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Research article

Differential expression of *Xoo-induced kinase 1 (XIK1)*, a *Xanthomonas oryzae* pv. *oryzae* responsive gene, in bacterial blight-susceptible and *Xa21*-mediated resistant indica rice cultivars

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Abstract

Bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a costly disease in rice that threatens global rice production. The *Xa21* gene is a broad spectrum BB-resistance gene that is extensively used for improving BB resistance in rice. The *Xoo* responsive gene, *Xoo-induced kinase 1* (*XIK1*), recently characterized in japonica rice, was also found to be involved in Xa21-mediated resistance. The current study investigated the differential expression of *XIK1* in the BB-susceptible indica rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21*/*Xa21*) in various growth stages and during *Xoo* inoculation. The results showed that the expression of *XIK1* was development-dependent and induced earlier in BB-resistant progenies BC_3F_3 than in the susceptible cultivar RD47 after *Xoo* inoculation. However, the expression levels of *XIK1* substantially dropped after a peak of expression. Interestingly, the *XIK1* expression tended to increase again in the succeeding hours post-inoculation in BC_3F_3 but not in RD47. Similar development and induction patterns were also observed when the expression of *XIK1* and *Xa21* was analyzed in the same runs in the BB-resistant BC_3F_3 under different growth stages and during *Xoo* inoculation. The findings suggested that XIK1 may also be involved in the Xa21-mediated resistance pathway of indica rice.

Introduction

Most of the world's population depends on rice (*Oryza sativa*) as the primary food source and this requires large-scale-production volumes to meet the growing demand (IRRI, 2006). However, limiting factors such as insect pests and diseases tend to reduce the yield by 30–80% (Reissig et al., 1985; IRRI, 2018a). Among the major diseases of rice, bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the costliest as it reduces production by up to 70% (IRRI, 2018b). Its symptoms include a vascular wilt at the seedling stage, a leaf blight, and unfilled panicles in mature plants which result from the invasion of the vascular system by *Xoo* bacteria (Mew, 1987). At the molecular level, *Xoo* secretes transcription activator-like (TAL) effectors which invade and highjack the host cells by activating the transcription of genes that enhance plant susceptibility and support bacterial virulence (Boch and Bonas, 2010; Bogdanove et al., 2010; Römer et al., 2010).

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In plants, the ability to recognize both general elicitors and specific pathogens through gene-mediated resistance is essential to their defense mechanisms (Andersen et al., 2018). Hence, studying the variations in the expression of various genes provides a perspective of the mechanisms of plant responses to BB. To date, more than 30 BB resistance genes have been identified in *Oryza sativa* and its closely related species; among these, *Xa21* has probably been the most commonly used for rice variety improvement as it provides for high level and broad spectrum BB resistance (Song et al., 1995; Win et al., 2012). *Xa21* codes for a plasma membrane which recognizes the tyrosine-sulfated protein RaxX and triggers the Xa21-mediated immunity (Pruitt et al., 2015). It is known that this response involves Xa21-binding proteins (Wang et al., 2006; Chen et al., 2010; Park et al., 2017) as well as the direct interaction of a cleaved XA21 subunit with the WRKY62 transcription factor (Peng et al., 2008; Park et al., 2012; He et al., 2017). Nonetheless, the precise mechanisms of *Xa21* resistance have not yet been completely elucidated.

The current study centered on the recently discovered *Xoo-induced kinase 1* (*XIK1*, Loc_Os02g4790) which encodes a LRR-RLK protein and is hypothesized to act as a co-receptor of *Xa21* since it positively regulates Xa21-mediated BB resistance (Hu et al., 2015). Since *XIK1* was previously identified and conserved in the japonica rice cultivar Kitaake, the current study characterized the expression levels of this gene in the BB-susceptible cultivar RD47 and its improved BB resistant progenies BC_3F_3 (*Xa21*/*Xa21*) in various growth and developmental stages and during *Xoo* inoculation.

Materials and Methods

Plant samples and growing conditions

Rice (*Oryza sativa* L. ssp. indica) cultivars RD47 and IRBB21 were provided by the Bureau of Rice Research and Development, Phitsanulok, Thailand. The *Xa21* gene from IRBB21 was introgressed in RD47 through backcross breeding and marker-assisted selection (data not shown) until homozygous-*Xa21* BC_3F_3 lines were obtained. Leaf samples were collected from the 2-leaf, 4-leaf, 6-leaf and reproductive stages, respectively.

Xoo isolate and inoculation test

BB-infected leaves were collected from paddy fields in Phitsanulok province and *Xoo* was isolated on nutrient agar (peptone-bovine-agar). The isolated bacteria was identified as *Xoo* through polymerase chain reaction (PCR) assays using *Xoo* specific primers TXT (Sakthivel et al., 2001) and Xoo80 (Lu et al., 2014). Before infection, the *Xoo* isolate labeled as "xoo16PK002" was re-streaked and incubated at 28°C for 48 hr. A *Xoo* inoculum (the optical density at 600 nm (OD600) of 0.2) was prepared and used to inoculate 60-day-old plants using the clipping method of inoculation (Kauffman, 1973). Mock (water) inoculation was used as a control. Samples corresponding to 5 cm of the leaves directly below the inoculation sites were collected at 0 hr, 1 hr, 2 hr, 6 hr and 24 hr post inoculation (hpi); leaf samples were frozen in liquid nitrogen immediately.

RNA extraction and cDNA synthesis

Total RNA was extracted from each 100 mg leaf sample using an RNAprep Pure Kit (Tiangen Biotech Ltd.; China) following the manufacturer's instructions. Each RNA sample was treated with Rnase-Free Dnase I (RBC Bioscience; Taiwan) to remove possible gDNA contaminants. Total RNA samples were quantified using a Synergy H1 microplate reader (Biotek; USA) and their integrity was assessed using agarose gel electrophoresis. The qScript™ XLT cDNA synthesis kit (QuantaBio; USA) was used to reverse transcribe 1 µg of total RNA templates in order to synthesize first strand cDNAs according to the manufacturer's protocol.

Polymerase chain reaction and cloning of XIK1 partial cDNA sequence

PCR was performed using a BioRad T100™ Thermal Cycler for 35 cycles (95°C for 10 s, 60°C for 10 s, 72°C for 20 s). The primer XIK1Ri (Hu et al., 2015) was used to amplify the *XIK1* gene. The PCR products were cloned using RBC TA cloning vector (RBC Bioscience; Taiwan) following the instructions in the manual. Plasmids carrying the *XIK1* gene were extracted and subjected to sequence analysis.

Quantitative real-time polymerase chain reaction

For the quantitative real-time polymerase chain reaction (qPCR) analyses, fast SYBR Green Master Mix (Quantabio; USA) was used to prepare 20 µl qRT-PCR reactions containing 1 µl of the cDNA templates and 0.5 µM of each primer. The specific primers used to amplify the genes of interest (GOIs) evaluated in this study are shown in Table 1. Non-RT PCR was performed to confirm no gDNA contamination. The specificity of the PCR and qPCR products was carefully assessed using gel electrophoresis and melting curve analysis, respectively. Technical triplicates and no template controls (NTCs) were run on an Eco 48 Real Time PCR System (PCR Max; United Kingdom) for 35 cycles (95°C for 10 s, 60°C for 10 s, 72°C for 20 s) followed by melting curve analysis.

Results

Expression of XIK1 and Xa21 in different growth stages

The amplified *XIK1* product using the primers based on japonica rice Kitaake showed 100% nucleotide identicality to the annotated *XIK1* sequence (Fig. 1). The relative expression of *XIK1* in different growth stages (2-leaf, 2S; 4-leaf, 4S; 6-leaf, 6S; reproductive stage, RS) showed that *XIK1* gene expression levels progressively increased from the 2S to 6S stages and eventually decreased in the RS stage in both the BB-susceptible cultivar (RD47) and its improved BB-resistant progenies (BC_3F_3) as shown in Fig. 2A and 2B, respectively. The expression of *Xa21* in the improved BB-resistant (BC_3F_3) progenies showed a similar trend to the *XIK1* gene (Fig. 2C). As expected, no amplification of *Xa21* was detected in the BB-susceptible cultivar RD47 (data not shown).

Table 1 List of genes evaluated in this study

Gene name	Primer sequence (5'-3')	Amplicon length	Reference
Xoo-induced kinase 1 (XIK1)	GACCAGGCGAAATCAACTTT ATGTAAGGCAGTGAGTTTAGTCAA	187 bp	Hu et al., 2015
Xa21	CAGAGTATGGCGTTGGGCT CGGGTCTGAATGTACTGTCA	114 bp	Promma et al., 2016
Triosephosphate isomerase (TI)	CGACATCATCAACTCCGCCAC CCTCTTCAGACATCTTCCCACG	83 bp	Wang et al., 2016
Endothelial differentiation factor (Edf)	TCCGAACCAGCAGATCATCG GCATGGTATCAAAAGACCCAGC	158 bp	Wang et al., 2016
Ubiquitin-5	CCAGTACCTCAGCCATGGA GGACACAATGATTAGGGATC	69 bp	Hu et al., 2015

Gene names and all their details are presented using the style in the reference cited.

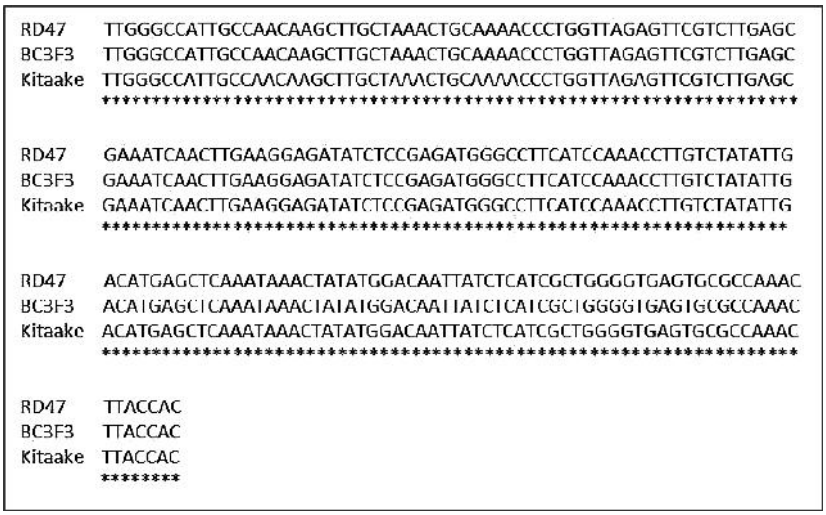

Fig. 1 Multiple sequence alignment of *XIK1* sequences in RD47 and improved BB-resistant BC_3F_3 progenies compared with the japonica cultivar Kitaake (CLUSTAL O 1.2.4).

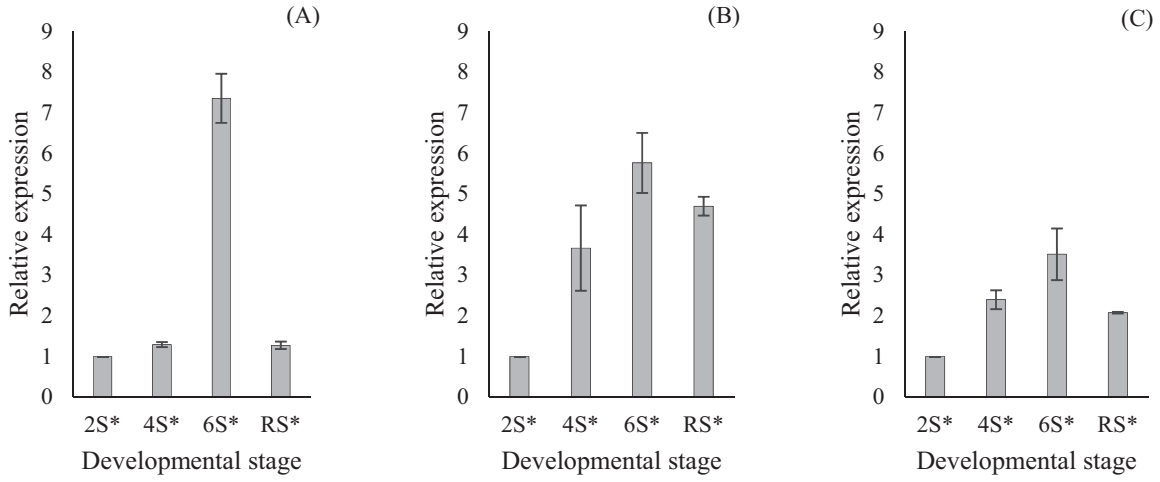

Fig. 2 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 progenies; (C) relative expression of *Xa21* in BC_3F_3 progenies in various growth stages, where 2S, 4S, 6S and RS represent the 2-leaf, 4-leaf, 6-leaf, and reproductive stages, respectively and asterisks indicate significant differences among datasets based on analysis of variance at $p < 0.05$ and error bars indicate SD of the analyzed data that have been normalized relative to *Ubiquitin 5*.

Response of XIK1 and Xa21 to Xoo infection

The 60-day-old RD47 and BC_3F_3 plants corresponding to the 6S stage were subjected to *Xoo* infection and samples were collected at different times post inoculation for qPCR analyses. The results showed that the expression of *XIK1* was induced by *Xoo* inoculation for both the RD47 and BC_3F_3 lines but in different times post inoculation. The expression of *XIK1* in the RD47 cultivar was suppressed in the first hour after *Xoo* inoculation and was later induced after 2 hpi. Notably, the expression level substantially dropped after the time of induction and there was no clear indication of up-regulation in the succeeding time post inoculation (Fig. 3A). On the other hand, the expression of *XIK1* in the BC_3F_3 lines was quickly induced after 1 hpi but the expression level suddenly decreased after 2 hpi. Interestingly, the expression of the gene was up-regulated again after 6 hpi until 24 hpi, thus indicating that the expression of *XIK1* tended to increase again (Fig. 3B). The expression of *Xa21* in BC_3F_3 was also significantly induced after 1 hpi and eventually decreased in the succeeding time post inoculation (Fig. 3C). No signal of *Xa21* up-regulation was observed, even after 24 hpi.

Discussion

The genetics of resistance to bacterial blight has been studied in depth and was first carried out using the resistance (R) gene *Xa21*. As there is diversity in the *Xoo* strains of different rice-producing countries, the mechanism of *Xa21*-mediated resistance has not yet been completely elucidated. Since *Xa21* functions as a pattern recognition receptor and a tyrosine-sulfated protein *RaxX* is required for the activation of Xa21-mediated immunity, the activity of *Xa21* in the plant's plasma membrane may accelerate subsequent responses to the conserved bacterial molecule of *Xoo* (Gómez-Gómez and Boller, 1999). Previous reports have reported that several Xa21-binding proteins are directly involved and play important roles in the early events of the *Xa21* signaling pathways (Wang et al., 2006; Lee et al., 2009; Chen et al., 2010; Park et al., 2010; Park et al., 2012). However, no changes of expression after *Xoo* inoculation were found in the genes encoding these proteins. Hu et al. (2015) found a BB-resistant gene (*XIK1*) that shared a structural motif with *Xa21* and positively regulated Xa21-mediated immunity. However, that study did not indicate whether the expression of the *XIK1*gene was stable in the time post inoculation after *Xoo* infection and whether the gene expression in the various leaf stages was the same for both BB-resistant and BB-susceptible cultivars. The current results showed that the expression levels of *XIK1* were induced earlier post inoculation by *Xoo* in both the BB-susceptible cultivar (RD47) and the BB-resistant BC_3F_3 progenies. However, the induction on the expression was earlier in BC_3F_3 than in RD47. Though the expression of *XIK1* significantly decreased after the induction, its expression tended to accumulate in the succeeding time post inoculation in the resistant BC_3F_3 but not in the susceptible RD47. These results suggested that *XIK1* is activated earlier and is progressively expressed in the succeeding hours after *Xoo* infection in BB-resistant BC_3F_3 but not in the BB-susceptible cultivar RD47. In addition, *XIK1* was either induced by *Xoo* or wounding (data not shown). Considering the reports that *Xa21* expression is development-dependent and its expression is either induced by *Xoo* or wounding (Mazzola et al., 1994; Century et al., 1999), the same findings for *XIK1* expression for both BB-susceptible and BB-resistant cultivars were also presented in this study.

The gradual increase in the expression of *XIK1* during the plant's growth and development shares similarity with the expression of *Xa21* and the responses of both genes to *Xoo* inoculation in the BB-resistant BC_3F_3 progenies carrying the *Xa21* gene are in commonality after 1 hpi and in the succeeding time post inoculation, Thus, *XIK*1 might act as a co-receptor of *Xa21* in regulating the early events of XA21-mediated signaling and thereby conforming

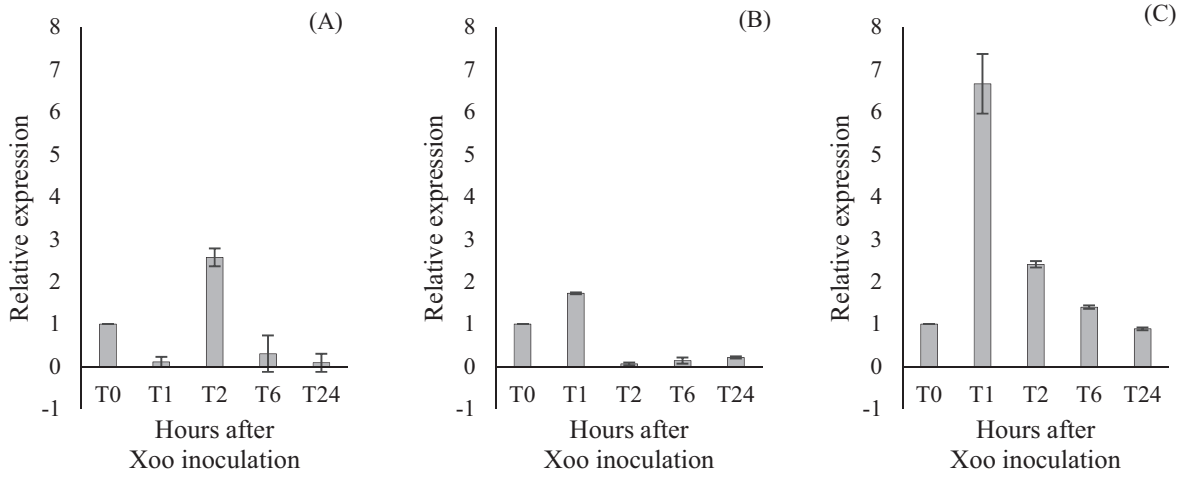

Fig. 3 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 lines; (C) relative expression of *Xa21* in BC_3F_3 lines after *Xoo* inoculation, where 0 hr, 1 hr, 2 hr, 6 hr and 24 hr post inoculation are presented as T0, T1, T2, T6, and T24, respectively and error bars indicate SD of the data analyzed that have been normalized with the reference genes *Edf* and *TI*.

to the findings of Hu et al. (2015). Data on the disease development by *Xoo* in both tested cultivars also confirmed that BC_3F_3 progenies expressing the genes *Xa21* and *XIK1* established resistance against the *Xoo* pathogen (Fig. 4). In this case, the current study supports the mechanism of defense for *Xa21*-mediated rice plants in both indica and japonica subspecies as characterized by *XIK1* and *Xa21* genes expression as being alike.

Fig. 4 Disease development of *Xoo* on indica rice cultivars RD47 and BC_3F_3 progenies 21 d post inoculation, where RD47 shows complete susceptibility while BC_3F_3 shows moderate resistance

The results revealed that the expression of *XIK1* was development-dependent and was induced by *Xoo* in the tested indica rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21*/*Xa21*). Furthermore, it was shown that the *XIK1* gene was induced earlier in the BB-resistant BC_3F_3 progenies than in the susceptible cultivar RD47 after *Xoo* inoculation, thus indicating that *XIK1* was activated earlier in resistant plants than in susceptible ones. Moreover, similar expression patterns of *Xa21* and *XIK1* were identified in the various growth stages and after *Xoo* inoculation.

Conflict of Interest

The authors declare that there were no conflicts of interest and all ideas reflected herein have the agreement of all authors.

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BIOCONVERSION OF LIGNOCELLULOSIC AGRICULTURAL BY-PRODUCTS BY MICROORGANISMS INTO HIGH MYCOPROTEIN FEEDS

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ABSTRACT

This research study was conducted to produce high mycoprotein feeds (HMPF) from lignocellulosic agricultural by-products through solid-substrate-fermentation. Six species of fungi were used as fermenting organisms and four agricultural wastes as substrates. The nutrient composition of the fermented wastes was determined through proximate analysis. Results of the study showed that the three macrofungi *Pleurotus* spp., *Ganoderma* spp., and *Psilocybe* spp. and the three microfungi *Aspergillus* spp1, *Trichoderma* spp., and *Aspergillus* spp2 were capable of producing HMPF through solid-substrate-fermentation of lignocellulosic agricultural by-products. Further, banana leaves, rice straw, corn cob and sugarcane bagasse are candidate substrates for the production of HMPF. Proximate analysis revealed that the crude protein (CP) of the fermented banana leaves obtained a four- to five-fold increase (24.41%-28.16%) in CP while an eight to nine-fold increase (19.66%-22.63%) in corn cobs after fermentation. The fermented sugarcane bagasse attained 11- to 13-fold (21.37%-25.83%) rise in the CP content while the fermented rice straw obtained two to five-fold increase (18.88%-29.51%) in the CP content. The ash contents (ACs) of the fermented products likewise increased while the crude fiber (CFr) and crude fat (CF) of the lignocellulosic agricultural by-products decreased after fermentation. The present results demonstrate the feasibility of utilizing lignocellulosic agricultural-wastes as substrates of fungal organisms to produce high protein feeds for animals.

Key words: protein biomass, high mycoprotein feeds (HMPF), lignocellulosic, solid-substrate-fermentation, macro and microfungi

INTRODUCTION

Agricultural wastes comprise a major proportion of agricultural production. These crop residues are made up mostly of lignocellulosic materials. These lignocellulosic crop residues are rich in dietary fibers (Valéro *et al*., 2012) which are great potential energy resources for livestock and poultry. However, they are characterized with very poor inherent

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feeding value due to low digestible dry matter and protein content (Nasehi *et al*., 2017). Likewise, these fiber-rich wastes have an enormous potential to be exploited for the production and recovery of several products and ingredients to improve animal nutrition and the worldwide supply of protein and calories for animal production in lesser environmental footprints. By using appropriate technologies, the economic value of lignocellulosic crop residues could be increased through nutrient enrichment and production of products that are safe not only for animal use but also for human feeding.

Animal production in the Philippines is a lucrative viable enterprise and progressively developing. However, the high cost of animal feeds due to the importation of expensive feed ingredients is a major problem that limits the sustainability of animal production. Aside from this, the extensive use of synthetic growth promoters and additives is now a worldwide concern because of its impact on consumers' health and disputes on multidrug resistance. While feed comprises the bulk of the total cost of production, efforts in the production of safe and cost-effective alternative feed ingredients could lead into a cost-efficient and successful animal production by not compromising the quality of feeds, nutrition of animals and health of consumers.

Microbial degradation of lignocellulosic materials brings a variety of changes in their bio-physicochemical properties. Microbial treatment can enhance the digestibility of various agricultural residues. Filamentous fungi are potential candidates that can improve the nutritional quality of lignocellulosic residues by degrading lignin with the use of the complex extracellular cellulolytic enzymes and converting these complex polysaccharides into simple sugars (Singh and Kumar, 2015). The use of fungal fermentation to cycle and recycle these residues will not only result in the reduction of pollution but also help to produce low cost and high-quality protein biomass. Further, production of protein biomass from fibrous wastes is an eco-friendly approach to recovering renewable energy resources from agri-crop production into valuable low-cost products. This research was conducted to produce high mycoprotein feeds from lignocellulosic agricultural by-products through solid-substrate-fermentation. Specifically, it aimed to identify species of filamentous macro and microfungi that could produce mycoprotein feeds and determine the proximate composition of the mycoprotein feeds produced from different agricultural by-products.

MATERIALS AND METHODS

Four independent studies were conducted utilizing four agricultural by-products as substrates and six fungal organisms. The agricultural by-products were sugarcane bagasse, rice straw, corn cobs and banana leaves. Each of the study was set-up with five replications to produce high mycoprotein feeds.

A 39 g potato dextrose agar (PDA) basal medium (TM Media) was weighed using a toploading analytical balance (National LCS-3000, 1200gx0.1g, Nagata Scale Co., Ltd, Taiwan). It was placed in a beaker and dissolved in 1 Li of distilled water. The beaker was placed on top of a hot plate (All American™ Corning™ PC-420 Fisher Scientific, USA) stirred continuously until the PDA was completely dissolved. A 20 ml mixture was dispensed in sterilized flat bottles. The bottles were covered immediately with cotton wool and aluminum foil to reduce contamination. Then, the bottles were sterilized for 15 min using an autoclave (All American™ Stove Top, Wisconsin Aluminum Foundry, USA) maintaining a pressure of 15 psi. After sterilization, the bottles were slanted and allowed the

basal medium to cool down and solidify.

The three macrofungi (*Pleurotus* spp., *Ganoderma* spp., *Psilocybe* spp.) were kindly given by Mr. Benjie L. Garcia of the Central Luzon State University and the three microfungi (*Aspergillus* spp1, *Trichoderma* spp., *Aspergillus* spp2) were obtained from the Department of Agriculture-Philippine Center for Postharvest Development and Mechanization (DA-PhilMech).

Sub-cultures of the six fungal organisms were prepared from pure cultures. Before inoculation, the glass chamber was surface sterilized to minimize contamination. Each pure culture of the fungal organisms was sub-cultured one at a time. The inocula of the pure culture of macro and microfungi were aseptically transferred to flat bottles containing the basal medium using an inoculating loop. After inoculation, the flat bottles containing the inoculants were properly labeled and arranged on the shelves and incubated at room temperature for 5 to 7 days until full mycelial ramification of the fungal mycelium is evident on the surface of the basal medium.

A standard nutrient solution (SNS) based on that used by Pham *et al*. (1992) with some modifications by Demo-os *et al*. (2013) were prepared. Urea, ammonium sulfate, ammonium phosphate, vinegar and sugar were dissolved and mixed in water.

The sugarcane bagasse was obtained from a sugarcane producer and processor in Sta. Ignacia, Tarlac while the banana leaves were obtained in San Jose, Tarlac. The corn cob, on the other hand, was obtained from Pindangan 2nd, Camiling, Tarlac and the rice straw was obtained in Sta. Ignacia, Tarlac. The fibrous agricultural by-products were shredded using a shredding machine, sundried and used as substrates. In the preparation of the substrates, the shredded agricultural by-products were weighed, and the SNS was mixed with the shredded agricultural by-products. For the corn cob, a ratio of 60 SNS:40 corn cob substrate was used while a ratio of 50 SNS:50 substrates was used for sugarcane bagasse, rice hay and banana leaves. The SNS was gradually added to the fibrous sources individually and mixed thoroughly until a uniform mixture was attained. The substrates were packed in an equal quantity of 1 kg in polypropylene bags fitted with polyvinyl chloride (PVC) necks. The mouth of the PVC was covered with cotton wool and used paper and tightly sealed with a rubber band to avoid contamination. The bagged substrates were sterilized in an autoclave for 30 minutes maintaining a pressure of 15 psi. The sterile substrates were placed in a tray to cool down and were arranged on the shelves before inoculation.

The substrates were inoculated using the previously prepared sub-cultures of the six fungal organisms. Each of the cultures was aseptically inoculated in the substrates using an inoculating loop. After inoculation, the substrates containing the inoculum were properly labeled, placed in fermenting shelves and incubated for 21 days at room temperature. Every week, the inoculated substrates were kneaded to mix the fungal colonies in the substrate for efficient colonization and fermentation.

After 21 days of incubation, the resulting products of fermentation were kneaded and were removed individually from the propylene bags, placed in trays and sun-dried to about 90% dry matter.

After sun-drying, a 250 g fermented product was compositely sampled from the five replicates of each substrate, placed in ziplock plastics and labeled for proximate analysis. The finished products were brought to the Department of Agriculture-Regional Feed Chemical Analysis Laboratory in San Fernando City, Pampanga for the analysis of the nutrient composition in terms of moisture content (MC), crude protein (CP), crude fat (CF),

ash content (AC), crude fiber (CFr) and dry matter (DM). Data were gathered from the average of two runs of proximate analyses of the same analytical condition.

RESULTS AND DISCUSSION

The results of the proximate analysis of banana leaves before and after fermentation by macrofungi is shown in Figure 1. It shows that unfermented banana leaves contain 4.07% CP, 1.87% CF, 41.43% CFr, 10.00% AC and 91.00% DM. The fermentation of *Pleurotus* spp. brought a four-fold increase in the crude protein. After fermentation, the CF decreased by 0.70% which is an indication of the use of fats by *Pleurotus* spp. as a source of energy. Moreover, a 0.55% rise in AC indicating an increase in the inorganic components of the fermented banana leaves. A lower %MC brought the high %DM. Similar results were observed in *Ganoderma* spp. and *Psilocybe* spp. when used as fermenting organisms. A remarkable four-fold increase in the protein content was obtained after 21 days of fermentation. The CF decreased by 0.57% and 0.59% for both fungal organisms, respectively. The ACs of the fermented products improved by 0.33% and 0.73%, respectively. DM content was 84.70% and 86.10%, respectively. Proximate analysis of the banana leaves fermented by microfungal species is presented in Figure 2. Fermentation of banana leaves by *Trichoderma* spp. and *Aspergillus* spp2 brought a four-fold increase in the CP of fermented banana leaves while a five-fold CP was attained when *Aspergillus* spp1 was used in fermenting the banana leaves. A slight decline in the %CF

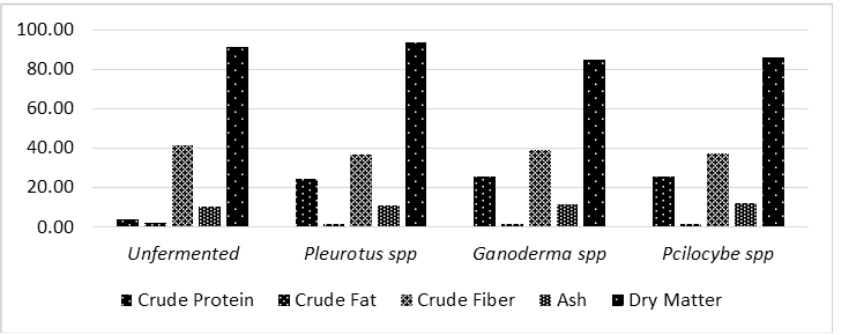

Figure 1. Proximate analysis of banana leaves before and after fermentation by macrofungi for 21 days at room temperature.

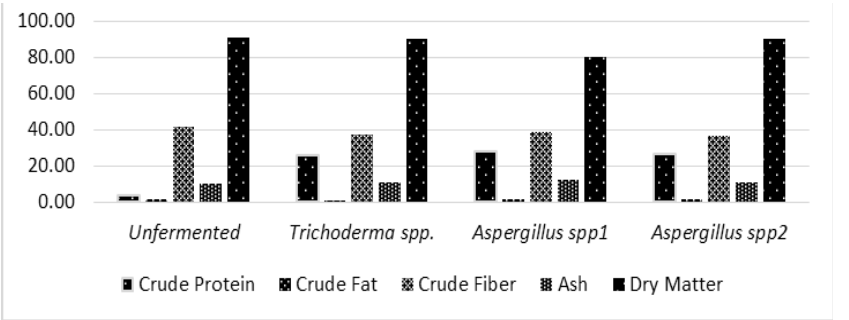

Figure 2. Proximate analysis of banana leaves before and after fermentation of microfungi for 21 days at room temperature.

of banana leaves was observed after fermentation by the three microfungal organisms. The CFr of fermented banana leaves decreased by 3.83% for *Trichoderma* spp., 2.76% for *Aspergillus* spp1 and 5.09% for *Aspergillus* spp2 when compared to unfermented banana leaves having a CFr of 41.43%. The AC increased by 1.23% in *Trichoderma* spp.-fermented banana leaves while 2.02% and 1.15% in the two *Aspergillus*-fermented banana leaves. There was a slight decline in the DM of the banana leaves fermented by *Trichoderma* spp. and *Aspergillus* spp2 while an 11.10% DM was attained in banana leaves fermented by *Aspergillus* spp1.

The results of the proximate analysis of corn cobs before and after fermentation of macrofungi is shown in Figure 3. It shows that unfermented corn cobs contain 2.09% CP, 0.8% CF, 41.90% CFr, 1.87% AC and 90.70% DM. The high organic matter, particularly the CFr, is an indication of high degradability and can be used as substrates for microbial fermentation converting complex carbohydrates into protein by several species of microorganisms. A nine-fold increase in the CP content was attained from the fermented corn cobs after 21 days of fermentation by *Pleurotus* spp. at 21.11%, *Ganoderma* spp. at 21.69% and *Psilocybe* spp. at 19.66%. The CF decreased by 0.63%, 0.49% and 0.35%, respectively, which indicates that the macrofungi utilized CF during the process of fermentation.

Moreover, a 0.5% rise in AC indicates an increase in the inorganic components of the fermented banana leaves. A lower %MC brought the high DM. Similar results were observed in *Ganoderma* spp. and *Psilocybe* spp. when used as fermenting organisms. A remarkable four-fold increase in the protein content was obtained after 21 days of fermentation. The CF of the corn cob decreased by 3.13%, 2.16% and 4.43% after fungal fermentation by *Pleurotus* spp., *Ganoderma* spp. and *Psilocybe* spp., respectively. There was also an improvement in AC of fermented corn cobs at 1.71%, 1.77% and 1.60% respectively, as compared to the unfermented corn cobs. The DM of *Pleurotus* spp. was 81%, 77% for *Ganoderma* spp. and 95% for *Psilocybe* spp.

On the other hand, Figure 4 shows that fermented corn cob by microfungal organisms had resulted in eight- to nine-fold rise in the %CP content of the fermented corn cobs. The CF content decreased by 0.43% when *Trichoderma* spp. was used in fermenting the corn cob while 0.64% and 0.76% decrease in CF when corn cob was fermented by *Aspergillus* spp1 and *Aspergillus* spp2, respectively. The CFr of fermented corn cob decreased by 2.51% for *Trichoderma* spp., 1.90% for *Aspergillus* spp1 and 3.79% for *Aspergillus* spp2 when compared to unfermented corn cob having a CFr of 41.90%. There was also a rise is the AC of the fermented corn cobs by the three fungal organisms compared with the unfermented substrates. The DM of the fermented corn cobs were 86.60% for *Trichoderma* spp., 83% for *Aspergillus* spp1 and 86.60% for *Aspergillus* spp2.

The results of the proximate analysis of unfermented and fermented sugarcane bagasse are shown in Figures 5 and 6. It shows that the unfermented sugarcane bagasse has very low CP, CF and AC but a very high DM constituting about 91.10% DM. After fermentation, there was a 12- to 13-fold increase in the CP content of the fermented sugarcane bagasse. The AC was likewise escalated from 2.41% unfermented sugarcane bagasse to 5.87%, 6.40% and 7.67% after fermentation by *Psilocybe* spp., *Ganoderma* spp., and *Pleurotus* spp., respectively. The CF of the fermented sugarcane bagasse, on the other hand, decreased when compared to the unfermented one whereas similar results were attained in the CFr of the unfermented sugarcane bagasse after fermentation. The fermentation of sugarcane bagasse by the microfungal organisms resulted to an 11- to 13-fold significant

increase in the CP and a 1.5 to 1.8-fold rise in the AC with a percent increase of 3.28% for *Trichoderma* spp., 4.16% for *Aspergillus* spp1 and 3.49% for *Aspergillus* spp2. The CF and the CFr of the fermented sugarcane bagasse also decreased.

Figures 7 and 8 show a snapshot of the proximate analysis of unfermented and fermented rice hay by several species of fungal organisms. The figures reveal that the unfermented rice straw has 5.60% CP, 0.99% CF, 37.14% CFr, 15.16% AC and 91% DM. After 21 days of fermentation, there was a two- to five-fold increase in the CP content of fermented products. Among the macrofungal isolates, *Psilocybe* spp. had produced the highest CP content of the fermented rice straw at 29.51%. This was followed by *Ganoderma* spp. with 22.09% CP and *Pleurotus* spp. with 19.83% CP. The CFr of rice straw fermented by *Pleurotus* spp. was 37.16%, *Ganoderma* spp. was 34.41% and *Psilocybe* spp. was 32.40%. The ACs of the fermented rice straw was 16.28%, 17.84% and 16.09%, respectively, slightly higher when compared to 15.16% of the unfermented rice hay. The DM of the fermented rice straw was 95.60% for *Pleurotus* spp., 86.90% for *Ganoderma* spp. and 93.20% for *Psilocybe* spp. Figure 8 further discloses that the fermentation of the three microfungal isolates resulted in a 2.37 to a 2.73-fold increase in the CP content of the fermented product. The CP of rice straw fermented by *Trichoderma* spp. was 20.19%. *Aspergillus* spp1 fermentation resulted in 18.88% CP and 19.65% CP by *Aspergillus* spp2. There was also an increase in the ACs of the fermented products that ranged from 0.93% to 2.68% by the three isolates. The CFr of rice straw fermented by *Trichoderma* spp. was 34.66% which was lower by 2.46% compared

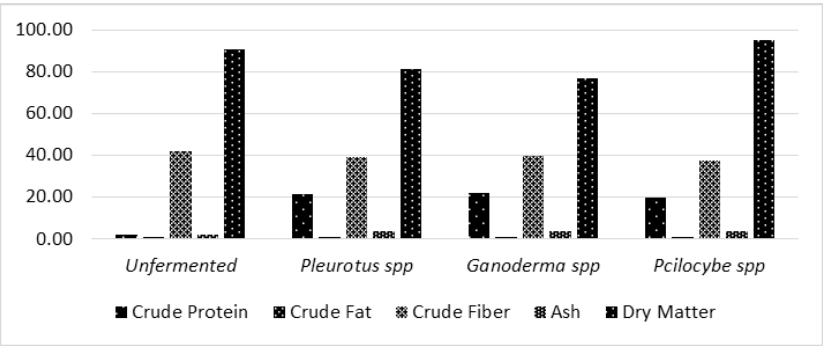

Figure 3. Proximate analysis of corn cob before and after fermentation of macrofungi for 21 days at room temperature.

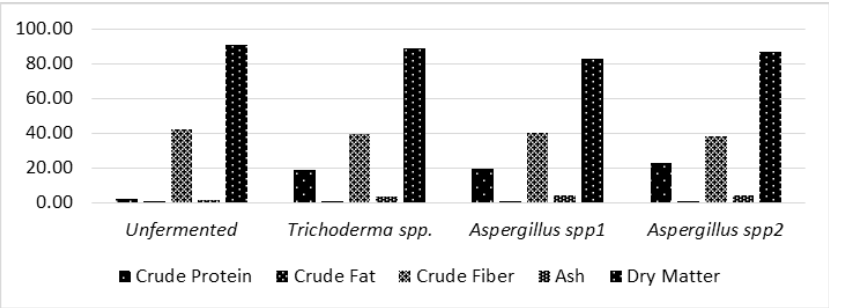

Figure 4. Proximate analysis of corn cob before and after fermentation of microfungi for 21 days at room temperature.

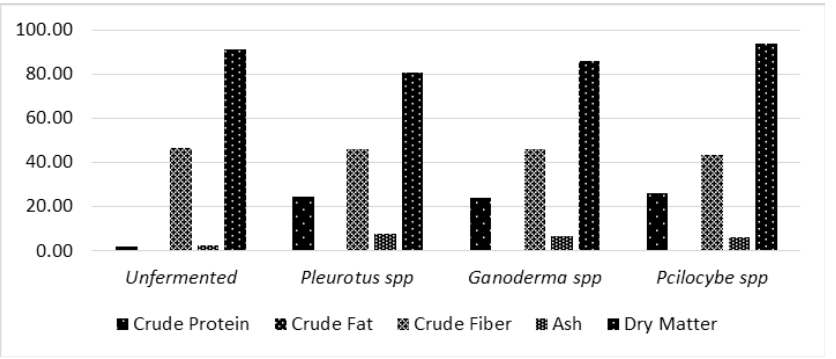

Figure 5. Proximate analysis of sugarcane bagasse before and after fermentation of macrofungi for 21 days at room temperature.

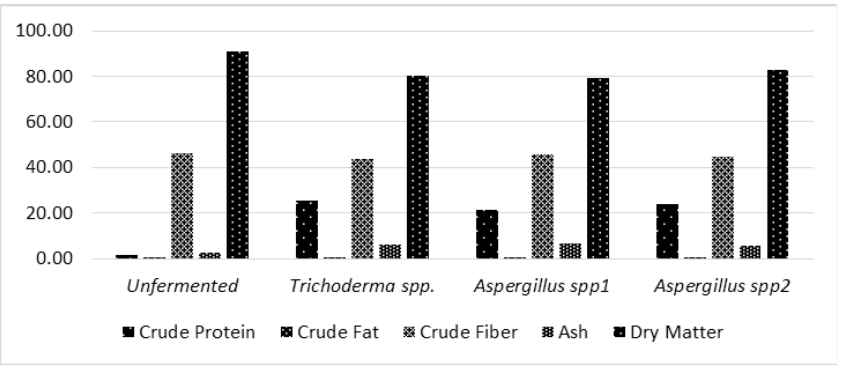

Figure 6. Proximate analysis of sugarcane bagasse before and after fermentation of microfungi for 21 days at room temperature.

to the unfermented one. The CFr obtained from *Aspergillus* spp1 fermented rice straw was 36.29% while rice straw fermented by *Aspergillus* spp2 attained 36.99% CFr.

Production of inexpensive high protein biomass from agricultural crop residues plays a substantial role in improving not only the economic utilization of agricultural by-products but also a tangible solution in replacing imported feed ingredients for animal production. Agricultural by-products like rice straw, sugarcane bagasse, banana leaves and corn cobs contain biologically important nutrients that require microbial bioconversion through biotechnological approaches for the efficient utilization of animals. Most of the agricultural crop residues used in the present study are generally made up of complex carbohydrates like cellulose, hemicellulose and lignin that are difficult to degrade. The physicochemical composition of crops residues is dependent on the types. In the case of rice straw, it is composed mainly of cellulose, hemicellulose, lignin, ash and other extractives. It contains on average between 30% to 45% cellulose, 20% to 25% hemicellulose, 15% to 20% lignin, as well as some minor organic compounds. Rice straw is poor in nitrogen, but relatively high in inorganic compounds, often referred to as ash (Boschma and Kwant, 2013). On the other hand, sugarcane bagasse, a by-product after extracting the juice for sugar production has similar chemical constituents to rice straw. However, it varied in percent composition. The cellulose, hemicellulose and lignin constitute 90% of the dry weight of the fiber (Rezende *et al*., 2011). The ash content is low which implies that the non-fiber extractive compounds

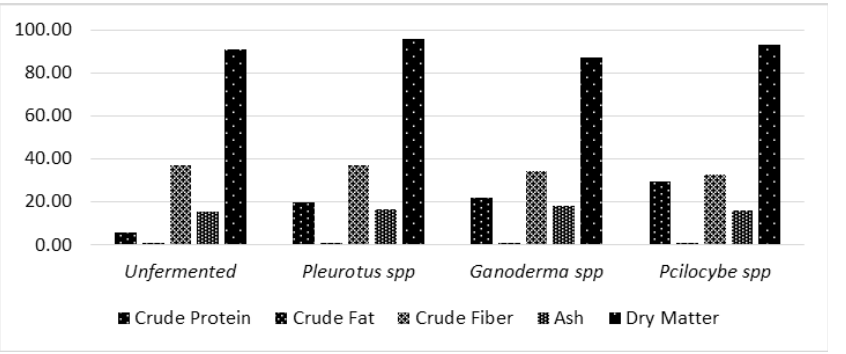

Figure 7. Proximate analysis of rice straw before and after fermentation of macrofungi for 21 days at room temperature.

