change and adaptation appraisal phases by serving as infomediaries. Infomediaries are individuals who facilitate access to information (Sein and Furuholt, 2012). They can either do this role with a fee or *pro bono*. In the case of this research, the infomediaries being referred to are *pro bono* or lay infomediaries (Abrahamson and Fisher, 2007). Access to information is important as it leads to having knowledge that can serve as inputs to decision-making. In the context of this paper, access to information can lead farmers to understand adaptive mechanisms available which are also applicable in their respective farms.

During the climate change appraisal phase, young people can provide information to farmers about weather forecasts. They can also feed information about the assessments on the severity of the forthcoming weather event based on their engagement with news stories on social media. There is evidence pertaining to news consumption among young people (Fish, 2016). These news stories however, could also be easily accessed by farmers on their own through radio or television; nonetheless, young people can help them. Young people's contributions widen in the adaptation appraisal phase for instance, young people can help search for adaptive options that a specific farmer can employ. Afterwards, it is expected to lead on enhancing the perceived adaptation options among farmers. Young people may also help enhance the self-efficacy perception of farmers by starting conversations on how adaptive options can be effectively and easily carried out. In terms of costs of the adaptive mechanisms, they may also assist by searching for ways in which the costs can be lessened or look for cheaper alternatives.

There are enablers for young people to effectively perform the roles mentioned above. First is **knowledge**, young people need to understand how climate change impacts agriculture, which is a source of livelihood for millions of farming families across the globe. Once young people have the knowledge, then they will be equipped with enough confidence to converse about the matter. They should primarily be introduced on the impacts of climate change on agriculture. Second is **exposure** to agricultural activities, which would help them appreciate agriculture and the relevant roles they can play in enhancing adaptive capacity of farmers. Equivalent to knowledge, exposure can provide young people the confidence to tackle the matter of enhancing adaptive capacity among farmers. Third is **motivation**. Young people must have the essential mindset that they can do something or they are competent in relation to enhancing farmers' adaptive capacity. Motivation may come from individual realization of the centrality of agriculture in the livelihood of their respective families or communities. These enablers help young people perform their roles in enhancing adaptive capacity of farmers towards climate change

impacts. An enhanced adaptive capacity may lead to farmers pursuing an adaptive response.

These enablers can be constrained or enhanced by the opportunities available for young people (Sumberg et al., 2019) and these opportunities vary from one context to another. Opportunities are influenced by different factors such as enabling policies at the global, national, and local levels (Sumberg et al., 2019). For instance, a national policy institutionalizing the teaching of rice production lessons in schools would greatly enhance the performance of young people serving as infomediaries. The level of infrastructure availability and other support mechanisms also determines the opportunities available for young people to develop themselves (Sumberg et al., 2019); or to help perform their roles as infomediaries as context of this paper. For example, young people who do not have access to good internet connection will not be an effective online infomediaries. Lastly, opportunities may also be affected by prevailing social norms (Sumberg et al., 2019). In societies where exchanging thoughts between older and younger people is unacceptable or being frowned upon, then being infomediary will not likely to succeed. For infomediaries to effectively perform their roles, a supportive environment is necessary.

The model is useful in communities where access to a reliable extension service is a challenge. The strength of the model is in tracing the effect or result of response to a climate stimulus. For example, one can say that a farmer opted to pursue certain response because it was affordable, i.e., after everything was considered.

One of the challenges in applying the model is performing it in communities where the opportunities afforded to young people or participants of this study are absent. For example, the insufficient support from school administrators in consideration with the different priorities of each school. With the hierarchical system, which is true in most organizations in the Philippines, it would be difficult to imagine how a school-based initiative such as this could take off without a support from an educational institute in a specific area. Another point that needs to be highlighted is the cultural dimension of this study. In the Philippines, young people having a discussion with adults is not so much of an issue. The model may prove challenging to apply in areas where young person and adult conversation receives unacceptable gestures within the community. This is an important point as the model somehow promotes the importance of young people's voice in the rice cultivation practices that are currently being employed.