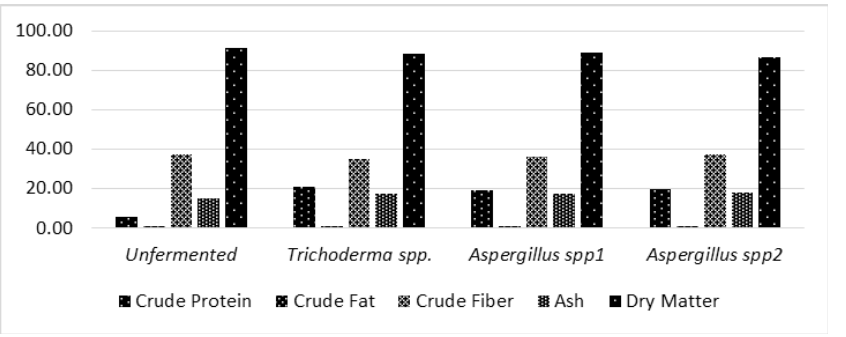

Figure 8. Proximate analysis of rice straw before and after fermentation of microfungi for 21 days at room temperature.

represent most of the dry weight of the sugarcane bagasse.

Furthermore, the polymeric fiber composition of corn cobs is made up mainly of monomeric molecules. Cellulose is made up of C6 sugars while hemicellulose is mainly made-up of the C5 sugars xylose and arabinose. Lignin consists of phenolic macromolecules (Pointner *et al*., 2014). Reports on the proximate composition of banana leaves revealed that it also contains a high amount of lignocellulosic materials mainly cellulose, lignin and hemicellulose. Banana leaves contain approximately 12% ash with very high organic matter of around 83% and around 5% carbon and very low nitrogen at 0.2%. However, the protein contents of these crop residues were found low. The high organic matter of the agricultural crop residues implies their high degradation potentials through enzymatic hydrolysis by several species of microorganisms. However, Mosier *et al*. (2004) and Menon and Rao (2012) revealed that the cellulose, hemicellulose and lignin that are embedded in a complex matrix are very resistant to enzymatic degradation. Screening and selection of several species of fungal organisms that are highly efficient in the biodegradation and conversion of the complex carbohydrates into protein are essential. Filamentous microfungi produce fibrous materials that can be easily converted to textured food products with protein content as high as 30% to 50%. The biomass produced by filamentous fungi can be used as food for animals without any further processing because it provides carbohydrates, lipids, minerals, vitamins and proteins. Also, nucleic acid contents of fungal protein are lower than that of yeast and bacteria (Chahal, 1982). White rot macrofungi, on the other hand, are efficient

lignocellulolytic decomposers capable of metabolizing plant cell constituents particularly cellulose, hemicellulose and lignin by their enzymes (Eriksson *et al*., 1990). Many species are capable of degrading lignin and can improve the nutrient composition of fodder for ruminant nutrition (Howard *et al*., 2003).

The intricate enzyme systems bring the ability of fungal organisms to degrade complex carbohydrates. According to Dashtban *et al*. (2009), the bioconversion of lignocellulosic residues could be done by microorganisms like fungi and bacteria that are capable of degrading lignocellulolytic materials through enzymatic hydrolysis. The conversion of cellulosic biomass to fermentable sugars requires the synergistic action of three cellulolytic enzymes namely 1,4 endoglucanase, -1,4 exoglucanase and -1,4 glucosidase. The most extensively studied cellulases are those produced by efficient lignocellulose-degrading fungi, particularly *Trichoderma* (Narsimha *et al*., 2006) and *Aspergillus* spp. (Baig, 2005). Such innate characteristic is an opportunity in the utilization of these organisms to produce protein biomass. Sibtain *et al*. (2017) revealed that using *Trichoderma harzianum* to produce fungal biomass protein from rice polishing resulted to a maximum of 49.50% crude protein, 32.00% true protein, 19.45% crude fiber, 9.62% ash, 11.50% cellulose content and 0.325% RNA content. The profile of amino acids of the final fungal protein biomass (FPB) exhibited that all essential amino acids were present in great quantities. The *T. harzianum* produces FPB with high nutritional value suitable as supplement for poultry animals.

It was reported by Patyshakuliyeva *et al*. (2016) that fungi such as *Trichoderma reesei* and *Aspergillus niger* produce enormous amounts of extracellular cellulolytic enzymes which is found to have synergistic interaction in degrading cellulose like the endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases. Meanwhile, similar enzymes called cellulosome associated with the cell wall are also secreted by bacteria and strains of fungal anaerobes capable of degrading cellulose. In basidiomycetes, lignocellulose degradation could be attributed to having unique oxidative systems and cellulolytic and hemicellulolytic activities (Dashtban *et al*., 2009).

Aspergillus niger and *Aspergillus oryzae* are also the most commonly used industrial *Aspergillus* species for the production of pharmaceuticals, food ingredients and enzymes (Berka *et al*., 1992; Pandey *et al*., 1999). *A. niger* and *A. oryzae* produce a broad range of enzymes related to the degradation of plant polysaccharides, such as cellulose, xylan, xyloglucan, galactomannan and pectin (de Vries and Visser, 2001). These enzymes are essential in converting natural carbon sources into smaller molecules easily permeable to cells. *A. oryzae* also produces some enzymes like proteases which rapidly reduce the viscosity of gelatin solutions and cause rapid digestion of the lower molecular weight components of gelatin have been detected in the culture filtrate. Esterase, phosphatase, amylase, sucrase and catalase were also liberated from the mycelium in sufficient quantity for convenient estimation. Enzymes catalyzing the liberation of inorganic phosphate from phytic acid and lecithin, various dipeptidases, lactase, maltase, salinase, nuclease and urease were also detected. White rot fungi is also an alternative and safe source of biologically active extracellular cellulolytic enzymes. It was reported that *Ganoderma* spp. possesses three major families of fungal lignin-modifying enzymes (LMEs) (Thurston, 1994). These include laccases, manganese-dependent peroxidases (MnPs) and lignin peroxidases (LiPs) (Youn *et al*., 1995). It is believed that these enzymes can degrade lignin that can be used in degrading lignocellulosic material as a renewable resource for the production of paper products, feeds, chemicals and fuels. These LMEs can oxidize phenolic compounds thereby

creating phenoxy radicals, while non-phenolic compounds are oxidized via cation radicals. LiP and MnP oxidize non-phenolic aromatic compounds with high oxidation-reduction potentials, the major components of the lignin polymer. Laccase oxidizes non-phenolic aromatic compounds with relatively low oxidation-reduction potentials (Kirk *et al*., 1987).

Reports also revealed that several species of *Pleurotus* spp. are among the most efficient in utilizing lignocellulosics (Zhang *et al*., 2002; Salmones *et al*., 2005; Albores *et al*., 2006). Similar studies on three *Pleurotus* spp., namely, *P. florida*, *P. ostreatus*, and *P. sajor-caju* for cellulolytic enzymes production showed that *P. florida*, produced the highest levels of enzyme activity, indicating high production of extracellular cellulases under optimum cultural and nutritional parameters using submerged fermentation conditions (Goyal and Soni, 2011).

Based from the results of the study, the three macrofungi *Pleurotus* spp., *Ganoderma* spp., and *Psilocybe* spp. and the three microfungi *Aspergillus* spp1, *Trichoderma* spp., and *Aspergillus* spp2 are capable of producing mycoprotein feeds from agricultural wastes of banana leaves, rice straw, corn cobs and sugarcane bagasse with an increase in CP content through solid-state fermentation. Further study could be conducted by varying moisture content of the substrates, inoculum size, and duration of fermentation, C/N sources concentration, qualitative protein and lipid content analysis, enzymatic analysis and feeding trials in livestock and poultry animals.

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The Effects of Plant Leaves Variants from The Philippines on Infective *Oesophagustonum dentatum* Larvae

Pengaruh Sembilan Tanaman Asal Filipina Terhadap Infeksi Larva Oesophagustonum dentatum

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ABSTRACT

Nine aqueous extracts of plant leaves from the Philippines were evaluated against *Oesaphagustomum dentatum* sensitive (ODSEns) larvae for their anthelmintic effects. The plant species evaluated were *Ananas comosus, Chrysophyllum cainito,Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii* Boerl,*Azadirachta indica, Trichantera gigantea* and *Leuacaena leucocephala*. The leaves of the plants gathered from Camiling, Tarlac, Philippines, were dried under room temperature for 30 days and then ground. Aqueous extracts of these plant leaves were prepared using migration buffer. *In vitro* migration and inhibition assays were used to evaluate the anthelmintic activity against exsheathed third stage larvae of *Oesophagustomum dentatum.* The experiments were put in 24-well plates at room temperature with four replications per treatment using different concentrations of 1, 2.5, 5 and 10 mg/ml. About 120 larvae per well were deposited on a larval migration apparatus consisting of 20 µm nylon mesh filters and incubated 20 hours under different treatments and another 2 hours to allow the migration of active motile larvae. The evaluation criteria were based on the average numbers of inhibited larvae under the different treatments. The percentage inhibition were statistically analyzed using Two-Way ANOVA (P=≤.0001) of the Graph Pad Prizm software (V5 San Diego, Ca). The most effective leaves with their highest inhibition level were from *Ananas comosus* (pineapple, 86.50%), *Gliricidia sepium* (madre de cacao, 84.50%), *Chrysophyllum cainito* (star apple, 83.75%), *Moringa oleifera* (horseradish,58.75%), *Annona squamosa* (sweet sop, 56.00%), *Tinospora rumphii* Boerl (\Makabuhay, 55.75%), and *Azadirachta indica* (Neem, 51.50%), whereby extremely significant (P<0.001) effect against *O. dentatum* larvae. Two leaves extracts, from *Trichantera gigantea* and *Leuacaena leucocephala* have no significant effect on *O. dentatum* larvae. The larval migration assay allows initial studies on the anthelmintic effects of the herbs.

Keywords: plant leaves, anthelmintic property, inhibition assay, Philippines

ABSTRAK

Sembilan ekstrak air daun tanaman dari Filipina dievaluasi terhadap sensitifitas larva *Oesaphagustomum dentatum*yang mempengaruhi anthelmintik. Spesies tanaman yang dievaluasi adalah *Ananas comosus, Chrysophyllum cainito, Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii Boerl, Azadirachta indica, Trichantera gigantea* dan *Leuacaena leucocephala*. Daun tanaman dikumpulkan dari Camiling, Tarlac, Filipina, dikeringkan selama 30 hari dalam suhu kamar dan kemudian ditumbuk. Ekstrak air dari daun tanaman tersebut disiapkan menggunakan buffer migrasi. Migrasi in vitro dan uji penghambatan dilakukan untuk mengetahui aktivitas antelmintik terhadap larva tahap ketiga *Oesophagustomum dentatum.* Eksperimen dilakukan dalam 24-sumuran *plate* pada suhu kamar dengan empat replikasi per perlakuan menggunakan konsentrasi yang berbeda dari 1, 2,5-, 5 dan 10 mg / ml. Sekitar 120 larva per sumur diendapkan pada peralatan migrasi larva yang terdiri dari 20 µm filter mesh nilon dan diinkubasi 20 jam dengan perlakuan berbeda dan 2 jam lagi untuk memungkinkan

migrasi larva motil aktif. Kriteria evaluasi didasarkan pada jumlah rata-rata larva yang dihambat di bawah perlakuan yang berbeda. Penghambatan persentase dianalisis secara statistik menggunakan Two-Way ANOVA (P = ≤,0001) menggunakan *software* Graph Pad Prizm (V5 San Diego, Ca). Daun yang paling efektif dengan tingkat penghambatan tertinggi berasal dari *Ananas comosus* (nanas, 86,50%), *Gliricidia sepium* (daun Gamal, 84,50%), *Chrysophyllum cainito* (daun sawo duren, 83,75%), *Moringa oleifera* (daun kelor, 58,75%), *Annona squamosa* (daun srikaya, 56,00%), *Tinospora rumphii Boerl*(Bratawali, 55,75%), dan *Azadirachta indica* (daun mimba, 51,50%), semuanya menunjukkan efek yang sangat signifikan (P<0,001) terhadap larva *O. dentatum*. Dua ekstrak daun yaitu *Trichantera gigantea*(daun cemara raja) dan *Leuacaena leucocephala*(daun lamtoro) tidak berpengaruh signifikan terhadap larva *O. dentatum*. Uji migrasi larva memungkinkan studi awal tentang efek anthelmintik dari herbal**.**

Kata kunci**:** daun tanaman, antihelmintik, uji penghambatan, Filipina

INTRODUCTION

The high cost of conventional anthelmintic drugs and the development of anthelmintic resistance lead researchers to evaluate the different plants as an alternative source of anthelmintics, especially in tropical countries where many plants abound throughout the year. The use of plants and plant extracts as a possible source of natural anthelmintics has received more interest in the present years.

The use of medicinal plants for the prevention and treatment of gastro-intestinal parasitism has its origin in ethnoveterinary medicine (Athanasiadou *et al*, 2007). For ages, plants have been used to control parasitism, and in many countries of the world, they are still used for this purpose. There have been reports around the world about the use of plants for their anthelmintic properties to treat human and animal parasitic infection (Bahaud, *et al*, 2006; Lopez-Aroche *et al*, 2008; Mali and Mehta, 2008; Bachaya *et al*, 2009; Marie-Magdeleine, *et al*, 2010; Narojini *et al*, 2011; Rabiu and Subhasish, 2011; Reuben *et al*, 2011; Deb Roy *et al*, 2012; Getachew *et al*, 2012 and Ferreira *et al*, 2013). The aim of this study was to test anthelmintic effects of nine plants aqueous extracts from the Philippines under controlled experimental conditions.

MATERIAL AND METHO DS

Plant leaves

Plants leaves were collected fresh, directly from naturally grown plants in Camiling, Tarlac, Philippines during the wet season (July and August). Leaves were dried for 30 days under room temperature and then sent to Iowa State University with an approved permit. The plant leaves were taxonomically identified as *Ananas comosus, Chrysophyllum cainito, Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii* Boerl, *Azadirachta indica, Trichantera gigantea* and *Leuacaena leucocephala.* The scientific names and common names are summarized in **Table 1**.

Plant extract preparations

The leaves were ground using an Oster® processor at high speed for two minutes and stored in 50 ml falcon tubes until later use. One gram of leaves of each species were suspended in 20 ml of migration buffer and were shaken for 24 hours using an Orbit1000® (Labnet Intl). The aqueous solution was then extracted using 20 μm nylon mesh (Elko®) filters thus making the stock solution in equivalent to 0.05 g/ml concentration. Each treatment was prepared adding 0.2 ml, 0.5 ml, 1ml and 2 ml and up to 10 ml of the migration buffer. The

concentrations were prepared at 1 mg, 2.5 mg, 5 mg and 10 mg/ml, respectively.

Oesophagustomum dentatum L_3

The *Oesophagustomum dentatum* larvae were obtained by fecal culture. The feces were mixed daily with wooden spatulas and incubated for 6 days to promote egg hatching and the development of L_3 infective larvae. The L larvae were recovered using the Baerman funnel technique, and filtered and washed by centrifuging in 40% sucrose.Afterwards, they were re-suspended in sterile distilled water and stored in culture flasks and maintained in a low temperature Incubator™ (Fisher Scientific) at 10.5^{o}C.

For exsheathment, the larvae were placed in sterile water in a5 ml Falcon® tube with one ml of 10% of sodium hypochlorite (Vertex CSS-5 Bleach®, St. Louis, MO) for 5-8 minutes at room temperature or as soon as 90-95% of the larvae were exsheathed when seen under a microscope (Nikon TMS™, MVI, Avon, Ma.). The mixture was spun (Eppendorf Centrifuge 5415, Brinkmann™) at 31,000 rpm for 2 minutes, rinsed and re-suspended in sterile water.

Anthelmintic Activity: Larval migration assay

For the larval migration assay, the procedure of Nagamori, *et al* (2013) was used. The concentrations of the larvae were at 120 larvae/well. After the larvae were exsheathed, they were re-suspended in appropriate positive and negative control drugs and with the different leaves extracts in aqueous solution at concentrations of 1mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml, and then incubated at room temperature (25-30^{O}C) for 20 hour treatment exposure time periods.

After twenty hours, the larvae were added to the migration apparatus consisting of 20 µm nylon mesh filters (Elko®) in a 24-well plate (Corning®, Corning, NY) and incubated for another 2 hours to allow migration/inhibition of the L3 active, motile larvae. After 2 hour migration periods, the larvae were washed with migration buffer into 3.5 cm petridishes . Both the number of migrated larvae from the 24-well plate and inhibited larvae from the dishes were recorded under a microscope (Nikon TMS™, MVI, Avon, MA), as well as the total number of larvae. The evaluation criteria were based on the average number of inhibited larvae under the different treatments. The formula was:

$$\% \text{ inhibited} = \frac{\text{No. of inhibited larvae}}{\text{Total No. of Larvae}} \times 100$$

Statistical analysis

Data were statistically analyzed using Two-Way ANOVA (P=≤.0001) of the Graph Pad Prizm software (V5 San Diego, Ca). The differences between the negative control and each of the treatments were analyzed using the Bonferroni post-test.

RESULTS AND DISCUSSION

Table 1 shows the mean percentage inhibition to various concentrations of nine different species aqueous leaves extracts after 20 hours of exposure to *O. dentatum* exsheathed larvae. At the highest leaf extracts concentration of 10mg/ml, *Ananas comosus* (pineapple)leaves produced the highest inhibition of 86.50% (P<0.0001), compared to *Gliricidia sepium* (gliciridia) at 84.50% and *Chrysophyllum cainito* (star apple)*at* 83.75% inhibition (P<0.0001). At the same level of concentration, three other plant extracts, *Annona squamosa*had 56.00% (P<0.001) inhibited larvae in the migration assay,

Moringa oleifera with 58.75% (P<0.001) and *Tinospora rumphii* Boerl with 55.75% (P<0.001).

Ananas comosus and *Chrysophyllum cainito* showed very significant effects even when given at a lower concentration of 1 mg/ml, with 49.25% (P<0.001) and 47.75 % (P<0.001) inhibition, respectively (Figure 1). With the study done by Fernandez *et al* (2013), *Chrysophyllum cainito* extract was mixed with other two leaves extracts, makabuhay (*Tinospora rumphii*), and makahiya (*Mimosa pudica*). Ethanol was the best solvent to extract the bioactive components of the three plants. Eighty percent (80%) of *Haemonchus contortus* larvae died 30 minutes after exposure with the cocktail.Likewise, in a number of tests, tannins were reported in the phytochemical analysis of *C. cainito* as being responsible for its observed anthelmintic effects (Fernandez *et al*, 2013). The beneficial effects of condensed tannins on livestock are the reduction of the detrimental effects of parasitism in grazing livestock (Hoste, *et al*, 2006). The consumption of high concentration of condensed tannins (>7% of DM) has been associated with detrimental effects of ruminants such as reduction of food intake, growth inhibition and interference with the morphology and the proteolytic activity of microbes in the rumen. Low and moderate concentrations of condensed tannins (<6% of DM) have resulted in positive effects in the herbivores like increased growth and increased milk production (Min, *et al*, 2003; Waghorn and McNabb, 2003).

Pineapple leaves have previously been used in the Philippines as anthelmintic preparations for livestock (Jovellanos, 1997; Baldo, 2001). According to Githiori (2004) and Debnath *et al* (2013) the pineapple plant contains cysteine proteases (bromelain), have been reported to have anthelmintic properties.

Table 1. List of medicinal plants used *in vitro* for anthelmintic evaluation against *O. dentatum:* their common name and scientific names

Scientific Name	Common Name
Ananas comosus	Pineapple (Engl); Pina (Spanish); Abacaxi (Portuguese); Annachi pazham (Tamil); Nanas (Malay); Aananas (Many languages)
Chrysophyllum cainito	Caimito (Tag., Span.), Cainito (Engl.) Star apple, Star plum (Engl.) Niu nai guo (Chin.)
Gliricidia sepium	Madre de Cacao (Guatemala) Kakawate (Tag), Cacao de nance, Cacahnanance, Mata Raton, Madriado (Honduras)
Annona squamosa	Custard apple, sugar apple, sweet sop (English); Gishta (Arabic); Kannelappel (dutch), Atis (Tag); Attier, Cachiman canelle, pomme de cannelle (French); Rahmapfel, Süßsack (German); Ata, Sharifa, Sitaphal (Hindi); Buah nona, Nona sri kaya, Sri kaya (Malaysian); Fan-li-chi (Mandarin); Fructa do conde (Portuguese); Anón, Anona, Anona blanca, Candongo, Chirimoya, fructo do conde, pinha, saramuya (Spanish); Lanang, Makkhiap, Noina (Thai); Mang câú ta, na (Vietnamese)
Moringa oleifera	Horseradish tree, Radish tree, Drumstick tree, Mother's Best Friend, West Indian ben (Engl); Bèn ailé, Benzolive, Ben oléifère, Arbre radis du cheval (French); Behenbaum (german); Sàndalo ceruleo (Italian); Acácia branca, Marungo (Portuguese); Árbol del ben, Ben, Morango, Moringa (Spanish); Kachang Kelur (Malasia, Indon); Mulanggay (Tag); Kaanaeng-doeng (Thai)
Tinospora rumphii Boerl	Heavenly elixir (Engl) Makabuhay (Tag., Ilk.); Giloya (Ind.); Fa leng teng (Chin.)
Azadirachta indica	Neem, Nimtree, Indian Lilac, Margosa Tree, Neem Chal (Engl)
Trichantera gigantea	Madre de Agua (Tag), Cenicero, Tuno, Naranjillo, and Palo de agua (Span)
Leuacaena leucocephala	Ipil-ipil (Tag) White Leadtree, White Popinac (Engl) ; Subabool (Indian)

Gliricidia sepium (madre de cacao) was also active with 41.25% inhibition when given at 1mg/ml (P<0.01). Its effect became very significant when exposed at a higher concentration of 5mg/ml, which showed 72.75% inhibition (P<0.001). This validates the efficacy of the leaves extract as it has been used in the Philippines to treat external parasitism. Fresh, clean leaves are pounded, with its juice extracted and applied to affected animal body parts, once to twice a day for one week. Fresh leaves are also used as a fresh feeds for deworming purposes, especially in goats by many farmers in the countryside.

Gliciridia was found to contain 40.7g of condensed tannins/kg dry matter.Tannins have been found to have potential antidiarrheic, antidysenteric, antimutagenic, antinephritic, antioxidant, antiradicular, antiviral, bactericide, cancer-preventive, hepatoprotective, pesticide, psychotropic, and viricide activities (Duke *et al,* 1994). There is little evidence of toxic effects with ruminants fed either fresh or wilted leaves (Stewart, *et al,* 1996). In a reportpublished by the Cornell University (2009), goats safely consumed larger quantities of plants with tannins than cattle and sheep because goats have a salivary protein that binds to the tannins that the other livestock lack. It is reported in Asia that *Gliciridia* has apparent palatability problems (Trung, 1989), but farmers are continuously feeding it, especially in goats, because of its availability. One advantage of *Gliricidia* is having more biomass than *L. leucocephala* (Stewart *et al.* 1992), with annual leaf dry matter production generally ranging from about 2 t/ha/year (Wong and Sharudin, 1986) to 20 t/ha/year (Sriskandarajah, 1987).

Moringa oleifera leaves extract showed 58.75% inhibition, a very significant effect (P<0.001) when given the highest dose of 10mg/ml, and a significant effect of 41.00% inhibition at 5mg/ml concentration (P<0.05). Moringa had been used for a wide range of various ailments (Sreelatha and Padma, 2009). The different parts of this plant such as the leaves, seed, roots, bark, flowers and fruits and immature pods have been found to act as agents for the following effects: cardiac and circulatory stimulus, antiulcer, antispasmodic, diuretic, antihypertensive, possess antitumor, antipyretic, antiepileptic, antiinflammatory, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and treatments of other various ailments in South Asia (Anwar *et al,* 2007). Although, there have been no report on its deworming activity, results of this study found that *Moringa* has potential in this regard.

Annona squamosa leaves extract showed 56.00% inhibition at the highest concentration of 10mg/ml and 48.00% at 5mg/ml (P<0.001) against *O. dentatum* larvae. This conforms with other research where *Annona squamosa*was found to reduce faecal egg counts (FEC) and total worm counts in experimental lambs (Githiori *et al,* 2004).The anthelmintic properties of *Annona squamosa* indicated the presence of phenolic compounds in its aqueous leaf extract (Kamaraj and Rahuman, 2011), which was observed also by Fereira *et al* (2013) when they tested the leaves extract against eggs, infective larvae and adult forms of *Haemonchus contortus* in sheep. Plant phenolic compounds are diverse forms of proanthocyanidins and condensed tannins. They were found to prevent bloat in cattle, reduce gastrointestinal nematode numbers and flystrike (Waghorn and McNabb, 2003).

Tinosphora rumphii or makabuhay leaves (*Tag*.) showed 55.75% inhibition at 10mg/ml concentration (P<0.001), an extremely significant effect, but no

significant effectsat lower concentrations against *O. dentatum* larvae. Earlier studies on crude extracts of *Tinosphora rumphii* were highly effective against *Haemonchus* larvae *in vitro* and in reducing worm egg counts and worm numbers in sheep and goats (Sani *et al*, 2004).

Likewise, *Azadiractha indica* or neem tree leaves produced 51.50% inhibition at 10mg/ml (P<0.01), a very significant effect, but similar to *T. rumphii*, no significant effect was seen when applied at lower concentrations. In tropical Asia, fresh neem tree leaves were fed to sheep, which lowered the infection against *H. contortus* fecalysis count (Sani, 2004). Its active compounds were found to be a high content of condensed tannins, which might be responsible for its anthelmintic activity (Chandrawathani *et al*, 2002 and 2006; Githori *et al*, 2004; Costa *et al*, 2006). **Table 2** presents a summary of several plants used in this study as to their naturally occurring agents that possess anthelmintic properties.

Table 2. Aqueous leaves extracts evaluated against *E. dentatum*, and theirmean percentages of inhibition at different concentrations after 20 hours incubation time under room temperature.

Treatments	% Mean Inhibition* at different concentrations			
	1 mg/ml	2.5 mg/ml	5 mg/ml	10 mg/ml
Migration Buffer (-) Control	18.50	23.75	24.50	29.75
Levamisole (+) Control	94.97	96.00	95.50	98.75
Ananas comosus	49.25***	64.00***	84.50***	86.50***
Gliricidia sepium	41.25**	45.25**	72.75***	84.50***
Chrysophyllum cainito	47.75***	52.50***	59.75***	83.75***
Moringa oleifera	28.75 ns	35.25 ns	41.00 *	58.75***
Annona squamosa	19.75 ns	21.00 ns	48.00***	56.00***
Tinospora rumphii Boerl	11.50 ns	25.75 ns	34.00 ns	55.75***
Azadirachta indica	17.00 ns	22.00 ns	35.00 ns	51.50**
Trichantera gigantea	28.50 ns	31.75 ns	37.25 ns	42.25 ns
Leuacaena leucocephala	5.00 ns	12.50 ns	18.00 ns	21.75 ns

* Bonferroni Post tests results as compared to the negative control (P value= <0.01).
** Significant result at P<0.001.
*** Highly significant results at P<0.0001.

In the data analyses, the interaction between the leaves extracts and the concentrations used account for 5.11% of the total variance (P<0.0001) and was extremely significant. The plant leaves extracts account for 71.41% of the total variance (P<0.0001) and the concentrations account for 15.58% (P<0.0001), both effects were also extremely significant. In summary, the most effective plant species leaves in producing the highest *O. dentatum* larvae inhibition levels were from *Ananas comosus* (pineapple, 86.50%), *Gliricidia sepium* (madre de cacao, 84.50%), *Chrysophyllum cainito* (Star apple, 83.75%), *Moringa oleifera* (horseradish,58.75%), *Annona squamosa* (sweet sop, 56.00%), *Tinospora rumphii* Boerl (Makabuhay, 55.75%),and *Azadirachta indica* (Neem, 51.50%), which all proved extremely significant (P<0.001). Two plant species leaves extracts, those from *Trichantera gigantea* and *Leuacaena leucocephala*,had no significant anthelmintic effects on *O. dentatum* larvae.

The active compounds are plant secondary metabolites (PSM) (Githiori et al, 2006), the plant products that have been associated with defensive mechanisms of plants against herbivore grazing (Mueller-Harvey and McAllan, 1992). Saponins, alkaloids, non-protein amino acids, tannins and other polyphenols, lignins, glycosides, are all

PSM and some of them have been considered responsible for the anti-parasitic effect of plants (Githiori, 2006).

Fresh roughages supplied to a 30 kgs goat,with 3% of its body weight dry matter (DM) requirement, needs 0.9 kg DM a day. If the roughage to legumes ratio was 75%:25%, the DM needed for roughages was 0.67 kg and 0.22 kg for legumes. Converted the DM into fresh roughage (20% DM), a goat needs about 3.37 kg of roughage and 0.90 kg fresh legumes (25% DM). The legume part was the replaced components by the different leaves in controlling the parasites. Given 10 mg of leaves (DM)/ml as the effective concentration with the highest inhibition effect to larvae, in a goat's rumen with 3 L capacity, at least 151 grams of fresh leaves must be given daily for three consecutive days to effectively control the parasites.

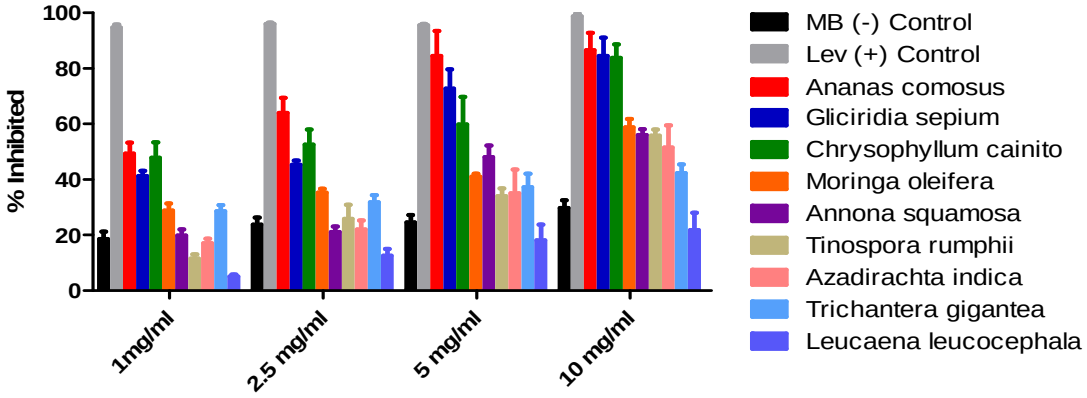

Figure 1. Inhibition percentage of nine aqueous leaves extract on *Oesophagostomum dentatum* larvae using larval migration assay.

CONCLUSIONS

This effort to scientifically screen and evaluate the effect of medicinal plants from the Philippines documented statistically significant inhibition of *O. dentatum* larvae by exposure to the leaf extracts of seven of the nine plant species examined. The results of this study validate the traditional use of these plants as having natural anthelmintic properties and they may be used with its pharmacological potentials for veterinary health management systems, especially in developing countries where these plants abound and the farmers typically lack the financial resources to routinely purchase commercial chemical treatments. The methods of this experiment also provide an additional resource to conduct future *in vivo* investigations on other parasites and with other plant species extracts. Furthermore, combination of DE and plant leaf extracts may worth investigation in the future combining the mechanical damage of the parasites with the natural effect of some plant leaves.

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Validation and molecular analysis of *β-1,3-GLU2 SNP marker* associated with resistance to *anthracnose* in Philippine carabao mango (*Mangifera indica* L. cv. 'Carabao')

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ABSTRACT

In the Philippines, mango anchors a million-dollar industry which is largely dependent on the export of a sole variety, the 'Carabao' mango. Varietal improvement of the 'Carabao' mango involves the introgression of anthracnose resistance to improve its yield and export quality. Molecular genomic tools such as single nucleotide polymorphism (SNP) markers provide a platform to accelerate breeding of resistant varieties through marker-assisted selection (MAS). Here, we developed and analyzed the molecular basis of an SNP marker within the pathogenesis-related β-1,3-glucanase 2 (β-1,3-GLU2) gene putatively linked with resistance to anthracnose. This SNP is an A/G transition causing a missense mutation (I196V) in glycosyl hydrolase family 17 (GH17), a highly conserved domain involved in physiologically important processes in plants, notably in response to biotic and abiotic stresses. Structural analysis suggested that the I196V mutation resulted in conformational changes in the enzyme's (β/α)8 TIM-barrel motif and catalytic groove by causing a steric clash with V261 residue, thereby possibly affecting the overall protein stability or catalytic activity and subsequently inhibit fungal defense response mechanisms. Our findings also demonstrated the association of this SNP marker with anthracnose resistance wherein mango accessions with the mutant allele 'G' showed significantly higher disease severity post-inoculation while those with wildtype allele 'A' showed phenotypic resistance against anthracnose. The positive correlation between the type of SNP allele present and reaction of mango to *C. gloeosporioides sensu lato* coupled with the ability of the marker to discriminate SNP alleles using a simple and cost-effective allele-specific PCR assay make it suitable for MAS. The results of this study support the utilization of the developed β-1,3-GLU2 SNP marker for routine screening of anthracnose resistant phenotypes as early as the seedling stage. This will help improve mango breeding efficiency and significantly reduce the expenses in field inputs, maintenance, and evaluation of material over years.

Keywords: AS-PCR, Carabao mango, *Colletotrichum gloeosporioides sensu lato*, Genotype-by-sequencing, Glucan endo-1,3-beta-glucosidase, Single nucleotide polymorphism

1. INTRODUCTION

Mango (*Mangifera indica* L.) is a commercially important fruit crop in the tropical and subtropical regions, particularly in Asia. It anchors a million-dollar industry ranking sixth in terms global fruit production after bananas, watermelons, apples, oranges, and grapes (FAO, 2019a). In 2018, the global production of mango fruit reached up to over 52 million metric tons, with more than one thousand varieties grown in Asia, Central and South America, and Africa (FAO, 2019b). Although there is increasing demand in developed countries, only 3-4% of the global production is traded internationally and the rest is traded and consumed domestically (Mitra, 2016). Despite its excellent qualities, the export potential of mangoes are not fully attained due to its short shelf-life, thin peel and low quality and production yield attributed to susceptibility to insect pests and pathogenic diseases. The high average temperature and relative humidity in tropical regions favor the rapid development of diseases at both the pre- and postharvest stages, which directly affect fruit quality and yield (Dodd et al., 1991). One of the most serious and destructive diseases of mango is anthracnose, which is primarily caused by the fungus *Colletotrichum gloeosporioides* (Dodd et al., 1991). This fungus causes leaf blight, blossom blight, mummified fruits, and dieback by infecting leaves, flowers and juvenile fruits (Arauz, 2000). During postharvest, germination of dormant fungal spores is induced by ripening and disease development becomes apparent when black, sunken, rapidly proliferating lesions develop on marketable fruits rendering them worthless and non-marketable (Akem, 2006).

Different disease management strategies have been developed to control the disease but these often involve excessive use of fungicides, which are expensive and damaging to the environment (Dodd et al., 1991). Another management method, the hot water treatment (HWT), needs to be revalidated for its fungistatic rather than fungicidal ability (Alvindia & Acda, 2015). In the absence of cheap, safe and efficient protection measures, breeding for resistance is valuable in solving the growing problem in the mango industry. However, traditional mango breeding programs are slow and challenging due to several

factors such as long juvenile stage, long generation time, high heterozygosity, and low crossing rates (0.1%). The detection of the disease poses additional problems in traditional mango breeding programs as infected fruits show no signs of the disease until the onset of ripening.

Advancements in molecular biology provide genomic tools such as DNA markers which can aid in the selection of target traits and to accelerate the breeding process of new varieties of mango. DNA markers are sequences with a known location in the genome and they can assist in breeding selection when found associated with a desired trait. Among the DNA markers, single nucleotide polymorphisms (SNPs) are advantageous in marker-assisted selection (MAS) due to its high density and abundance across the genome (Syvänen, 2005). SNPs are single base changes in the DNA, which allow a higher probability of finding an SNP-based marker within the gene of interest. SNP marker detection can be automated, enabling high-throughput analysis appropriate for breeding programs involving large populations (J. Kumar et al., 2011; Syvänen, 2005; Y. Xu & Crouch, 2008). Although several studies have already used SNPs in linkage mapping and estimation of genetic diversity in mango (Iquebal et al., 2017; D.N. Kuhn et al., 2016; David N. Kuhn et al., 2019; Sherman et al., 2015; Singh et al., 2016; Warschefsky & von Wettberg, 2019), SNP markers associated with important horticultural traits, including disease resistance, are yet to be developed and used for marker-assisted selection in mango breeding.

In the present study, a previously identified SNP marker located within a putative defense-related gene associated with resistance against *Colletotricum gloeosporioides* infection was developed and validated for use in marker assisted selection (MAS) of anthracnose resistant mango phenotypes. We hypothesize that this SNP plays a role in *C. gloeosporioides* pathogenicity on mango fruit that may confer resistance to anthracnose disease.

2. MATERIALS AND METHODS

2.1 Plant materials

A total of 143 mango varieties and strains from commercial growing areas and research institutions in the Philippines were used in the present study. Genetic resources in this collection include 130 'Carabao' mango strains, 9 commercial varieties, and 4 unknown cultivars. Scions of mango collections showing promising traits were collected and asexually propagated by cleft-grafting and maintained at the fruit crops nursery of the Institute of Plant Breeding, University of the Philippines Los Baños (UPLB), Laguna, Philippines.

2.2 Phenotypic screening for anthracnose resistance

Preliminary screening of mango accessions for anthracnose resistance was performed using the pathogen *Colletotrichum gloeosporioides sensu lato* isolated from mango samples collected from various provinces in the Philippines through tissue planting technique. The most aggressive isolate (Mg12) was used in the study (Figure). Molecular identity of this fungal isolate was established through PCR assay using Col1/Col2 (Martinez-Culebras et al., 2003) and CgInt/ITS4 (Mills et al., 1992) primer pairs. Fungal spores were harvested by adding 5 mL sterile distilled water on 7-day old culture and scraped using flamed L-spreader. Spore suspension was filtered on a four-layered sterile gauze cloth to filter only the fungal spores. Spore count was adjusted to 10^6 spores/mL using hemacytometer. Fruits were artificially evaluated *in vitro* via spore suspension droplet inoculation technique. Samples were disinfected using 20% sodium hypochlorite for 10 minutes, rinsed with sterile distilled water twice and blot dried using sterile tissue paper. Disinfected fruits were placed in a moisture chamber with moistened sterile cotton to maintain humid condition (Alcasid et al., 2018; Torres-Calzada et al., 2013). Each fruit was pricked with sterile 1 mL syringe to create wound and inoculated with 20 µL of spore suspension (10^6 spores/mL). Reaction to mango anthracnose was observed after 12 days of incubation following the disease rating scale (Table 1. Disease rating scale used for the phenotypic screening in this study.) and disease severity was calculated using the formula previously described by Paull (2002).

Table 1. Disease rating scale used for the phenotypic screening in this study.

Rating Scale	Description	Reaction
0	No infection	Immune
1	1-5% of the total fruit surface infected	Highly Resistant
3	6-15% of the total fruit surface infected	Resistant
5	16-30% of the total fruit surface infected	Intermediate
7	31-50% of the total fruit surface infected	Susceptible
9	More than 50% infection	Highly Susceptible

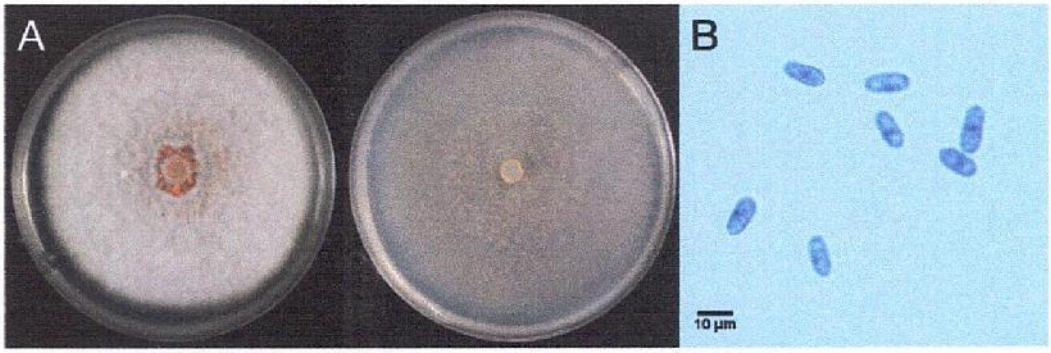

Figure 1. (A) Colony morphology of *C. gloeosporioides sensu lato* isolate MG12 and its (B) spores under 40X magnification.

Confirmatory evaluation of resistance was conducted for accessions that initially showed resistant and intermediate reactions in the preliminary trials. Approximately 120-day old mango fruits were disinfected and reactions were evaluated following the methods described above.

2.3 Genomic DNA isolation

Genomic DNA was isolated from the youngest, fully mature and green leaves following the modified CTAB protocol of Lachica et al. (2019). DNA stock solutions were quantified using Epoch Microplate Spectrophotometer (BioTek® Instruments, Inc., USA) and diluted to 50 ng/µL with sterile nanopure water. All extracted DNA and working stocks were stored at -20°C until use.

2.4 *β-1,3-GLU2* marker design and validation

A significant mango SNP (AlleleID 21881933) was recently identified by the Institute of Plant Breeding–Physiology Laboratory of UPLB (unpublished data) using Genotyping-by-Sequencing (GBS) generated by DArTseq™ platform (Diversity Arrays Technology Pty Ltd., Canberra, Australia). Gene annotation reveals that SNP 21881933 (A>G) is within a putative Glucan endo-1,3-beta-glucosidase 2 or *β-1,3-glucanase 2* (*β-1,3-GLU2*) gene. This SNP was developed into an allele-specific PCR (AS-PCR) marker for experimental validation. Gene-specific primers were designed based on the *Citrus sinensis* glucan endo-1,3-beta-glucosidase 2 (top hit) conserved regions flanking the SNP site using NCBI Primer-BLAST (Altschul et al., 1990). The gene specific forward primer (Glu2-F) was 5'—ACTGCAGCTAATTGGG—3' while the reverse primer (Glu2-R) was 5'—GGTTTGTAGTATCATTTGCT—3'. In addition, an allele-specific forward primer (AS-F), 5'—GTTCGTATCTCATGCTCAAAG—3', with a 3'-terminal nucleotide corresponding to the target mutant SNP allele "G" was designed using the Web-based Allele Specific Primer (WASP) tool (Wangkumhang et al., 2007). All primers were synthesized by Integrated DNA Technologies (IDT), Singapore.