Another challenge in applying this model is on quantitative assessment of impact of mobilizing young people to serve as infomediaries. Hence, it would be good to look into how to quantify the impacts from the infomediation process. Another one is considering that the response on this model is subtle or not very obvious. In climate change adaptation, responses are diverse, and there are responses that are not so obvious. A not so obvious response is to not do anything (Evangelista et al., 2016), which is entirely possible if a farmer decides to just resign from the situation. It remains to be seen if the current model is equipped to capture such kind of responses.

Future researchers might want to consider doing a parallel study in a different context. Communities where there are observable and sudden change in land utilization such as transforming agricultural land to industrial ones might be a good area or context.

In terms of research types, this model would be best to use in action research studies where there is an intent of youth involvement towards climate change adaptation while studies with instrumental aims in particular would be best served by this framework. This study framework would also be useful in operational research studies with the objective of youth involvement in enhancing farmers' adaptive capacity in relation with climate change impacts. For example, most projects in recent years by international organizations and even by government institutions such as the Department of Agriculture have youth component in their climate change adaptation projects.

Conclusion

Despite the limitations of the model, it brings several insights to the fore. First, is the need to seriously consider the youth engagement in agriculture efforts. This paper has shown that proper mobilization and opportunities for the young people to do something good for their communities can be a vital force in enhancing farmers' adaptive capacity towards climate change impacts. The model brings youth engagement in climate action, a step further by banking on tangible actions as opposed to being solely vocal critics of global inaction. The current paper goes beyond bringing climate awareness.

This paper, which is heavily anchored on the *Community-Youth Development Theory* (Brennan and Barnett, 2009), espouses that, indeed, young people should be seen as assets that should be mobilized and not problems that must be solved. Second, is the need to ensure that climate change and agriculture topics are integrated in every school curriculum. Third, which grounded from the previous insights, is the need to focus on attaining fundamental social change (Sumberg et al., 2019) to ensure that young people in agricultural communities are supported and being considered so they can do something good for their respective communities.

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COMPARISON OF THE TOTAL PHENOLIC CONTENT AND ANTIOXIDANT CAPACITIES OF SOME PLANT FOODS AS AFFECTED BY DRYING TREATMENTS

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Abstract

Drying or dehydration is a critical step in the analytical evaluation of antioxidant content and capacity of plant samples, particularly in foods with potential health-promoting or therapeutic properties. This work assessed the effects of oven drying and lyophilization or freeze-drying on the phytochemical content and antioxidant capacity of some locally cultivated plant foods. Raw dried samples using convection oven (40°C) and lyophilizer (-108°C) were evaluated for their total phenolic content (TPC) and antioxidant capacities using the 2,2-diphenylpicrylhydrazyl (DPPH) and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS) assays. Lyophilization generally had higher phenolic and antioxidant levels with turmeric having the most TPC (180.77 mg GAE/g), DPPH (750.58 µmol TE/g), and ABTS (824.96 µmol TE/g) activities. Meanwhile, oven-dried eggplant showed higher TPC and DPPH values (924% and 488%, respectively) than its lyophilized counterpart. Lyophilization could better retain the levels and kinds of antioxidant compounds but oven-drying is considered more economical. Correlation analyses between the TPC and antioxidant capacity values were significantly positive using both dehydration methods. Therefore, oven drying can be utilized for routine analysis. However, researchers, food processors, and pharmacologists can utilize specific drying methods for plant materials based on their needs, such as isolation of healthful antioxidants for routine extraction of phytochemicals for further analysis or incorporation in food products and dietary supplements. The effects of drying treatments on specific antioxidants per plant sample and their levels, as well as the potential impact on the bioactive properties of these compounds are worth exploring.

Keywords: *Antioxidants, Drying, Freeze-drying, Lyophilization, Oven drying, Phenolics*

Introduction

Plants are known as rich sources of phytochemicals or biologically active substances that usually function as antioxidants. Antioxidants provide health benefits such as slowing down the aging process, strengthening the immune system, and preventing chronic diseases. Thus, high intake of vegetables and fruits has been associated with lower risks of life-threatening diseases such as cardiovascular diseases, diabetes mellitus, and various forms of cancer, which are among the leading causes of mortality in the Philippines (PSA, 2022). The Philippines has a rich and diverse collection of plant resources, which may contain high levels of antioxidants to prevent treatment of non-communicable diseases, whether these plants are in the form of food or as nutraceuticals. It is one of the reasons why considerable interest is devoted to the exploration and discovery of plants with potentially high levels of biologically active components.

In quantitative analysis, sample preparation is an important step to ensure the accuracy of results to be obtained. Drying or dehydration is commonly the first step employed in preparing samples for subsequent analytical tests. It is a process of removing moisture through successive heat and mass transfer (Ertekin and Yaldiz, 2004). It greatly influences the level and even nature of bioactives extracted from plant samples (Orphanides et al., 2013) and depends on the chemical properties of the compounds present (Durazzo, 2017). Some compounds are non-extractable and are bound to carbohydrates, proteins, and macromolecules in the food matrix, which can be released in the cell wall during drying (Chang et al., 2006; Perez-Jimenez and Torres, 2011; Singh et al., 2015). Therefore, this first step influences the end results of analysis and its purpose such as generation of information for public dissemination, use of the plant bioactives in foods and nutraceuticals, or policy formulation.

Chemical composition, physical characteristics, and temperature significantly affect the extraction of antioxidants in a sample, particularly phenolics (Baiano, 2014; Durazzo, 2017; Luthria, 2006; Tomas-Barberan and Espin, 2001). Studies have dealt with the analysis of antioxidants in local crops, but are usually focused on the antioxidants levels such as

ascorbic acid and beta-carotene. There are limited information on the effects of drying methods on the antioxidant levels and capacity of different plant foods that are locally available for consumption. Convective drying methods like oven drying are widely and commonly used in dehydrating plant materials, but the high temperatures and long drying times can have negative effects on the nutrients and phytochemicals of the final product (Mundala et al., 2010; Rodriguez et al., 2013). Lyophilization or freeze-drying is often considered to be the best drying method for preserving heat-sensitive compounds including phytochemicals (Shofian et al., 2011), and reducing sensorial degradation. However, it is known to be the least economical method of drying foods (Ratti, 2001). In recommending a suitable dehydration method for analytical determinations specifically in laboratories, which daily evaluates large number of samples, the quality of the final product should not be compromised by the status and processes of economy; instead, these aspects should be balanced. Hence, this work evaluated two drying techniques as analytical preparatory step in the total phenolic content and antioxidant capacity analysis of selected locally available plant foods.

Materials and Methods

Test Samples and Chemicals

Nine plant foods (Table 1) collected during the 2016 dry season from the Science City of Muñoz, Nueva Ecija were used as test samples. These samples were selected representing different types of plant foods locally available.

Folin-Ciocalteu phenol reagent, DPPH, ABTS, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid (GA), and potassium persulfate were from Sigma Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

Sample Processing

Dirt and inedible parts of the freshly procured dry samples were removed while edible ones were weighed. After washing twice with tap water and once with the distilled, the samples were then drained.

Drying Methods

To evaluate the effect of drying methods on the phytochemical and antioxidant levels of different crops, the samples were subjected to oven drying (T_1) and lyophilization (T_2).

Oven Drying

The processed samples were chopped/diced into small pieces, spread on net bags, and then placed on aluminum trays. The samples were dried in a forced air oven (DKN-812C, Yamato Scientific Co., Ltd., Tokyo, JP) set at 40°C for a minimum of 16 h and afterwards stored at -20°C until further processing.

Lyophilization

The washed samples were placed on pre-weighed polyethylene containers and kept at -4°C overnight. The frozen samples were lyophilized at -108°C for a minimum of 16 h using a LaboGene CS 110-4 System freeze-dryer (LaboGene A/S, Allerød, DK) and later on stored at -20°C until further processing.

Processing of Dried Samples

The dried samples were weighed, powdered, and then stored at -20°C.