PCR reactions were performed in a final mix of 10 µL using the iNtRON PCR Kit (iNtRON Biotechnology, Inc., South Korea) containing 1X PCR Buffer A, 0.2 mM dNTPs, 0.5 U i-Taq™ DNA Polymerase, 0.2 mM of each primer, 10 ng of template DNA, and sterile nanopure water. The following optimized thermal cycling conditions were performed on T100 Thermal Cyclers (BioRad® Laboratories, USA): 2 min at 94 °C, followed by 30 cycles of 20 s at 94 °C, 10 s at 50 °C (annealing), 30 s at 72 °C, and 1 cycle of 5 min at 72 °C. PCR products were visualized in 2% agarose gel electrophoresis using the GelDoc™ XR+ Gel Documentation System (BioRad® Laboratories, USA). The presence of the SNP allele 'G' was based on the detection of a 507-bp fragment in the AS-PCR assay. Amplifications that did not contain this fragment indicates the presence of the SNP allele 'A'. An 805-bp fragment, serving as internal control, indicates the successful amplification of the *β-1,3-GLU2* gene fragment. Analysis of variance was performed for the available phenotypic data, and the association between the SNP alleles and reaction to anthracnose (% disease severity) was analyzed by performing a Pearson correlation and linear regression analysis using IBM SPSS Statistics v23.0 software (SPSS, Chicago, USA).

2.5 Sequence analysis and molecular modelling of mango *β-1,3-GLU2 and SNP 21881933*

Amplified *β-1,3-GLU2* gene fragments from identified resistant and susceptible accessions were sequenced using Sanger technology (1st BASE, Selangor, Malaysia) to further validate the presence of the SNP and the identity of the target gene. Multiple sequence alignment (MSA) and identification of open reading frames (ORF) were performed using MEGA7 (S. Kumar et al., 2016) and UniPro UGENE v33.0 (Okonechnikov et al., 2012), respectively. Protein sequence analysis utilized the standard protein BLAST (BLASTp) tool and the Conserved Domain Database (CDD) of NCBI. Analysis and identification of conserved and functional residues were performed using ConSurf (Glaser et al., 2003; Landau et al., 2005). ORFs were translated using ExPasy (Gasteiger et al., 2003) and protein structure modeling was performed using Phyre2

(Protein Homology/analogY Recognition Engine version 2.0) (Kelley et al., 2015). The coordinates corresponding to the crystal structure of the glycoallergen endo-beta-1,3-glucanase (Hev b 2) from *Hevea brasiliensis* (top hit) was used to generate the model of *β-1,3-GLU2* in this study. UCSF Chimera software version 1.15 (http://www.cgl.ucsf.edu/chimera) was utilized for 3D model visualization, and analysis of molecular structures and mutation analysis, using default settings and criteria.

3. RESULTS

3.1 Phenotypic evaluation of anthracnose resistance

Isolate Mg12 used in this study amplified a single band product using the Col1/Col2 primer pair establishing that the isolate belongs to genus *Colletotrichum*. Using CgInt/ITS4 primer pair, a 450 bp product was amplified corresponding to the expected amplicon size for *Colletotrichum gloeosporioides sensu lato*. No amplification was observed in the control group.

Of the total 143 mango accessions evaluated, thirty (30) initially exhibited intermediate resistance (IR) and resistant (R) reactions, while the rest showed susceptible (S) and highly susceptible (HS) reactions in the preliminary screenings. Confirmatory evaluations showed that nine (9) accessions exhibited potential resistance to mango anthracnose after three trials. None of the 30 accessions exhibited immune reaction or 0% infection. The remaining 21 accessions showed intermediate and susceptible reactions to mango anthracnose (Table 2).

Table 2. Confirmatory evaluation of 30 mango accessions which initially showed intermediate resistance (IR) and resistant (R) reactions to *Colletotrichum gloeosporioides sensu lato*.

Sample	Accession	Variety	Source	Mean Disease Severity (%)
1	ADMINX	Unknown	Laguna	14
2	12-182	Haden	Laguna	11
3	12-070	Unknown	Laguna	14.2
4	16-016	Haden	Guimaras	11
5	Haden Hawaii	Haden	Laguna	11
6	Tommy Atkins	Tommy Atkins	Laguna	12
7	12-265	Haden	Davao	11
8	Gouveia	Gouveia	Guimaras	15
9	12-112	Carabao	La Union	12.5
10	12-052	Carabao	Laguna	36.67
11	12-080	Carabao	Quezon	30
12	12-088	Carabao	Zambales	29
13	12-171	Carabao	Laguna	25
14	GES 73	GES 73	Guimaras	25
15	12-209	Carabao	Quezon	25.33
16	12-013	Carabao	Laguna	44.67
17	12-014	Carabao	Laguna	43
18	12-027	Carabao	Laguna	31
19	12-075	Carabao	Quezon	36
20	12-081	Carabao	Quezon	33.67
21	12-090	Carabao	Zambales	37
22	12-104	Carabao	La Union	36.67
23	12-106	Carabao	La Union	33
24	12-111	Carabao	La Union	36
25	12-166	Carabao	Laguna	47
26	12-170	Carabao	Laguna	37
27	12-179	Carabao	Laguna	42
28	12-092	Carabao	Zambales	56
29	12-094	Carabao	Zambales	56
30	12-178	Carabao	Laguna	58

3.2 Experimental validation by AS-PCR

The AS-PCR assay optimized in this study yields a polymorphic banding pattern that can be used to differentiate genotypes between mango accessions. Results show that a 507-bp allele G-specific PCR product was amplified in 22 of the 30 accessions tested using the allele-specific primer pair (AS-F and Glu2-R). The remaining 8 accessions, namely 'ADMINX', '12-182', '12-070', '16-016', 'Haden Hawaii', 'Tommy Atkins', '12-265', and 'Gouveia', did not amplify this PCR product (Figure). The specific nucleotide at the 3' end of the AS-F primer will only allow amplification in the presence of the allele G and consequently prevents the amplification of a mismatched allele A. This means that the 22 accessions that amplified the 507-bp PCR product contain the G allele, while the 8 other accessions contain the A allele. To further ensure the accuracy of the AS-PCR, gene-specific primers Glu2-F and Glu2-R were introduced in the reaction to amplify an 805-bp *β-1,3-GLU2* gene fragment. This PCR product was successfully amplified in all tested accessions indicating a successful PCR reaction and confirming the presence of the *β-1,3-GLU2* gene in all samples.

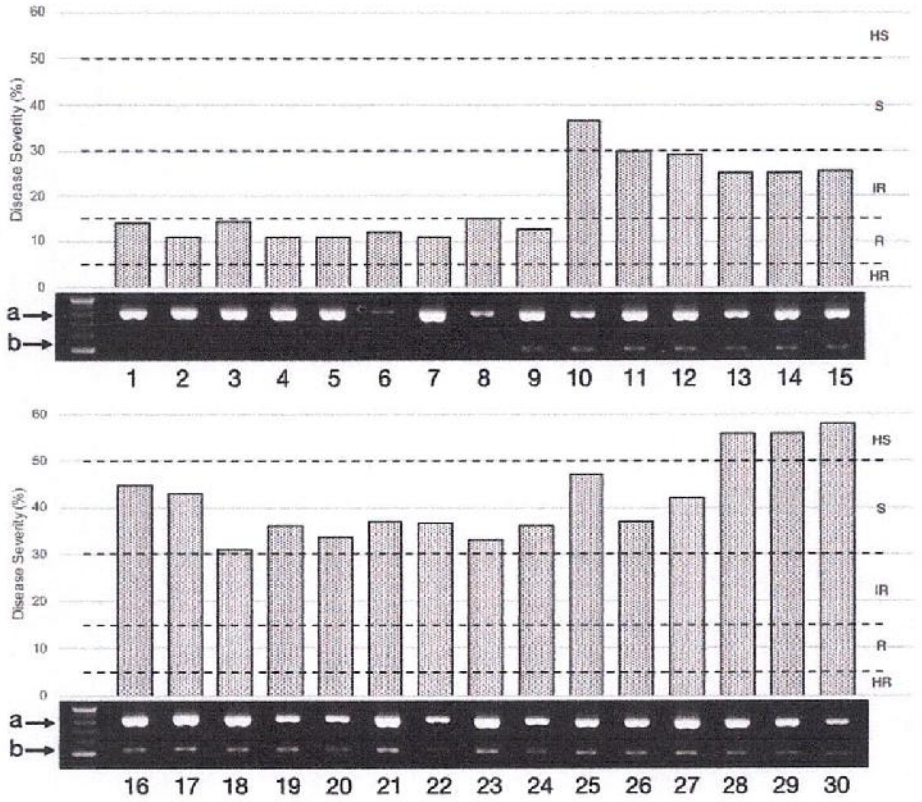

Figure 3. Association of anthracnose resistance phenotypes (% disease severity) with the banding patterns from the SNP 21881933 derived AS-PCR assay of different mango accessions. The upper band (a) is the amplification of the *β-1,3-GLU2* fragment and serves as a positive control. The lower band (b) is an allele-specific amplification. Lanes 1-9 are identified resistant accessions while lanes 10-30 are identified susceptible to intermediately resistant accessions based on Table 2. (HS) highly susceptible; (S) susceptible; (IR) intermediate; (R) resistant; (HR) highly resistant

3.3 Association of SNP 21881933 alleles with phenotypic anthracnose resistance

All but one of the 9 resistant accessions tested showed a consistent genotype–phenotype relationship wherein resistance to anthracnose was associated with the *β-1,3-GLU2* allele A. On the other hand, intermediate resistance and susceptibility to anthracnose were associated with the *β-1,3-GLU2* allele G, as shown by its presence in all accessions with higher % disease severity (>30%). Only the resistant accession '12-112' yielded an inconsistent association between genotype and phenotype, which may be due to a different mode of resistance. Moreover, analysis of phenotype variance (one-way ANOVA) showed that the level of resistance is significantly different between the alleles A and G genotypes ($P < 0.01$). In terms of genotype-phenotype association analysis, Pearson correlation and regression analysis showed that SNP 21881933 alleles is significantly correlated to anthracnose resistance phenotypes ($r = 0.74$; $P < 0.01$).

3.4 Molecular analysis of SNP 21881933 and mango *β-1,3-GLU2* gene

PCR products amplified by the gene-specific primers in this study were sequenced to confirm its identity and the presence of SNP 21881933 in mango accessions. All amplicon sequences significantly matched to a *β-1,3-glucanase 2* (*β-1,3-GLU2*) protein in the NCBI RefSeq database. Sequence comparisons with *β-1,3-GLU2* of closely related fruit tree crops confirms

an A > G nucleotide transition at the SNP 21881933 site (Figure 1-A). This leads to a missense mutation in the translated *β-1,3-GLU2* protein with an isoleucine to valine substitution within the conserved Glycosyl hydrolase family 17 (GH17) domain (Figure 1-B). ConSurf analysis predicts that this specific isoleucine residue (I196) is a structural residue that is highly conserved among closely related fruit tree crops (Figure 1-C, D).

The predicted protein structure of mango *β-1,3-GLU2*, which was modelled based on the crystal structure of *H. brasiliensis* endo-beta-1,3-glucanase (Hev b 2), exhibits the canonical $(\beta/\alpha)_8$ TIM-barrel motif and the catalytic groove across the protein surface found in other glucan endohydrolases (Receveur-Bréchot et al., 2006) (Figure 1-E). Superimposition of the structure models with A and G alleles also revealed changes in the predicted folding pattern of residues 82-90 (α-helix), 119-127 (extended loop), and 293-298 (extended loop) of the mutant *β-1,3-GLU2* (Figure 1-F). Based on the predicted model, the I196V mutation is situated near the catalytic groove where the binding site of substrates containing β-1,3-linked glucose residues is situated (Figure 1-G). Since I196 is predicted to be a structural residue, replacement with a valine residue at this position might result to structural changes in the catalytic groove that may affect the substrate binding specificity of the protein. To investigate the possible effects of this mutation, I196V was introduced using UCSF Chimera v.1.15 and predicted a significant clash with V261 residue having an overlap of 0.780 Å and distance of 2.980 Å (Figure 1-I).

4. DISCUSSION

Fruit tree breeding methods conventionally rely on phenotypic evaluations of parental lines to be used for varietal improvement. Such selection practices are subjective and are highly variable depending on the environment of the plant. Marker-assisted selection (MAS) provides a more rapid, accurate and discriminative way in identifying individuals as parentals for breeding and development of plant varieties with desired agronomic characteristics. In this study, an SNP marker within the β-1,3-glucanase 2 (*β-1,3-GLU2*) gene in mango was found to be associated with resistance to anthracnose and developed for MAS using a simple AS-PCR assay.

β-1,3-glucanases (E.C. 3.2.1.39) are enzymes widely found in bacteria, fungi, viruses, and plants that catalyze the hydrolysis of 1,3-β-D-glucosidic linkages between β-1,3-glucans – a major component of fungal and plant cell walls (Torres et al., 2015; X. Xu et al., 2016). In plants, β-1,3-Glucanase genes (GLUs) forms complex and diverse gene families playing important roles in physiological and developmental processes, including pathogen defense mechanisms (Doxey et al., 2007). GLUs and other pathogenesis-related proteins like chitinase and phenylalanine ammonia-lyase (PAL) are considered key enzymes in the control of plant disease in resistant systems (Zeng et al., 2006). Specifically, plant GLUs defend against pathogen infections by hydrolyzing β-1,3-glucans in fungal cell walls or by promoting the release of cell wall-associated immune elicitors that further stimulate defense reactions (X. Xu et al., 2016).

Previous studies have shown evidences of β-1,3-glucanase's role in *Colletotrichum* infection. In maize, nine GLUs were significantly upregulated in leaves during *Colletotricum graminicola* infection as a result of pathogen-associated molecular patterns (PAMP)-triggered defense response (Oliveira-Garcia & Deising, 2013). Similarly, strawberry plants infected with *C. fragariae* or *C. acutatum* induced the expression of two GLUs to over a thousand fold (Shi et al., 2006). In mango, Zhang et al. (2013) previously reported that β-aminobutyric acid (BABA) treatment in mango fruits significantly enhanced the activities of β-1,3-glucanases and effectively suppressed anthracnose caused by *C. gloeosporioides* during storage at 25°C. Increased β-1,3-glucanase activities was also attributed to reduction of postharvest anthracnose and enhancement of disease resistance in mango fruit after exogenous nitric oxide (NO) treatment (Hu et al., 2014). These previous findings indicate that β-1,3-glucanases significantly respond to *Colletotrichum* infection and possibly elicit defense response mechanisms involving the enhancement of defense-related enzyme activities to confer resistance to the pathogen.

Our results describe a possible mechanism of resistance against *C. gloeosporioides* in mango involving β-1,3-glucanases. A positive correlation between the presence of SNP 21881933 mutation and increased susceptibility to the disease implies that this specific mutation might have significantly reduced the activity of β-1,3-glucanase 2 enzymes, which have a direct role in defense response. As a PR protein, β-1,3-glucanases have been shown to directly defend against fungi infection by hydrolyzing fungal cell walls leading to fungal cell lysis (de la Cruz et al., 1995; Sandhu et al., 2017). While the mutant allele G found in susceptible accessions could have deleterious effect on the enzyme function rendering it vulnerable against infection and disease development, the β-1,3-GLU2 allele A, on the other hand, could be inferred as a biologically-active protein retaining its hydrolyzing function. This conclusion is supported by the fact that there is a significantly higher disease severity in all accessions with the G allele post-inoculation, while accessions with A allele had significantly reduced infection.

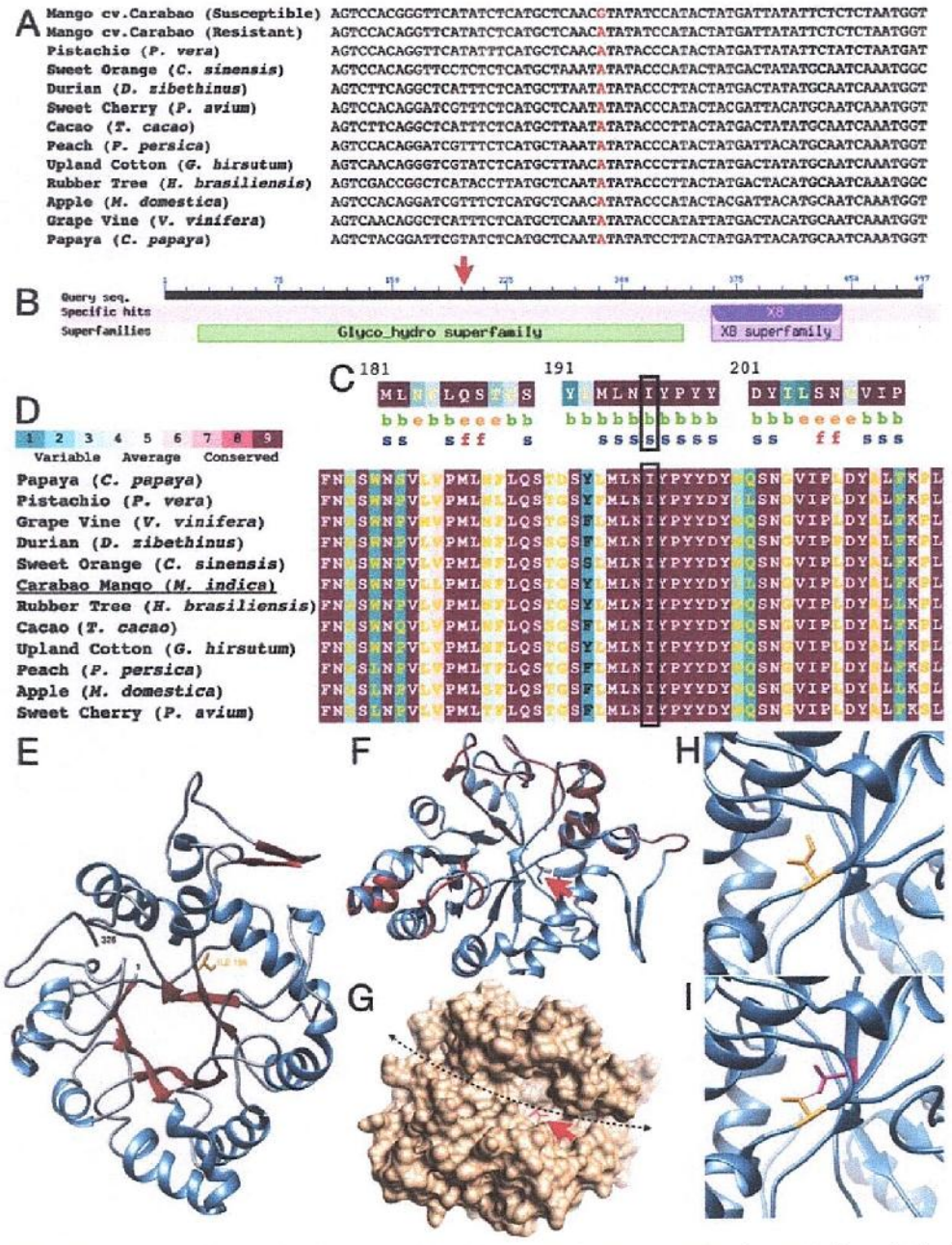

Figure 1. SNP 21881933 is a A > G transition in mango *β-1,3-GLU2* leading to an Isoleucine to Valine substitution. (A) MSA showing the SNP 21881933 site at position 6:17813013 (indicated in red) in mango *β-1,3-GLU2* and its homologs. The adenine [A] base is conserved between mango, apple, cacao and other fruit crops. (B) The isoleucine to valine substitution (I196V) caused by SNP 21881933 is predicted to be within the conserved protein domain family Glycosyl hydrolases family 17 (GH17; pfam00332) through the NCBI conserved domain search service (CD-search). A red arrow indicates site of I196V mutation relative to the GH17 domain. (C) ConSurf analysis of the mango *β-1,3-GLU2* protein sequence shows that 196I is highly conserved and predicted to be a buried residue (b), and thus likely to be a structural residue (s). (D) 196I in mango is highly conserved in β-1,3-glucanases of other fruit tree crops based on ConSurf Color-Coded MSA. Yellow letters in (B,C) indicates insufficient data where calculation of conservation was performed on less than 10% of the sequences. (E) Complete 3D structural model of the wildtype mango *β-1,3-GLU2* protein based on Phyre2 modelling from the *H. brasiliensis* β-1,3-glucanase crystal structure. The β-sheet strands (red) surrounded by the α-helices (blue) form the (β/α)8 TIM-barrel motif referred to in the text. Numbers 1 and 326 indicate the N- and C-terminal ends of the polypeptide chain, respectively. 'ILE 196' indicates the position of the isoleucin residue at the mutation site. (F) Superimposition of the structural models from mango *β-1,3-GLU2* with allele A (blue) and allele G (red) reveals some structural differences, with 97.52% identity. A red arrow points to the I196V substitution caused by SNP 21881933. (G) Molecular surface model of the mango WT *β-1,3-GLU2* protein showing the proximity of 196I residue (red arrow) to the catalytic groove indicated by the curved dashed line. (H,I) The effect of the mutation on the *β-1,3-GLU2* atomic interactions (clashes/contacts) is shown in H and I. (H) In the wildtype A allele, Ile196 residue (yellow) does not interact with any other residues within 5.0 Å. (I) In the SNP 21881933 mutation, Val196 (yellow) forms a steric clash (pink line) with Val261 (magenta) indicating a direct unfavorable interaction.

Missense mutations caused by SNPs occurring at gene coding regions generally affect protein stability, protein-protein interactions, and critical components of biological reaction (Zhe Zhang et al., 2012). SNP 21881933 described in this study has been found to be located within the highly conserved Glycosyl hydrolases family 17 (GH17) domain. This domain family is involved in physiologically important processes in plants, such as response to biotic and abiotic stresses, defense against herbivores, activation of phytohormones, lignification, and cell wall remodeling (Opassiri et al., 2006). Interestingly, another conserved domain called C-terminal X8 family carbohydrate-binding domain is also present in the expressed *β-1,3-GLU2* gene close to the SNP site. This domain is primarily involved in carbohydrate binding and cleaving (1,3)-beta-D-glucosidic linkages along with GH17 family (Marchler-Bauer et al., 2014). These suggest that the SNP is situated near or within the active site region of the enzyme and mutations within this domain could affect any of these functions. Any conformational change altering the active sites of proteins, as well as mutations quite close to it, can affect biochemical reactions because catalytic reactions are very sensitive to the precise geometry of these active sites for both of the products and reactants (Zhe Zhang et al., 2012).

To analyze the possible structural effects of the SNP mutation, we generated the predicted protein structure of the isolated mango *β-1,3-GLU2* and introduced the mutation in silico. Based on the three-dimensional structure model, the resulting I196V mutation is situated within the typical eightfold β/α TIM-barrel motif and the catalytic groove across the protein surface strictly conserved in other glucan endohydrolases of the GH17 family. This motif consists of an internal crown of eight β-strands connected to an outer crown of eight α-helices. According to Receveur-Bréchot et al. (2006), the active site of these enzymes consists of two glutamate residues in strands *β4 and β7* acting as proton donor nucleophile residue, respectively. These correspond to residues E50 and E240 in mango *β-1,3-GLU2*. Moreover, the three-dimensional molecular surface model showed that the I196V substitution constitute a part of the catalytic groove where β-1,3 linked glucan trisaccharide substrates are accommodated (Receveur-Bréchot et al., 2006). These observations suggest that I196, being a conserved structural residue, is necessary in maintaining the precise conformation of the enzyme's active site.

We hypothesized that the replacement of I196 mango *β-1,3-GLU2* with a valine residue caused contacts/clashes with the surrounding residues. Based on mutation analysis, a steric clash between V196 and V261 side chains was predicted with an overlap of less than 0.8 Å. This indicates a direct unfavorable interaction wherein atoms are too close together allowing van der Waals forces between the two residues. Such molecular interactions could result to structural perturbations. Since the I196V mutation is proximal to the active site and catalytic groove of mango *β-1,3-GLU2*, a clash between V196 and V261 could affect conformational stability and topology of the region possibly inhibiting the interaction of the substrate with the active site.

Together, these results suggest that the mutation caused by SNP 21881933 and its predicted structural changes most likely affected the catalytic activity of the wildtype *β-1,3-GLU2* in defense response against *C. gloeosporioides* infection. Although our findings provided an insight in the structural impact of the SNP mutation, functional studies are needed in order to confirm and elucidate further these mechanisms of resistance. Moreover, despite a significant positive correlation between *β-1,3-GLU2* alleles and degree of *C. gloeosporioides* infection, the biological function of β-1,3-glucanases alone cannot be conclusively associated to anthracnose resistance. Plant defense response against fungi is complex and requires involvement of regulatory factors that determines the susceptibility or resistance of plant to a particular pathogen.

The developed *β-1,3-GLU2* SNP marker reported in this study will enable marker-assisted selection of anthracnose-resistant mango as early as the seedling stage. We recommend this functional marker for routine genotyping of parental and hybrid mango to facilitate and improve the efficiency of mango breeding programs.

5. CONCLUSION

In crop production where specific quality traits dictate either significant economic losses or gains, more efficient breeding strategies for food crops are needed. Advancements in molecular biology provide genomic tools such as molecular markers which can aid in the selection of target traits to shorten selection times and to accelerate the breeding process of new varieties of mango. As demonstrated by this study, SNP markers can be used to identify mango with phenotypic anthracnose resistance as early as the seedling stage to significantly reduce the expenses in field inputs, maintenance, and evaluation of material over years. AS-PCR is a simple and effective method to employ such markers, but these can be also used in high-throughput genotyping technologies for large-scale screening and analysis. Our results show that *β-1,3-GLU2* is a reliable marker for screening and developing anthracnose resistant breeding lines that can complement the conventional mango breeding approach. However, due to the limited sample size and phenotypic data available in this study, it is recommended

to further employ the developed marker in a segregating population with a large sample size to strengthen the genotype-phenotype association analysis.

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Molecular characterization and association of lactoferrin gene to subclinical mastitis in goats (*Capra hircus*)

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ABSTRACT. The study characterized the lactoferrin (Lf) mRNA gene in different goat breeds in the Philippines and determined its association with subclinical mastitis (SCM). The study involved collection of milk at second week of lactation (n=75) and blood samples (n=5) to obtain extracted RNA and using cDNA to amplify Lf gene through polymerase chain reaction. The nucleotide and amino acid sequences were determined and used as reference in the evaluation of phylogenetic relationship. Amplified products were utilized for RFLP analysis before determining the association of the gene with SCM. Results of the study demonstrated that Lf gene in goats registered a molecular weight of 2135. Nucleotide and amino acid sequence of Lf gene revealed high similarity (99%) in Saanen, Anglo-Nubian and Philippine native goats with that of *Capra hircus* (U53857) Lf gene submitted to GenBank. Phylogenetic studies showed that Lf gene of Anglo-Nubian, Saanen and Native goats clade together with Lf gene of *C. hircus* (U53857). Three genotypes in goats were documented using the restriction enzymes *AluI* and *HaeIII*. Based on the Statistical analysis, association (comp 5.65, p = 0.0308) has been established between the Lf genes of goats with genotype BB to SCM using *HaeIII* restriction enzyme.

Keywords: Anglo-Nubian; Lf gene; Philippine native goat; Saanen.

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Introduction

Milk coming from dairy animals meets the basic requirements of the body. The significant increase of milk, milk fat, and protein in dairy animals is a result of advanced researches in the past two decades (Tsuda et al., 2000).

Mastitis is an economically significant infectious disease of dairy animals. Losses are due to decreased quantity and quality of milk, heightened by medicine and labor cost (Hogeveen &Van der Voort, 2017; Waminal, Tubalinal, & Mingala, 2019). Limiting the occurrence of subclinical mastitis (SCM), therefore, can be a vital strategy in reducing economic losses. To understand the mechanism on the susceptibility of animals to mastitis, genetic traits for milk production should be considered. The use of genetic markers in the selection of animals for breeding has also be considered (Rupp & Boichard, 2003; Gholizadeh, Mianji, & Zadeh, 2018).

Lactoferrin (Lf) gene, also known as 'red protein from milk', is an iron-binding protein that is found in most fluids in the body (Marchweka, Roterman, Strus, Spiewak, & Majka, 2012) and is the most important iron-binding protein in milk (Collins, Flores, Wang, & Anderson, 2018). Neutrophils and inflamed tissues release Lf and considered to have direct antimicrobial properties (limits bacterial proliferation and adhesion to microbes) and have a role in innate immunity (Van Der Strate, Belijaars, & Molema, 2001; Valenti & Antonini, 2005; Legrand et al., 2008; Walker, 2010; Siqueiros-Cendon et al., 2014). Furthermore, Lf possesses abundant health benefits such as anti-fungal, anti-parasitic, anti-viral, and anti-tumor properties (Niaz et al., 2019).

The potential of Lf gene (Lonnerdal & Iyer, 1995) as genetic marker for mastitis resistance in dairy animals needs to be elucidated in order to validate its role in mastitis (Pawlik, Sender, & Korwin-Kossakowska, 2009). The selection of superior individuals that show resistance or susceptibility to mastitis based on data generated through molecular studies of the Lf gene will be necessary for the selection of animals for breeding (Sharma, Chakraborty, & Gupta, 2015). This strategy may boost the economic condition of the farmer and nurture the dairy industry as a whole while promoting a mastitis-free dairy herd.

Currently, the information about Lf gene in goats (*Capra hircus*) is limited. Genetic characterization of Lf gene would augment their possible role in occurrence, onset and disease resistance to SCM (Pawlik et al., 2009). The potential of Lf gene as a marker for mastitis resistance in dairy animals is important and will augment existing marker-assisted breeding and selection resulting in improved dairy performance of dairy animals (Shimazaki & Kawai, 2017; Waminal et al., 2019). In cattle production, molecular characterization and genetic diversity of Lf gene have been associated with different traits and disease resistance and have been included in their breeding and selection techniques (Sharma et al., 2015).

The main goal of this study is to characterize and identify Lf gene as a genetic marker for SCM resistance in goats through molecular analysis and association of nucleotide polymorphisms with the presence of SCM.

Material and methods

The collection of samples for analysis was conducted from goats in selected provinces in Central Luzon, the Philippines.

Sample collection

A total of 75 goats (30 Anglo-Nubian, 30 Saanen and 15 mixed breeds) on their second week of lactation onwards and 5 Philippine native goats (not lactating) were selected from previously identified goat farms in Luzon (Farm 1 – 15.6310157, 120.5991522; Farm 2 – 15.4557851, 121.3377025; Farm 3 – 15.7409607, 120.9273197). Housing, feeding, and overall management of the animals per farm were not altered or changed for the conduct of this study. In addition, the farms included in the study housed their animals in concrete housing in group pens with elevations. It was during the summer season (March-May 2018) in the Philippines during the collection of the samples. The test animals were manually milked from the udder and pooled to collect 30 mL of milk sample for ribonucleic acid (RNA) extraction. The milk was collected in a 50 mL conical tube and was placed in a cooler with ice transported to the laboratory for processing. In case the milk was not processed immediately, it was placed in a refrigerator and processed the following day. For the five (5) native goats, RNA was extracted from blood as these animals were not lactating. Five (5) mL of blood was collected from the jugular veins of the animals using EDTA tube. Samples were processed that day after collection.

RNA Extraction

RNA extraction from milk and blood samples from the goats was done following the Promega protocol with some modifications. For milk, 30 mL of raw milk was centrifuged at 4,000 rpm for 20 min. After centrifugation, the fat layer and the supernatant were discarded, and afterwards the pellet was suspended in one (1) mL PBS (phosphate-buffer-saline, a pH of 7.4). This suspension was centrifuged again at 4,000 rpm for 10 min. After this step, the pellet was collected into 2 µL microtube after removing the supernatant fluid. One (1) mL of PBS was mixed again with the pellet and was centrifuged for 14,000 rpm for 1 min. This process was repeated twice and the supernatant was removed to collect the pellet.

For blood, 500 µL of the buffy coat was aspired after centrifugation at 4,000 rpm for 5 min and transferred into the 2 µL microtube, and then added with 2-3 volumes of 0.14 NH_4Cl. The mixture was mixed by sonication and centrifuged at 14,000 rpm for 1 min. Supernatant was removed and the process was repeated twice to collect the pellet.

The pellet from the milk and the blood was added with 1 mL cell lysis solution and was subjected to sonication to mix the pellet and cell lysis. After which, it was centrifuged to 14,000 rpm in 1 min. The supernatant was discarded and 500 µL of nuclei lysis and 300 µL protein precipitation solution was added next and centrifuged for 14,000 rpm for 1 min. After discarding the supernatant fluid, isolated cells were dissolved with 1 mL Trizol reagent and incubated for 10 min at room temperature. Then 200 µL of chloroform was added. The mixture was centrifuged at 14,000 rpm for 15 min. at 4°C. The solution was decanted and pellets were washed by 1 mL 75% ethanol and centrifuged for 5 min at 8,000 rpm at 4°C. Pellets were dried and reconstituted with 30 µL diethyl pyrocarbonate (DEPC) treated water.

Reverse-transcription - polymerase chain reaction (RT- PCR)

RT-PCR kit (TaKara™) was used to synthesize the cDNAs from the total RNA samples extracted. One µL of dT random primers were mixed with 1 µL dNTP, 5 µL of RNAse free water and 3 µL of RNA template. The solution was incubated for 5 min at 65°C before running for PCR. The prepared RNA primer mix was mixed

with 4 µL 5x buffer, 0.5 µL RNAse inhibitor, 1 µL reverse transcriptase, and 4.3 µL RNAse free water. This was subjected to PCR run for segment 1, 10 min. at 30°C; segment 2, 45 min. at 50°C; and segment 3, 5 min. at 95°C. The β-actin was used to determine if the DNA has been produced through PCR.

PCR assay

Primers (Table 1) were designed using Primer3 server and Primer-BLAST using the Caprine Lf gene (Accession number: U53857) stored from National Center for Biotechnology Information (NCBI).

Table 1. Primers used for the amplification of cDNA.

Gene	Accession Number	Primer Sequence (5'-3')	Fragment Length (bp)
	Primer 1	Forward- AGACATGAAGCTCTTCGT Reverse- GAGTACGGACAACACCGGGC	834
	Primer 2	Forward- ATCCTTCGCCCGTTCTTGAG Reverse- CGCCGAATCTACTTTTGAGG	561
	Primer 3	Forward- GCCCAGGTCCCTTCTCATG Reverse- GGGCACAGCTCTGACTAAAG	736
	Primer 4	Forward- AGAAAGCAAATGAGGGGCTC Reverse- TTACCTCGTCAGGAAGGCCG	749

All PCR assays were performed in 20 µL reaction volume containing 2 µL of genomic DNA template, 10 pmol of each primer and PCR master mix. The amplification of the Lf gene was carried out in a thermocycler (SimpliAmp, Thermofisher) under the optimized conditions. Briefly, initial denaturation at 94˚C for 5 min., followed by 40 cycles consisting of denaturation at 94˚C for 1 min., annealing at 58˚C for 1 min. and extension at 72˚C for 1 min., with final extension at 72˚C for 5 min.

After amplification, 1 µL of the PCR product was electrophoresed in 1.5 % agarose gel containing 1X TAE buffer at 70 volts for 30 min. and visualized under ultraviolet light using UV transillumination advance imaging system. To ensure that amplification products are of the expected size, a 1 kb plus DNA ladder was run simultaneously as a marker.

Gene sequencing and phylogenetic tree construction

Lf products were submitted for sequencing. Sequences were assembled using Mega 7 software. The forward and reverse sequences for each primer were assembled to form contigs of the respective regions. The gene sequences were compared with the Lf mRNA sequences to annotate different exonic regions putatively to identify SNPs in respective regions. The contiguous Lf gene nucleotide sequence was subjected to Basic Local Alignment Search Tool (BLAST) at NCBI database to determine the sequence similarity with the corresponding regions of other species. DNA nucleotide sequence was also conceptually translated using MEGA 7 software and compared with that of NCBI Genbank for Caprine and Bubaline Lf gene to detect amino acid changes. Phylogenetic trees were derived using MEGA 7 software using Maximum Likelihod method with 1000 bootsrap replications (Tamura et al., 2011).

Restriction fragment length polymorphism (RFLP) analysis

The Lf PCR products using primer 2 (23 µL) were subjected to endonuclease digestion in goat to amplify exon 2, 3 and 4 of Lf gene with an amplicon size of 561 bp.

Restriction enzymes (Table 2) that could cut the fragments were analyzed using Sequence Manipulation Suite: Restriction map (Stothard, 2000). RFLP was conducted by preparing the reaction mixture composed of 7 µL of PCR product, 5.15 µL of sd2H_20, 0.35 RFLP buffer and 0.075 µL for each enzyme. Samples were incubated at 37°C for 4h. Restriction fragments were resolved on 2% agarose gel in horizontal electrophoresis. The restriction-digested gene fragments were visualized and documented using a UV transilluminator (FlourChemE by ProteinSimple ™). The differences in fragment yielded by various restriction enzymes that would indicate polymorphism in a particular gene were analyzed and compared.

Table 2. Restriction enzymes used for the RFLP analysis.

Restriction Enzymes	Lf Gene Loci
AluI (AG/CT)	21, 53, 78, 295, 418, 465, 472
HaeIII (GG/CC)	353, 380, 492

Association of Lf gene with SCM

Samples of amplified Lf gene from the milk of goats where polymorphisms shown by different genotypes of the target gene previously exhibited after RFLP were taken as reference in tracing back the goats suspected for SCM and for California mastitis test (CMT) evaluation utilizing their milk as samples. A total of 69 goat samples were used in the study that associated expression of Lf gene and in the occurrence of SCM. Physical assessment for SCM covered the evaluation of the mammary gland and milk that involved visual observations and palpation.

CMT was used as an indirect method of measuring SCC as the indicator of intramammary infection as it has the advantage of being a quick, cheap, and simple 'animal side' test (Persson & Olofsson, 2011). CMT scores for all animal subjects were classified as non-mastitic if CMT score result is 1 or lower and mastitic if CMT score result is 2 or higher.

Statistical analysis

Univariate analysis on the possible association between the genotypic frequency and the occurrence of SCM was examined using Chi-square ($\chi 2$) by a certified statistician (Petrie & Watson, 2006).

Results and discussion

Of the 80 samples collected, only 4 (5%) samples produced complete Lf mRNA sequences in goats. It has been shown that the mRNA sequence of Lf gene was detected in the milk samples of Anglo-Nubian and Saanen and in the blood samples of the Native goats. Target primers 1, 2, 3, and 4 were amplified and generated an amplicon size of 834 bp, 561 bp, 736 bp, and 749 bp, respectively.

Native goat Lf mRNA sequence resulted in an average of 2133 bp while there were 2134 bp in Anglo-Nubian and 2135 bp in Saanen. Goat Lf nucleotide sequences were aligned with other ruminants and other species. Statistical nucleotide pair frequency of the 14 aligned nucleotide sequences showed an average of 1334 identical pairs, 751 transitional pairs, and 60 transversional pairs. Among the different goat breeds' nucleotide pair frequencies, the average identical pairs were 2118 with 2 transitional pairs and 6 transversional pairs. There was a high similarity of nucleotide sequences between the breeds of goats being studied (Table 3). It shows that there is a high similarity in the nucleotide and amino acid sequence of different goats. This may be due to the highly conserved region that can be found in the Lf gene. This is essential to maintain their distinctiveness from other species.

Table 3. Nucleotide and amino acid sequence percentage similarity of Native, Anglo-Nubian and Saanen breeds in reference to other ruminant species.

Species (Accession No.)	Nucleotide Sequence			Amino Acid Sequence		
	Native	Anglo Nubian	Saanen	Native	Anglo Nubian	Saanen
C. hircus (U53857)	99.24%	99.44%	99.58%	99%	99%	99%
O. aries (NM_001024862.1)	97.74 %	97.97 %	98.03 %	97 %	98 %	98 %
B. grunniens (DQ387455.1)	93.94 %	94.03 %	94.26%	93%	94 %	94 %
B. bubalis (JF825526.1)	94 .09%	94.28%	94.38%	93%	94 %	94 %
B. indicus (GU059864.1)	93.91%	94.14%	94.14%	93%	93%	94 %
B. Taurus (FJ589071.1)	94.04%	94.05%	94.29%	93%	93%	94 %
E. caballus (NM_001163974.1)	77.12%	77.09%	77.19%	77 %	77 %	77 %
S. scrofa (M81327.1)	72.80%	72.97%	73.02%	72%	72%	72%
H. sapiens (U076343)	73.28%	73.11%	73 %	72%	72%	72%

Maximum Likelihood algorithm with 1000 NJ bootstrap resampling revealed the clustering of all breeds of goats' Lf nucleotide sequence with that of *C. hircus* (U53857.1). Anglo-Nubian and Saanen claded together while Native goats claded with other breeds (Anglo-Nubian and Saanen). All breeds of goats' Lf sequences claded together with *C. hircus* (U53857) sequence. *O. aries* (NM_001024862.1) Lf sequence, on the other hand, separated from the clade of goats. While *B. taurus* (FJ589071.1), *B. indicus, B. grunniens,* and *B. bubalis* (JF825526.1) Lf sequences have much greater separation from the clade of small ruminants. *S. scrofa* (M81327.1), *E. caballus* (NM_001163974), and *H. sapiens* (U07643.1) Lf nucleotide sequence have been used to show an outlier from other ruminants (Figure 1).

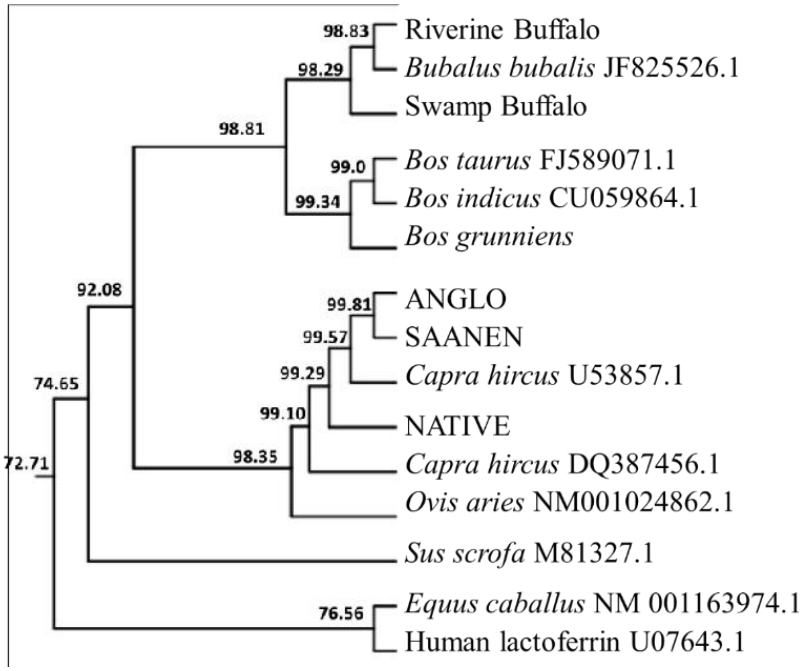

Figure 1. Phylogenetic tree showing the relationship of Lf gene between breed of goats, water buffaloes, other ruminants' and human.

The coding sequence of Lf gene in goats was analyzed for differences in nucleotides and functional amino acid substitution. Furthermore, restriction enzyme capable of digesting the sequence to identify the nucleotide polymorphism and distinguish the genotype has been identified, and the results are herein presented. Sequence analysis revealed seven (7) nucleotide polymorphism in native in the coding sequence of Lf in native goat at locus 551, 552, 553, 701, 910, 939 and 1069 with 5 amino acid substitutions, three (3) in Saanen at locus 1069, 1526 and 1527 with 2 amino acid substitutions and four (4) in Anglo-Nubian breed at locus 910, 1069, 1526 and 1527 with 3 amino acid substitutions.

The nucleotide polymorphism at locus 1069 of Native, Saanen, and Anglo Nubian goat Lf gene sequences resulted in a functional amino acid substitution from leucine to valine. Kaminski et al. (2008) reported that the higher milk protein yield was related to polymorphism at position +216 when it occurs along with another polymorphism. The substitution of valine which is a growth hormone receptor gene can increase the milk yield as well as elevate the milk protein and fat yield.