Antioxidant Extraction

Powdered sample (0.5 g) was added with a 10 mL of hexane. The tube was thoroughly mixed using a mechanical shaker (KS-260 Basic, IKA, Staufen, DE) at 300 rpm for 2 h and then centrifuged at 3,000 rpm for 15 min. The supernatant was discarded while the pellet was added with 10 mL of 85% methanol, shaken overnight at 300 rpm for 12 hr, and centrifuged at 3000 rpm for 15 min, and centrifuged (3000 rpm, 15 min). The methanol extract was kept in the dark at 4°C until analyzed.

Table 1. The selected crops used in the experiment.

Rice-based Crop	Scientific Name	Variety/ Description	Part Used
Chili	*Capsicum frutescens*		Leaves
Eggplant	*Solanum melongena*	Round/ Green	Fruit
Green pepper	*Capsicum annuum*	Django	Fruit
Red coral lettuce	*Lactuca sativa*	Lollo Rosso	Leaves
Squash	*Cucurbita maxima*	Suprema	Flower and Fruit
Sweetpotato	*Ipomoea batatas*	Yellow fleshed	Tuber
Turmeric	*Cucurma longa*		Rhizome
Water spinach	*Ipomoea aquatica*	Lowland	Leaves and edible stems

Total Phenolic Content

The total phenolic contents of the hydrophilic extracts from various rice-based crops were determined based on the method of Singleton et al. (1998) simple, and require only common equipment and have produced a large body of comparable data. Under proper conditions, the assay is inclusive of monophenols and gives predictable reactions with the types of phenols found in nature. Because different phenols react at different degrees, expression of the results as a single number, such as milligrams per liter gallic acid equivalence, is necessarily arbitrary. Because the reaction is independent, quantitative, and predictable, analysis of a mixture of phenols can be recalculated in terms of any other standard. The assay measures all compounds readily oxidizable under the reaction conditions and its very inclusiveness allows certain substances to also react that are either not phenols or seldom thought of as phenols (e.g., proteins with few modifications. In a dim place at ambient temperature, 0.5 mL of extract was added a 2.5 mL Folin-Ciocalteu reagent (1:10 dilution) and thoroughly mixed the tube using a vortex. After allowing to stand for 15 min, 2 mL of 7.5% Na_2CO_3 solution was added to the solution and mixed it again thoroughly. The sample was allowed to stand for 1 h at room temperature and then read at 765 nm using a spectrophotometer (DU 730, Beckman Coulter, CA, USA). A reagent blank (85% MeOH) and gallic acid (GA) standards (0, 10, 20, 40, 50, 60, 80 and 100 µg/L) were analyzed along with the samples. Results were expressed as milligrams gallic acid equivalents per gram of dry sample (mg GAE/g).

Antioxidant Capacity Analysis

DPPH Radical Scavenging Activity

The DPPH radical scavenging activities of the vegetable extracts were evaluated using the method of Brand Williams et al. (1995) with modifications. Briefly, 0.5 mL of sample extract and 0.01 M DPPH solution were added. The contents of the tube were mixed thoroughly, let to stand for 1 h and read at 517 nm. A reagent blank (85% MeOH) and Trolox standards (0, 20, 40, 60, 100 160, 200, 240, 280, and 500 µmol/L) were analyzed with the samples. Results were expressed as micromole Trolox equivalent per gram of dry sample (µmol TE/g).

ABTS Radical Cation Scavenging Activity

The ABTS radical cation scavenging activity of the sample extracts were evaluated based on the procedure of Pellegrini et al. (2003) and Moore et al. (2005) with modifications. An ABTS working solution was prepared by mixing 2.4 mM potassium persulfate and 7 mM aqueous ABTS. The contents were left standing for 12 - 16 h and then diluted to a ratio of 1 : 45. The solution was mixed thoroughly and allowed to react for 1 min. The absorbance was read at 734 nm against distilled water as blank and Trolox standards (0, 20, 40, 60, 100, 160, 200, 240, 280, and 320 µmol/L). Results were also expressed as µmol TE/g of sample, in dry weight basis.

Statistical Analysis

Using IBM SPSS Statistics version 20, ANOVA was performed on the antioxidant and TPC values while multiple comparisons were carried out using the Tukey's studentized range test. T-test was performed through Statistical Analysis Software (SAS) version 9.1 while the correlation analysis was done using the MS Excel Analysis ToolPak. The significance level used was $p<0.05$. All measurements were performed in triplicates.

Results and Discussion

Total Phenolic Content

Phenolic compounds have been reported as major contributors for high radical scavenging activities in plants (Kameya et al., 2014; Takebayashi et al., 2013). Most phenolic compounds are unstable and subject to changes from drying treatments (Lim and Murtijaya, 2007). The TPC of the oven-dried and lyophilized vegetables are shown in Table 2.

Table 2. TPC of oven-dried and lyophilized vegetables.

Vegetable	Total Phenolic Content (mg GAE g^{-1})		Difference
	Oven-Dried	Lyophilized	
Chili leaves	37.88 ± 3.45^{c}	36.99 ± 1.91^{bc}	0.89
Eggplant (round green)	24.46 ± 0.46^{d}	2.39 ± 0.30^{d}	22.08*
Green pepper	9.14 ± 0.04^{e}	11.32 ± 0.32^{d}	2.19*
Red coral lettuce	55.30 ± 1.28^{b}	46.33 ± 0.33^{b}	8.97*
Squash flower	11.31 ± 0.16^{e}	11.54 ± 0.36^{d}	0.23
Squash fruit	1.13 ± 0.00^{f}	1.78 ± 0.05^{d}	0.65*
Sweet potato (yellow flesh)	1.70 ± 0.17^{f}	0.17 ± 0.03^{d}	1.53*
Turmeric	79.62 ± 0.96^{a}	187.50 ± 18.64^{a}	107.88*
Water spinach (lowland)	28.35 ± 2.23^{d}	27.07 ± 2.44^{c}	1.28

Mean ± SD (n=3). Mean values with the same letter within the same column are not significantly different at $p<0.05$.

The TPC of the crops ranged 1.13 - 79.62 mg and 0.17 - 188.77 mg GAE g^{-1} for oven-dried and lyophilized samples, respectively. The highest levels observed for both treatments were those of turmeric rhizome and the leafy vegetables. The lowest values were exhibited by squash fruit and sweet potato. Two drying methods did not affect the TPC of chili leaves, squash flower, and lowland water spinach (p<0.05) while lyophilized green pepper and turmeric had significantly higher TPC than the oven-dried counterparts, with turmeric having 135% higher TPC. In a previous study of Hirun et al. (2014) on turmeric, the polyphenol oxidase (PPO) was inactivated at temperatures greater than 60^{o}C, which was higher than the oven drying procedure used in this study. PPO results in the oxidation of phenolic compounds forming condensation products and colored complex polymers, which reduce the measured phenolic content (Yoruk and Marshall, 2003). Enzymes, substrates, and activators may also be released that could potentially alter the measurable phenolics (Chang et al., 2006). In addition, no thermal degradation of polyphenols and no action of degradative enzymes occur in lyophilization, which are evident in the basically unaltered physical appearances of the vegetables after the dehydration process. Moreover, higher TPC in lyophilized samples, which could reflect their actual TPC, could be the result of phenolic compounds released from cell walls that ruptured when ice crystals were formed during freezing. Breaking of the cell wall could also make the cellular components more accessible to extraction solvents (Asami et al., 2003; Chan et al., 2013; Das et al., 2012,). Hence, lyophilized plants often have comparable quality with the fresh products (Chan et al., 2013). Oven-dried red coral lettuce and eggplant, however, recorded higher TPC with eggplant having 924% more TPC than the lyophilized sample. This can be associated to the formation of simpler phenolic compounds from complex ones (Singh et al., 2015).

DPPH Radical Scavenging Activity

DPPH is among the most widely used methods to measure antioxidant capacity of various samples because of its sensitivity and that it only requires a small sample (Kulisic et al., 2004). It relies on losing the purple color of the radical solution through the reduction of the DPPH radical using antioxidant as the hydrogen atom donor. However, its usefulness is undermined by its sensitivity to acidic pH and slow reaction (Shalaby and Shanab, 2013). The DPPH activities of the oven-dried and lyophilized vegetables are shown in Table 3.