This is in consonance with the previous studies (Lee et al., 1997; Martin-Burriel, Osta, Baredse, & Zaragosa, 1997; Li & Chen, 1999; Kaminski, Oleński, Brym, Malewski, & Sazanov, 2006; Daly, Ross, Giblin, & Buckley, 2006) that polymorphisms in Lf gene reportedly occur in the coding and regulatory regions and polymorphisms are encoded in exons and introns. To develop rapid recognition of polymorphic site of Lf gene, restriction enzymes *AluI* and *HaeIII* were used to digest 561 bp that was amplified by primer 2. This 561 bp segment covered exons 2 and 3 of the coding region based on the SMS Restriction Digest application of 4 samples with complete Lf gene nucleotide sequence.

The availability of these restriction patterns on extracted Lf gene was examined by using PCR product that was subjected to *AluI* (Figure 2) and *HaeIII* (Figure 3) digestion and electrophoresed. Results showed restriction patterns that were almost the same based on the conceptualized expected pattern (Chang-hong, Gaoming, Yanliang, & Zhaoxia, 2008).

The presence of three restriction patterns using *AluI* seen in gel conferred that there were more polymorphic nucleotides for Lf gene and had to be discovered. High polymorphism in Lf was quite expected because of its function to bind peptides derived from exogenous antigens and in mediating immune response. *AluI* produced cut at AGT/ACT site. Results showed no cut in genotype AA producing 561 bp fragment size of the gene, while genotype AB had one cut producing two bands with 300 bp and 217 bp amplicon size products. Genotype BB produced 3 bands with 561 bp, 300 bp, and 217 bp fragment sizes.

Another restriction enzyme used to test the restriction pattern of Lf gene in goats was *HaeIII* which produced cuts at AGT/ACT site. Results showed no cut in genotype AA producing 561 bp fragment size of the gene, while genotype AB had 5 cuts producing four bands with 561 bp, 312 bp, 122 bp, and 70 bp amplicon size products. Genotype BB produced 3 bands with 312 bp, 122 bp, and 70 bp fragment sizes (Figure 3)

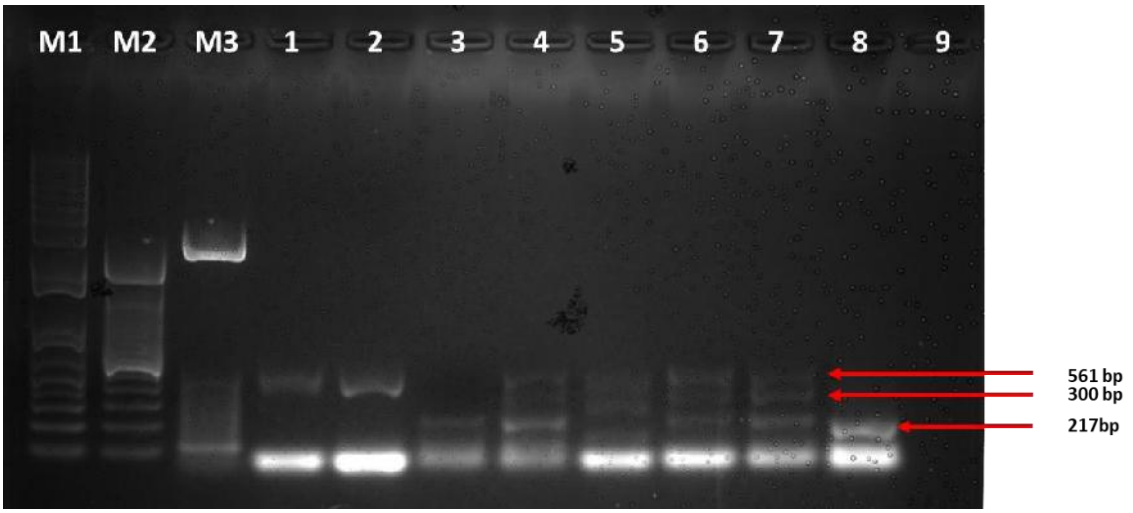

Figure 2. Restriction patterns of caprine Lf gene PCR products using *AluI* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder M3 25 bp ladder, Lane 1 and 7-genotype AA, Lanes 2 to 5 genotype AB, Lanes 6 and 8 –genotype BB.

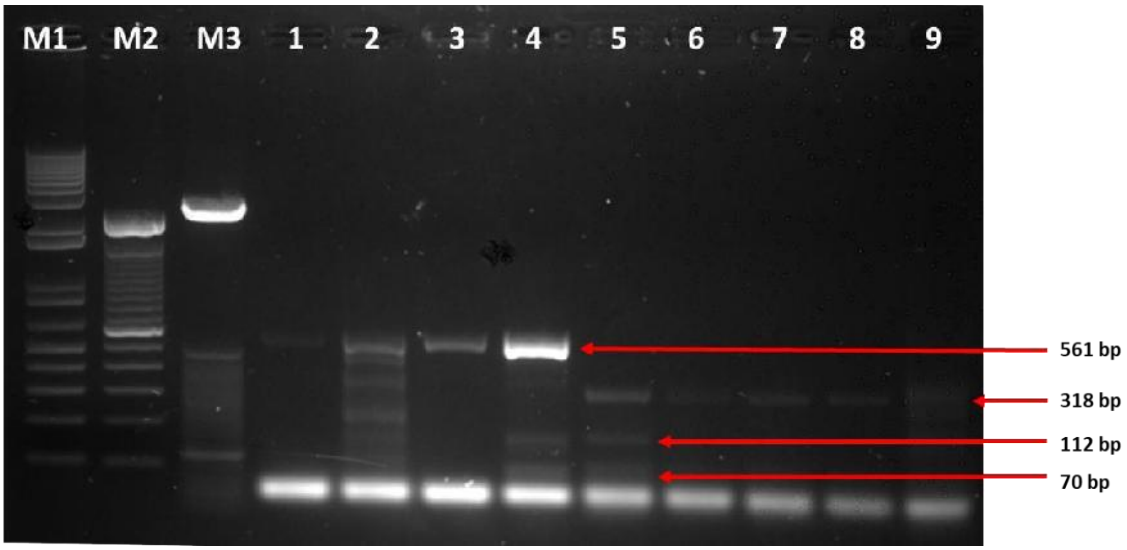

Figure 3. Restriction patterns of caprine Lf gene PCR products using *HaeIII* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder. M3 25 bp ladder, Lanes 1 and 3-genotype AA, Lanes 2 and 4-genotype AB, Lanes 5 to 9–genotype BB.

Restriction enzyme *Alul* that can cut AG/CT nucleotide site was selected to examine restriction fragment pattern in the nucleotide sequence of Lf gene based on the SMS Restriction Digest application of the Lf complete nucleotide sequence.

The different genotypes found in the Lf gene are not significantly associated with the occurrence of mastitis using other restriction enzymes. No prior studies have been conducted in support of the three different genotypes found in goats using *HaeIII* and *AluI* restriction enzyme, hence this study will be the baseline for other researchers in identifying mastitic resistance genotypes.

The 561 bp was used for the association study. It showed the frequency of Lf genotypes in non-mastitic and SCM goats. Although chi-square test revealed no significant effect to make an association between genotypes and mastitic incidence (Table 4 and 5), it was clearly presented that genotype BB had a high frequency in SCM compared to non-mastitic animals. Animals having a Genotype BB may or may not increase the tendency on the occurrence of SCM as compared to genotype AA and AB.

Table 4. Chi-Square Analysis on the Association of Genotypes and Sub-clinical mastitis in Goats using Restriction Enzyme *AluI*.

Genotype	Expected Frequencies			Chi-square			Chi-square computed	Chi-square tabulated
	AA	AB	BB					
Non-mastitic	11.88	17.23	11.88	0.10	0.18	0.69	2.43	5.99
Sub-clinically Mastitic	8.11	11.76	8.11	0.15	0.26	1.02	2.43	5.99

Table 5. Chi-Square Analysis on the Association of Genotypes and Sub-clinical mastitis in Goats using Restriction Enzyme *HaeIII*.

Genotype	Expected Frequencies			Chi-square			Chi-square computed	Chi-square tabulated
	AA	AB	BB					
Non-mastitic	10.84	16.57	16.57	0.43	0.35	1.26	5.658	5.99
Sub-clinically Mastitic	6.15	9.42	9.42	0.75	0.62	2.22	5.65	5.99

Percentage frequencies of Lf *AluI* and *HaeIII*-based genotype in non-mastitic and SCM goats are presented in Table 6 and 7.

Table 6. Percentage frequency of Lf *Alu1*-based genotype in non- mastitic and sub-clinically mastitic goats.

Genotype (base pair size)	Category of Animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA (561bp)	65.00	13	35.00	7	28.99	20
AB (300 bp, 217 bp)	65.58	19	34.48	10	42.02	29
BB (561 bp, 300 bp, 217 bp)	45.00	9	55.00	11	28.99	20
Total	59.42	(41)	40.58	(28)	100	69

Table 7. Percentage frequency of Lf *HaeIII*-based genotype in non-mastitic and sub- clinically mastitic goats.

Genotype (base pair size)	Category Of Animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA (561bp)	76.47	13	23.53	4	24.64	17
AB (561bp, 318bp, 112bp, 70bp)	73.08	19	26.92	7	37.68	26
BB (318bp, 112 bp, 70 bp)	46.15	12	53.85	14	37.68	26
Total	63.77	44	36.23	25	100	69

Association analysis based on the frequency of genotypes revealed that there is an association between genotypes and mastitic incidence. The frequency of the occurrence of digestion patterns were produced by *HaeIII* enzyme with SCM and non- mastitis regardless of breed. The Lf gene in goats has a bactericidal and bacteriostatic activity that could lessen the bacterial population in the milk through phagocytic killing (Tsuda et al., 2000). However, this may not be enough to sequester the microbial flora in the mammary gland of the animals. Underlying factors such as climate, housing system, type of bedding, and rainfall and wetness in the vicinity of dairy premises interact to influence the degree of exposure of teat and tend to increase mastitis pathogens that cause SCM (McEwen & Cooper, 1947).

Conclusion

The whole mRNA coding sequence of goats had an average molecular weight of 2134 bp. Both nucleotide and translated amino acid sequences of Lf were highly similar (98-99%), and phylogenetic analysis found an evolutionary relationship with goats from NCBI GenBank.

The Lf gene of goat established three different genotypes (*AA, AB, and BB*) using restriction enzymes *Alu1* and *HaeIII;* however, Chi-square analysis revealed no association between Lf gene and the occurrence of SCM using *HaeIII* restriction enzymes.

These initial findings are applicable in the field of immunity and disease resistance. The incidence of association between restriction sites and clinical parameters converts PCR-RFLP into a powerful tool in relating specific amino acid substitution at a critical position to possible disease resistance. Moreover, the selection of disease resistance genes will provide potential avenues for improving the health status of the animal and increasing productivity.

The polymorphic nature of the genes merits further investigation for other nucleotide loci and their significance to disease resistance or susceptibility. Identification of the blood composition of the upgraded goats would help establish the bloodline of animals. Expression analysis can also assist in the understanding of how the genes progress in disease protection.

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CULTURE CONDITIONS OF SPIRULINA IN FRESHWATER MEDIUM AND ITS EFFECT ON CELL GROWTH

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Abstract

Spirulina is a multicellular and filamentous microalgae that is considered a promising protein source alternative and absolute food and feed supplement for growth and nutrition. Optimizing the culture conditions of Spirulina is detrimental to maximizing biomass yield and minimizing cost by modifying the commercial culture media. This study was conducted to determine the chemical culture conditions *of Spirulina platensis* concerning biomass yield. Chemical conditions of the liquid culture such as the pH level and salt concentration of the cultivated Spirulina were determined. Moreover, the cell length (µm) of Spirulina as affected by pH and salt concentration was measured using Image J. The optimum quality and quantity of DNA as affected by preparation was determined. Results revealed that Spirulina thrived in alkaline conditions and significantly yielded a biomass of 75.43g and 67.47g at pH 8 and 9, respectively. The fresh weight harvest yield was significantly best observed at 1% salt concentration (w/v). In preparing and extracting the DNA of Spirulina using the modified CTAB DNA Extraction protocol, the Non-grinded sample of the DNA yielded the highest nucleic acid concentration of 437.8 ng/µl and had rendered pure DNA quality with an A260/280 ratio of 1.95 nm absorbance as compared with the ground samples. The size in length (µm) of the rod-shaped Spirulina cells cultivated in freshwater media was significantly long (626.75 µg and 494.64 µg) when cultivated at pH 8 and 9, respectively. Similarly, the spiral-shaped Spirulina cells were significantly long at pH 8 (356.67 µm) and pH 9 (348.53 µm). Moreover, the size length of the rod-shaped Spirulina cells is longer when cultivated at 1% (w/v) salt concentration with a length of 568.75 µm while the spiral-shaped cells were significantly long at 5% with a length of 193.68 µm.

Keywords: Spirulina, Culture Media, Production, Microalgae

INTRODUCTION

Cultivation and production of Spirulina have gained so much interest among researchers, industries, and other stakeholders due to its cardinal role in human and animal nutrition, environmental protection, bio-circular economy, food, and feed industry, aquaculture, nutraceutical, and pharmaceutical application (Saranraj, et al, 2014; Soni, R.A. 2017; Lafarga et al., 2021). Its wide array of content of excellent nutritional values like high protein, vitamins, and minerals has made spirulina useful in many nutritional and industrial applications In addition, this photosynthetic, multicellular, and filamentous blue-green algae is easy to grow and harvest.

Much progress has been achieved during the past decade in developing appropriate technology for microalgal mass cultivation. Many studies on optimizing the culture condition such as the chemical culture media, light intensity requirement, ambient temperature, aeration, agitation, etc. have been conducted to obtain maximized algal biomass yield at a considerable input cost. Spirulina, under controlled conditions, grows best using Zarrouk's medium. It is the standard substrate used for growing Spirulina however it incurs higher costs due to the expensive

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components needed in concocting the medium. Hence, studies of finding the alternative cost-effective source of modifying Zarrouk's medium either one or more of its components have been and are continuously being explored. Despite the enormous information on the optimum culture condition of Spirulina that will maximize its biomass yield, little is reported on the effect of the pH level and salinity concentration on the growth (cell length) of the Spirulina. This study has attempted to determine the optimal pH level and salt concentration of the culture medium using freshwater and its effect on the cell growth (length in μm) of Spirulina. Moreover, the DNA extraction method of Spirulina cell that will yield a suitable good quality and quantity of DNA was also explored.

METHODOLOGY

Cultivation Conditions

The strain of Spirulina was maintained in Zarrouks's medium at ambient temperature with 12 h light and 14 h dark photoperiod using a standard light and manually aerated in the flask. All reagents used for preparing the media were technical grade.

Experimental Set up

Five (5) liters of freshwater were placed in ten (10) –liter laboratory open glass containers. An anti-chlorine reagent was added to the water to ensure the removal of the chlorine which is detrimental to the survival of the Spirulina culture. The anti-chlorine reagent was left to be mixed in the water for an hour. Air pumps and accessories such as air hose, hose clamp, and air stone were assembled and installed in the experimental set-up as source of oxygen supply. After some time, carbonates and chloride salts were added to the freshwater to obtain the desired pH levels and salt concentrations. Thorough mixing was employed to ensure proper dissolution of the salts. When careful mixing of the liquid media was done.

Experimental Procedure

Effects of various pH levels and salt concentrations were determined by fresh weight of Spirulina biomass. The optimum quality and quantity of Spirulina through modified CTAB DNA Extraction protocol and the cell length through Image J application.

Inoculum. A concentration of 10 g/l of Spirulina scum were added into the labeled containers representing various pH levels and salt concentrations.

Lighting. Since Spirulina requires lots of sunlight, the experiment was set up outside the laboratory where the right amount of sunlight with minimum direct radiation was ensured. An optimum of 1000 -4500 lux is the best range for growth of spirulina (Sukenik et al., 1991).

Aeration system: Proper aeration is important to meet the CO2 requirement and prevent algae to settle down and form layer at the bottom. Aeration was achieved using manual stirring 3x a day and continuous air pump system. pH and temperature measuring device: PCSTestr 35 multi-parameter instrument and pH strips are used to determine pH value and temperature of media.

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Harvesting. After a month of cultivation, (while maintaining 5L in each of the containers by adding more water), the algal scum was harvested using fine cheese cloth, and the fresh weight was measured for data analysis

DNA Extraction Protocol

Three hundred milligrams (300mg) of pure Spirulina cells were placed in an Eppendorf tube labeled as grinded and non-grinded using a pipette tip. Prior to the grinding of the cells, liquid nitrogen was added to the tube labeled as grinded and none to the non-grinded tube. The tubes were placed in an ice box to ensure the integrity of the cells and the DNA as well. The 600µl of pre-warmed (at 65°C) cationic detergent, Cetyl Trimethyl Ammonium Bromide (CTAB) buffer, then was mixed by inverting tubes several times vigorously. The tubes were then incubated for at least 30 minutes at 65°C using a dry bath. The samples were cooled a bit before adding an equal volume of phenol-chloroform isoamyl alcohol, 24:25:1, and then mix by inverting the tubes several times vigorously.

The samples were incubated for 5 minutes leaving the tubes laid down on the bench, then centrifuged at 13000 xg for 10 minutes at room temperature. The supernatant was transferred to a new 2-ml microfuge tube this time, an equal volume of chloroform isoamyl alcohol, 24:1 was added. The tubes were then mixed by inverting the tube several times vigorously and incubating for 5 minutes leaving the tubes laid down on the bench. After which, the tubes were again centrifuged at a speed of 13000 rpm for 10 minutes at room temperature. The aqueous phase was transferred to a new 1.5 microfuge tube before adding 5µl RNAse a (10mg/ml) and was then incubated for 30 minutes at 37°C. An equal volume of cold 2-Propanol was added, then gently mixed by inversion, and was incubated once more at room temperature for 15 minutes. Tubes were again centrifuged at 13000 xg for 5 minutes. The supernatant, before adding 1ml of 70% ethanol (to wash the DNA pellet) was shaken and tubes were tapped to dislodge the DNA pellet. Once more, the tubes were centrifuged once more at 13000 xg for 2 minutes. Ethanol was decanted and tubes were re-centrifuged for 20-30 seconds to bring down traces of ethanol before removing the remaining liquid using a pipette, taking care not to disturb the pellet. The DNA pellet was allowed to dry for 10-15 minutes (avoid over-drying as the pellet will be hard to dissolve) in the fume hood. The DNA pellet was reconstituted in 70µl TE buffer (1x comprised of Tris-HCl and EDTA, pH 8.0). At this point, the samples were kept on ice. The quality and quantity of the 2µl genomic DNA were checked and recorded using the Nanodrop. The samples were stored in a -20°C freezer for further use.

Measuring the Length of Spirulina Cell

A drop of Spirulina culture, from each replicate per treatment was placed on a glass slide and was covered with a coverslip to keep the specimen flat while under microscopic examination. Using a compound microscope (Olympus) and accessories, the pictures of the Spirulina cells were taken at ROI conditions of Height – 800 µm, Width - 800µm, 20x magnification optics, and 30ms. The images taken were measured using the Image J Application. Before, measuring, a calibrated image was used to standardize the calibration. Ten images per treatment of both the rod-shaped and spiral-shaped were randomly taken and measured.

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Determination of quantity and purity of Spirulina DNA Concentration

DNA concentration was quantified using Nanodrop (ND-100) spectrophotometer. To calibrate the Nanodrop, about 1 µl of molecular biology grade water was pipetted onto the nozzle of the machine while the arm was closed to initiate spectral measurements using the operating software in the computer. When a prompt of "load your sample" on the screen was flashed, 1µL of DNA samples was pipetted on the nozzle while the arm was closed to determine the DNA concentration sample expressed in ng/µL. The purity of the DNA was determined by the A260:A280 (1.8-2.0). The quantity and quality of the DNA were detected by spectrophotometer while the integrity was validated using 1% agarose gel electrophoresis stained with SYBR safe.

DNA Analysis by Agarose Gel Electrophoresis

The genomic DNA was analyzed using 1% agarose gel electrophoresis in 1X TAE buffer (0.04M Tris-HCl pH 8.0, 0.04M acetic acid, 0.001M EDTA pH 8.0) as described by Sambrook et al. (1989). A 10µL sample was loaded with 5 µL loading dye (0.25% bromphenol blue and 30% glycerol) and was loaded in the gel wells. Electrophoresis was performed at a constant 130 V for 45 min. After electrophoresis, the gel was visualized by UV light trans-illumination, with Flour S™ MultiImager (Bio-RAD). The size of the DNA was compared against the 1 Kb ladder, of which 8 µL will be loaded in the gel before the samples.

RESULTS AND DISCUSSION

Determination the pH Levels and Salinity Concentration of Spirulina Culture

Table 1 shows the fresh weight of the Spirulina in grams in different pH levels, 8, 9, and 10, respectively.

Table 1: Fresh weight of Spirulina in different pH Levels

Treatment	Fresh weight (g)
T1 – 8	75.43 *a*
T2 - 9	67.47 *ab*
T3 – 10	59.43 *b*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD.

Spirulina best thrives in alkaline and saline solution (Vonshak, 1997). However, how alkaline and how saline the liquid culture media is posing a concern of interest. Based on the results, pH 8 significantly rendered the highest yield (75.43g) of fresh Spirulina biomass, although it is equally comparable with the biomass harvested in a culture media of pH 9 (67.47). Bicarbonate salts increase the pH level of the freshwater liquid media. Spirulina feeds on the bicarbonates which explains the alkaline requirement in the liquid media. However, increasing the pH level of the media causes apoptosis. This may be attributed to the hypertonic reaction of the Spirulina cells.

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Table 2: Fresh weight of Spirulina in different salinity concentrations (% w/v)

Treatment	Fresh weight (g)
T1 – 1%	59.73 *c*
T2 – 3%	69.97 *b*
T3 – 5%	84.30 *a*
T4 – 8%	0.00 *d*
T5- 11%	0.00 *d*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD

Data in Table 2 reveals that Spirulina cells thrive in saline freshwater conditions. In addition, results show that from among the salt concentrations, the Spirulina biomass is significantly high at 5% salt concentration. The data denotes that the 5% (w/v) salt concentration is the optimum salt requirement for Spirulina cell growth. Obviously, when the salt concentration is increased from 8% and above, cell death of Spirulina is observed. Apoptosis may be attributed to the osmotic reaction of the cell in which in this case, a hypertonic reaction is observed.

Determination of the length of Spirulina based on the Different pH levels

Table 3 shows the lengths (µm) of the rod-shaped and spiral-shaped of Spirulina cell cultivated in different pH levels.

Table 3: Size in length (µm) of t rod-shaped and spiral-shaped of Spirulina cell cultivated in different pH levels

pH Level	Rod-shaped Spirulina Cell (µm)	Spiral-shaped Spirulina Cell (µm)
T1- 8	626.75 *a*	356.67 *a*
T2 - 9	494.64 *a*	348.53 *a*
T3 - 10	240.44 *b*	135.08 *b*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD

Spirulina platensis is morphologically rod-shaped or spiral-shaped. In this study, both forms were observed (Figure 1). Whether both forms were of the same species or not, that information is not included in this study. Although there are images that may provide information that both forms are of the same species; the rod-shaped Spirulina cell during the log/exponential growth phase and becomes spiral when it reaches the mature and reproductive stage (Figures 2 and 3). Interesting findings these may seem. However, until proven with substantial evidence, then this study may be the first to report such a phenomenon.

The length of both forms was measured using the Image J Application. Based on the results, the rod-shaped Spirulina cells are significantly longer in pH 8 and 9. This result may explain the highest yield of Spirulina biomass that was harvested as shown in Table 1. In terms of the Spirulina spiral-shaped cell growth (length), pH 8 and 9 also rendered the optimum growth of Spirulina cells.

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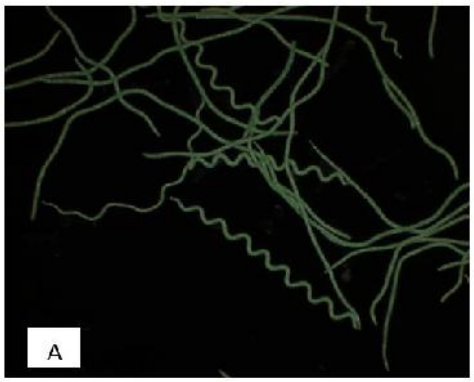

Figure 1A: Rod-shaped and Spiral-shaped Spirulina cells at 10x magnification

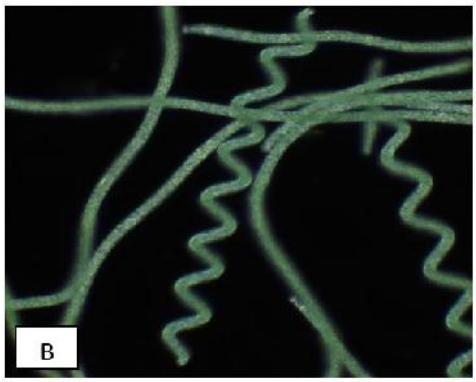

Figure 1B: Rod-shaped and Spiral-shaped Spirulina cells at 20x magnification

Figure 2: Rod-shaped Spirulina cells at 20x magnification

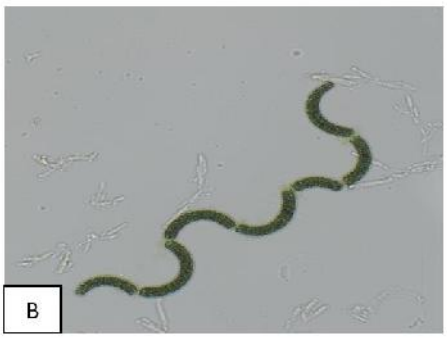

Figure 3: Fission of spiral-shaped Spirulina cells at reproductive stage at 20x magnification

Determination of the size in length of Spirulina based on the different salinity concentration

Table 4 depicts the size in length of Spirulina cells cultivated in different salt concentrations for both the rod-shaped and the spiral-shaped Spirulina cells.

Table 4: Size in length (µm) of t rod-shaped and spiral-shaped of Spirulina cell cultivated in different salinity concentration (% w/v)

Salinity Concentration (%w/v)	Rod-shaped Spirulina Cell (µm)	Spiral-shaped Spirulina Cell (µm)
T1- 1%	568.75 *a*	140.78 *b*
T2 – 3%	239.69 *b*	128.28 *b*
T3 – 5%	224.35 *b*	193.68 *a*
T4 – 8%	0.00 *c*	0.00 *c*
T5 – 11%	0.00 *c*	0.00 *c*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD

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Results in Table 4 reveal that the cell growth of rod-shaped Spirulina in length (μm) is significantly high at 1% salt concentration while 5% with that of the spiral-shaped Spirulina cells. The data may depict that since Spirulina feeds on bicarbonate salts, additional chloride salts may seem to supplement the bicarbonates which is why the cell growth of the rod-shaped Spirulina requires a minimal amount of other sources of salts. In the instance of the spiral-shaped Spirulina cell, this form required a much higher concentration of salts (5%) than that of the rod-shaped. If this study hypothesizes that the spiral-shaped is the reproductive stage, hence it can be interpolated that the additional salts source is much needed in preparation for the cell fission of the Spirulina (Figure 3).

Quantity and Quality Analysis of Genomic DNA of Spirulina

Table 5 shows the quality and quantity of the DNA of Spirulina using spectrophotometer and Agarose Gel Electrophoresis.

Table 5: Quantity and Quality of the Genomic DNA of Spirulina between Grinded and Non-grinded preparation of Spirulina Cell

Spirulina Cell Preparation	Nucleic Acid Concentration (nm/μL)	A260/A280 Absorbance (nm)
T1- Grinded	116. 5	1.95
T2 – Non-Grinded	437.8	1.94

The table shows the purity of the DNA extracted from Spirulina cells. Between the two preparations, the non-grinded yielded a greater amount of nucleic acid concentration as compared with the grinded. The grinding may have ruptured the cell wall as well as the nucleus where the DNA is found which is why the amount of the nucleic acid is lower than that of the non-grinded. In terms of the quality of the DNA, the purity of the DNA from both preparations is almost pure or of good quality as the DNA A260/280 ratio obtained was at the absorbance of 1.95 nm for the grinded and 1.94 nm for the non-grinded preparation. When the A260/280 ratio of DNA is ~ 2.0, the DNA is considered pure. These ratios are commonly used to assess the amount of protein contamination that is left from the nucleic acid isolation process since proteins are absorbed at 280 nm.

CONCLUSIONS

Spirulina platensis, a filamentous blue-green algae that is cultivated in the Tarlac Agricultural University thrived in an alkaline and saline freshwater medium, which is similar to other *Arthrospira sp*. That is found worldwide. Two forms of Spirulina cells, rod-shaped and spiral-shaped cells were observed under a microscope. This finding suggests a hypothesis that these two forms may be of the same species and represent two developmental stages. Based on microscopical studies, the rod-shaped Spirulina cells may be in the log or exponential phase whereas the spiral-shaped cells may be in the reproductive or lag phase. However, until this hypothesis is further substantiated, no conclusive findings may be reported. Paradoxically, if this finding may be proven true, then this study is the first to report this phenomenon based on our knowledge. Despite the differences in forms, both exhibit optimal growth at pH 8 and 9 and 1% and 5% (w/v) salt concentration. The preparation of Spirulina cells for DNA extraction

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plays a crucial role. Mechanical stress like grinding using pipette tips may not seem to yield a high amount of nucleic acid concentration, although it gives a pure DNA A260/280 ratio. The denaturing agent is sufficient to rupture the cell wall of the Spirulina cells.

Conflict of interest

The author declares No conflict of interest to this manuscript.

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AGRICULTURAL CHEMISTRY AND FERTILITY OF SOILS

Delineation of Micronutrient Deficient Zones in Agricultural Soils of Santa Ignacia, Tarlac

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Abstract—Delineation of nutrient-limited zones in agricultural soils is delicate in achieving balanced fertilization and appropriate land management. Due to the lack of detailed information regarding micronutrient availability in Santa Ignacia, Tarlac, Central Luzon Philippines, a study was conducted to investigate the spatial distribution of Zn, Cu, Fe and Mn in the municipality. Soil test results were subjected to descriptive statistics and geo-analytical technique through best fit semivariogram based on highest coefficient of determination. Then, ordinary kriging was employed using the optimum model to generate spatial variability maps and eventually delineate nutrient limited areas. Micronutrient concentration in the area followed the order Fe > Mn > Cu > Zn with moderate to strong variability. Spatial dependence factors (SDf) were found to be moderate for Zn and Cu, while strong SDf were recorded for Fe and Mn. Zn deficient zones accounting to 2517 ha were delineated in the extreme northern and southern region of the municipality. Cu deficient zone covering 1893 ha was delineated at the east-central region of the study area. Meanwhile, no limited zones were delineated for Fe and Mn indicating the relative abundance of these nutrients in Santa Ignacia, Tarlac. Therefore, interventions to increase Zn and Cu availability in deficient zones are recommended to further improve crop yields in the municipality.

Keywords: kriging, micronutrient, semivariogram, spatial variability, Inceptisols

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INTRODUCTION

Essential micronutrients primarily serve as catalyst in enzyme systems and are required in numerous metabolic functions [37]. Although they are needed in minute amounts, the deficiency or abundance of these elements directly affects crop nutrition and environmental pollution [4, 10]. Intensified crop production in past years have resulted in the decline of fertility and overall quality of soils in the Philippines. The Department of Agriculture and prior research have reported cases of micronutrient deficiency in agricultural soils, though its distribution has not been thoroughly analyzed and studied [24, 31]. The lack of micronutrient management system employed in farmlands coupled with indiscreet use of chemical inputs have caused the imbalance of soil nutrients [48]. These issues amplify the call to conduct investigation on the fertility status of soil resources in the country.

The interplay of weathering process, natural events and anthropogenic factors may generate levels of spatial variation among trace elements in different agroecosystems [17, 41]. Zonal delineation on the availability of soil nutrients is a key element of precise agriculture. The identification of nutrient limited and sufficient zones is required to formulate an appropriate and site specific nutrient management scheme [36]. Although fertility maps are available in the region, it is limited to some macronutrients namely, nitrogen, phosphorus and potassium. Characterization and distribution of trace elements in local soils are often neglected. Thus, there is an urgent need to precisely describe the spatial distribution of micronutrients in the study area.

In this regard, geostatistical techniques have become a standard in the analysis of spatial differentiation concerning soil nutrients. Integrating spatial information in geostatistical models can refine estimation and improve map accuracy [26]. Among the various geostatistical methods employed by the researchers for generating soil fertility maps, ordinary kriging remains to be the most commonly utilized interpolation technique [6, 36, 44]. It has the potential to provide the best unbiased linear predictions and generate information regarding estimation errors while reducing the local error variance [27]. It is proven to be fast, cost-efficient and reliable method of producing thematic maps that will serve as basis for appropriate land management. Therefore this study aims to (i) assess the level of micronutrient elements (Zn, Cu, Mn, Fe) in the study area and (ii) delineate micronutrient deficient zones using optimal semivariogram (ordinary kriging) models.

Table 1. Micronutrients rating based on soil test values, mg kg^{-1}

Soil test	Deficient	Sufficient		
		moderate	high	extremely high
Zinc	<0.5	0.5–1.0	1.0–3.0	>3.0
Copper	<0.2	0.2–1.0	1.0–1.8	>1.8
Iron	<4.5	–	–	–
Manganese	<5.0	5.0–15.0	15.0–30.0	>30.0

MATERIALS AND METHODS

Site description. The study was conducted in the Municipality of Santa Ignacia, Tarlac. It is within the Central Luzon region of the Philippines. The capital of the area is geographically located at 15.5841° N and 120.4688° E. It is bounded on the north by the town of Camiling, on the south by San Jose, on the west by Mayantoc and on the east by Gerona, Tarlac. The municipality has a relatively flat terrain in the central and eastern portion, while moderately hilly on the western side. The total land area is 14 189 ha, and most of it is involved in agriculture (Fig. 1). It produces a wide range of crops, including rice, fruits and vegetables. The dominant soil type in the area is fine loamy textured inceptisol characterized by isohyperthermic temperature regime with incipient development towards maturity but not fully developed diagnostic horizons. This soil has minimum complexity towards horizonation which is commonly found in areas with pronounced wet and dry seasons [5, 33]. Soil pH in the area ranges from extremely acidic to slightly alkaline while organic matter (OM) varies from low to medium levels. Finally, the levels of phosphorus (P) and potassium (K) range from low to high concentrations. Based on Corona Climate Classification, Santa Ignacia belongs to Climate Class I which has pronounced wet and dry periods. Annual average precipitation sits at 715 mm where maximum rainfall occurs in the months of July and August. Temperature in the area ranges from 22–31°C, maximum temperature occurs during April and May while minimum temperature is felt in the months of January and February.

Soil sampling and analysis. A total of 336 soil samples at a depth of 0–30 cm were randomly collected in the study area. The geographic location of each sampling point was recorded using a handheld GPS. Distribution of sampling points is shown in Fig. 1. Each soil sample was air dried while impurities were removed by hand before passing through a 2 mm mesh and eventually stored in a clean plastic container. Micronutrients including Fe, Cu, Zn and Mn were analyzed through diethylenetriaminepentaacetic acid extraction–atomic absorption spectrometry (DTPA-AAS) using of 1 : 2 soil:extractant ratio as described in FAO–Standard Operating Procedure for Soil Available Micronutrients and Heavy Metals [8, 18]. In addition, soil pH, organic matter and phosphorus were also analyzed using 1 : 1 soil and water suspension [12], Walkley and Black [45] and Olsen extractant method [30], respectively. Soil test values were interpreted and clustered based on micronutrient fertility standards of Philippine Council for Agriculture and Resources Research and other relevant literature [36, 48].

Data processing and analysis. Descriptive statistical analysis was executed using SPSS 20.0 software. Semivariance analyses and model fitting were conducted using GS + 9.0 software. Models with maximum coefficient of determination (R^2) were identified as optimal model parameters [16]. The best fit model was then used as basis for ordinary kriging interpolation using ArcGIS 10.4 (ESRI, Redlands, CA, USA). Semivariance is expressed as follows:

$$r(h) = \frac{1}{2N(h)} \sum_{i=1}^{n(h)} [z(Xi) - z(Xi + h]^2,$$

where $r(h)$ is the semivariogram, h is the separation distance, $N(h)$ is the pairwise number of data points separated by distance h, $Z(x)$ is the value of a regionalized variable at spatial position x, and $Z(x + h)$ is the value of a regionalized variable at position $x + h$. Nugget, sill and range are key parameters of semivariogram models to describe spatial structure. Furthermore, the nugget/sill ratio and spatial dependence factor (SDf) represents the spatial autocorrelation between variables. SDf < 25% indicates high spatial correlation, in which natural factors are the principal drivers; SDf = 25–75% represents moderate spatial correlation which might be affected by both natural and anthropogenic factors; and SDf > 75% indicates low spatial correlation, where the variation is attributed to random factors [36, 48].

RESULTS AND DISCUSSION

Statistical characteristics of soil micronutrients. Descriptive statistics of the soil test values are presented in Table 2. The micronutrient concentration in the area followed the order Fe > Mn > Cu > Zn. The Zn level in the study area ranges from 0.06–21.16 mg kg^{-1} with a mean value standing at 1.21 mg kg^{-1}. It means that Zn concentrations are qualitatively classified as deficient to extremely sufficient. The Cu concentration ranges from 0.02 to 41.09 mg kg^{-1} with a mean value at

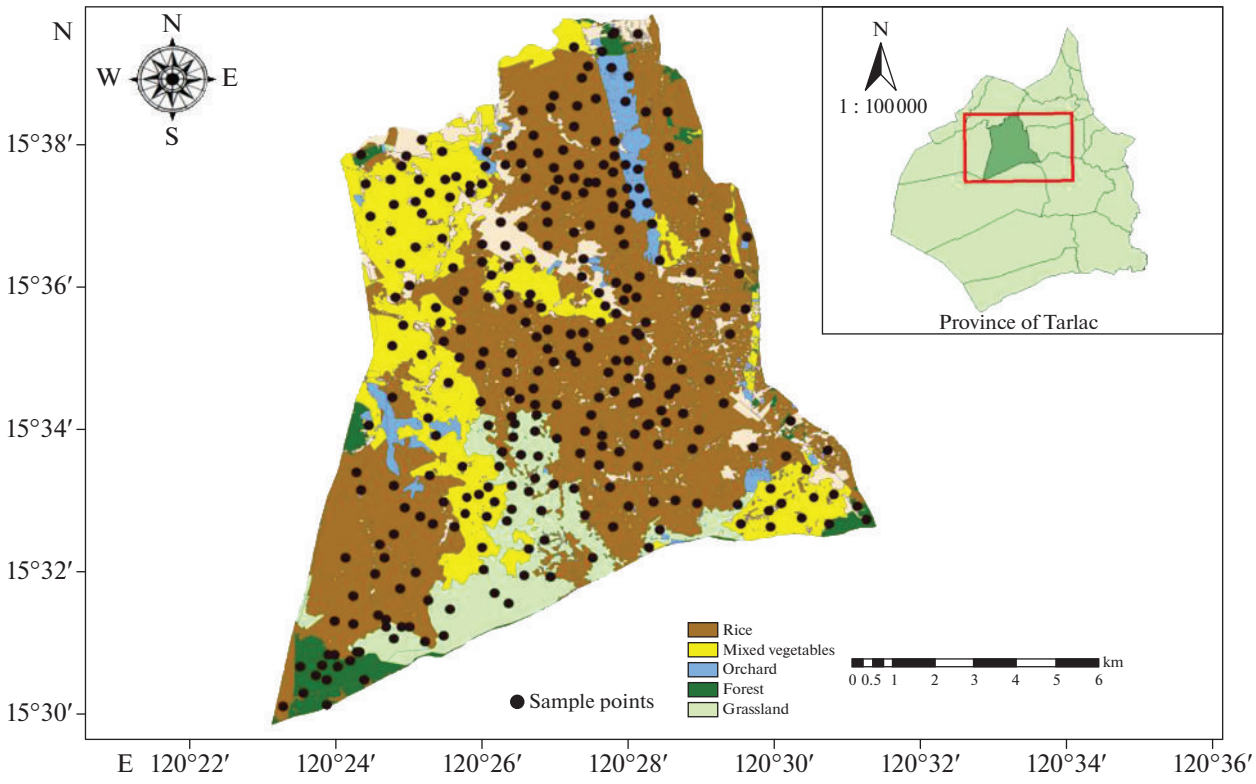

Fig. 1. Land use map and distribution of sampling points in the study area.

5.58 mg kg^{-1}. Likewise, clusters of soil test results are classified as Cu deficient to extremely sufficient. The levels of Fe and Mn ranges 13.97–237.28 and 15.95–246.97 mg kg^{-1} respectively. These values are considered extremely sufficient, which indicates the relative abundance of Fe and Mn in the study area.

One of the very important statistical indices to reflect spatial variation is the CV. A CV of >1 is considered to be strong, while a CV of 0.1–1 and <0.1 is considered moderate and weak variation, respectively [25, 26, 31]. The CV of the subject elements is considered moderate to strong which ranges from 0.6 to 1.97. The order of variation among the nutrients is recorded as Zn > Cu > Mn > Fe. The considerable variation of micronutrients is a common finding especially in agriculture based ecosystems. Aside from parent materials, the nutrient variability in agricultural soils may be attributed to different land utilization types which requires varying fertilizer application, pest control, and other crop management systems [16, 46]. Taking into account the lack of micronutrient fertilization guide and indiscreet use of chemical inputs, it is not surprising to observe moderate to strong variability of soil nutrients in the study area.

Aside from soil micronutrients, different edaphic properties in the study area were also analyzed to determine their possible relationship. The correlation map of Zn, Cu, Fe, Mn, P, K, pH, and OM is summarized in Table 3. Cu concentration is correlated with pH and OM level. Cu concentrations vary with soil pH due to the dissolution of Cu insoluble composts or adsorption on the surfaces of Fe and Al oxides or precipitated in hydroxyl forms depending on the level of acidity [28]. Furthermore, OM has a dynamic relationship with the mobility of Cu in soils. The complex-

Table 2. Descriptive statistics of essential micronutrient elements, mg kg^{-1}

Elements	Max	Min	Mean	Std. Dev.	Skewness	Kurtosis	CV
Zn	21.16	0.06	1.21	2.39	7.18	58.71	1.97
Cu	41.09	0.02	5.85	7.24	2.38	7.15	1.23
Fe	237.28	13.97	89.11	53.13	0.87	0.13	0.60
Mn	246.97	15.95	60.39	43.58	1.61	3.32	0.72

Std. Dev, Standard Deviation; CV, Coefficient of Variation.

Table 3. Correlation matrix of soil trace elements

Variables	pH	OM	P	K	Z	Cu	Mn	Fe
pH	**1**	**0.463**	–0.148	0.185	0.083	**–0.421**	–0.195	**–0.380**
OM	**0.463**	**1**	–0.114	0.119	0.095	**0.337**	–0.100	–0.091
P	–0.148	–0.114	**1**	–0.015	0.133	–0.094	–0.196	0.142
K	0.185	0.119	–0.015	**1**	–0.045	0.081	–0.056	0.036
Z	0.083	0.095	0.133	–0.045	**1**	**–0.216**	**–0.217**	–0.088
Cu	**–0.421**	**0.337**	–0.094	0.081	**–0.216**	**1**	0.185	–0.019
Mn	–0.195	–0.100	–0.196	–0.056	**–0.217**	0.185	**1**	0.062
Fe	**–0.380**	–0.091	0.142	0.036	–0.088	–0.019	0.062	**1**

Values in bold are different from 0 with a significance level $\alpha = 0.05$.