The turmeric consistently yielded high DPPH value in both oven-dried (303.24 μmol TE g^{-1}) and lyophilized (750.58 μmol TE g^{-1}) forms (Table 3). The leafy vegetables red coral lettuce, chili leaves, and water spinach also exhibited high DPPH radical scavenging activities, with red coral lettuce having the highest value in oven-dried form. Except for green pepper, differences between the measured DPPH radical scavenging activities of oven-dried ang lyophilized samples were significant (p<0.05). Differences in the DPPH values between samples dried using the two treatments were variable. Significantly higher values were recorded in lyophilized turmeric, chili leaves, lowland water spinach, and squash fruit. In turmeric, the release of phenolic compounds upon rupturing of cell walls during freeze drying may have caused the higher DPPH values of lyophilized samples. Thermal degradation of polyphenolic compounds and PPO conversion to non-phenolic colored compounds have been reported to reduce TPC and antioxidant capacities in heated samples (Chan et al., 2013; Lim and Murtijaya, 2007; Suvarnakuta et al., 2011). However, red collar lettuce, eggplant, squash flower, and sweet potato had higher DPPH values when oven-dried. This can be attributed to the formation of other compounds with antioxidant properties such as free flavonols upon heating (Stewart et al., 2000).

Table 3. DPPH of oven-dried and lyophilized vegetables.

Vegetable	DPPH Radical Scavenging Activity (μmol TE g^{-1})		
	Oven-dried	Lyophilized	Difference
Chili leaves	197.33 ± 2.62^{c}	363.46 ± 18.94^{b}	166.12*
Eggplant (round green)	141.31 ± 1.47^{e}	24.05 ± 1.58^{e}	117.26*
Green pepper	40.85 ± 0.27^{f}	38.00 ± 0.87^{e}	2.85
Red coral lettuce	380.57 ± 14.72^{a}	201.59 ± 2.45^{d}	178.99*
Squash flower	28.94 ± 1.02^{f}	18.74 ± 0.20^{e}	10.19*
Squash fruit	1.24 ± 0.05^{g}	1.90 ± 0.02^{e}	0.66*
Sweetpotato (yellow flesh)	10.03 ± 0.31^{g}	1.04 ± 0.13^{e}	9.00*
Turmeric	303.24 ± 8.99^{b}	750.58 ± 26.04^{a}	447.34*
Water spinach (lowland)	170.83 ± 5.04^{d}	244.55 ± 12.03^{c}	73.72*

Mean ± SD (n=3). Mean values with the same letter within the same column are not significantly different at p<0.05.

ABTS Radical Cation Scavenging Activity

ABTS has the same hydrogen atom and single electron transfer mechanism of DPPH assay (Kasote et al., 2015). However, the study of Floegel et al. (2011) on 50 antioxidant-rich foods showed that ABTS can better reflect the antioxidant content in a variety of foods, fruits, vegetables, and beverages than DPPH assay. They attributed this result to the improved applicability of ABTS to both hydrophilic and lipophilic systems, while DPPH is more applicable to the latter only. The ABTS radical scavenging activities of the oven-dried and lyophilized vegetables are shown in Table 4.

The results generally show significant differences in the antioxidant capacities of most of the dried vegetables using the two treatments (Table 4). It is important that there were no significant differences in the measured ABTS activity of eggplant and squash flower compared with DPPH. Antioxidants in a biological sample could have additive and synergistic effects with other compounds present in the sample (Brighenti et al., 2005; Puchau et al., 2009). Another possible reason is that antioxidants with high DPPH values have higher ability in donating hydrogen to free radicals like lipid peroxides or hydroperoxide radicals (Bamfort et al., 1993), while high molecular weight phenolics like tannins have greater ability to quench ABTS free radicals (Hagerman et al., 1998).

Correlation of TPC and Antioxidant Capacities

Correlation analysis between the TPC and the antioxidant capacities of the oven-dried and lyophilized plants showed very strong positive relationships, which were significant at $p<0.05$ (Figure 1A and 1B). In the oven-dried samples, the TPC had a strong association with DPPH, in which 85% of the variability in the DPPH radical scavenging activities are accounted for by the TPC of the samples. Likewise, TPC is strongly associated with the ABTS radical scavenging activities, with 95% of the variation in the antioxidant capacity explained by the TPC.