Table 4. Geostatistics of the fitted semivariogram models of micronutrients

Elements	Model	Nugget	Sill	NSR	R^2	SpD
Zinc	Spherical	1.25	2.26	0.55	0.73	55.30
Copper	Spherical	1.19	1.92	0.62	0.69	61.90
Iron	Exponential	115	483	0.23	0.81	23.80
Manganese	Exponential	80	423	0.18	0.89	18.91

NSR—nugget to sill ratio; R^2—R-squared; SpD—spatial dependence factor.

ation of OM with Cu is an important mechanism in its retention and bio-availability [25]. Meanwhile, Fe is negatively correlated with pH. Fe is commonly affected by pH due to the latter's direct effect on the precipitation of soluble Fe into insoluble forms [13]. Interestingly, Zn has negative correlation to Cu and Mn, which suggests negative interactions among these nutrients. Alloway [2] reported that the relationships between Cu and Zn are often antagonistic because of their competitive adsorption on soil colloids and absorption sites of plant roots. Cu fertilization has been shown to alter Zn dynamics in soil or vice versa [20, 21]. Interestingly, Rahman et al. [35] reported that Cu and Zn were mostly speciated as carbonate phases under P-deficient condition. The complexation of these elements with carbonate and phyllosilicate minerals is likely the controlling factor of their bio-availability. In addition, Vasu et al. [43] identified antagonistic nutrient interaction as one of the primary drivers of the concentration and spatial characteristics of micronutrients under semi-arid tropical environment. Thus, co-fertilization trial of Cu and Zn may be explored to further characterize the antagonistic behavior of these nutrients in local soils of Santa Ignacia.

Spatial structure and variability of micronutrients. Spherical model was the optimum semivariogram function for Zn and Cu, while exponential model was optimum for Fe and Mn. The highest coefficient of determination (R^2) was observed for Mn at 0.89, and Cu has the least R^2 at 0.69. A strong spatial dependence factor was observed for Fe (23.80) and Mn (18.91). It indicates that spatial variation of Fe and Mn in the study area is primarily affected by soil forming factors, including parent material, terrain and other structural parameters. Same results were reported by Kavitha et al. [16], Shukla et al. [38] and Zou et al. [48] regarding strong spatial dependence of these nutrients. However, contrary results were reported by Odoi et al. [30] indicating weak SpD of Fe and Mn within the industrial zone of Ghana. They identified the discharge of industrial waste as main factor on the spatial structure of such nutrients. Thus, the absence of heavy industrialization and other similar activities in Santa Ignacia might have caused the dominance of structural factors on the spatial characteristics of Fe and Mn.

On the other hand, moderate spatial dependence factor (SpD) was observed for Zn (55.30) and Cu (61.90). Moderate SpD suggest that spatial variation of these nutrients is primarily affected by the simultaneous action of structural and random factors, including land use, crop management and anthropogenic pollution [48]. Moderate spatial dependence was also observed by Shukla et al. [38] and Liu et al. [19] for Cu and Zn respectively. They hypothesized that the spatial characteristics of these nutrients may be attributed to the imbalance utilization of heavy metal containing inputs in crop production. Furthermore, weak SpD for Zn and Cu was even observed in various crop producing areas which suggest the significant effect of farm management on the variability of these nutrients [34, 38]. Such findings on spatial autocorrelation indicate the need to conduct a deeper analysis on the specific causes of spatial variability of soil micronutrients in the study area.

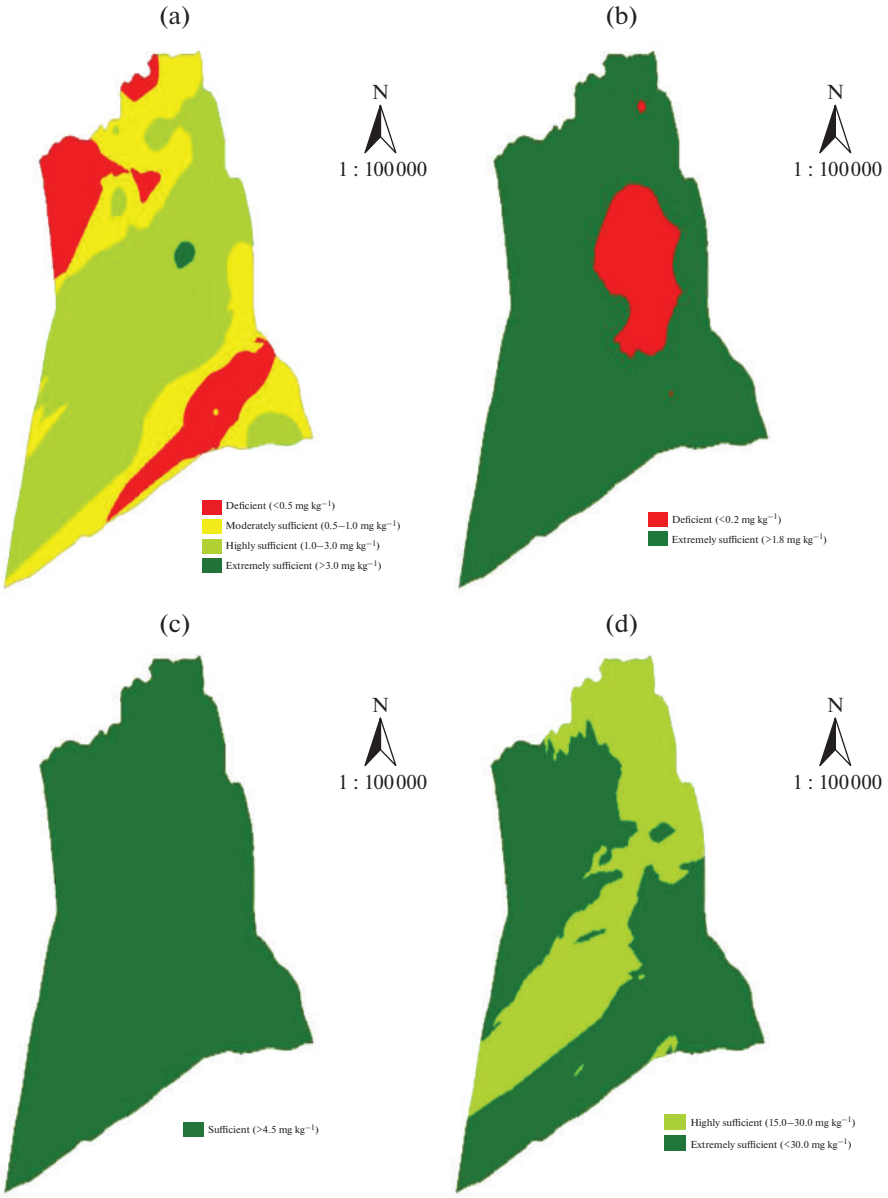

Fig. 2. Spatial variability map of micronutrients (a) zinc, (b) copper, (c) iron, (d) manganese.

The spatial variability of micronutrient concentration in the study area is displayed in Fig. 2. Zn deficient zones accounting to 2517 ha were delineated in the extreme northern and southern region of the municipality, while the remaining portion varies from highly to extremely sufficient. The application of zinc sulphate is recommended to correct the deficiency in the concerned areas. Under flooded conditions, zinc

Table 5. Distribution of micronutrient availability classes

Fertility class	Zn		Cu		Fe		Mn	
	area, ha	%	area, ha	%	area, ha	%	area, ha	%
Deficient	2517	18	1893	13	–	–	–	–
Moderate	3469	24	–	–	–	–	–	
High	8061	57	–	–	14189	100	5788	41
Extremely High	142	1	12296	87	–	–	8401	59

solubility often decreases due to mechanisms associated with soil redox status [1, 3]. Since paddy rice is the dominant crop in the area, water management system to improve aeration and drainage may be employed to increase zinc solubility [13]. Other options, such as utilization of ammonium sulfate and applying organic fertilizer to further increase the availability of zinc may also be considered [11]. Cu deficient zone covering 1893 ha of the study area was delineated at the east-central region. The remaining portion of the area is classified as highly sufficient for Cu. The addition of CuO or $CuSO_4$ in deficient areas is recommended to balance Cu levels. The amendment of Cu-enriched foliar fertilizer and elemental sulfur is likewise recommended to increase Cu absorption by plants [11, 14]. Finally, no deficient zones were delineated for Fe and Mn. The levels of these nutrients are found to be highly sufficient in the study area.

The results of the study concur with prior research regarding micronutrient status of farmlands in the Philippines. Yoshida et al. [47] estimated around 500 000 ha of Zn deficient rice areas alone. Katyal and Vlek [15] also reported Zn deficiency in some hydrosols and gleysols in the country. Mahagud et al. [22] likewise assessed widespread Zn deficiency especially in lowland areas. Furthermore, the Philippines was also included in a group of countries with serious Zn deficiency problem in their agricultural soils [2]. Meanwhile, Descalsota et al. [7] reported Cu deficiency in particular areas of Central Luzon including some parts of Muñoz, Llanera, and Zaragosa in Nueva Ecija; La Paz in Tarlac and Santa Cruz in Zambales. Finally, Mahagud et al. [23] reported high levels of Fe and Mn in agricultural areas of Central Luzon. Toxic accumulations of Fe and Mn (300 and 1000 mg kg^{-1} respectively) are observed in rice plants evidencing the abundance of these elements in the area.

CONCLUSIONS

The study provides the first micronutrient availability map of Santa Ignacia, Tarlac. Moderate to strong variability of micronutrients was observed in the area. Zn (18%) and Cu (13%) deficient zones have been delineated in multiple regions of the municipality. The employment of field measures to increase Zn and Cu availability in deficient areas is recommended to further increase crop yields in the municipality. Meanwhile, Fe and Mn are found to be entirely sufficient suggesting their relative abundance in the study area. Geo-statistical method is indeed a time and cost-effective tool in mapping various soil properties. Finally, the results of this study can be used to formulate site specific nutrient management and other relevant land policies in Santa Ignacia, Tarlac.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in the conduct of this research.

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Hatchability of duck eggs as affected by types of incubators under varying relative humidity

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Abstract The mean percentage hatchability was not significantly affected by types of incubators, two levels (70% and 80%) of relative humidity, and the interaction effect of types of incubators and relative humidity. However, the percentage of egg hatching was significantly affected by types of incubators such that the means of the percentage hatch in Cabinet-Type Electric Incubator (M=43.95%) and in Bamboo or "Garong"-Type Incubator (M=41.88%) were significantly higher from "Lawanit" Board-Type Incubator (M=27.68%). There was no significant difference in the percentage hatch as affected by two different levels of relative humidity and the interactions of types of incubators and varying percent relative humidity. In this study, the use of Bamboo or "Garong"- Type Incubator indicated the lowest cost (₱0.83) to hatch a duckling, the cheapest (₱17.70) to produce a duckling, and highest ROI of 2.00%.Thus, the Bamboo or "Garong"-Type Incubator was the most economical to use among the three types of incubators.Among the three types of incubators under the two levels of relative humidity, it was observed that the cost to hatch and the cost to produce a duckling is lower under 80% relative humidity with an average cost of ₱1.89 and ₱20.00 respectively. Higher ROI (1.56%) was also observed when the eggs were incubated under 80% RH than 70% RH with an average ROI of 1.54%. Furtheremore, the Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubator were identified to be the most efficient types of incubators. All the hatching parameters were not significantly affected by two levels (70% and 80%) of relative humidity.

Keywords: Bamboo or "Garong" incubator, Duck egg incubation, Parched rice incubation, Rice husk incubation technique

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Introduction

Ducks are commonly raised by rural farmers in the Philippines mostly in Central Luzon and some part of Western Visayas. Around 429,700 families derive their livelihood from it (Santiago, 2018). These farmers, who have less than 100 heads of ducks, contribute to 70% of the ducks in the country (Philippine Statistics Authority [PSA], 2015) as reported by Arrosa (2018). Ducks are preferred by small-holders in the communities compared to other fowls because ducks can adapt and survive under a wide range of climatic conditions, can feed on a variety of feedstuffs, and are resistant to diseases. Raising Mallard Duck (*Anas platyrhynchos*), being known for egg production, provides a good source of income to farmers through its products such as *balut* (embryonated egg) and salted egg. These ducks can also be sold as cull after two years of egg production when their laying performance begins to decline. However, there are some issues and concerns about the duck industry. Results from a farm survey on duck egg production in the Philippines showed that duck farmers generally lack the technical know-how and extension services as well as insufficient supply and high cost of producing good quality ducklings (Chang *et al*., 2008). Most of these problems, particularly on technical and production and supply aspects, are brought about by the issues concerning the type of incubator used and the physical factors to which the eggs were subjected before and during the incubation period.

The duck industry has a promising future given the high demand for salted eggs and *balut* which accounts for 90% of the total egg production in the country (Beltran, 2015). The vast knowledge of local farmers in raising ducks, the availability of complete feeds at different stages of growth (from brooding to laying), and the government programs on strengthening mallard duck production (*Itik Pinas*) all over the country make the duck raising more encouraging. Furthermore, it is one of the special programs of the Bureau of Animal Industry under the Department of Agriculture that aims to contribute to attaining one of the goals of President Duterte on food sufficiency. However, there are other problems with the industry such as the fluctuating prices of eggs, limited space for free-range operations, and inadequate research studies being conducted on duck raising ("Native", 2016).

At present, commercial incubators of varying capacities are being used by *balut* and duck producers in the country. Most of these incubators are operating with electricity for heating and other mechanical functions. However, small-holders experience issues in the cost of procurement, maintenance, and operations. According to Boleli *et al.* (2016), the main focus of research at present is the manipulation of thermal incubation conditions and the integrated

effect of factors that influence incubation. Commercial hatcheries use modern state-of-the-art incubators but one of the questions that need to be answered is how effective and cost-efficient the incubators are in terms of promoting greater hatchability and better chick quality.

Currently, artificial incubators made from locally available materials, using parched rice and rice husk, have been used by hatcheries in Central Luzon and National Capital Region. However, the technology was not widely adopted and little is known about the efficiency of using this type of incubator. Furthermore, literature and studies suggest a wide range of humidity levels inside the incubator and different egg turning frequencies to produce a good hatch. Therefore, it is important to find a method and technique of duck egg incubation that is efficient, less expensive, uses locally available resources, and can easily be adopted by both backyard and commercial raisers.

Thus, this study was conducted to determine the hatchability of duck eggs using different types of incubators under two levels of relative humidity. Also, this study aimed to determine which among the three types of incubators is the most efficient and most economical to be used and be recommended for small-hold, backyard duck raisers, or commercial hatcheries. The results of this study could contribute to the determination of optimum relative humidity which would be needed to attain better hatchability of duck eggs using different types of incubators. Moreover, the results may lead to finding better techniques for hatching duck eggs through the use of locally available resources that would reduce the production cost of quality hatchlings. Through the adoption of technology to be generated, more duck raisers especially the small-hold raisers would be benefited and be able to produce their own ducklings instead of buying them from commercial hatcheries or other suppliers. Furtheremore, the results of this study may be used as a basis for further researches about duck egg incubation.

Materials and methods

Time and place of research

The experiment was conducted at the duck egg hatchery and “balutan” of Mr. Renato C. Ramos in Brgy. Carmen, Zaragosa, Nueva Ecija from June 2020 to July 2020.

Research design

The study was laid out into treatment combinations using a Completely Randomized Design following the 3 x 2 factorial arrangements using three

types of incubators under varying relative humidity. Factor A served as the three types of incubators (Cabinet-Type Electric Incubator, Bamboo or "Garong"-Type Incubator, and "Lawanit" Board-Type Incubator) while the Factor B served as the two levels of relative humidity (70% RH and 80%RH).

Experimental treatments and layout

Five thousand and four hundred duck eggs were used in the study. These were randomly divided into six treatment combinations based on the experimental factor. Each composed of 900 eggs. Each treatment combination was further subdivided into three replications with 300 eggs per replicate.

Setting-up of cabinet-type incubator

Six cabinet-type forced-air electric incubators were used. These were prepared by cleaning especially in the interior area. Thermometers and hygrometers were checked and ensured that these were functioning. The experimental eggs were incubated at a temperature ranging from 37.22-37.78^{o}C.

Setting-up of bamboo or "Garong" and "Lawanit" board incubator

Six "Garong" and six "Lawanit" Board Incubators were also used to complete the types of incubators needed in the study. Each incubator had a diameter of 45-50 cm and a height of 85-90 cm. Nylon net with a size of 75 cm x 70 cm was used to contain 100 eggs and also to contain 1.5 kg of unpolished rice/parched rice as a source of heat.

The rice was heated twice a day to about 42^{0}C to 43^{0}C using a vat or cauldron or "kawa" following the procedures in making *balut* (ATBP.PH, 2016). A pan of water was placed at the bottom of the incubator. Bamboo slats were placed on top of the pan before making a pile of heated rice and duck eggs. Five bags of heated unpolished rice (1.5 kg per bag) and three bags of preheated eggs (100 eggs per bag) were piled in an alternating position having the rice at the bottom and on top when the pile was completed.

The incubators were arranged at a distance of at least four inches from each other. Rice hull was used to fill up spaces between incubators. It served as an insulator and to conserve heat energy.

Egg collection

Large-sized eggs, 65-70 grams, were collected from mated flocks in a selected house on the farm. Eggs laid not later than three days were selected as experimental materials.

Egg setting

The 300 eggs were set in each replication of the three types of incubators. Before setting, eggs were pre-heated under the sun for two to three hours with a temperature ranging from 23.9-26.1^{o}C following the procedures stated in Hatchery Tips (2017).

Incubation duration

Experimental eggs were incubated (with a source of heat) for 15 days. On the 16th day after setting, eggs were transferred onto a table in a closed room (no window) until they were hatched.

Egg turning

Eggs in "Garong" and "Lawanit" board were manually turned two times a day following the procedures in making *balut* by ATBP.PH (2016). On the other hand, eggs in electric incubators were turned by switching on the "turn" button. The eggs were turned twice a day until the 15th day of incubation. From 16 days to hatching, eggs were turned four times within 24 hours.

Candling

The first candling was done on the 10th day after egg setting to determine the number of fertile eggs and infertile eggs. The second candling was done on the 15th day of incubation to select fertile eggs but would fail to hatch due to the following reasons: (1) dead embryo, (2) presence of a red ring or blood around the embryo, (3) enlarged blood vessels, and (4) presence of oozing substance (Smith, 2018).

Relative humidity and temperature control

Relative humidity in the "Garong"-Type and "Lawanit" Board-Type Incubators was controlled by placing a moisture pan inside. Rice was heated two times a day until the 15th day of hatching. After 15 days, rice or palay bags were not heated anymore since the embryos could generate enough heat to keep them warm. However, the humidity in Cabinet-Type Electric Incubator was controlled by placing also moisture pan inside. The temperature was set into 37.5 ^{0}C until 15 days of hatching.

Data analysis

The data from the experiment were subjected to analysis of variance in 3x2 factorial in Completely Randomized Design (Gomez and Gomez, 1984). When significant differences were obtained, means were compared using the Least Significant Difference (LSD) at 5% probability. To facilitate calculations and analysis of experimental data, the computer program Statistical Tool for Agricultural Research was used.

Results

Percentage hatchability

The mean of the percentage hatchability of duck eggs as affected by types of incubators under varying relative humidity is shown in Table 1. The results of the study revealed that percent hatchability of duck eggs was not affected by the types of incubators as indicated by their means (M=48.42%, M=50.40%, and M=37.17%) having no significant difference, $F(2,12) = 2.78$, $p = 0.1020$ when analyzed for variance. The percentage hatchability was not affected also by the two levels (70% and 80%) of relative humidity wherein their means (M=44.98% and M=45.68%) were comparable. As for the effect of the interactions of types of incubators and varying percent relative humidity, it was found out that these interactions did not affect the percent hatchability of duck eggs, $F(2,12) = 1.81$, $p = 0.2057$.

Table 1. Mean of the percentage hatchability of duck eggs as affected by types of incubators under varying relative humidity

Factor A – Types of Incubators	**Factor B – Relative Humidity**		**Factor A Mean**
	70% RH	**80% RH**	
Cabinet-Type Electric Incubator	44.83	52.00	**48.42**
Bamboo or “Garong”-Type Incubator	56.70	44.10	**50.40**
“Lawanit” Board-Type Incubator	33.40	40.93	**37.17**
Factor B Mean	**44.98**	**45.68**	

Percentage hatch

The mean of the percentage hatch of duck eggs as affected by types of incubators under varying relative humidity is presented in Table 2. In this study, the percentage hatch of duck eggs was significantly affected by the types

of incubators, F(2,12) = 5.73, p = 0.0179.The mean percentage hatch in Cabinet-Type Electric Incubators (M=43.95%) and Bamboo or "Garong"-Type Incubators (M=41.88%) was significantly higher than the mean percentage hatch in "Lawanit"Board-Type Incubator (M=27.68%).

However, the percentage hatch was not affected by the two levels (70% and 80%) of relative humidity with which their means (M=38.03% and M=37.65%) were not significantly different, F(1,12) = 0.01, p = 0.9290. As for the effect of the interaction of types of incubators and varying percent relative humidity, it was revealed that these interactions did not affect the percent hatch of duck eggs.

Table 2. Mean of the percentage hatch of duck eggs as affected by types of incubators under varying relative humidity

Factor A – Types of Incubators	Factor B – Relative Humidity		Factor A Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	39.00	48.90	**43.95 a**
Bamboo or "Garong"-Type Incubator	48.00	35.77	**41.88 a**
"Lawanit" Board-Type Incubator	27.10	28.27	**27.68 b**
Factor B Mean	**38.03**	**37.65**	

Note: Means followed by the same letter are not significantly different at 5% level of significance by LSD

The most economical type of incubator

The mean of the average cost to hatch a duckling among three types of incubators for 70% and 80% relative humidity is shown in Table 3. In this study, the lowest average cost (₱0.83) to hatch a duckling was determined when using the Bamboo or "Garong"-Type Incubator. It was lower than the cost of using the "Lawanit" Board-Type Incubator and Cabinet-Type Electric Incubator with the average costs of ₱1.12 and ₱4.11, respectively.

Among the three types of incubators under the two levels of relative humidity, it was observed that the lowest average cost (₱0.75) to hatch a duckling was with the use of Bamboo or "Garong"-Type Incubator under 70% while the highest average cost (₱4.47) was by using Cabinet-Type Electric Incubator also under 70% relative humidity. Furthermore, the cost to hatch a ducking was lower (₱1.89) under 80% relative humidity than 70% relative humidity with an average of ₱2.59.

Table 3. Mean average cost (₱) to hatch a duckling using three types of incubators under 70% and 80% relative humidity

Incubator type	Cost to hatch a duckling (₱)		Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	4.47	3.75	**4.11**
Bamboo or "Garong"-Type Incubator	0.75	0.91	**0.83**
"Lawanit" Board-Type Incubator	1.22	1.01	**1.12**
Mean	**2.15**	**1.89**	

The mean of the average cost to produce a duckling among three types of incubators for 70% and 80% relative humidity is shown in Table 4. The study revealed that the Bamboo or "Garong"-Type Incubator was the cheapest to use among the three types of incubators to produce a duckling with an average cost of ₱17.70. However, a higher average cost to produce a duckling was realized with the use ofCabinet-Type Electric Incubator and "Lawanit" Board-Type Incubator with the average costs of ₱21.04 and ₱23.72, respectively.

Among the three types of incubators under the two levels of relative humidity, it was observed that the lowest average cost (₱16.02) to produce a duckling was by using Bamboo or "Garong"-Type Incubator under 70% while the highest average cost (₱26.02) was by using the "Lawanit" Board-Type Incubator also under 70% relative humidity. Moreover, it was determined that it was cheaper ((₱20.00) to produce a duckling under 80% relative humidity than under 70% relative humidity with an average cost of ₱21.64.

Table 4. Mean of the average cost (₱) to produce a duckling among three types of incubators for 70% and 80% relative humidity

Incubator type	Cost to hatch a duckling (₱)		Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	22.87	19.21	**21.04**
Bamboo or "Garong"-Type Incubator	16.02	19.37	**17.70**
"Lawanit" Board-Type Incubator	26.02	21.42	**23.72**
Mean	**21.64**	**20.00**	

The mean of the average percentage ROI among the three types of incubators for 70% and 80% relative humidity is illustrated in Table 5. Among the three types of incubators, the Bamboo or "Garong"-Type Incubator obtained the highest average percentage return on investment (2.00%) while the "Lawanit" Board-Type Incubator obtained the lowest average ROI (1.21%).

Among the three types of incubators under the two levels of relative humidity, the highest average percentage ROI (2.37%) was obtained when Bamboo or "Garong"-Type Incubator was used under 70%. However, the

lowest average percentage ROI (0.99%) was attained when "Lawanit" Board-Type Incubator was used under 70% relative humidity. On the other hand, higher ROI (1.56%) was realized under 80% relative humidity than under 70% relative humidity with an average ROI of 1.54%.

Table 5. Mean of the average percentage (%) return on investment among three types of incubators for 70% and 80% Relative Humidity

Incubator type	% ROI		Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	1.25	1.62	**1.44**
Bamboo or "Garong"-Type Incubator	2.37	1.62	**2.00**
"Lawanit" Board-Type Incubator	0.99	1.43	**1.21**
Mean	**1.54**	**1.56**	

The most efficient type of incubator

Based on the results of the study, only the percentage hatch was affected by a certain factor - the types of the incubators. The analysis of variance yielded a main effect for the type of incubator, $F(2,12) = 5.73$, $p < .05$, such that the average percentage hatch was significantly higher inCabinet-Type Electric Incubator (M=43.95%) and Bamboo or "Garong"-Type Incubator (M=41.88%) than in"Lawanit"Board-Type Incubator (M=27.68%) (see Table 2). The main effects of humidity and interaction were non-significant, $F(1,12) = 0.01$, $p > .05$ and $F(2,12) = 2.27$, $p > .05$, respectively. Therefore, the most efficient types of incubators are the Cabinet-Type Electric Incubator and the Bamboo or "Garong"-Type Incubator.

Discussion

Commercial incubators of varying capacities are being used by *balut* and duck producers. However, the cost of procurement and operation, and the effectiveness and cost-efficiency in terms of promoting greater hatchability of these incubators are the major concerns needed to be answered. Artificial incubators made from locally available materials are being used by hatcheries in Central Luzon and National Capital Region. However, the technology was not widely adopted and little is known about the efficiency of using this type of incubator. The study was conducted to determine the effect of types of incubators under varying relative humidity on the different hatching parameters.

This study demonstrated that the hatchability of fertile duck eggs was not affected by types of incubators ($p = 0.1020$), by the two levels (70% and 80%)

of relative humidity, and the interactions of types of incubators and varying percent relative humidity (p = 0.2057) when analyzed for variance.This result is parallel with the study of Indarsih *et al.* (2019) which revealed that the sawdust incubator gave similar fertility, hatchability, and embryonic mortality values as the electric incubator. Also, this result is associated with the findings of the study Bruzual *et al.* (2000), whichpointed out that fertile hatchability was optimum when incubated at 53% relative humidity. The findings of Bruzual *et al.* (2000), is supported by Hitchener (2017) and Daniels (2020) who recommended that the ideal relative humidity is at 55%.

The percentage hatch of duck eggs was significantly affected by the types of incubators (p = 0.0179). In this experiment, the mean percentage hatch in Cabinet-Type Electric incubators (M=43.95%) and Bamboo or "Garong"-Type Incubators (M=41.88%) was significantly higher than the mean percentage hatch in "Lawanit" Board-Type Incubator (M=27.68%). Boleli *et al*. (2016) explained that this is because the latter provides better incubation physical conditions such as ventilation, egg turning, and egg position, which may affect hatchability. However, the percentage hatch was not affected by the two levels (70% and 80%) of relative humidity and the interaction of types of incubators and varying percent relative humidity. These results could be correlated to the statement of Paniago (2005) as specified by Boleli *et al.* (2016) that despite the technological advances of modern incubation machines, still, the quality of labor both inside and outside the hatcheries determines the success of incubation.

The most economical type of incubator was determined based on the following aspects: (1) cost to hatch a duckling; (2) cost to produce a duckling; and (3) percentage ROI.In this study, the use of Bamboo or "Garong"-Type Incubator indicated the lowest cost (₱0.83) to hatch a duckling, the cheapest (₱17.70) to produce a duckling, and has the highest ROI of 2.00%.

Among the three types of incubators under the two levels of relative humidity, it was observed that the cost to hatch and the cost to produce a duckling was lower under 80% relative humiditywith an average cost of ₱1.89 and ₱20.00 respectively against ₱2.15 and ₱21.64 under 70% relative humidity. Higher ROI (1.56%) was also observed when the eggs were incubated under 80% RH than 70% RH with an average ROI of 1.54%. Moreover, the determination of most economical type of incubator is greatly affected by the hatchability of fertile duck eggs from the specific incubator. This means that the higher the hatchability of an egg from a certain incubator, the lower the cost that may incur to hatch or to produce a duckling. These results are supported by the results of the experiments conducted by El-Hanoun and Mossad (2008) pointing out that the hatchability of fertile Pekin Duck eggs could be improved

by raising the relative humidity (RH) to 80% during the period of 14-28 days of incubation. Their experiments are related to the study of Onbasilar *et al*. (2014) which revealed that hatchability of set and fertile eggs of Pekin Ducks were higher when incubated at 37.5^0C and sprayed with warm water ($25\text{-}28^0$C) from day 4 to day 25 of incubation.

The most efficient type of incubator was determined only when the types of incubators, relative humidity, and the interaction effect of incubator and humidity have a significant effect on the hatching parameters. These conditions were discussed by Boleli *et al.* (2016) in their article regarding optimizing production efficiency that includes manipulation of thermal incubation conditions and the integrated effect of factors that influence incubation. In this present study, only the percentage hatch was affected by a certain factor, the types of the incubators, which revealed that a significantly higher percentage hatch was obtained in Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubator than in "Lawanit" Board-Type Incubator. In addition, all the hatching parameters were not significantly affected by two levels of relative humidity. Therefore, the effects of 70% and 80% relative humiditiesare comparative.

In summary, the study showed that the Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubator yield a significantly higher number of hatch eggs than "Lawanit" Board-Type Incubator. Also, the Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubators were the most efficient types of incubators. Bamboo or "Garong"-Type Incubator was the most economical (lowest cost to hatch and to produce duckling and highest % ROI) type of incubator to use.

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Climate-Resilient Agri-fisheries (CRA) Assessment, Targeting & Prioritization for the Adaptation and Mitigation Initiative for Tarlac Province

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Abstract. This study is part of the Adaptation and Mitigation Initiative in Agri-fisheries (AMIA) Project of the Department of Agriculture (DA) and International Center for Tropical Agriculture (CIAT) to operationalize the goal of making agriculture and its stakeholders adapt and mitigate the effects of climate change. The outputs of this study were vital in the implementation of the next phase of AMIA: climate-risk vulnerability (CRVA) map and investment brief. The CRVA map was developed to identify the vulnerable areas in Tarlac. The CRVA map considered three factors: climate-risks sensitivity, exposure, and adaptive capacity given a 15-15-70 weighted percentages, respectively. The municipalities of Ramos, La Paz, Bamban and Victoria were identified as highly vulnerable to the effects of climate change. Three investment briefs were prepared for policy makers for possible funding and implementation. They were developed by identifying climate-resilient agricultural practices in the province which are the use of climate smart varieties, crop rotation-minimum tillage combination, and alternate wetting and drying method. These practices were evaluated using the CBA Tool and were found to be sustainable. Social net present value was also determined taking into account the externalities, which was given a value through interview of experts.

INTRODUCTION

The Philippines is highly vulnerable to the adverse impacts of climate change as the country's backbone is agriculture [1]. The effect of intense and longer droughts, increase in temperature, erratic rainfall distribution and natural hazards that affects earth's ecosystem as well as health, livelihood, social systems and economy. Agriculture is one of the most vulnerable sectors on the impact of climate change and farmers who are directly dependent on their lands for survival are the most affected. Climate change mitigation and adaptation to climate change is crucial particularly in agriculture sector, thus, different adaptive strategies are being done to cope up with its impacts. One of the activities in the strategic actions of food security for 2011-2028 is to enhance site-specific knowledge on the vulnerability of agriculture and fisheries [2].

The Adaptation and Mitigation Initiative in Agriculture (AMIA) seeks to enable the Department of Agriculture (DA) to plan and implement strategies to support local communities in managing climate risks – from extreme weather events to long-term climatic shifts. Spearheaded by the DA System-wide Climate Change Office (DA SWCCO), AMIA Phase 1 in 2015-16 to implement activities to strengthen DA's capacity to mainstream climate change adaptation and mitigation strategies locally and in national scale. With AMIA Phase 2, making climate-resilient agri-fisheries (CRA) an operational approach through field-level action that directly involves, and influences on the livelihoods of farming communities is one of the next big task for the program. AMIA2 aims to invest in the launching of CRA communities in Tarlac province as the initial target site for action learning, supported by an integrated package of climate services within a broader food value chain setting. The program is launching an integrated and multi-stakeholder effort to operationalize CRA at the community level in 10 target regions. Successful implementation of AMIA2 at the regional level requires the strong collaboration and support of key research and development institutions within the region. This proposed project enables AMIA2 to establish and mobilize regional teams, each led by a local State University/College (SUC), and in partnership with the corresponding Department of Agriculture - Regional Field Office (DA-RFOs).

Climate-resilient agriculture/agri-fisheries (CRA) aims to achieve food security and broader development goals under a changing climate and increasing food demand [3]. With the three pillars: productivity, adaptation, and mitigation, CRA initiates sustainably increase productivity, enhance resilience, and require planning to address tradeoffs and synergies [4]. To enable to assess, plan and pilot climate-risk prone agri-fisheries communities in pursuing sustainable livelihoods while effectively managing the impacts of climate variability. Thus, one of the Region to initiates the CRA strategic framework was Region 3 or Central Luzon specifically in

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Tarlac province to identify key climate risk and vulnerable areas and to assess the current status of CRA as well as the cost and benefits of these practices and technologies. Therefore, the study aims to establish and mobilized team in Region III for AMIA 2 in order to operationalized AMIA strategies in managing climate risk in Tarlac province. Specifically is to enhanced capacities of AMIA partner organizations in the Region, developed geospatially referenced data on climate-risks in Tarlac, generate profile on community's CRA strategies, and perform costs-benefits & trade-offs for these CRA practices. The result of the study will serve as guide in piloting community action research in establishing community-level research and development interventions.

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MATERIALS AND METHODS

Study Area

The study was established in the province of Tarlac. The study covered an area of 273,660 hectare and is located between latitude 15º 10'15" N to 15º 52'52" N, longitude 120º 8'4" E to 120º 46'27" E. The study area has flat to undulating topography, with the eastern part of the province being plain and the western part to be hilly to mountainous. Tarlac has two distinct seasons, the wet and the dry seasons. It has unimodal rainfall pattern, having high monsoon peaks in the wet season (WS) and negligible rainfall in the dry season (DS). Recorded annual rainfall varies from 2,030 mm to 4,060 mm in the northwestern portion [5].

Tarlac is basically an agriculture-based economy, located in the heart of Central Luzon with a total land area of 305,345 ha, constitutes 16.75% of the regional land area and 1.0 % of the total national land area with 112,997.57 hectares concentrated on agricultural production. Rice and corn are the top 2 commodities planted in the province planted in 2 to 3 cropping a year. There are 102,178.06 ha planted to rice, which are irrigated, rainfed and in upland areas. On the other hand, there are 16,458.98 ha planted to corn. Of these, only a small portion, are planted with white corn while the rest are planted with the yellow corn. With this vast track of land concentrated in agriculture, Tarlac likewise grows lowland vegetables and root crops. Of the lowland vegetables grown in the region, tomato occupies the largest area with 215.81 has while sweet potato is the largely grown rootcrops with a production area of 3,641.58 ha. Both crops are grown after rice usually during the onset of the dry season when rice has been harvested. Orchard occupies 10,498.65 hectares planted with our local fruit trees. The most common is mango that is planted in an area totaling to 25,660.03 hectares [6].

Framework of AMIA 2 Project

The project seeks to contribute to the overall AMIA2 program framework, by contributing specific outputs to targeted national-level research projects. It has four key components: (1) Capacity strengthening for CRA research & development, (2) Geospatial assessment of climate risks, (3) Stakeholders' participation in climate adaptation planning, and (4) Documenting & analyzing CRA practices. These project components were designed to be directly aligned with the research agenda of three AMIA2 projects: 1) climate-risk vulnerability assessment (CRVA), 2) decision-support platform for CRA, and 3) institutional and policy innovations. Figure 1 shows the framework for this study.

Component 1 - Capacity strengthening for CRA research & development

The regional project team participated in a series of trainings, workshops and learning events organized by AMIA2 projects. These were focused on three key methodologies: 1) CRVA, 2) CRA prioritization, and 3) CRA M&E. The project provided training support to key research and development stakeholders in the region, by organizing an intra-regional training that covers key learning contents from the national-level trainings. The CRA monitoring and evaluation was later included in the phase two of the AMIA2 which is currently handled by the Department of Agriculture Regional Field Office 3 in Victoria, Tarlac. The town was one of the identified vulnerable areas in the province.

Component 2 - Geospatial assessment of climate risks

The project team collected and organized geo-referenced data on vulnerability to climate risks of the region's agri-fisheries sector. These datasets, from both primary and secondary sources, were used on the methodological guidelines provided by the AMIA2 CRVA project – covering climate-risk exposure, sensitivity and adaptive capacity. Preliminary analysis – using GIS software and climate modelling tools – was undertaken at the regional level. The project team also participated in a national-team level joint analysis of cross-regional data.

Component 3 - Stakeholders' participation in climate adaptation planning

The regional project team organized a series of stakeholders' meetings and focus group discussions to collect supplementary data and validate preliminary results of CRVA, as well as in identifying CRA prioritization and planning. These activities were guided by process facilitation using the MaxEnt and CBA Tool developed by the AMIA2 projects on CRVA and CRA decision-support platform.

Component 4 - Documenting & analyzing CRA practices

A semi-structured survey with local stakeholders was conducted to identify and document CRA practices, as well as collect existing CRA-relevant statistical and other secondary data. Focus group discussion with farmers, farmer-leaders, technician, municipal and provincial agriculturists, and representative of provincial government of Tarlac were done on four separate occasions to gather data, validate and present the results, and revalidation of results. These data was systematized and analysed using cost-benefit and trade-off analyses tools as input to AMIA2 CRA prioritization and investment planning. A CBA Tool was made available online by the CIAT to facilitate the computation of cost, benefits, and others. These contributed to developing knowledge products, such as searchable online portal, under the AMIA2 project on CRVA decision-support platform.

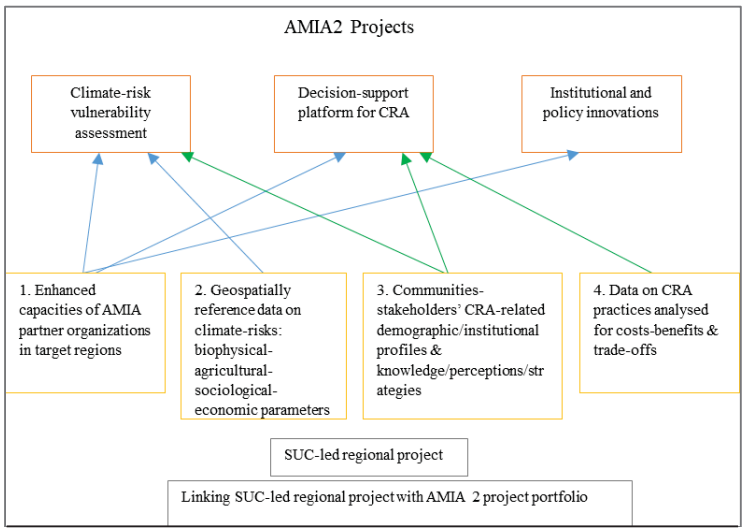

FIGURE 1. Framework of the Study

Data Acquisition

Collection of secondary data for exposure-sensitivity and secondary-primary data for adaptive capacity was done from different agencies such as the Provincial Agriculture Office, Municipal Agriculture Offices, and data from the AMIA2 outputs. Data collection started with gathering of primary and secondary data, that includes those from focus group discussion (FGD), key informant interviews (KII) and municipality surveys.

CRVA Framework

The vulnerability mapping was done which follows the Climate-Risk Vulnerability Assessment (CRVA) framework. It starts with the identification of the vulnerability determinants (hazard, sensitivity, and adaptive capacity) and their respective indicators. This framework was the standardized procedure followed by different SUCs involved in the project. Data collection started in gathering the primary and secondary data, that includes those from focus group discussion (FGD), key informant interviews (KII) and municipality surveys. The identified priority crops of the province are rice, corn, tomato, and mango. Sweet potato was an additional crop prioritized by the team because it is one of the crops planted on the second cropping season after rice and corn.

CRA Practices

Key informant survey on climate-resilient agri-fisheries (CRA) practices in the province of Tarlac. These were gathered from a series of Focus Group Discussions with municipal agriculturists, representatives of the Office of the Provincial Agriculturists and Local Farmer Technicians. From the list of CRA practices, key

informants identified three priority practices. The interview guide provided by CIAT was modified to fit the scope of the study before it was given to the group of key informants. Data gathered were used in the evaluation of CRA practices using the CBA Tool. CRA practices with the incorporation of externalities was given priority in the study.

RESULTS AND DISCUSSIONS

Enhanced Capacities of AMIA Partner Organizations in the Region

Series of trainings, workshops and learning events were organized for AMIA2 project teams in an intra-regional training that covers key learning contents from the national-level trainings as shown in Table 1.

Climate Risk Vulnerability Assessment (CRVA)

The vulnerability mapping was based on the Climate-Risk Vulnerability Assessment (CRVA) framework. It started with the identification of the vulnerability determinants (sensitivity, hazards, and adaptive capacity) and their respective indicators.

Sensitivity Analysis

In developing the impact of climate change to crop suitability, a crop distribution model was used and the factors associated are the 20 bioclimatic variables and the existing crop location. Sensitivity index was used in the sensitivity analysis to determine the sensitivity of crops to changes in temperature and precipitation.

The aggregated sensitivity of crops for rice, maize/corn, tomato, mango, and sweetpotato are given equal weights of 20% as shown in Figure 2 (a). It appears that the sensitivity index of the province generally ranges from -5% to -50% which means that it is sensitive to highly sensitive as influenced by the bioclimatic variables. On the other hand, improved varieties of crops, water conservation and soil conservation technologies that are being practiced by farmers mitigate the effect on crop yield.

Hazards Vulnerability

The natural hazards that were added to come up with the hazard vulnerability index are tropical cyclone/typhoon, flood, drought and erosion which are given the corresponding weights of 35%, 35%, 27% and 3%, respectively. The areas of San Manuel, Anao, Moncada, and La Paz are highly vulnerable to hazards as shown in Figure 2 (b). These areas experiences flooding on the onset of monsoon rain especially during typhoon because of geographically low areas of the province and become the catchment basin of the nearby provinces.

Adaptive Capacity Analysis

The adaptive capacity indicators considered in the assessment of the province's readiness to withstand the effects of climate change. These seven capitals are economic, natural, human, physical, anticipatory, social and institutional. Figure 2 (c) on the adaptive capacity illustrates the economic, natural, human, physical, anticipatory, social and institutional capital of every municipality. These seven (7) capitals of the adaptive capacity were given the same weight to come up with the aggregated adaptive capacity map.

The adaptive capacity of the province shows the readiness to adapt to climate risk. Concepcion and Gerona were found to have very high adaptive capacity while the municipalities of Ramos, San Jose, La Paz, and Bamban have very low adaptive capacity.

Climate-Risk Vulnerability

The climate-risk vulnerability map was developed by adding the sensitivity index, hazard index and the adaptive capacity index with their corresponding weights. A national experts' meeting composed of agriculturists, policy makers, and scientist, agreed the 15-15-70 percentage of weight for the sensitivity, hazard and adaptive capacity, respectively. The formula used in the development of climate-risk vulnerability map is stated in Equation 1:

$$\begin{aligned}\text{Climate - risk vulnerability} = {} & \text{Sensitivity index} * 0.15 + \\ & \text{Hazard index} * 0.15 + \\ & \text{Adaptive capacity index} * 0.70\end{aligned} \quad (1)$$

Figure 3 show the vulnerability to climate-risk is very high in the municipalities of Ramos, Bamban and La Paz; high in San Jose, Victoria and Pura; lowest in Tarlac City. The factor that has the major contribution in the vulnerability assessment is the adaptive capacity given a weight of 70% compared to sensitivity and hazards with 15% weights each. The perspective of giving a high percentage to the adaptive capacity is the thought of the ability of every municipality being able to cope with extreme events like temperature, rainfall, typhoon, flood, drought, erosion and other natural hazards because these municipalities are equipped with facilities and structures, and services for the adaptation.

The maps developed in the CRVA assessment was presented to a focus group discussion (FGD) with the stakeholders from the Provincial Agriculture Office (PAO), Municipal Agriculture Office (MAO), Farmers and other agencies. From the FGD and field visit conducted, the participants agreed that the maps developed are similar to the actual situation in their municipality. Vulnerable areas due to risks of climate was mentioned in the study of Dikitanan, et al. (2017).

TABLE 1. Capability Building Training, Seminars and Workshop Attended by the Regional Team

Title	Date	Venue
Training on Climate Risk Vulnerability Assessment	June 6-8, 2016	Torre Venezia Hotel, Quezon City
Cost- Benefit Analysis (CBA) on Climate Resilient Agriculture Practices	August 6-8, 2016	Torre Venezia Hotel, Quezon City
Methodology for Evaluating Social and Environmental Benefits, in Agricultural Systems	December 2, 2017	Tarlac Agricultural University, Camiling, Tarlac
Climate Risk Vulnerability Assessment (CRVA) Mapping & and Adaptive Capacity Mapping	January 10-12, 2017	SEARCA, UPLB, Los Banos, Laguna
AMIA2-CIAT Project: Results Sharing and Validation Workshop on CRVA & CRA Decision Support	February 6-7,2017	Parklane International Hotel, Cebu City
Workshop on Finalizing Results on CRA and Prioritization and Extended CBA	March 1-3, 2017	B Hotel, Quezon City
Completion Review of BAR Funded Climate Change Projects	May31-June 2, 2017	Partido State University, Goa, Camarines Sur
AMIA2-CIAT Project: Workshop on Outcome Monitoring and Evaluation of Community-Based Action Research	June 21-22, 2017	Sequoia Hotel, Quezon City

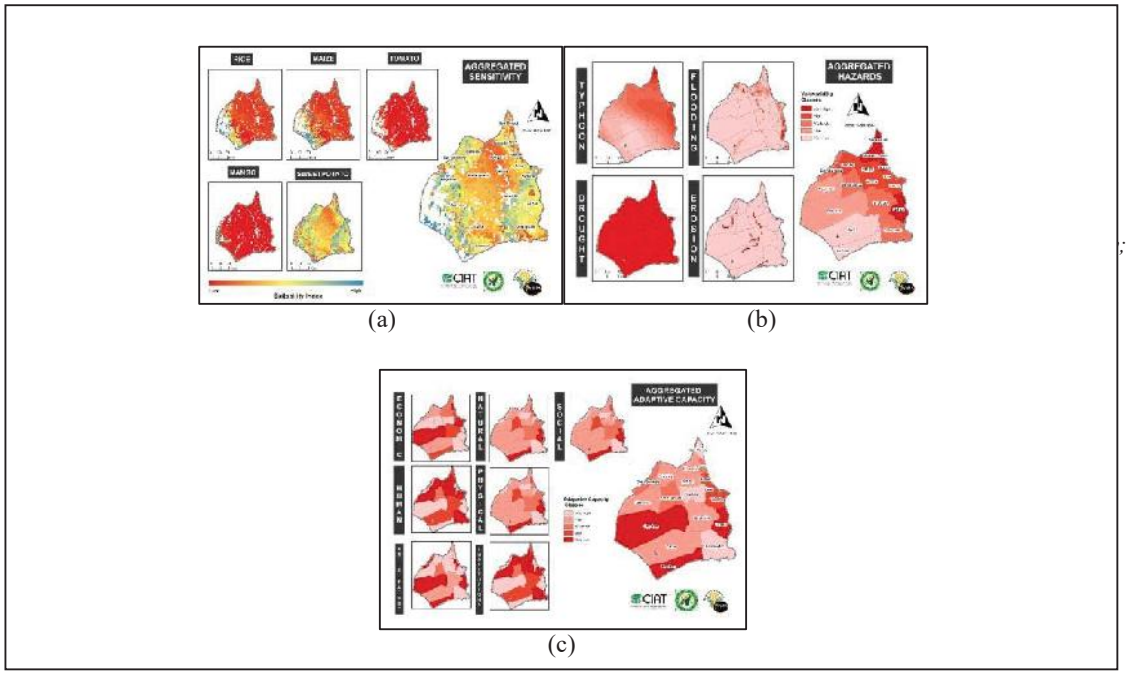

FIGURE 2. Maps on sensitivity, hazards and adaptive capacity to produce CRVA map: (a) Aggregated Sensitivity; (b) Aggregated Hazards; and (c) Aggregated Adaptive Capacity

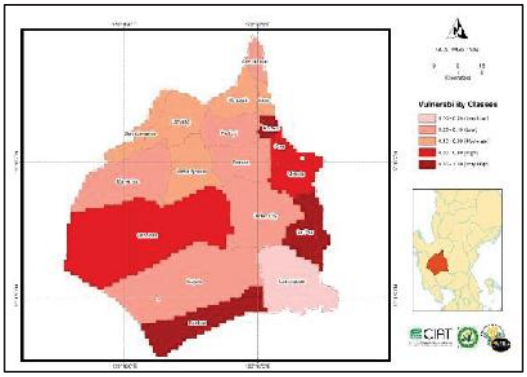

FIGURE 3. Climate-Risk Vulnerability Map of Tarlac

Cost-Benefit Analysis (CBA) of CRA Practices

The identified climate-resilient practices in the province of Tarlac which are the following: Organic agriculture, climate smart varieties/lines, water conservation technology, adaptive crop calendar/crop switching, soil conservation technology, intercropping/crop rotation, community-based management, crop insurance/weather adverse condition insurance, and improved housing for animals. The identification of CRA practices were gathered through interview. The list was trimmed and validated through a series of FGDs with municipal agriculturists, representatives of the Office of the Provincial Agriculturists, local farmer technicians and farmers. CRA practices have also been priorities in the study of Dikitanan, et al. (2017).

From the list of CRA practices, key informants identified three priority practices: climate smart varieties, crop rotation, and water conservation technology particularly the alternate wet and dry method (AWD). The interview guide provided by CIAT was modified to fit the scope of the study before it was given to the group of key informants. Data gathered were used in the evaluation of CRA practices using the CBA Tool. Validation of data was done by seventy one (71) municipal agriculturists, farmer leaders and farmer technicians participated in the Focus Group Discussions. The cost and benefits derived from the CRA practices were confirmed. These data were used to analyse the profitability and sustainability of the CRA practice. The three prioritized practices are the climate smart varieties, crop rotation and water conservation technology because of the immediate effects and perceived potential benefits to the farmers, food security and, mitigation and adaptation to climate change. Investment prioritization brief were also prepared for policy makers to support the climate-resilient agriculture practices in the provinces as shown in Figure 4. Researchers and extensions' personnel must use the investment brief for dissemination and reference for further validation or research in a specific area.

Climate Smart Varieties

Yield of crops is affected by extreme changes in climatic conditions such as flooding, and drought and attack of insect pests and diseases. Farmers and agricultural technologists favor climate smart varieties because of its high yield and its capacity to withstand varied climatic conditions. The Green Super Rice lines (GSR 8, 15, 21, and 22) earned special mention among farmers who have experienced growing it due to its resiliency especially during typhoons and floods, drought, and attack of insect pests and diseases. Farmers who planted GSR lines attested the resilience of these lines to typhoon and submergence. Yield was not likewise affected.

Crop Rotation

Crop rotation was chosen mainly because farmers could switch from rice to other crops (corn, sweet potato, etc.) depending on availability of water, soil conditions, and other climactic factors. Crop rotation also means reduction in pest occurrence due to non-availability of the host.

Water Conservation Technology

To address problems on scarcity of water supply or limited access to water sources in the upland, rainfed, and other areas, water conservation technologies are resorted to. The Alternate Wet and Dry (AWD) Method was specially mentioned because it utilizes materials readily available and can be constructed easily by the farmer. Some parts of the province depend on shallow tube wells and Tarlac Ground (TG) water as their main

source of irrigation. In these areas, one of the main expenses incurred by farmers is gasoline that is used to run pumps. This practice was chosen because it allows farmers to irrigate at the proper time. The intermittent drying of fields enables the farmers to save on time and money.

FIGURE 4. Investment Brief for Climate Smart Agriculture Practices

CONCLUSION

The two major output of this study are: climate-risks vulnerability map and investments briefs. The map was used to identify pilot area of climate smart village. The Department of Agriculture Regional Field Office 3 chose the municipality of Victoria as the pilot climate-smart village in the province of Tarlac. DA RFO3 based the selection on the result of the climate-risk vulnerability mapping and willingness of the local government unit to support the project. Victoria is considered as one of the high vulnerable municipalities that is sensitive to changes in climate, vulnerable to hazards and has less adaptive capacity to cope up with the phenomenon. Victoria is a 2nd class municipality of the province of Tarlac with 26 barangays and is located at the eastern part of the province. Majority of the total land area is devoted to agricultural activities and livestock production.

The climate resilient agriculture practices identified in the prioritization is being adopted in areas in the province for further testing and evaluation in the suitability of a CRA practices in an area. The investment briefs were presented to Department of Agriculture and Office of the President as a support for decision- and policy-making.

ACKNOWLEDGEMENT

We would like to give thanks to Department of Agriculture (DA) System-wide Climate Change Office (SWCCO) and International Center for Tropical Agriculture (CIAT) for the AMIA2 project.

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Small Farm Reservoir Suitability Analysis in Tarlac Province, Philippines

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Abstract Small farm reservoir (SFR) suitability analysis is useful in water resources management and development assistance of government and non-government agencies for farmers and farmer-groups. The researcher utilizes the geographic information system to analyze the suitable areas for the construction and management of small water impounding to store and conserve rainwater in rainfed areas. The factors on rainfall, soil texture, slope, land use, irrigation status, groundwater availability and distance from river were considered for the suitability mapping of SFRs. The following factors have their corresponding weights which are derived from using the analytical hierarchy process (AHP) procedure. The testing of the model was done by determining the suitability value (S) of each sample SFR. The research findings showed the areas in the province potentially suitable for SFRs of the total land area of Tarlac: 47% are not suitable, 25% are marginally suitable, 13% are moderately suitable and 15% are highly suitable.

Keywords Suitability, Small Farm Reservoir, Geographic Information System, Rainfed Areas, AHP

1. Introduction

The Philippines has 41% total rainfed cropped area that mostly relies on rainfall; however its availability is lesser in dry season (Moya *et al.,* 1994). In addition, development of facilities for conventional irrigation is unlikely because of undulating topography, surface drainage and monetary constraints. Rainfed farmers suffer frequently from drought because of the inadequate water together with poor management practices of irrigation water. To mitigate the effect of drought in these areas, farmers with small farms are collecting rainfall and runoff and storing rainwater in small farm reservoir to be used for the wet and dry season crops (Guerra *et al*., 1994). Small farm reservoir (SFR) is an earth dam structure used to harvest and store rainfall and runoff for irrigation. It is the smallest version of small water impounding project with an embankment height of less than 4 meter (Ines *et al*, 2018). Studies showed that small farm reservoirs (SFRs) serve as an economically viable means for storing and conserving rainwater to lessen the effect of drought and cropping intensification in rainfed drought-prone areas. However, information about this technology is very limited making a hindrance to researchers, technical implementers and government agencies in utilizing its maximum potential in rainfed areas. Generating information system about SFRs with the aid of geographic information system (GIS) technology can be used as a basis for areas suited for SFRs as an effective water management scheme for individual farmer and farmer groups to improve crop production. Furthermore in the water resources development planning strategies of the government for the national, regional and local levels, as GIS has often used for the geographic concerns on agriculture. Thus, the objective of the study was to generate suitability maps for SFR construction in the province of Tarlac.

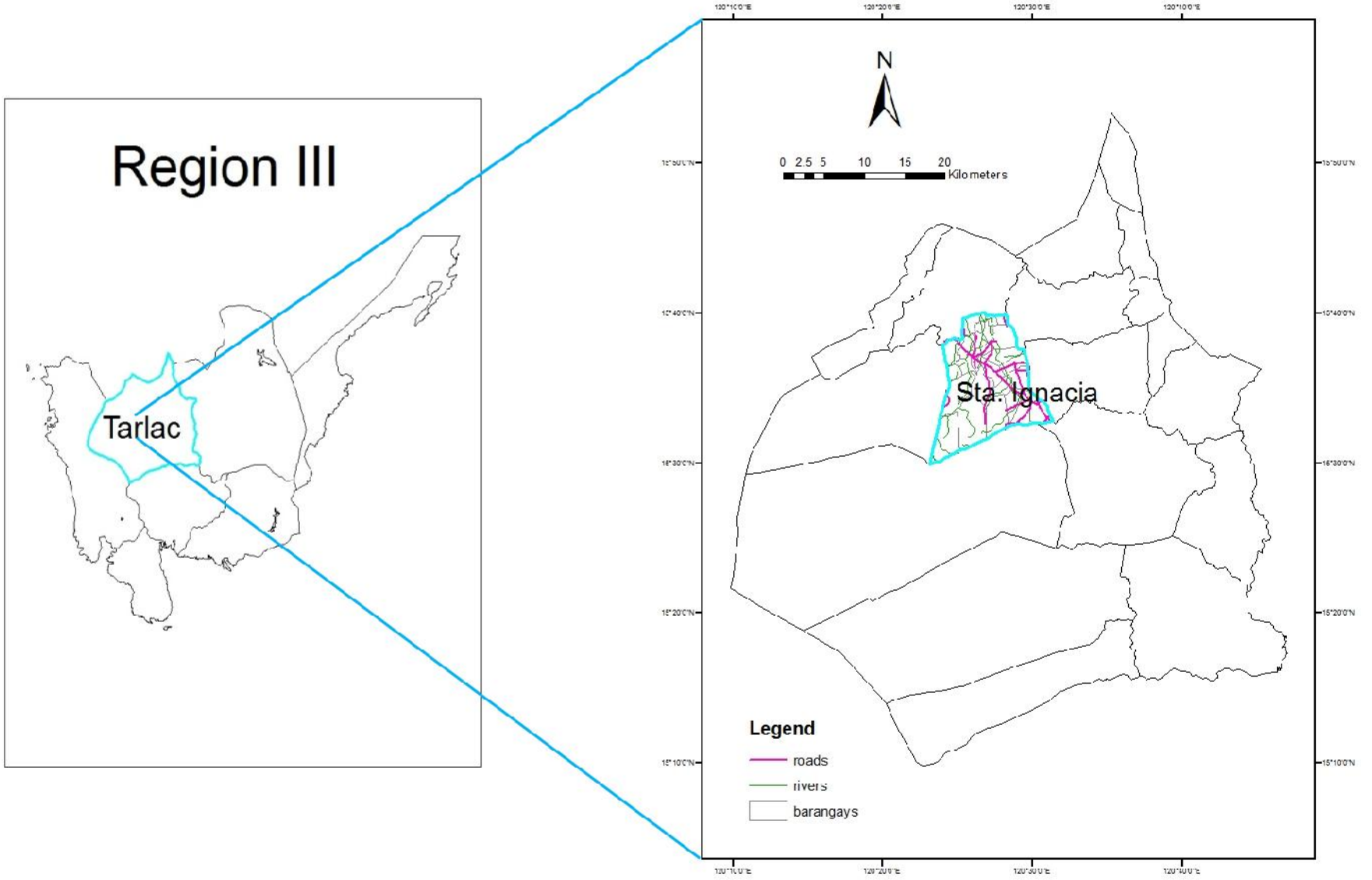


Figure 1. Map of Tarlac Province, Philippines

2. Materials and Methods

Study Area

The study was established in the province of Tarlac (Figure 1). The study covers an area of 273,660 hectares and is located between latitude 15º 10'15" N to 15º 52'52" N, longitude 120º 8'4" E to 120º 46'27" E. The study area has flat to undulating topography, with the eastern part of the province being plain and the western part to be hilly to mountainous. Tarlac has two distinct seasons, the wet and the dry seasons. It has unimodal rainfall pattern, having high monsoon rains in wet season (WS) and lesser amount of rainfall in dry season (DS). Recorded annual rainfall varies from 2,030 mm to 4,060 mm in the northwestern portion.

Data Acquisition

Rainfall map, soil texture map, slope map, land use map, irrigation status map, groundwater availability map and distance from river map were acquired from corresponding agencies namely in local agrometeorological station, Mines and Geosciences Bureau (MGB), National Mapping and Resource Information Authority (NAMRIA), National Irrigation Administration (NIA), National Water Resources Board (NWRB) and Department of Agriculture – Bureau of Agricultural Research (DA-BAR), respectively.

Suitability Factors

The data on rainfall, soil texture, slope, land use, irrigation status, groundwater availability and distance from river were used as the factors in the suitability mapping of SFRs. In the study of Cacayan *et.al* (2019), the factors considered are average annual rainfall, soil texture, slope and irrigation status while the past study of Galang *et.al* (1994), the criteria used at macrolevel are land use, slope, road network, municipal boundaries; however rainfall and soil type are excluded in the study. On the study of De Guzman (2013), the factors on rainfall, soil texture, slope, land use, irrigation status, groundwater availability and distance from river were used. The corresponding weights of these factors and its suitability ratings were determined. The factor maps derived from the seven thematic maps were integrated to the GIS (ArcGIS) software to develop a final suitability map to show the potential sites for SFRs construction. The methodology for identifying potential sites for SFRs is summarized in Figure 2.

Suitability Model for Evaluation of the Potential SFR Sites

Identification of the potential areas involves finding the areas that will satisfy a chosen set of criteria for establishment of SFR. Testing considers the impact of the system adoption.

The small farm reservoir suitability model (S) (equation 1) was derived from combining the factors with their corresponding weights for determining the potential areas for SFR in the final suitability map. Every location in the map had a suitability value. The formula below was used in calculating the suitability value of each grid cells:

S = [(Rainfall x rf) + (Soil texture x stf) + (Groundwater availability x gf) + (Slope x sf) + (Land use x lf) + (Irrigation status x if) (Distance x af)] (1)

where:

S = Suitability value for small farm reservoir
Rainfall = Rainfall factor map
Soil texture = Soil texture factor map
Slope = Slope factor map
Land use = Land use factor map
Irrigation status = Irrigation status factor map
Groundwater availability = Groundwater availability factor map
Distance = distance from river factor map
rf = rainfall weight
stf = soil texture weight
sf = slope weight
lf = land use weight
if = irrigation status weight
gf = groundwater availability weight
af = distance from river weight

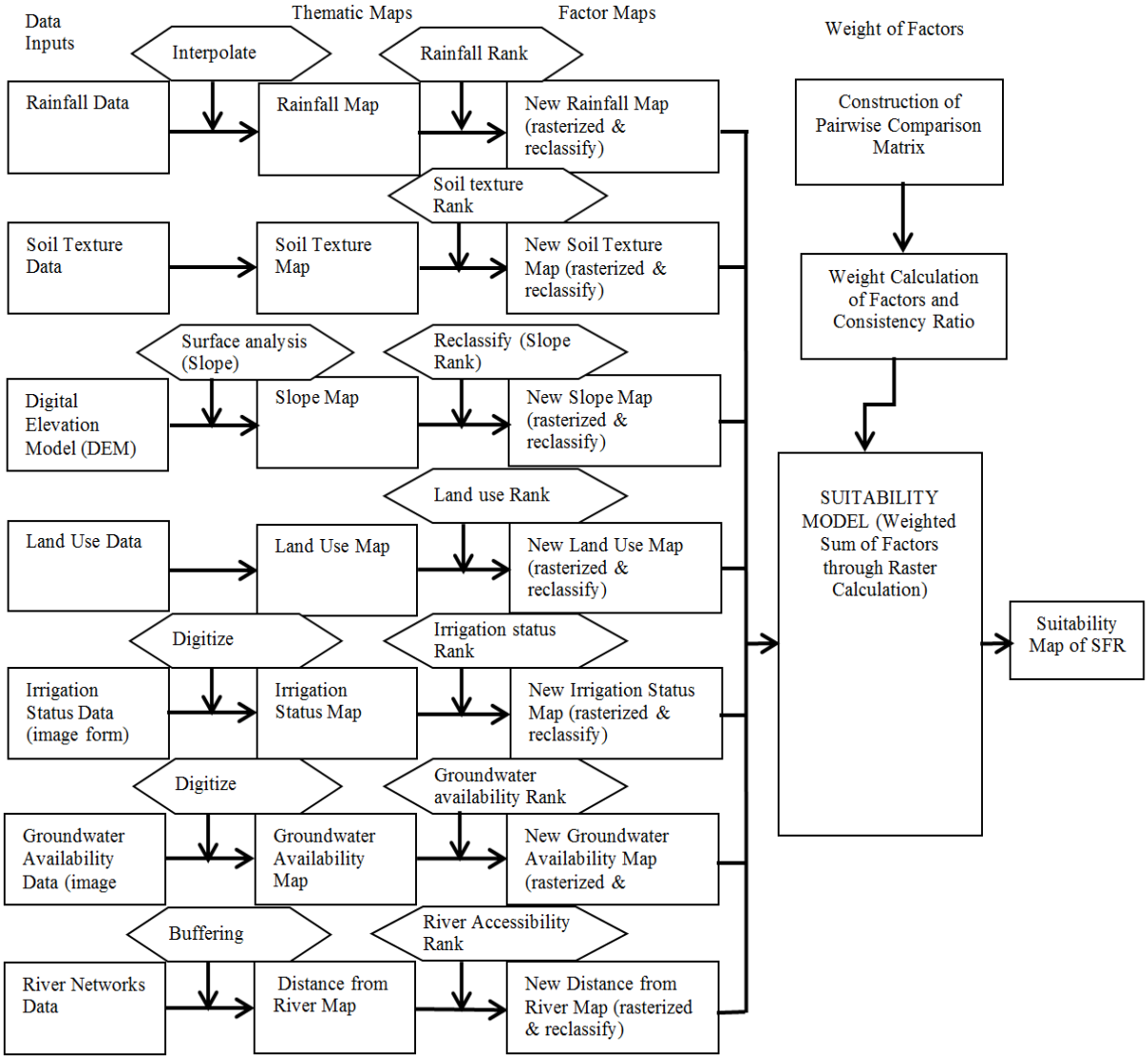


Figure 2. Methodology for identifying potential sites for SFRs

Table 1. Ranking of factors for SFRs

Factors	Description	Suitability Scale
Rainfall [a]	<1000 mm	1
	1000 - 1200 mm	2
	>1200 mm	3
Soil texture [b]	other class {sand, silt loam, silt, clay, Mountain soil (undifferentiated), Angeles soil (undifferentiated), Tarlac soils}	0
	sandy loam	1
	sandy clay loam	2
	clayloam & silty clayloam	3
Slope [c]	3- 8% (gently sloping to undulating)	3
	0 - 3% (level to nearly level)	2
	8 - 18% (undulating to rolling)	2
	18 - 30% (rolling to moderately steep)	1
	>30% (steep to very steep)	0
Land use	other land uses {built-up, closed forest, forest plantation, inland water, open forest, wooded lands}	0
	barren land	1
	Grassland	1
	cultivated land	3
Irrigation status	non-irrigated area	3
	irrigated area [d]	0
Groundwater availability [e]	deep well areas	3
	shallow well areas	0
	difficult areas [f]	0
Distance from river	> 200 m	3
	100 - 200 m	2
	50 -100 m	1
	0 -50 m	0

Note: [a] Rainfall description based from the category used by Galang et al. (1994)

[b] Soil texture description based from different soil types used by BSWM (1997) wherein only soil types under loamy soils is considered

[c] Slope class used by Galang et al. (1994) based from the slope category of DA-BAR

[d] Irrigated area of BBMP acquired from NIA-Tarlac

[e] Groundwater availability map acquired from NWRB

[f] Forested area with deep well areas that unsuitable for groundwater extraction

Ranking of Factor

Each map layer has individual values in each class. To be able to perform arithmetic operation, values must be assigned from a numeric evaluation scale referred to as suitability scale or preference from best to worst. Each factor was ranked by how suitable it is and is done through the process of reclassifying.

Table 1 shows the ranking of the factors for potential areas of SFRs wherein suitability scale of 0-3 was used, 3 being the highest value. Ranking of factors was based from the following four suitability ratings: not suitable (0), marginally suitable (1), moderately suitable (2) and highly suitable (3).

Weighting of Factor

Some factors are more important than the others in the suitability model. Therefore, percent influence or weight is assigned to each factor based from its importance. Calculating the weight of each factor was done using analytical hierarchy process (AHP). From the AHP procedures of Coyle (1989), the three steps used are as follows: (1) construction of a single pair-wise comparison matrix; (2) calculating the list of relative weights, importance, or value of the factors; and, (3) calculating and checking of the Consistency Ratio (CR).

The study of Al-Ruzouq *et al.* (2019) used the AHP in determining the importance of parameters such as precipitation, drainage stream density, geomorphology,

geology, curve number, total dissolve solids, elevation, slope and major fracture Euclidean distance for dam site suitability mapping and analysis.

Suitability Rating

Table 2 shows the suitability rating having its corresponding ranges of each class. The interpretation of suitability classes for each factor was classified on a scale from 0 to 3 as follows: not suitable, marginally suitable, moderately suitable, and highly suitable.

Table 2. Suitability rating

Suitability Levels	Range
Not suitable	0.0000 - 2.0000
Marginally Suitable	2.0001 - 2.5000
Moderately suitable	2.5001 - 2.7500
Highly suitable	2.7501 - 3.0000

Testing of the Suitability Model

The selected SFRs locations in the study area were overlayed in the final suitability map for SFRs and the testing of the model (equation 1) was done by determining the suitability value (S) of each sample SFR. There are one hundred fifty (150) SFR samples that are randomly selected on the municipality of Sta. Ignacia, Tarlac. Locations of SFRs are done using global positioning system (GPS).

3. Results and Discussion

Finding the best location for SFRs was accomplished in the suitability mapping of SFR sites. Each factor map used was reclassified according to its suitability and these maps were combined with their corresponding percent influence to produce the final suitability map of SFRs. Suitability value of every location in the map was obtained after the creation of the final suitability map for SFRs.

Thematic Maps

The thematic maps (rainfall, soil texture, slope, land use, irrigation status, groundwater availability and distance from river) used as the factors of the study that are essential for identifying the potential sites for SFR are presented in Figure 4. These are the preliminary maps used with their corresponding attributes.

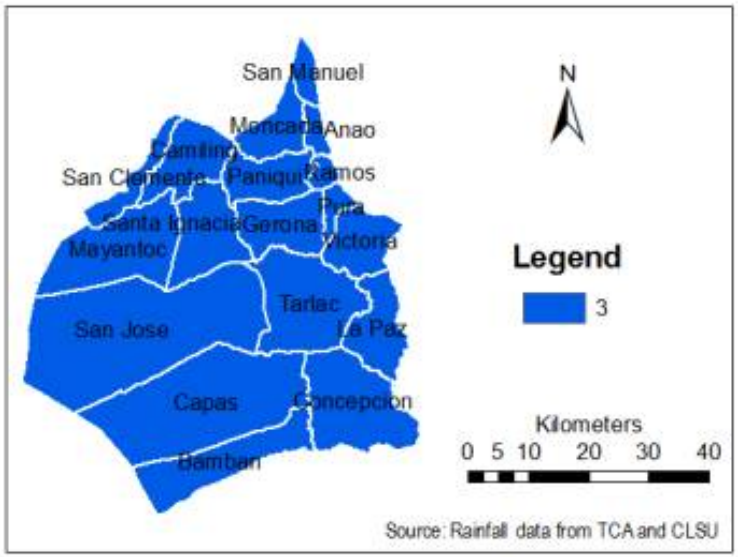
Factor Maps

Each thematic map having individual values in each class was assigned a value from the numeric evaluation scale known as suitability scale or preferences, from best to worst, to be able to perform arithmetic operation in the suitability analysis. These thematic maps are ranked according to suitability through reclassification. Ranking of the factors was done by assigning a scale of 0 to 3, 3 being the highest value. The factor maps were the resulting maps after reclassification of the thematic maps shown in Figure 5.

Determination of the Relative Important Weights of Factor

Assigning weights or percent influence to each factor was needed because of the fact that some factors are more important in the suitability model than others. This was done through analytical hierarchy process (AHP). If the factors are of equal importance then assign the same weight to each one.

In SFR adoption, the factor considered as most preferable is land use, and the next are rainfall, irrigation status and distance from river followed by soil texture and lastly, slope. Table 3 shows the pair-wise comparison matrix for assessing the relative important weights of each factor in creating the suitable areas for SFRs.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Source: Rainfall data from TCA and CLSU

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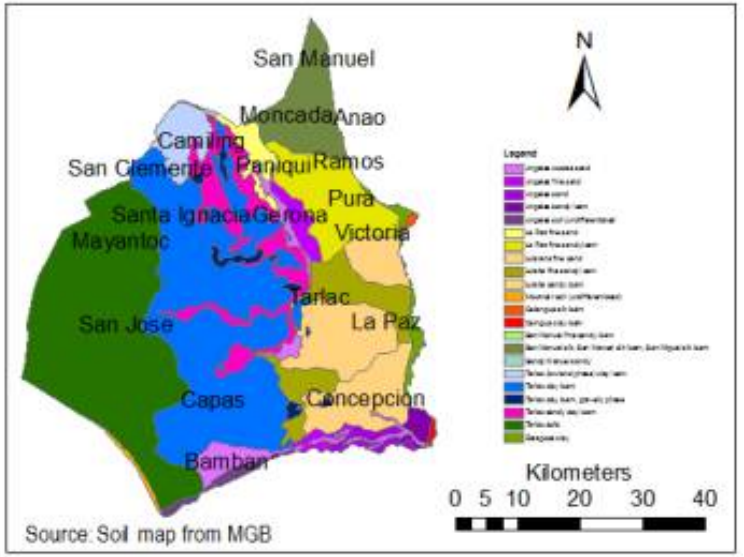
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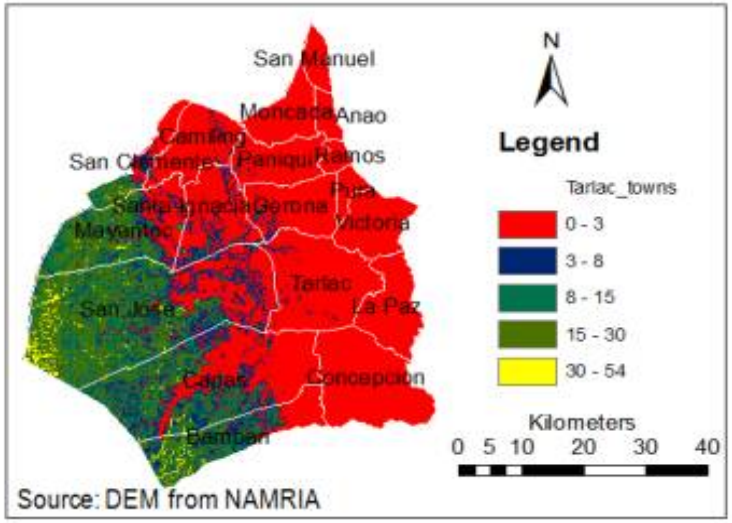
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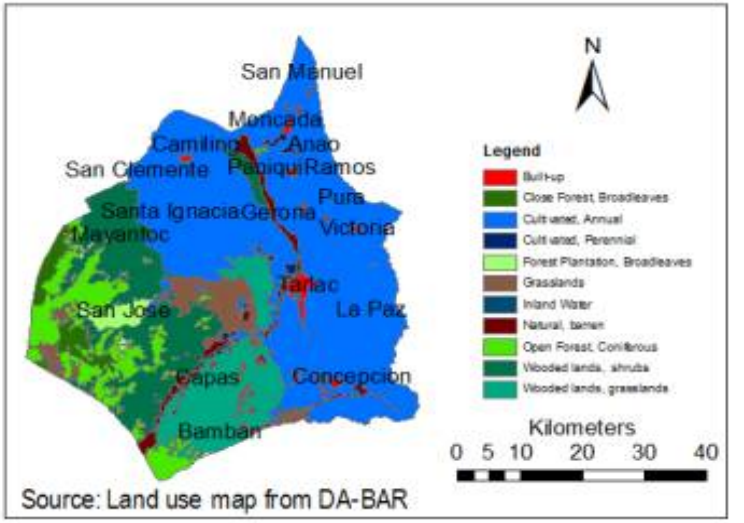
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Cultivated, Perennial
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Source: Land use map from DA-BAR

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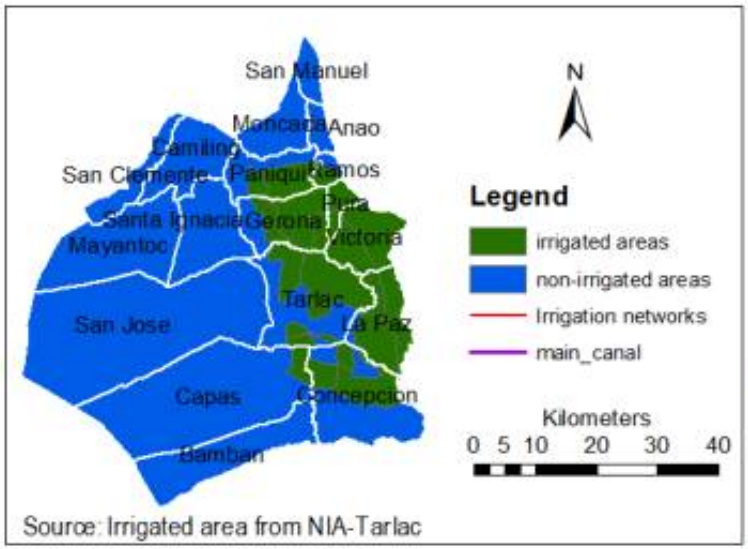

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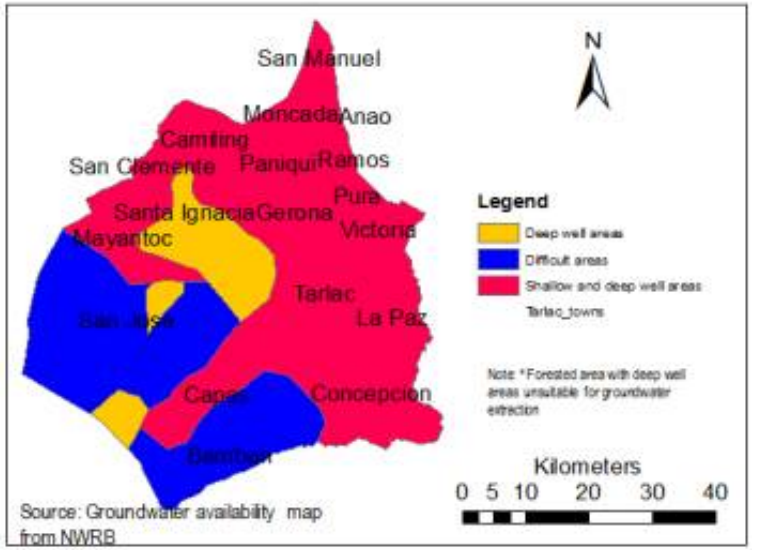

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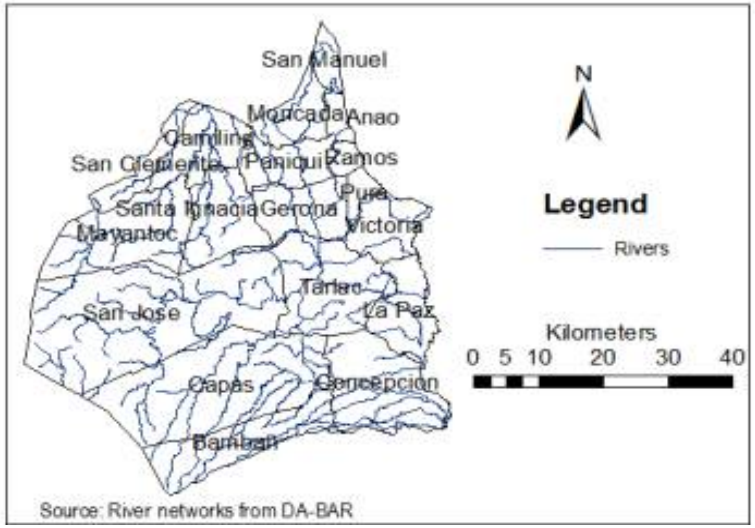
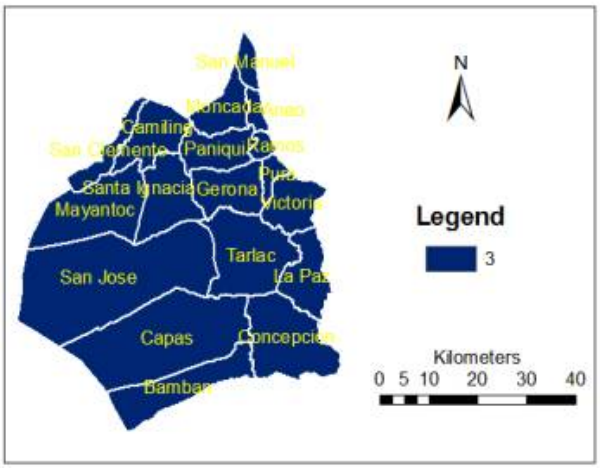


(g)

Figure 4. The thematic maps used in suitability mapping (a) Rainfall map (b) Soil texture map (c) Slope map (d) Land use map (e) Irrigation status map (f) Groundwater availability map and (g) River network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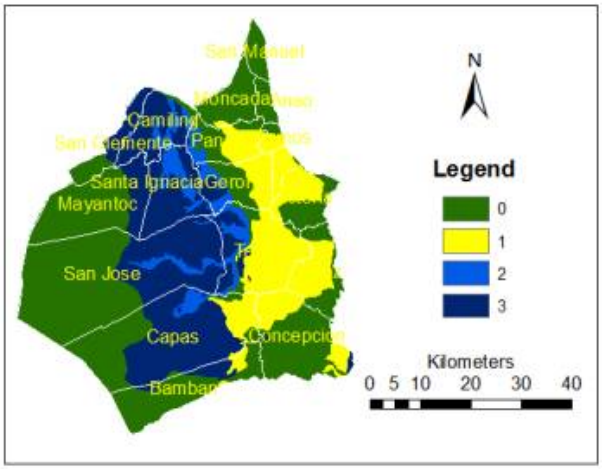
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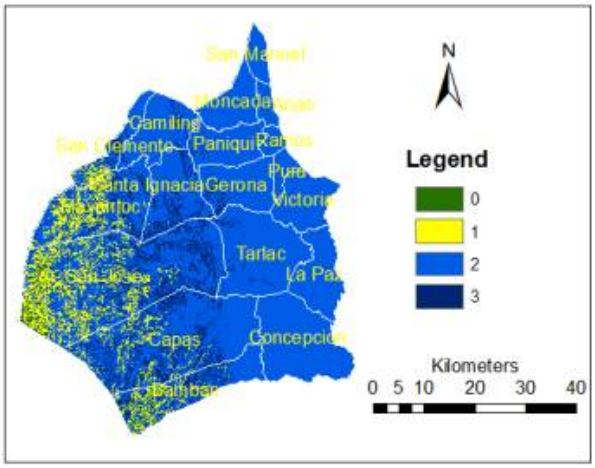
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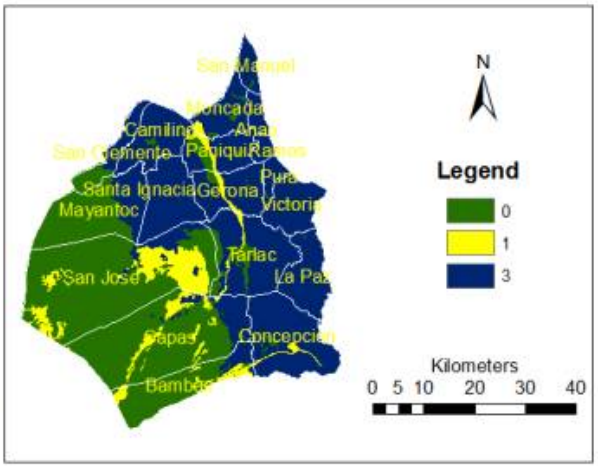
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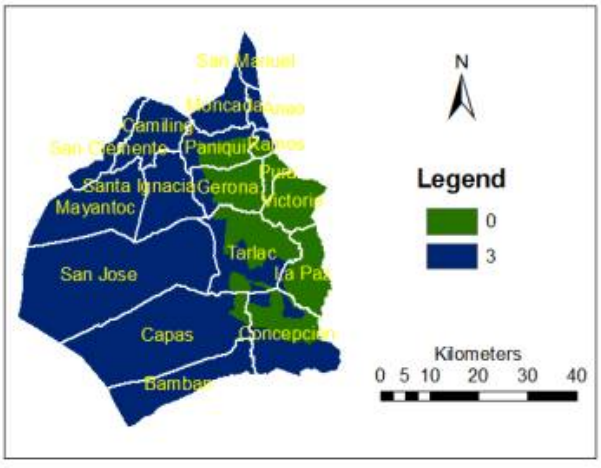
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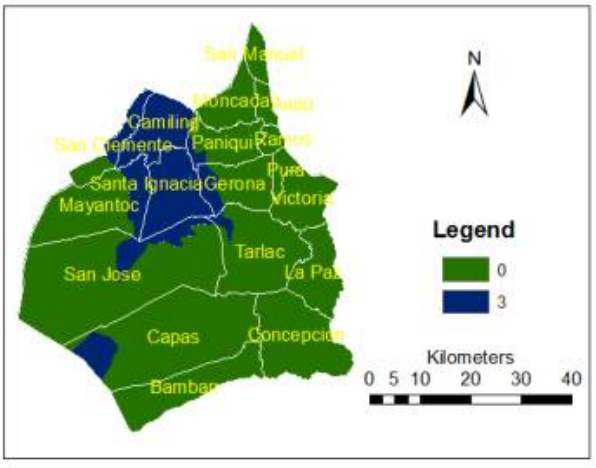
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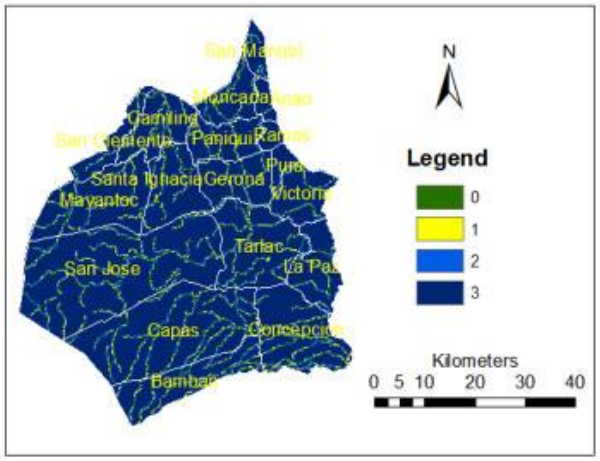

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Figure 5. The different factor maps produced from the thematic maps (a) Rainfall factor map (b) Soil texture factor map (c) Slope factor map (d) Land use factor map (e) Irrigation status factor map (f) Groundwater availability factor map and (g) Distance from river factor map

Table 3. Pair-wise comparison matrix for assessing the relative weight of factors

	Rainfall	Soil texture	Slope	Land use	Irrigation status	Groundwater availability	Distance from river	RIW
Rainfall	1	5	7	1/3	1	3	1	0.167
Soil texture	1/5	1	3	1/7	1/5	1/3	1/5	0.038
Slope	1/7	1/3	1	1/9	1/7	1/5	1/7	0.022
Land use	3	7	9	1	3	5	3	0.366
Irrigation status	1	5	7	1/3	1	3	1	0.167
Groundwater availability	1/3	3	5	1/5	1/3	1	1/3	0.073
Distance from river	1	5	7	1/3	1	3	1	0.167

Consistency ratio (CR): 0.03

RIW = Relative Important Weight

Final Suitability for SFRs

Overlaying of the different factor maps produced the suitable sites for SFRs. Combining these factor maps was done by the use of raster calculator from spatial analyst tool after reclassifying each map. Each factor maps was multiplied with its corresponding weights and added together to produce the final suitability map.