Correlation analysis on lyophilized samples (Figure 1B) were similar with the oven-dried samples (Figure 1A). Both antioxidant capacities were strongly associated with the TPC with higher correlation coefficient obtained between TPC and ABTS (Figure 1B). The results indicate that phenolics have a strong contribution to the antioxidant capacities displayed by the plants, consistent in the previous studies (Takebayashi et al., 2013; Kameya et al., 2014).

The analyses show that the two drying techniques have variable effects on the phenolic content and antioxidant capacities of the different plant samples. Correlation analyses, however, exhibited the consistency of samples in terms of TPC and antioxidant capacities regardless of the drying treatment. Lyophilization could be a better choice because this process allows the plant matrix to be accessible for bioactive component extraction and the result prevents the production of new antioxidant compounds. However, it is an expensive process (Ratti, 2001). In this study, lyophilization required the overnight freezing of material and dehydration up to 24 h. The equipment and its maintenance are expensive. Hence, a lyophilizer may not be available in all laboratories. In contrast, oven is used for various analytical procedures and is therefore available in all laboratories. For analytical evaluations and isolation of healthful antioxidants or utilization in certain food products or dietary supplements, lyophilization can be the method of choice. For instance, Chan et al., (2009) conclude that lyophilization could better retain the phenolic compound and antioxidant capacity levels in four species of tea. However, as shown by the strong correlations in this study, sample preparation for routine antioxidant determination may employ oven drying. It should be noted that oven-drying must be done at temperatures below 50ºC because

Table 4. ABTS of oven-dried and lyophilized vegetables.

Vegetable	ABTS Radical Cation Scavenging Activity (μmol TE g^{-1})		Difference
	Oven-dried	Lyophilized	
Chili leaves	135.41 ± 2.66^{c}	148.37 ± 2.04^{c}	12.96
Eggplant (round green)	28.94 ± 2.35^{e}	27.93 ± 1.40^{e}	1.01
Green pepper	30.72 ± 1.78^{e}	21.21 ± 0.25^{ef}	9.51*
Red coral lettuce	180.88 ± 3.61^{b}	179.99 ± 0.52^{b}	0.89
Squash flower	18.54 ± 0.26^{f}	19.97 ± 1.40^{ef}	1.43
Squash fruit	2.10 ± 0.02^{g}	2.85 ± 0.02^{ef}	0.76*
Sweet potato (yellow flesh)	4.40 ± 0.17^{g}	0.71 ± 0.25^{f}	3.69*
Turmeric	322.67 ± 7.25^{a}	824.96 ± 26.32^{a}	502.29*
Water spinach (lowland)	101.10 ± 0.68^{d}	104.34 ± 1.52^{d}	3.25

Mean ± SD (n=3). Mean values with the same letter within the same column are not significantly different at p<0.05.