The formula in equation 2 used in the suitability model was substituted by the computed values of weights or percent influence to each factor as shown below:

S = [(rainfall factor x 0.167) + (soil texture x 0.038) + (slope x 0.022) + (land use x 0.366) + (irrigation status x 0.167) + (groundwater availability 0.073) + (distance x 0.167)]

Figure 6 shows the suitability map for SFR sites in Tarlac classified into four suitability classes; 0-not suitable, 1-marginally suitable, 2-moderately suitable and 3-highly suitable. Not suitable areas have the highest value of 142,353 hectare or 47% of the total land area of the province. Marginally suitable areas were 73,839 hectare or 25% of the total area of the province. Moderately suitable areas had the smallest area of 40,129 hectare or 13% of the total land area of the province. Highly suitable areas for SFRs got 44,813 hectare or 15% of the total land area of the province.

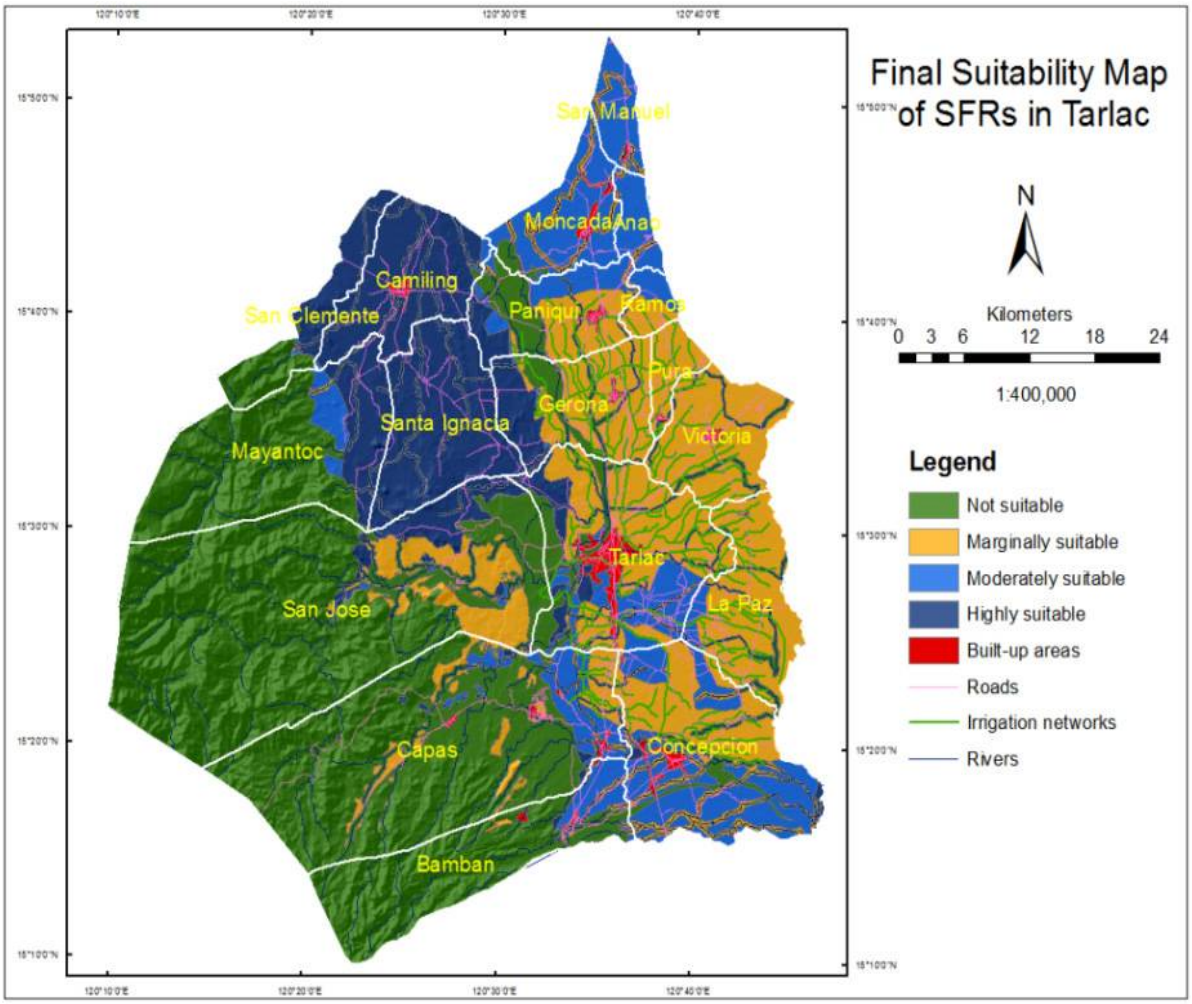

Figure 6. Final Suitability Map of SFRs in Tarlac

Testing of the Suitability Model

Testing of the suitability model was done by determining the suitability (S) value of each SFR overlay in the final suitability map. The summary of the suitability value of each SFR is shown in Table 4.

Table 4. Summary of the suitability (S) value of selected SFRs

Suitability Class	Range of Suitability Value (S)	Frequency (n=150)	Percentage (%)
Not suitable	0.0000 -2.0000	11	8
Marginally suitable	2.0001 -2.5000	0	0
Moderately suitable	2.5001 -2.7500	2	1
Highly suitable	2.7501 -3.0000	137	91

4. Conclusions

Based on the result, the usage of SFRs as a source of water for irrigation in wet and dry season intensifies cropping in drought-prone rainfed areas. The availability of information system on SFRs can be used by authorities or sectors responsible for water resources management and development.

GIS-aided decision support system for SFRs can be a viable means in determining the areas suited for SFR construction as well as the location of existing SFRs to maximize their full utilization.

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Survey of Physical, Chemical and Microbial Water Quality of Irrigation Sources in Tarlac, Philippines

Edmar N. Franquera, Cielito A. Beltran, Ma. Asuncion G. Beltran and Ruth Thesa B. Franquera

Abstract The main sources of irrigation water for irrigating crops comes from major rivers. Usually these water sources which can be used for irrigating various crops could be very vulnerable to contamination. The aim of the study was to determine the physical, chemical and microbial water quality of the different irrigation sources in Tarlac and to compare it with the existing water quality guidelines stipulated in the DENR AO 08 Series of 2016. The water samples collected from the surface water of different rivers were subjected to laboratory analysis. Higher TSS was found to be during wet season as compared during the dry season. Higher COD was found both in dry and wet seasons in Benig river. All of the major rivers have a less than 0.05 mg/l lead and 0.0002 mg/l mercury based from the result of the laboratory analysis. The highest dissolved oxygen was found to be within the Tarlac River both during the dry and wet season. Comparing with the National standards from the DENR the major rivers of Tarlac surpasses the minimum standards of classification of water bodies with dissolved oxygen ranging from 2 to 6 mg/l. The lowest dissolved oxygen was found in Concepcion River during the dry season (5.0 mg/l) and in Rio Chico River (4.8 mg/l) during the wet season. Higher total dissolved solids were observed in the different rivers during the dry season which ranges from 300 to 560 mg/l as compared during the wet season which ranges from 169 to 540 mg/l respectively. The nitrate concentrations of the different rivers in Tarlac shows to be within the range of the National Standards of the DENR. Higher concentrations of *E. coli* and fecal coliform count were also noted within the different rivers of Tarlac.

Keywords Water quality · River · Irrigation · Tarlac

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1 Introduction

Water is life. All living organisms on earth need fresh water. The major user of freshwater in most countries is agriculture. The largest single user of freshwater in the world today which consumes an average of 70% globally is accounted in agriculture.[1] However, the availability of freshwater is already decreasing due to water pollution. Agriculture is considered to be a casualty of water pollution but it also causes and contributes to water pollution due to excess nutrients by too much application of fertilizers, excessive use of pesticides and other pollutants. Globally, agriculture is also considered to be the major cause of degradation of surface including groundwater resources as a result of erosion, excessive farming contaminating freshwater like wastewater coming from large poultries and piggeries, chemical run off and other indiscriminate human activities and improper agricultural management practices. Waste coming from swine is significant source of fecal pollution leading to water pollution by contaminating of ground and surface water from lagoon overflow and the use of lagoon surface water for irrigation. Thus, it is important to test a system or test a technology such as potential aquatic plants to decontaminate the wastewaters so that this will resolve the problem.

In the Philippines, agriculture wastewater is one of the major sources of water pollution which accounted 37%.[2] In addition, only 10% of wastewater is treated while 58% of groundwater is contaminated. Regions which had unsatisfactory ratings for their water quality criteria include National Capital Region (NCR), Southern Tagalog Region, Central Luzon (Region 3) and Central Visayas. Hence, there is a need to address the global implications of water quality and there is a need for wastewater treatments. In central Luzon, the agricultural land area is 653,607 km^2 and 9.1% contributed to the agricultural BOD generation, 9.0% industrial BOD generation and 9.9% domestic BOD generation leading to water quality degradation and contamination.[3]

Generally, the availability of clean freshwater is becoming a primary limitation to human activities expansion and also the scope or capacity of our agricultural lands to feed the tremendous population growth not only in the Philippines but globally. There are an estimated 2.2 million metric tons of organic water pollution that occur in the Philippines each year and the annual economic losses caused by water pollution are estimated at Php67 Billion which is equivalent to more or less US$1.3 billion.[4] Hence, this study aims to quantify the physical, chemical and microbiological water qualities of the different river waters in Tarlac, Philippines.

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2 Methodology

2.1 Gathering/Collection of Data of Existing Irrigation Water Sources in Tarlac

The existing data on the type of irrigation systems and the irrigation sources were gathered. This was done in collaboration with National Irrigation Administration (NIA). The water qualities that were gathered were compared to the existing standards of the Department of Environment and Natural Resources (DENR).

2.2 Water Sample Collection

Representative water samples were collected in seven major rivers of Tarlac based from the data of the National Irrigation Administration (NIA) and the Department of Environment and Natural Resources and the collection was done from 9:00 AM in the morning until 4:00 PM in the afternoon. A total of six liters of water samples were collected in each sampling sites based from the recommendation of the Department of Science and Technology. The water sampling collection was done on the onset of 2018 dry and wet season productions of rice.

2.3 Water Quality Analysis

Collected water samples were analyzed for its physical, chemical and microbiological qualities (Total suspended solids, chemical oxygen demand, total coliform bacteria, *E. coli*, lead and mercury content). These parameters were analyzed using the standard methods in analysis of water samples. Portable instruments were used for the analysis of the following parameters such as dissolved oxygen (portable oxygen meter), pH (HM pH-200) total dissolved solids and electrical conductivity (HM COM-100). For the nitrate quantification a Horiba portable nitrate meter was used.

2.4 Analysis of Data

Laboratory results from the collected water samples were analyzed and compared with the Water Quality Guidelines and General Effluent Standards of 2016 based on the Department of Environment and Natural resources (DENR) Administrative Order No. 08 Series of 2016.

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3 Results and Discussions

See Table 1.

3.1 *Total Soluble Solids and Chemical Oxygen Demand*

Table 2 presents the data of the different major rivers of Tarlac in terms of the total soluble solids and chemical oxygen demand. Results showed that the different river water has a varied total suspended solids and chemical oxygen demand. Higher TSS was found to be during wet season as compared during the dry season. This was also evident in terms of the chemical oxygen demand except for the two rivers, the Rio Chico and the Camiling river which exhibited a lower COD during the wet season with less than. For the TSS, based from the standard water qualifications, Tarlac and Concepcion rivers exceeded the numerical value which a body of water could be classified ranging only from 25 to 110 but for the two rivers it has both 169 mg/l total suspended solids during the wet season. Higher COD was found both in dry and wet seasons in Benig river with 27 and 22 mg/l respectively. Result of the COD laboratory test from the Benig river was also in consonance with the result of research conducted by Fernandez and David (2008) [5] which also shows high COD in Benig River. This implies that the higher COD in the sampling area, the higher level of water pollution. The wastewater discharge coming from the different industries within the area such as the presence of piggery farms could contribute to the higher COD of the water samples which maybe contributed to the deterioration of water quality within the sampling area (Al-Badaii et al. 2013).

3.2 *Heavy Metals (Lead and Mercury)*

The heavy metal concentrations (lead and mercury) in the different major rivers of Tarlac are presented in Table 3. All of the major rivers have a less than 0.05 mg/l lead and 0.0002 mg/l mercury based from the result of the laboratory analysis. Compared to the standards for the water quality the result both of the lead and mercury content of all the major rivers showed lesser than that of the standards. This implies that the rivers were not contaminated with heavy metals. This could be due to the non-presence of mining sites within the areas where the different rivers were located. Heavy metals were considered to be toxic and dangerous. The presence of higher concentrations of heavy metals in rivers as source of irrigation for the crops could lead also to the decline in production and these heavy metals could bio accumulate affecting also the humans whom will consume the crops irrigated with higher concentrations of heavy

[5] www.bgr.bund.de.Veranstaltungen. Last accessed 15 Dec 2017.

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Table 1 Water quality guidelines (DENR AO 08 Series 2016)

Parameter	Water body qualifications								
	AA	A	B	C	D	SA	SB	SC	SD
Dissolved oxygen (mg/l)	5	5	5	5	2	6	6	5	2
Fecal coliform (MPN/100 ml)	<1.1	<1.1	100	200	400	<1.1	100	200	400
Nitrate (mg/l)	7	7	7	7	15	10	10	10	15
pH	6.5–8.5	6.5–8.5	6.5–8.5	6.5–9.0	6.5–9.0	7.0–8.5	7.0–8.5	6.5–8.5	6.5–9.0
TSS	25	50	65	80	110	25	50	80	110
Lead (mg/l)	0.01	0.01	0.01	0.05	0.1	0.01	0.01	0.05	0.01
Mercury (mg/l)	0.001	0.001	0.001	0.002	0.004	0.001	0.001	0.002	0.004

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Table 2 Total soluble solids and chemical oxygen demand data of different major rivers of Tarlac province, Philippines during wet and dry season of 2018

River	Total suspended solids (mg/l)		Chemical oxygen demand (mg/l)	
	Dry season	Wet season	Dry season	Wet season
Benig	32	40	27	22
Tarlac	40	169	10	14
Bamban	58	32	11	15
Concepcion	52	169	21	19
Lapaz	223	91	11	28
Rio Chico	103	66	10	<10
Camiling	17	45	6.9	<10

Table 3 Heavy metals concentration of different major rivers of Tarlac province, Philippines during wet and dry season of 2018

River	Lead (mg/l)		Mercury (mg/l)	
	Dry season	Wet season	Dry season	Wet season
Benig	<0.05	<0.05	<0.0002	<0.0002
Tarlac	<0.05	<0.05	<0.0002	<0.0002
Bamban	<0.05	<0.05	<0.0002	<0.0002
Concepcion	<0.05	<0.05	<0.0002	<0.0002
Lapaz	<0.05	<0.05	<0.0002	<0.0002
Rio Chico	<0.05	<0.05	<0.0002	<0.0002
Camiling	<0.05	<0.05	<0.0002	<0.0002

metals. When crops were irrigated with water contaminated with heavy metals, the soils will also be polluted (Verma and Dwivedi 2013).

3.3 Dissolved Oxygen and pH

Table 4 presents the data on the dissolved oxygen and pH of the different major rivers of Tarlac province Philippines. Based from the result the highest dissolved oxygen was found to be within the Tarlac River both during the dry and wet season with 16.0 and 14.8 mg/l respectively.

The lowest dissolved oxygen was found in Concepcion River during the dry season (5.0 mg/l) and in Rio Chico River (4.8 mg/l) during the wet season. Comparing with the National standards from the DENR the major rivers of Tarlac surpasses the minimum standards of classification of water bodies with dissolved oxygen ranging from 2 to 6 mg/l. Low DO is also caused by fertilizer and manure runoff from streets, lawns and farms. The growth of too much algae which could be due to the overuse of fertilizers and the presence of fecal matters causes the speeding up of using the

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Table 4 Dissolve oxygen and pH of different major rivers of Tarlac province, Philippines during wet and dry season of 2018

River	Dissolved oxygen (mg/l)		pH	
	Dry season	Wet season	Dry season	Wet season
Benig	5.3	5.4	8.0	8.26
Tarlac	16.0	14.8	8.1	8.29
Bamban	9.2	6.0	8.0	7.96
Concepcion	5.0	5.0	7.0	6.78
Lapaz	8.0	5.0	7.2	7.98
Rio Chico	7.9	4.8	7.3	7.96
Camiling	15.0	14.0	8.0	8.26

oxygen quickly resulting to a lower DO.[6] The dissolved oxygen which drops below 5.0 mg/l causes stress to many aquatic lives. However based from the results, all of the rivers surpass or equal to 5.0 mg/l except for the Rio Chico River during the wet season with 4.8 mg/l.[7] In terms of pH, the major rivers of Tarlac are within the minimum and maximum standard of pH range within the DENR standards. The pH ranges from 6.78 to 8.29 during the wet season and 7.0–8.1 during the dry season.

3.4 Total Dissolved Solids and Electrical Conductivity

Higher total dissolved solids were observed in the different rivers during the dry season which ranges from 300 to 560 mg/l as compared during the wet season which ranges from 169 to 540 mg/l respectively. Too high or too low concentrations of TDS may limit the growth and may lead to the death of many aquatic organisms.[8] The reduction of water clarity, which contributes to a decrease in photosynthesis and lead to an increase in water temperature, could be due to the high concentrations of TDS. The EC during the dry season ranges from 389 to 423 while during the wet season it ranges from 280 to 420 respectively (Table 5).

3.5 Nitrate

The nitrate concentrations of the different rivers in Tarlac shows to be within the range indicated in Table 1. During the dry season, the nitrate concentrations from

[6] http://www.ririvers.org/wsp/CLASS_3/DissolvedOxygen.htm. Last accessed 30 Nov 2017.

[7] http://www.mymobilebay.com/stationdata/whatisDO.htm. Last accessed 30 Nov 2017.

[8] http://www.ei.lehigh.edu/envirosci/watershed/wq/wqbackground/tdsbg.html. Last accessed 15 Dec 2017.

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Table 5 Total dissolved solids and electrical conductivity of different major rivers of Tarlac province, Philippines during wet and dry season of 2018

River	Total dissolved solids (mg/l)		Electrical conductivity (μS)	
	Dry season	Wet season	Dry season	Wet season
Benig	323	218	400	323
Tarlac	308	169	420	416
Bamban	300	254	418	375
Concepcion	560	540	423	420
Lapaz	300	220	400	291
Rio Chico	305	250	412	281
Camiling	320	200	389	280

Table 6 Nitrate content of different major rivers of Tarlac province, Philippines during wet and dry season of 2018

River	Nitrate (mg/l)	
	Dry season	Wet season
Benig	14	59
Tarlac	10	48
Bamban	10	17
Concepcion	10	48
Lapaz	14	38
Rio Chico	10	45
Camiling	10	38

the different major rivers had a range of 10–14 mg/l. While during the dry season, it ranges from 17 to 59 mg/l with Benig River as the highest. The higher nutrient concentrations within the area could be due to the wastewater from the swine farm lagoons which may be discharged from the nearby farms within the area. Less than 5 mg/l N has little effect, even on nitrogen sensitive crops, but may stimulate nuisance growth of algae and aquatic plants in streams, lakes, canals and drainage ditches (Table 6).[9]

3.6 *Fecal Coliform and* E. coli

In terms of the microbiological parameters such as fecal coliforms and *E. coli*, the different river waters of Tarlac was higher than the standards particularly in Benig River with 11,000 MPN/100 ml and within the Concepcion river which exceeds the National standards for safe water with fecal coliform count of 140,000. Higher concentrations of *E. coli* were also noted in Benig and Concepcion River both with

[9]http://www.fao.org/docrep/003/T0234E/T0234E06.htm. Last accessed 15 Dec 2017.

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Table 7 Fecal coliform and *E. coli* concentration of different major rivers of Tarlac province, Philippines during wet and dry season of 2018

River	Fecal coliform (MPN/100 ml)	*E. coli* (MPN/100 ml)
	Wet season	Wet season
Benig	11,000	1700
Tarlac	390	21
Bamban	270	17
Concepcion	140,000	1700
Lapaz	2600	170
Rio Chico	2800	330
Camiling	330	<1.8

1700 MPN/100 ml. The high concentrations within the said rivers could be due to the wastewater discharged from the nearby areas contributing to the higher Fecal coliform and *E. coli* in the said areas of concern. The higher concentrations as observed in the two rivers could have a potential to reduce the water quality thus reducing also the recreational value (Table 7).[10]

4 Conclusions

The water samples collected from major rivers of Tarlac revealed that there were variations in the results in terms of the different parameters used to quantify the concentrations of the physical, chemical and microbiological quality of the river waters for irrigation purposes. Based from the result, the different river waters were also in accordance with the National Standards set by the Department of Environment and Natural Resources (DENR).

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[10]https://pubs.usgs.gov/wri/wri004139/pdf/wrir00-4139.pdf. Last accessed 15 Dec 2017.

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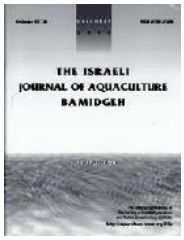

Fermented Sweet Potato Meal, a Sustainable Dietary Protein Ingredient for Juvenile *Penaeus vannamei,* Boone 1931.

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Abstract

Fermentation-biotechnology to produce high-protein agricultural biomass with potential as a feed ingredient is well-established. However, practical applicability of this technology in aquaculture has not been fully realized. The present work evaluates the nutritional and feed value of fermented sweet potato meal (ProEn-K™) to replace soybean meal in the diet of juvenile *Penaeus vannamei*. Four experimental diets containing graded levels of ProEn-K™ replacing 0 (%), 25 (%), 50 (%) and 100 (%) of soybean meal were formulated and fed to *P. vannamei* for 8 weeks. Results showed that 100 (%) of soybean meal can be replaced by fermented sweet potato and 50 (%) replacement elicited growth promoting effects. Survival, feed conversion, and body composition were similar in all treatments. Dietary inclusion of fermented sweet potato promotes better ratio of the n-3/n-6 fatty acid and lowers the total gut bacteria as well as total *Vibrio*. Collectively these results suggest that fermented sweet potato meal could fully replace soybean meal in *P. vannamei* diet. The use of this feed ingredient is a practical approach to meet the increasing needs of proteins in feeds for the expansion and sustainability of *P. vannamei* aquaculture.

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Introduction

Aquaculture of *Penaeus vannamei* in the Philippines is currently expanding due to the rising global market demands and the high profit gains of this farming system. (BFAR, 2016). The increase in production of cultured shrimp goes in tandem with the rising need for quality feed-protein ingredients, considered the most important and costly component of formulated feeds. The limited supply of feed-protein is considered the limiting factor in the sustainability and economic viability of this industry.

Significant research efforts have been directed to find alternative sources of feed protein for aquaculture since fish meal is a limited resource (Tacon et al., 2006). Use of soybean meal was found to lower the fishmeal inclusion in aquaculture feeds (Kaushik et al., 1995). However, livestock and aquaculture industries compete in the use of soybean meal which has resulted in the increase in prices and erratic supply of this ingredient. Feed prices and supply are therefore factors that dictate the sustainability and economic viability of aquaculture in the future (Tacon et al., 2006).

The application of biotechnology, specifically microbial-based solid state fermentation (SSF), has high potential in the production of cheap and sustainable feed ingredients for aquaculture. Technologies on SSF to convert agricultural biomass to a high-protein feed material are well-documented, feasible, and globally acknowledged (Apines-Amar et al. 2016, Zhang et al., 2018). In earlier reports it was shown that through SSF, the nutritional value and protein content of agricultural by-products such as copra meal was improved (Haryati et al., 2006; Dairo and Fasuyi, 2008; Hatta et al., 2014). However, information regarding the feed value and biological testing of these materials as a feed ingredient for aquatic animals is limited. In the present study we evaluated the feed value of SSF sweet potato as a replacement of soy bean meal in the diet of juvenile *P. vannamei*, a shrimp commonly cultured in industrial scale in the archipelagic countries of Southeast Asia and the Pacific region.

Materials and Methods

Diet Formulation

Fermented sweet potato meal (ProEn-K^{TM}) was produced and obtained from Agricultural Biomass Fermentation Laboratory of the Tarlac Agricultural University, Philippines. This ingredient was produced by solid state fermentation (SSF) of sweet potato with mixed consortium of microbes and fungi following a process described by Hatta et al, (2014) and Haryati et al., (2006). All other ingredients were purchased from the South East Asian Fisheries Development Center, Aquaculture Department (SEAFDEC-AQD) Feed mill Laboratory Tigbauan, Iloilo Philippines.

Four experimental diets were formulated containing increasing dietary inclusion levels of ProEn-K^{TM} to replace soybean meal by weight at 0% (TC), 25% (T25), 50% (T50), and 100% (T100) respectively (Table 1). Prior to diet formulation all the dry ingredients were sieved through a 100 µm mesh to standardize the ingredient particle size. These dry feed ingredients including the ProEn-K^{TM}, vitamins, and mineral mix were weighed and thoroughly mixed in a mechanical food mixer (Hobart, USA). The wet ingredients including lecithin, fish oil including oil soluble vitamins, were prepared and gradually added and mixed with the dry ingredients. An adequate amount of water was then added to the compounded dry ingredients to form a moist dough. The resulting dough was pelleted by cold extrusion using a laboratory pelletizer (Hobart, USA). The pellets were collected, oven-dried at 60^{0}C, cut to appropriate size, and stored at 8^{0}C until use. Composition and nutrient contents of the experimental diets are presented in Table 1.

Table 1. Composition and Biochemical analyses of the experimental diets.

Ingredients	*Soybean meal Replacement Level*			
	TC (0 %)	T25 (25%)	T50 (50%)	T100 (100%)
Fish Meal	15.00	15.00	15.00	15.00
Plankton meal (mysids)	5.00	5.00	5.00	5.00
Soybean meal (defatted)	45.00	33.75	22.50	0.00
ProEn-K	0.00	11.25	22.50	45.00
Cod liver oil	2.00	2.00	2.00	2.00
Soybean oil	1.00	1.00	1.00	1.00
Lecithin	1.00	1.00	1.00	1.00
Wheat Flour	25.00	25.00	25.00	25.00
Vitamin mix [a]	1.00	1.00	1.00	1.00
Mineral mix [b]	2.00	2.00	2.00	2.00
Gluten (Binder)	5.00	5.00	5.00	5.00
Total	100.00	100.00	100.00	100.00
Proximate Composition (g/100g diet, Dry Weight)				
Crude Protein	40.18	38.81	38.68	38.87
Crude lipid	9.18	11.01	9.85	11.04
Crude fiber	5.14	5.34	5.62	5.57
Ash	6.96	6.98	6.97	7.01
NFE	38.54	37.86	38.88	37.51
Total	100.00	100.00	100.00	100.00

[a] Vitamin premix (mg.kg^{-1} of diet): B-carotene, 36; cholecalciferol, 3; thiamin, 72 ; riboflavin, 144; pyridoxine, 132 ; cyanocobalamin, 0.4 ; alpha-tocopherol, 330;menadione, 48 ; niacin, 288 ; pantothenic acid,80; biotin, 0.4 ; folic acid, 24 ;inositol, 600; stay C, 2000.
[b]Mineral premix (mg.kg^{-1} of diet):Mg, 300; Fe, 30; Zn, 84; Cu, 42; K, 1500; Co, 22; Mn, 32; Se, 0.02; Mo,0.01; Al, 0.5; I, 8.

Feeding Trial and Growth Evaluation

P. vannamei juveniles were obtained from a private shrimp hatchery at Car-car City, Philippines. The experimental animals were stocked in holding tanks (5-ton capacity), fed with commercial shrimp pellets and acclimated to laboratory conditions for 2 weeks. Prior to the experiment, random samples of shrimp were collected and sent to Fish Health Department of SEAFDEC-AQD to check for the presence of shrimp pathogens and to ensure that the experimental animals were in prime condition. Molecular analysis (PCR) and examination indicate that the shrimp proved negative for white spot syndrome virus (WSSV) and other *Vibrio* pathogens.

Three hundred and sixty shrimps weighing 3.48 ± 0.17 g were randomly assigned to twelve 75L capacity polyethylene aquaria (12 shrimp/aquaria), equipped with individual aeration in a closed recirculating system. The treatment groups were arranged following a Complete Randomized Design. Each experimental diet was allocated to each treatment group, applied at a feeding rate of 3% body weight. Feed was given daily at 08:00, 11:00, 14:00, and 17:00 h for 8 weeks. Water parameters were ensured to be optimum for requirements of the shrimp throughout the experimental period. Water temperature, salinity, dissolved oxygen and pH was monitored daily at 8:00 and 16:00 h.

Sampling for growth and adjustment of feed allocation were carried out every 15 days. During sampling, shrimp in a replicate tank were collected and bulked weighed. Complete change of the recirculating reservoir water and total cleanup of the tanks to prevent algal and bacterial biofilm growth were also conducted. At the end of the feeding trial, shrimp were collected, weighed, and counted. Overall growth performance in response to the dietary treatments was assessed in terms of biological response indices calculated as follows:

Specific Growth Rate (SGR) = $\frac{ln\,(final\ body\ weight) - ln\,(initial\ body\ weight)}{number\ of\ days} \times 100$

Feed Conversion Ratio (FCR) = $\frac{total\ feed\ intake\ (g)}{weight\ gain\ (g)}$

Protein Efficiency Ratio (PER) = $\frac{weight\ gain\ (g)}{protein\ intake\ (g)}$

Percent Weight Gain (WG %) = $\frac{final\ weight(g) - Initial\ weight(g)}{Initial\ weight\ (g)} \times 100$

Percent Survival (S %) = $\frac{Final\ number\ of\ fish}{Initial\ number\ of\ fish} \times 100$

Protein Efficiency Ratio (PER) = $\frac{weight\ gain\ (g)}{total\ protein\ intake\ (g)} \times 100$

Protein Retention (PR) = $\frac{final\ body\ protein\ (g) - initial\ body\ protein\ (g)}{total\ protein\ intake\ (g)} \times 100$

Lipid Retention (LR) = $\frac{final\ body\ lipid\ (g) - initial\ body\ lipid\ (g)}{total\ lipid\ intake\ (g)} \times 100$

Biochemical Analyses

All analyses per sample were conducted in triplicate. Proximate composition analyses of the diets and carcass were conducted following the established methods of AOAC (1986). Crude protein was quantified by Kjeldahl total protein Nitrogen analysis (Foss Tecator™ Digestion and Foss Kjeltec ™ 8200 Auto Distillation). Total lipid was quantified by Soxhlet extraction with petroleum ether as solvent (Foss Soxtec ™ 2050 Automatic System) while total fiber was analyzed using Foss Fibertec ™ 2010 System employing the Ceramic Fiber Filter Method for crude fiber quantification. Moisture was analyzed using the infrared drying method (Mettler Toledo® Halogen Moisture Analyzer). Ash was quantified by furnace combustion method at 600°C (AOAC, 1996).

Total Amino acid profiling of ProEn-K™ was conducted using Prominence High Performance Liquid Chromatography Amino Acid Analysis System (Shimadzu, Japan), following the method detailed in the AOAC Official Method 994.12, Amino acids in feeds (Llames & Fontaine, 1994). Fatty acid profiling was only done in the control and in the treatment group exhibiting optimal growth responses in relation to the experimental treatment. Total fatty acid profiling of the experimental animals fed with the experimental dietary ingredient was performed using the Gas Chromatography/Mass Spectroscopy (GCMS) (Perkin Elmer Clarus 600) following the method described by Michael et al., (2006). Individual fatty acids were identified based on their retention times and equivalent chain length.

Antibacterial activity of fermented sweet potato extracts was conducted following the antibacterial disc assay described by Annie et al., (2009). The extract was prepared by soaking the dried fermented biomass with ethyl acetate for 24h and insoluble materials

removed through filtration. The collected solute was evaporated in a rotary evaporator. The residue was collected, dried, weighed, and dissolved in a similar solvent to prepare a 100 µg/ml solution. A 10-mm sterilized paper disc was prepared, added with 50 µl of the extract solution and dried at room temperature to remove the solvent. The control disc was prepared using only the solvent with no extract. The discs were then laid on the spread-plate culture of *Vibrio harveyi* (10^7 CFU/ml) in Luria-Bertani media containing 2% NaCl. Following the 24h incubation, diameters of the clear halo zones around the discs were measured as bactericidal zone of inhibition.

Similar to the fatty acid analysis, gut Vibrio and total bacterial loads were only quantified in the control and in the treatment group exhibiting optimal growth responses. To quantify the shrimp total gut *Vibrios* in response the test diet, the stomachs of shrimp were dissected aseptically collected and weighed. Sterile saline solution (1.5 % NaCl in distilled water) was added to the collected tissues which were then homogenized with a sterile tissue homogenizer. Ten-fold serial dilutions were prepared from the tissue homogenate and 100 µl aliquots were plated to the bacterial media, incubated at room temperature for 18-24 h and growing colonies were counted. Thiosulfate Citrate Bile Salt (TCBS) media was used to specifically quantify *Vibrio* colonies both the sucrose fermenters (yellow colonies) and the non-sucrose fermenters (green colonies). Total bacteria were counted using the general media Nutrient Agar (NA, Merck, Germany) containing 1.5% NaCl (Barcenal et al., 2015).

Statistical Analysis.

If applicable, data obtained were subjected to one-way analysis of variance (ANOVA). Significant differences observed among the treatment groups were resolved using Tukey's post hoc test. T-test was used to resolve the differences in comparing two treatment groups. Probability values in all test is set at a significance level of 0.05. Statistical analysis was carried out using the SPSS statistical package for windows version 18.

Results

Following the 8-week feeding trial, survival values among treatments were high and were not influenced by the dietary levels of fermented sweet potato meal. Significant improvement in weight gain in comparison to the control and the other treatment groups was exhibited in the T50 group. Weight gain of the other treatments, T25 and T100 were similar to the control group. Specific growth rate was also highest in the T50 group while TC, T25 and T100 groups exhibited similar values but were lower than those in T50. No significant treatment effects were observed in other biological growth indices including FCR, PER, and Nutrient Retentions (Table 2). Correspondingly, no treatment effects and significant changes were observed in terms of shrimp tissue biochemical composition even at the highest soybean meal replacement level (Table 3).

Table 2. Growth performance and Nutrient Utilization indices of *P. vannamei* fed experimental diets

Growth Indices	Soybean meal Replacement Levels			
	TC	T25	T50	T100
S (%)	80.00 ± 4.44	75.56 ± 3.22	91.7 ± 1.01	90.20 ± 2.01
WG (%)	332.00 ± 13.00^{a}	368.00 ± 2.30^{a}	446.00 ± 11.00^{b}	349.00 ± 3.00^{a}
SGR	2.44 ± 0.05^{a}	2.57 ± 0.03^{b}	2.85 ± 0.03^{c}	2.50 ± 0.01^{ab}
FCR	1.44 ± 0.02	1.47 ± 0.05	1.52 ± 0.02	1.40 ± 0.02
PER	1.86 ± 0.05	1.83 ± 0.07	1.78 ± 0.05	1.86 ± 0.03
PR	17.41 ± 1.37	22.17 ± 2.16	18.08 ± 2.89	16.67 ± 1.12
LR	9.99 ± 0.84	11.22 ± 1.03	9.77 ± 0.62	9.99 ± 0.84

***Where S(%) is the percent survival, WG(%) is the percent weight gain, SGR is the specific growth rate, FCR is the feed conversion ratio, PER is the protein efficiency ratio, PR is the protein retention and LR is the lipid retention*.**

Table 3. Whole body proximate compositions of *P. vannamei* after the 8-week feeding trial and the antibacterial activity of ProEn-K ethyl acetate extract with *V. harveyi* as test bacteria.

	Treatment Groups			
	TC	*T25*	*T50*	*T100*
Biochemical Composition (% Dry Weight)				
Total Protein	77.15±1.67	77.28±4.05	78.23±1.12	74.80 ±2.38
Total Lipid	11.22±1.71	10.51±2.23	9.98±1.52	10.91±1.11
Ash	10.72±0.08	10.17±0.18	8.81±0.38	9.81±0.21
ProEn-K Antibacterial Activity				
[a]Bacterial Zone of Inhibition (mm)				
[b]ProEn-K extract	23.00±1.2*			
Control (solvent only)	0.00			

[a] *Vibrio harveyi* (10^7CFU/ml) was used as the test bacteria.

[b] Ethyl acetate was used as the extraction solvent; Extract applied at a dose of 100µg/ml.

***Indicates significant statistical difference at α=0.05.**

Analysis of the nutritional composition showed that the fermented sweet potato meal had protein content of 40%, lipid content of 0.4%, ash content of 9%, fiber content of 4.2 %, and carbohydrate content of 46.4%. This ingredient also had complete content of essential amino acids. In comparison with the essential amino acid content of *P. vananmei* tissue protein, each amino acid comprising the fermented sweet potato protein, except for Lysine, exhibited greater than 90 chemical score value (Table 4).

Table 4. Essential amino acid profile *of P. vannam*ei muscle proteins, ProEn-K proteins and the essential amino acid chemical score index of this ingredient.

Essential Amino Acids	*Fermented Sweet Potato Essential Amino acid[1] (% protein)*	*P. vannamei Essential Amino acid[2] (% protein)*	*Fermented Sweet Potato Essential Amino acid Chemical Score[3]*
Phenylalanine & Tryptophan	5.18	5.39	96.10
Valine	4.74	3.30	143.63
Threonine	2.63	2.52	104.36
Isoleucine	2.72	2.65	102.64
Methionine & Cystine	5.78	2.67	216.47
Histidine	3.6	1.62	222.22
Arginine	5.79	6.10	94.91
Leucine	5.18	4.69	110.44
Lysine	1.23	4.84	25.41
[4]Fermented Sweet Potato Chemical Score Index	25.41		

[1]Fermented Sweet Potato Essential Amino acid (% protein): Actual analyzed values.
[2]*P. vannamei* Essential Amino acid (% protein): from Forster et al., 2002.
[^3]Essential Amino Acid Chemical Score= {(Essential amino acid amount (g) in 100 g *PECM™* protein) /(Essential amino acid amount (g) in 100 g shrimp protein)} ×100.
[4]CSI (Protein Chemical Score Index) = It is the chemical score value of an amino acid exhibiting the lowest essential amino acid chemical score.

The overall chemical score of this ingredient is 25 with Lysine as the most limiting amino acid. Fatty acid composition of the experimental animals maintained with T50 exhibited a similar profile than in the control group. However in terms of n-3/n-6 fatty acid ratio the T50 group had higher values than that of the control group (Table 5).

Table 5. Tissue fatty acid profile of the shrimp fed the control diet and those fed diets containing 50% ProEn-K as a replacement of soy bean meal for 8 weeks.

	T0 (Control) (FAME)	*T50 (FAME)*
Fatty Acid Methyl Esters (FAME)	g/100g FAME	
Decenoic acid	0.29±0.03	0.00±0.00
Dodecanoic acid	0.10±0.00	0.07±0.00
Tetradecanoic acid,	0.53±0.03	0.72±0.06
Pentadecanoic acid	0.33±0.02	0.24±0.01
Hexadecenoic acid	1.49±0.17	1.55±0.32
Hexadecanoic acid	12.00±0.41	18.57±4.35
Heptadecanoic acid	1.90±0.29	0.90±0.09
Octadecadienoic acid (linoleic, N-6)	10.80±0.20	4.97±0.02
Octadecenoic acid	25.93±2.50	20.52±4.64
Octadecanoic acid	8.01±0.12	9.50±2.45
Eicosatetraenoic acid, (arachidonic, N-6)	2.71±0.04	1.66±0.04
Eicosapentaenoic acid (EPA, N-3)	12.20±0.41	11.47±0.64
Eicosadienoic	2.73±0.42	1.00±0.12
Eicosenoic acid,	3.50±0.12	3.53±0.08
Eicosanoic acid	0.51±0.02	0.25±0.00
Docosahexaenoic acid (DHA, N-3)	15.21±1.47	10.74±2.03
Docosenoic	1.37±0.08	14.14±1.04
Docosanoic	0.40±0.02	0.19±0.02
N-3 / N-6 ratio	2.02	3.35

Assessment of the antibacterial activity of the fermented sweet potato extracted with ethyl acetate showed that the extract at 100µg/ml exhibited an inhibition diameter zone of 23.00±1.2 mm with *Vibrio harveyi* as the test bacteria. No inhibition zone is observable in the control group (Table 3). Also, a 10-fold reduction in gut Vibrio was exhibited in the test group as compared to the control (Figure 1). Accordingly, the level of gut bacteria was found significantly lower in those receiving the test diet as compared to the control (Figure 2).

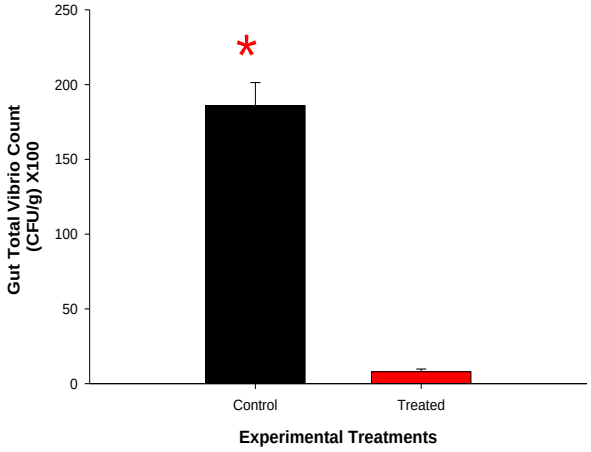

Figure 1. Total gut Vibrio load of the shrimp, *P. vannamei* fed with diets containing fermented sweet potato and the control. Values are mean ± standard error. Mean values with a star superscript are significantly different, T test, α=0.05.