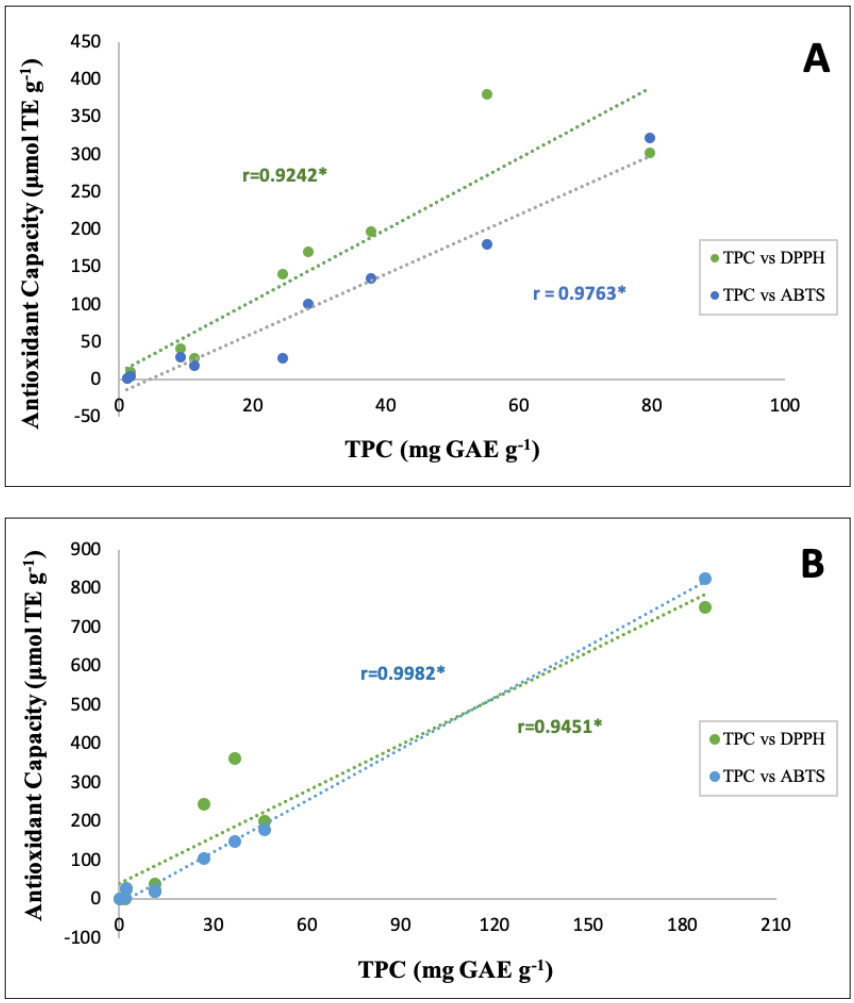

Figure 1. The correlations (p<0.05) of TPC versus antioxidant capacities of oven-dried (A) and lyophilized (B) raw vegetables.

higher value will drastically reduce the TPC of plant samples (Roshanak et al., 2016). On the other hand, drying at lower temperature (25ºC) required longer drying time and resulted in considerable reduction on the levels of bioactive compounds (Nguyen et al., 2015). In mortiño fruit, increasing the temperature and drying time reduced its antioxidant capacity and phenolic content (López-Vidaña et al., 2017). The duration of drying process using an oven must also be carefully chosen such that no microbial growth will be observed after drying. In this experiment, 16 h at 40°C was the lowest possible time-temperature combination that could yield samples with <10% moisture content (MC) and without signs of microbial growth that could severely impact the antioxidant levels in the samples. Foods with MC range of 5 - 15% are less prone to microbial spoilage especially during storage (deMan, 1999). These results could therefore guide the laboratories on the proper selection of dehydration method depending on matrices of sample to be analyzed, the purpose of the analysis, and resource availability in the laboratory.

Conclusion and Recommendations

Several plant foods are known to possess high bioactive components and antioxidant capacities with potential beneficial effects on the body. Accurate analytical determination of these compounds depends on reliable experimental procedures such as sample preparation, which includes drying or dehydration. This work showed that different drying techniques generally yield different TPC and antioxidant capacities in several plants. Turmeric consistently had the highest TPC and antioxidant capacities with lyophilized samples displaying significantly higher values at most 135% improvement in TPC, 148% in DPPH, and 156% in ABTS levels. Leaves with high TPC and antioxidant capacities had varying reactions to drying treatments. Oven drying of eggplant favored the formation of phenolic compounds (24.46 mg GAE g^{-1}) and higher DPPH antioxidant capacity (141.31 µmol TE g^{-1}), but similar ABTS reading. Antioxidant properties in most of the samples might be better retain through lyophilization; however,

oven drying is more practical, especially for routine determinations. Correlation analyses consistently indicated a strong positive association between TPC and antioxidant capacities regardless of drying treatment. These results suggest that oven-drying of plant species can be used in routine antioxidant determination depending on the purpose of the analysis. Dehydration methods can also be optimized for every plant material to ensure the maximum retention of their antioxidants. Further studies are recommended to validate the effects of the different drying treatments on specific antioxidants present in each vegetable, their levels, and the potential impact on their bioactivities.

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