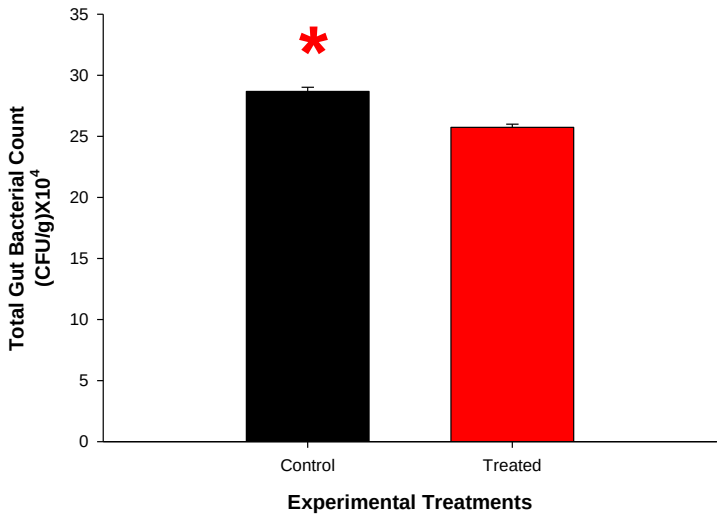

Figure 2. Total gut bacterial load of the shrimp, *P. vannamei* fed with diets containing fermented sweet potato and the control. Values are mean ± standard error. Mean values with a star superscript are significantly different, T test, α=0.05.

Discussion

Solid state fermentation aimed to improve the nutritional value of cellulosic and carbohydrate-rich agricultural biomass is considered a sustainable approach to meet the growing demands of feed ingredients for both the aquatic and the terrestrial animal-growing industries (Iyayi & Aderolu, 2004, Yousef & Alam, 2013, Ferreira et al., 2016). The enhancement of protein content of sweet potato to about 40% though SSF in the present study concurs with these earlier findings. Composition of the fermented material protein indicates complete and well-balanced essential amino acid content. All the essential amino acid exhibits a Chemical Score higher than 90 except for Lysine which scored lowest and is considered to be the most limiting essential amino acid. Similar to our results, improvement of the protein content and essential amino acid profile was also documented in wheat, soybean, and rice bran fermented with *Bacillus coagulans* and *Aspergillus niger* (Joseph et al., 2008). The amino acid profile of the fermented material is dictated by the microbial species and the biomass type used as substrate in the fermentation (Denardi-Souza et al., 2018). The low content of lysine in the present study could be attributed to the sweet potato used as substrate and the microbial species used in fermentation.

The feeding trial results confirm the viability of the fermented ingredient to completely replace soybean meal in the diet of juvenile *P. vannamei*. Growth response in treatments with complete replacement of soybean meal was found to be similar to that of the control. Moreover, significant growth enhancement was observed in the treatment receiving 50% soybean meal replacement. Earlier studies also indicate significant growth enhancement in broiler chickens fed diets containing yeast fermented products (Sulhattin et al., 2017), with fermented soybeans (Chah et al., 1975) and with fermented cereals (Sulhattin, 2015). In *P. vannamei*, feeding with fermented guar meal at 2.5% fish meal replacement was also reported to significantly enhance growth (Jannathulla et al., 2016). Growth promotion associated with fermented ingredients was attributed to the presence of small peptides in the fermented products, degradation of anti-nutritional factors and enhanced nutrient digestion due to the presence of residual microbial enzymes (Chah et al., 1975, Jannathulla et al., 2016, Sulhattin et al., 2017). Though not measured in the present study, these aspects may explain the growth enhancement effects of fermented sweet potato meal in *P. vannamei* as observed in this study.

The observed growth enhancement in the present study could also be attributed to improved gut health. Lower counts of gut associated bacteria and Vibrios were observed in the treated group (T50) as compared to the control group (TC). Furthermore, the ethyl acetate extract of the fermented material exhibits a potent antibacterial activity,

supporting the observed effects in lowering the gut microbial load of the treated shrimp groups. Similar to our findings, bacterial inhibitory activity of fermented feeds on gut microflora is well-documented in terrestrial animals including pigs (van Winsen et al, 2001) and broiler chickens (Missotten et al, 2013). Our present findings on the influence of fermented feeds on gut microflora are unprecedented in aquatic animals specifically in cultured shrimp.

Depressed growth in cultured shrimp is commonly attributed to the dominance of Vibrios in gut microflora. Gut infection of Vibrios in shrimp impairs digestive and absorptive processes and occasionally results to slow growth, infection, and eventually death (Kewadugama et al. 2017, Lavilla-Pitogo et al.1998). The decrease in the gut bacterial load may promote better nutrient absorption and assimilation, resulting to overall growth improvement as observed in the present study.

No negative influence of the fermented ingredient on the tissue chemical composition of the shrimp was observed in the present study even at the highest inclusion level. However significant alterations in terms of N-3 and N-6 fatty acids were observed in groups receiving the fermented ingredient. The treated group exhibited a better profile of the N-3/N-6 ratio compared to the control group, indicating lower N-6 fatty acid tissue accumulation. To date, the significant decline in tissue N-6 fatty acids in animals as influenced by the fermented dietary ingredient has not been not previously documented in any other animal species and our work is the first report regarding this aspect. In vertebrates the heightened biosynthesis of N-6 fatty acids specifically arachidonic acid is known to be triggered by inflammatory responses due to infection (Eberhard et al, 2002). Similarly, in insects (Stanley-Samuelson et al, 1991**)** and in crustaceans (Heckmann et al, 2008) arachidonic acid (N-6) is also utilized as a precursor in the synthesis of eicosanoids an important immunity signaling molecule that plays a vital role during infections. In relation to the present findings, it is tempting to speculate that the lower N-6 fatty acid content of the treated group could be due to the decreased gut Vibrio content that reduces inflammatory responses leading to minimal synthesis and tissue accumulation of N-6 fatty acids. However, the mechanism on how the fermented ingredient influences the shrimp tissue fatty acid composition is not fully understood and this aspect requires additional thorough investigation.

Collectively our findings indicate that fermented sweet potato meal could completely substitute soybean meal and elicits a growth promoting effect if utilized as 50% substitution of soybean meal in the diet *P. vannamei*. Use of this fermented ingredient also lowers the gut Vibrio contents and improves the N-3/N-6 tissue fatty acid profile of the cultured shrimp. Utilization of this feed ingredient is a practical approach to improve the quality of farmed shrimp, lessen the risk of Vibriosis and promote the sustainability of available feed-protein supply for the shrimp culture industry.

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Validation of suitable reference genes for normalization of quantitative reverse transcriptase- polymerase chain reaction in rice infected by *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a costly disease in rice that threatens global rice production. Gene expression analysis by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) allows the study of the underlying mechanisms of both BB pathogenesis and resistance. In relative quantification, reference genes are often used to normalize the results to remove technical variations allowing the determination of true biological changes in a pilot experiment. However, variations in the expression of these reference genes can lead to erroneous and unreliable results. Thus, choosing the most stable reference genes for any specific experimental condition is of utmost importance in qRT-PCR experiments. Here, we used geNorm, NormFinder, Bestkeeper, Delta-Ct and RefFinder programs and/or methods to analyze the stability of the expression of eleven candidate reference genes namely: *18S ribosomal RNA* (*18S rRNA*), *Actin-1* (*ACT1*), *ADP-Ribosylation Factor* (*ARF*), *Endothelial differentiation factor* (*Edf*), *eukaryotic Elongation Factor-1α* (*eEF-1α*), *eukaryotic Initiation Factor-4a* (*eIF-4a*), *Profilin 2* (*Prof2*), *Nucleic Acid Binding Protein* (*NABP*), *Triosephosphate Isomerase* (*TI*), *Ubiquitin 5* (*UBQ5*) and *Ubiquitin 10* (*UBQ10*) in cDNA samples from BB-susceptible and Xa21-mediated resistant rice cultivars collected at various times after *Xoo* inoculation. Under our experimental conditions, *Edf* and *TI* were the most stable reference genes while the common housekeeping genes *18S rRNA*, and *UBQ5* were among the least stable genes. Though using either *Edf* or *TI* as internal control is adequate for gene expression analysis, we suggest using both genes to normalize the data of qRT-PCR assays for rice subjected to *Xoo* inoculation.

1. Introduction

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a threatening rice disease responsible for the large percentage of yield reduction in all rice growing areas. Its symptom includes a vascular wilt at the seedling stage, a leaf blight, and unfilled panicles in mature plants which resulted from the invasion of the vascular system by *Xoo* bacteria (Mew, 1987). At the molecular level, *Xoo* secretes transcription activator-like (TAL) effectors which invade and highjack the host cells by activating the transcription of genes that enhance plant susceptibility and support bacterial virulence (Boch and Bonas, 2010; Römer et al., 2010). To date, more than 30 BB resistance genes have been identified in *Oryza sativa* and its closely related species. Among them, *Xa21* has probably been the most commonly used for rice variety improvement as it provides high level and broad-spectrum BB resistance (Nguyen et al., 2018; Singh et al., 2001; Zhang et al., 2006). *Xa21* codes for a plasma membrane receptor which recognizes the tyrosine-sulfated protein RaxX and triggers the Xa21-mediated immunity (Pruitt et al., 2015). It is known that this response involves Xa21-binding proteins (Chen et al., 2010; Park et al., 2010; Wang et al., 2007) as well as the direct interaction of a cleaved Xa21 subunit with the WRKY62 transcription factor (Park and Ronald, 2012; Peng et al., 2008), nonetheless, the precise mechanisms of the resistance are still not yet completely elucidated.

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Studying the variations in the expression of candidate genes provides perspectives of the mechanisms of plant responses to BB. The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) remains a tool of choice to quantify mRNA expression and thus confirm the involvement of various candidate genes in a specific cellular response due to the sensitivity, precision and reproducibility of its results (Derveaux et al., 2010; Hellemans et al., 2007). However, the veracity and reliability of qRT-PCR results are essentially altered by the variations between samples, RNA quality and quantity, and the different reverse transcription and PCR efficiencies (Bustin et al., 2009; Derveaux et al., 2010). To cope with this variability, expression levels of the genes of interest (GOIs) are usually normalized against a stably and uniformly expressed reference genes according to the $\Delta\Delta CT$ method in order to provide reliable relative expression data (Rao et al., 2013). Though, in this method, the selection of reference genes is critical since variations in their expression can completely overturn the final data especially when the variations in GOI expression are restrained. The expression of the reference genes used in qRT-PCR analysis should remain stable across all experimental samples and under different conditions (Derveaux et al., 2010; Huggett et al., 2005). Yet, previous reports on the stability of the usual reference genes has shown that no universally stable reference gene could be found, thus implicating that reference genes' stability in qRT-PCR experiments needs to be validated for specific and suitable experimental conditions and purposes (Kozera and Rapacz, 2013; Laurent et al., 2008).

Several software and programs have been established to evaluate the stability of the reference genes expression. Among the most commonly used are the geNorm (Vandesompele et al., 2002a), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) algorithms, and the Delta-Ct method (Silver et al., 2006) and RefFinder system (Xie et al., 2012). Using these tools, various studies on rice have highlighted that proper validation of reference genes is essential to determine their stability and thus recommending the suitability of each reference gene for various experiments in rice. For example, *Ubiquitin 5* (*UBQ5*), *Ubiquitin-conjugating enzyme E2* (*UBC-E2*), *Endothelial differentiation factor* (*Edf*) and *eukaryotic Elongation Factor-1α* (*EF-1α*) were found to be suitable for growth and development, environmental conditions, and organ-specific gene expression analyses (Auler et al., 2017; Narsai et al., 2010; Wang et al., 2016). For hormonal and abiotic treatments and stresses, *18S ribosomal RNA* (*18S rRNA*), *25S ribosomal RNA* (*25S rRNA*), *Ubiquitin 10* (*UBQ10*) and *Ubiquitin conjugating enzyme* (*UBC*) were the most stable reference genes (Almas and Kamrodi, 2018; Jain et al., 2006; Kim et al., 2003; Moraes de Freitas et al., 2015) while for a few biotic stress like blast infection *18S rRNA, Actin, and 40S 27a ribosomal subunit (40S 27a)* were found to be the most suitable reference genes (Bevitori et al., 2014; Che Omar et al., 2016). To our knowledge, no resources are currently available on the suitable reference genes for gene expression analysis involving *Xoo* inoculation experiments. Hence, in this paper, we analyzed 11 candidate reference genes in terms of their expression stability in both BB-susceptible rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21/Xa21*) (Sagun, Suachaowna et al., unpublished) at different times post *Xoo* inoculation.

2. Materials and methods

2.1. Plant material and growth conditions

Rice (*Oryza sativa* L. ssp. *indica*) cultivars RD47 and IRBB21 were provided by the Bureau of Rice Research and Development, Phitsanulok, Thailand. The *Xa21* gene originated from the wild species *O. longistaminata* was transferred through wide hybridization in IR24, resulting in the near-isogenic line, IRBB21. In tests for disease resistance, IRBB21 has been reported to be resistant to many *Xoo* strains from the Philippines and India (Khush et al., 1990). Rice cultivar RD47, an elite Thai cultivar, was derived from three-line cross between Suphanburi 1 and IR64 then with CNT86074–25–9-1 at Chainat Rice Research Center, Thailand (the Rice Department, Ministry of Agriculture and Cooperatives, Thailand).

The *Xa21* gene from IRBB21 was introgressed in RD47 through backcross breeding and Marker Assisted Selection (data not shown) until homozygous-*Xa21* BC_3F_3 lines were obtained.

2.2. Xoo isolation and inoculation test

Since Thailand biosafety regulations limit the import of living microorganisms especially those causing diseases in major commodities like rice, the authors made use of the local strains of *Xoo* in this study and validated them through PCR using specific universal primers.

BB infected leaves were collected from paddy fields in Phitsanulok province and *Xoo* was isolated on nutrient agar (peptone-bovine-agar). The isolated bacteria, xoo16PK002, was identified as *Xoo* through PCR assays using *Xoo* specific primers TXT (Sakthivel et al., 2001) and *Xoo* specific primers Xoo80 (Lu et al., 2014). Furthermore, preliminary pathogenicity tests on 60 days old rice plants were done and had shown that RD47 plants were rather susceptible to highly susceptible to xoo16PK002 with clear BB lesions lengths (LL) ranging 25-29 cm ± 1.28 at 21 days after inoculation, whereas RD47's near isogenic lines BC_3F_3 and the IRBB21 cultivar were more resistant to the *Xoo* strain with LL ranging from 5 to 9 cm ± 0.26 (Sagun, Suochawna, Puttasem et al., unpublished). For the infection experiments presented here, the *Xoo* isolate xoo16PK002 was re-streaked and incubated at 28 °C for 48 h. A *Xoo* inoculum (OD600 of 0.2) was prepared and used to inoculate 60-day-old plants according to the clipping method (Kauffman, 1973). Mock (water) inoculation was used as a control. Samples corresponding to 5 cm of the leaves directly below the inoculation sites were collected at 0, 2, and 24 h post inoculation, respectively, and leaf samples were frozen in liquid nitrogen immediately.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from each 100 mg leaf sample using RNAprep Pure Kit (Tiangen Biotech LTD., China) following manufacturer's instructions. Total RNAs were quantified using Synergy H1 microplate reader (Biotek, USA) and their integrity was assessed through agarose gel electrophoresis. The qScript™ XLT cDNA synthesis kit (QuantaBio, USA) was used to reverse transcribe 1 μg of total RNA templates in order to synthesize first strand cDNAs according to manufacturer's protocol.

2.4. Quantitative Real-time PCR

For qPCR analyses, the fast SYBR Green Master Mix (Quantabio, USA) was used to prepare 20 μl qRT-PCR reactions containing 1 μl of the cDNA templates and 0.5 μM of each primer pairs. Technical triplicates and No Template Controls (NTCs) were run through the Eco48 Real-Time PCR system (PCRmax, UK) for 35 cycles (95 °C for 10s, 60 °C for 10s and 72 °C for 20s) followed by a melting curve analysis. Ct values were determined using the Eco™ 48 Study Software installed in the Eco48 Real-Time PCR system. The Eco™ 48 Study software allows for normalized data combination from multiple Eco 48 experiments into a single analysis, and supports standard curve analysis and relative quantification experiments, which were used in this study. The comparative Ct method was used to transform generated Ct values in relative quantities with the highest relative quantity for each gene set up to 1.0. All data were subjected to analysis using geNorm v3.4 (Vandesompele et al., 2002a), NormFinder v20 (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), Delta-Ct method (Silver et al., 2006) and RefFinder (Xie et al., 2012) programs.

Table 1
List of candidate reference genes validated in this study.

Candidate reference gene	Primer sequence	Reference
Endothelial differentiation factor(Edf)	5′-TCCGAACCAGCAGATCATCG-3′	Wang et al., 2016
LOC_Os08g27850	5′-GCATGGTATCAAAAGACCCAGC-3′	
Triosephosphate Isomerase (TI)	5′-CGACATCATCAACTCCGCCAC-3′	Wang et al., 2016
LOC_Os01g05490	5′-CCTCTTCAGACATCTTCCCACG-3′	
eukaryotic Initiation Factor-4a (eIF-4a)	5′-TTGTGCTGGATGAAGCTGATG-3′	Wang et al., 2016
LOC_Os03g08020	5′-GGAAGGAGCTGGAAGATATCATAGA-3′	
Profilin-2 (Prof2)	5′-CCAACTGGTCTTTTCCTTGGG-3′	Wang et al., 2016
LOC_Os06g05880	5′-GGGGTCATCGGCTCATCATAG-3′	
ADP-ribosylation factor (ARF)	5′-ATGAAAGGAAGACATGGCGG-3′	Wang et al., 2016
LOC_Os05g41060	5′-TGGTGGTGGAAGCTAAAGAGC-3′	
Nucleic acid binding protein (NABP)	5′-GGAATGTGGACGGTGACACT-3′	Narsai et al., 2010
LOC_Os03g25980.1	5′-TCAAAATAGAGTCCAGTAGATTTGTCA-3′	
eukaryotic Elongation Factor-1a (eEF-1α)	5′-TTTCACTCTTGGTGTGAAGCAGAT-3′	Jain et al., 2006
LOC_Os03g08020	5′-GACTTCCTTCACGATTTCATCGTAA-3′	
Ubiquitin 10 (UBQ10)	5′-TGGTCAGTAATCAGCCAGTTTGG-3′	Jain et al., 2006
LOC_Os02g06640	5′-GCACGACAAATACTTGAGGAAGAG-3′	
Actin-1 (ACT1)	5′-CTTCATAGGAATGGAAGCTGCGGGTA-3′	Narsai et al., 2010
LOC_Os05g36290.1	5′-CGACGACCTTGATCTTCATGCTGCTA-3′	
18S ribosomal RNA (18SrRNA)	5′-CTACGTCCCTGCCCTTTGTACA-3′	Jain et al., 2006
Locus ID: AK059783	5′-ACACTTCACCGGACCATTCAA-3′	
Ubiquitin-5 (UBQ5)	5′-CCAGTACCTCAGCCATGG A-3′	Jain et al., 2006
Locus ID: AK061988	5′-GGACAGAATGATTAGGGATC-3′	

Gene names and all their details are presented the way they are reflected in the reference cited.

3. Results

3.1. qRT-PCR of candidate reference genes

A set of 11 candidate reference genes and their specific primers was selected from previous studies on reference gene validation in rice (Table 1). The expression levels of these genes were measured by qRT-PCR in 8 different samples corresponding to the leaves from the BB susceptible RD47 (no *Xa21*) and its BB resistant progeny BC_3F_3 (homozygous *Xa21*) collected at 0, 2 and 24 h after *Xoo* inoculation and 2 h after mock inoculation. For all the tested candidate genes, NTCs showed no amplification, and the sample melting curve analysis generated single peaks indicating that a specific PCR product for each gene was amplified. Moreover, electrophoresis of qRT-PCR products showed a single band of the expected size for each candidate gene (Fig. 1). After confirming the specificity of the qRT-PCR primers, the Ct values were determined for each technical triplicate in all samples. The mean Ct values (Supplemental Table 1) were then transformed into relative quantity values, which was later used in the geNorm and NormFinder programs by fixing the highest relative quantity for each candidate gene to 1 and using the comparative Ct method. For Bestkeeeper, Delta-Ct and RefFinder analyses, the raw Ct values were used.

3.2. Stability of candidate reference genes using geNorm analysis

The geNorm software (Vandesompele et al., 2002a) uses the principle that the expression ratio of perfect reference genes should remain constant across different experimental treatments. It determines the gene expression stability measure (M) of reference genes as well as the average pairwise variation for that gene as compared to other tested reference genes. Stepwise exclusion of the gene with the highest M value allows to select the two most stable genes. The two most stable genes are determined by sequentially removing the least stable gene with the highest M value (Vandesompele et al., 2002a). Initially, analysis of the samples from the BB susceptible RD47 (Fig. 2a) and the BB resistant BC_3F_3 plants (Fig. 2b) was undertaken separately. For the RD47 samples, *Edf* and *eIF-4a* had the lowest M values, hence, they were the most stable reference genes, and followed by *TI*. Meanwhile, *ARF*, and *18S rRNA* yielded the highest M values and were the least stable genes (Fig. 2a). In the BC_3F_3 samples, *Edf* and *TI* were the most stable genes while *eIF-4a* was ranked fourth. Meanwhile, *ARF*, *UBQ5*, and *18S rRNA* were among the three least stable reference genes (Fig. 2b). Finally, when all the samples were analyzed together for the expression stability of the candidate genes (Fig. 2c), *Edf* and *TI* were the most stable genes, followed by *eIF-4a* while *ARF*, *18S rRNA*, and *UBQ5* were still among the least stable genes.

3.3. Determination of the optimal number of reference genes by geNorm

Although most published studies on gene expression suggest a single internal control for qRT-PCR normalization, it is also known that increasing the number of reference genes results in more reliable and more accurate data (Jain et al., 2018; Vandesompele et al., 2002b; Zhao et al., 2016). However, there is a trade-off between accuracy and practical considerations when selecting reference genes to be used. Pairwise variation (V) analysis calculates between two sequential normalization factors containing an increasing number of reference genes; geNorm also provides the tool in generating the optimal reference genes to be used. The program suggests that if the value of V is below the 0.15 cut-off value, the last added reference gene may not need to be included for the data normalization. In this study, the V2/3 value of 0.136 indicates that the third most stable reference gene, which is *eIF-4a*, is not required and thus, the use of the two most stable reference genes, *Edf* and *TI*, is already optimal for accurate normalization (Fig. 3).

3.4. Stability of candidate reference genes using NormFinder analysis

To validate the results of the geNorm analysis, we also assessed the expression stability of the candidate genes in our samples with the NormFinder software. NormFinder directly calculates for each gene a stability value based on its inter- and intragroup variations of expression which can prevent the selection of co-regulated genes (Andersen et al., 2004). As the NormFinder software is limited to only 10 genes for analysis, the top 10-ranked stable genes from the geNorm analysis were used for the calculation. Results of the analysis showed that *Edf* and *TI*, with the lowest individual stability values of 0.036 and 0.059, respectively, were again selected as the most stable genes with a combined stability value of 0.029. The reference gene *Edf*, in particular, was deemed to be the best reference gene as its variation values for both intra- and inter- groups were lowest while *TI* still showed more variation than *eIF-4a* in the RD47 group (Table 2). The *UBQ5* and *18S rRNA* genes, respectively, were ranked 8th and 10th among the 10 tested

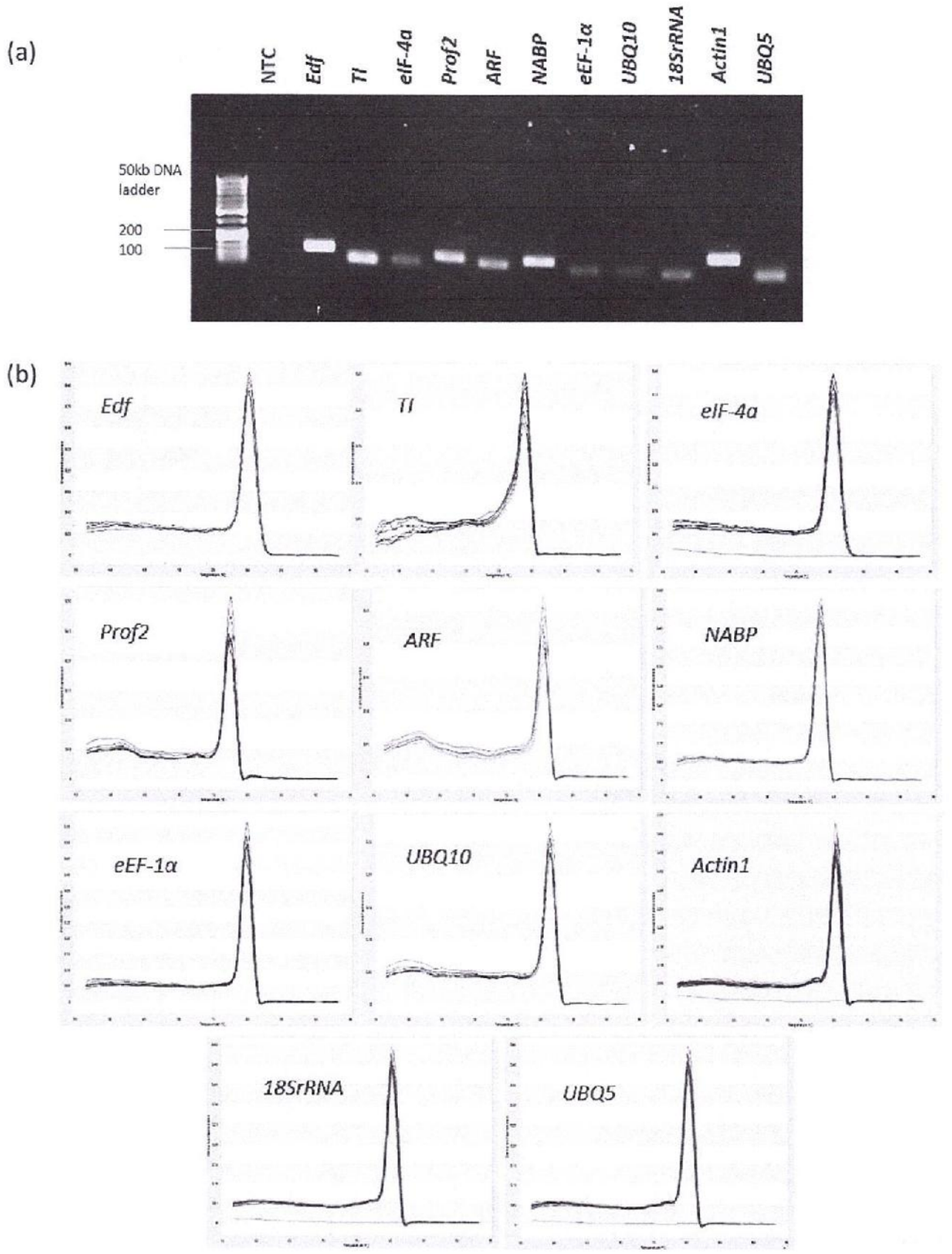

Fig. 1. Specificity of gene amplification products tested on the 6-leaf stage rice cultivar RD47 at 0 h after *Xoo* inoculation. (a) Gel migration of qRT-PCR products. A single DNA amplicon of the projected size is shown for each gene tested in this study. Agarose gel (2%). NTC-Non-Template Control; and (b) Dissociation curves of qRT-PCR products for all candidate reference genes validated in this study. Analysis of dissociation curves, also called melt curves, was used to assess whether the candidate reference genes produced single, specific products. The single peaks of dissociation curves in each candidate reference gene represented a pure, single

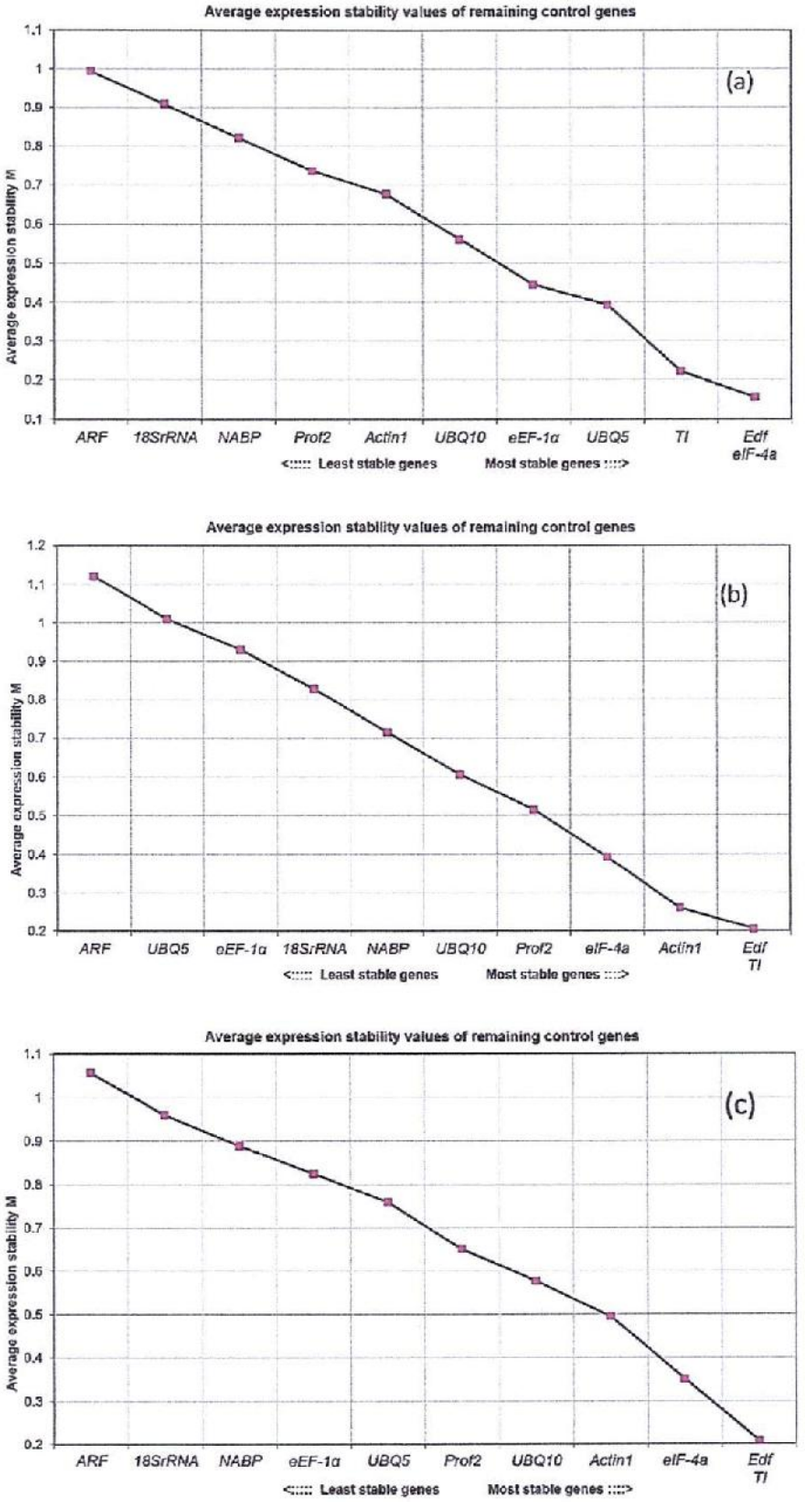

Fig. 2. Expression stability and ranking of reference genes by geNorm: (a) RD47; (b) BC_3F_3 (*Xa21-Xa21*) progeny; and (c) all samples. Gene(s) with lower average expression stability M denotes more stable expression.

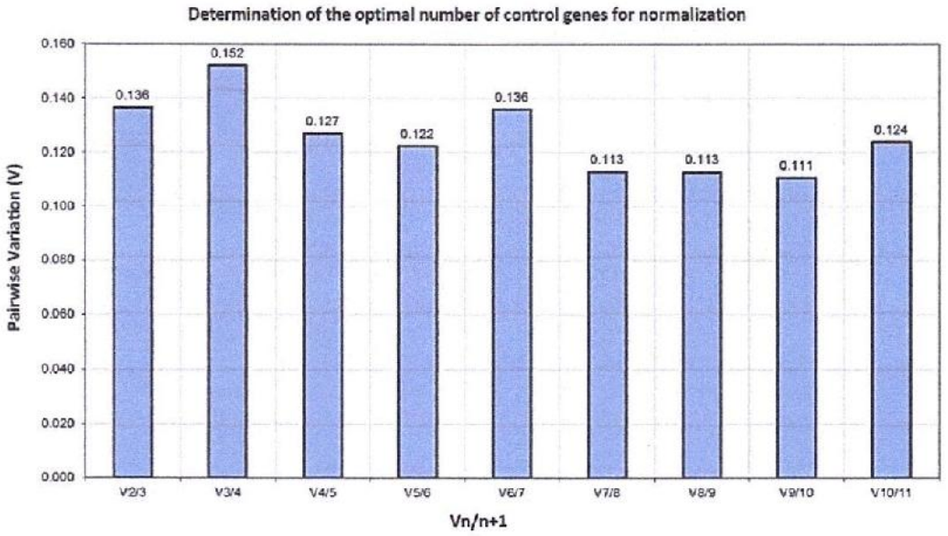

Fig. 3. Determination of the optimal number of control genes for accurate normalization by geNorm pairwise variation analysis. Pairwise variation analysis (Vn/n + 1) between the normalization factors NFn, including the n most stable reference genes, and NFn + 1, corresponding to the stepwise inclusion of the next most stable reference gene, were calculated. A large variation means the last added reference gene has a significant effect and should be included while Vn/n + 1 with a value below the 0.15 cutoff indicates n as the optimal number of reference genes.

Table 2
NormFinder analysis of top 10-ranked stable reference genes from the geNorm analysis. Gene(s) with lower stability value denotes more stable expression.

Candidate reference gene	Stability value	Intra-group variation		Inter-group variation
		RD47	BC_3F_3	
Edf	0.031	0.003	0.005	0.022
TI	0.048	0.015	0.005	0.032
eIF-4a	0.072	0.003	0.054	0.169
ACT1	0.184	0.319	0.029	0.061
UBQ10	0.208	0.135	0.217	0.037
Prof2	0.289	0.386	0.286	0.130
eEF-1α	0.322	0.280	0.575	0.297
UBQ5	0.326	0.242	0.659	0.178
NABP	0.339	0.396	0.530	0.033
18S rRNA	0.390	0.625	0.595	0.178
Best gene				*Edf*
Stability Value for Best Gene				0.031
Best combination of two genes				*Edf and TI*
Stability value for best combination of two genes				0.029

candidate reference genes indicating very low expression stability.

3.5. Stability of candidate reference genes using BestKeeper analysis

The BestKeeper software determines the best suited standards of reference genes and combines them into an index. The index is used to decide whether reference genes are differentially expressed under an applied treatment. All data processing for this software is based on crossing points and determines the optimal housekeeping genes employing the pair-wise correlation analysis of all pairs of candidate genes and calculates the geometric mean of the 'best' suited ones (Pfaffl et al., 2004). In this study, though *18S rRNA* and *ARF* were among the least stable reference genes selected by geNorm and NormFinder, the BestKeeper software identified and placed these two genes in rank 1 and 2 as the most stable genes since they have the lowest variation compared to the other genes as determined by their standard deviation (SD) of the crossing point values (CV) at 0.58 and 0.59, respectively. On the other hand, *Prof2* and *eEF1α* were among the least stable reference genes with SD values of 1.39 and 1.78, respectively (Table 3). For BestKeeper analysis, any SD values higher than 1 is considered unstable.

Table 3
Crossing point data of candidate reference genes by BestKeeper. Results of data analysis were taken from raw Ct values of RD47 and BC_3F_3 rice samples under 0, 2 and 24 h post inoculation, respectively, including mock inoculation.

	18S	*ARF*	*UBQ10*	*NABP*	*TI*	*Edf*	*UBQ5*	*ACT1*	*eEF4α*	*Prof2*	*eIF1α*
N	24	24	24	24	24	24	8	24	24	24	24
Geo mean [CP]	9.19	17.35	18.66	27.59	20.14	20.4	22.35	23	23.64	22.7	23.03
AR mean [CP]	9.21	17.37	18.69	27.62	20.18	20.4	22.39	23.1	23.69	22.8	23.12
Min [CP]	8.33	15.91	17.21	26.05	18.16	18.2	20.28	20.2	21.21	19.7	20.1
Max [CP]	10.51	18.24	20.58	29.59	21.92	22.2	25.33	25	26.12	25.1	26.63
Std dev [+/− CP]	0.58	0.59	0.89	0.97	1.09	1.12	1.22	1.26	1.28	1.39	1.78
CV [% CP]	6.3	3.39	4.77	3.49	5.43	5.47	5.44	5.46	5.41	6.09	7.7
Min [x-fold]	−1.81	−2.72	−2.74	−2.92	−3.95	−4.56	−4.18	−7.13	−5.42	−8.25	−7.6
Max [x-fold]	2.5	1.85	3.79	3.99	3.43	3.52	7.89	4.01	5.57	5.13	12.16
Std dev [+/− x-fold]	1.5	1.5	1.85	1.95	2.14	2.17	2.33	2.4	2.43	2.62	3.43

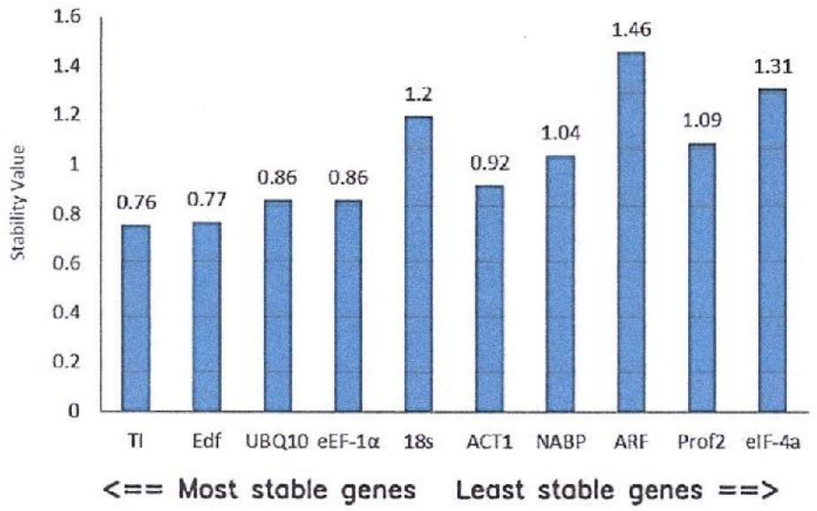

Fig. 4. Expression stability and ranking of candidate reference genes as determined by Delta-Ct method. Low gene stability values denote more stable expression.

3.6. Stability of candidate reference genes using the Delta-Ct (ΔCt) method

The ΔCt method compares relative expression of gene pairs within each sample to identify useful housekeeping genes. If the calculated value between the two tested genes remains constant in different RNA samples, it means that the expression of either both genes are stable among those samples, and if the ΔCt changes, then either one or both genes are inconsistently expressed (Silver et al., 2006). In our results, *TI* (0.77) and *Edf* (0.772) were identified as the most stable reference genes, the same genes identified by geNorm and NormFinder, while *ARF* (1.272) and *eEF1α* (1.487) were among the least stable genes (Fig. 4).

3.7. Stability of candidate reference genes using RefFinder analysis

To come up with a more comprehensive report on the expression stability of reference genes relative to our experimental conditions, we subjected our data series for RefFinder analysis to pull out a general recommendation. RefFinder is a web-based comprehensive tool developed to evaluate and screen reference genes from a widespread experimental dataset. It integrates the currently available major computational programs such as geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method. The system compares and ranks the tested candidate reference genes based on assigned appropriate weight of each individual gene and calculated geometric mean of their weights for the overall final ranking.

Results of the analysis showed that *TI* and *Edf* were among the most stably reference genes since they have the lowest stability values at 1.778 and 1.861, respectively. These two genes were consistent in all validation analyses except for BestKeeper. The *18S rRNA* (5.335) and *ARF* (7.183) which were the most stable genes determined by BestKeeper were ranked 6th and 8th, respectively. *Prof2, UBQ5,* and eEF1α were identified as the least stable reference genes with gene stability values of 7.364, 7.483, and 9.685, respectively (Table 4).

Table 4
Ranking of candidate reference gene expression stability by RefFinder.

Candidate reference gene	geNorm	NormFinder	BestKeeper	Delta Ct	RefFinder	Rank
TI	0.208	0.048	1.09	0.76	1.5	1
Edf	0.208	0.031	1.12	0.77	2.21	2
UBQ10	0.576	0.208	0.89	0.86	3.66	3
eEF-1α	0.351	0.322	1.28	0.86	4.12	4
18S rRNA	0.962	0.39	0.58	1.2	4.9	5
ACT1	0.494	0.184	1.26	0.92	5.14	6
NABP	0.891	0.339	0.97	1.04	5.63	7
ARF	1.057	N/A*	0.59	1.46	6.69	8
Prof2	0.65	0.289	1.39	1.09	7.17	9
UBQ5	0.76	0.326	1.22	1.2	7.48	10
eIF-4a	0.827	0.072	1.78	1.31	8.97	11

* N/A - not applicable.

4. Discussion

Earlier studies reported that the expression stability of various reference genes in rice vary under different experimental conditions and emphasized the need for a proper validation of the stability of reference genes for any gene expression analysis to come up with accurate and reliable results. The geNorm method was the first released algorithm in evaluating the stability of the expression of candidate reference genes and has established itself as the golden standard with more than 10,000 citations to date. However, as the expression stability value M for a gene generated from geNorm is dependent upon the other tested genes, concern has been raised about the possible selection of co-regulated genes instead of the stable ones. In this case, the NormFinder software (Andersen et al., 2004) uses a different approach which can cope with this problem. Thus, it is not rare that both of these methods alongside with BestKeeper, Delta-Ct method and RefFinder programs are usually used together to determine the best reference genes for a specific gene expression assay (Auler et al., 2017; Bevitori et al., 2014; Wang et al., 2016). In several studies, the genes selected by these algorithms are often slightly different even in most cases, the most stable reference genes selected by one program still belongs to the relatively stable genes category in the other analysis. In our study, *Edf* and *TI* were found to be the best reference genes across all approaches except for Bestkeeper thereby suggesting that their expressions are stable under *Xoo* inoculation.

Traditional reference genes like the *Actin1* (*ACT1*), *eEF-1α, β-tubulin, UBQ10, UBC-E2, UBQ5, 18S rRNA,* and *25S rRNA* were commonly used as internal controls in various experiments in rice focusing on growth and development stages, different tissue samples, and various treatments due to their recognized stability. Among these reference genes, *UBC* and *UBQ10* were found to be the most stable in rice plant responses to heavy metal stress (Almas and Kamrodi, 2018); *UBC* was the

most stable in rice treated with different nitrogen levels (Benemann et al., 2017); *18S rRNA* was the most suitable reference gene under various growth stages of etiolated seedlings, different cultivars, and various times after UV-irradiation treatment compared to *glyceraldehyde-3-phosphate dehydrogenase, actin,* and *tubulin* (Kim et al., 2003). However, other studies have also stressed that the transcription levels of these traditional reference genes may change depending on the plant developmental processes, environmental conditions and treatment sets, and expression differences of the genes of interest could be attributed to the expression variation of inappropriate reference genes (Gutierrez et al., 2008a; Gutierrez et al., 2008b; Wang et al., 2016). In the report of Jain et al. (2006), *UBQ5* and *eEF-1α* were found to be the most stable whereas *18S rRNA* and *UBQ10* were among the least stable in these commonly used reference genes when analyzed across all their samples and in developmental series. Li et al. (2009) also analyzed the stability of these commonly used reference genes during rice seed development and found out that *eIF-4a* and *ACT1* were the most suitable reference genes while again *18S and 25S rRNAs* were among the least stable in almost all the tested samples from two rice varieties at different developmental stages, and a total of 6 reference genes was optimal for qPCR calibration using most of their tissue groups.

As no holistic stable reference gene could be found among the traditionally used housekeeping genes, significant efforts have been created to find novel and more stable reference genes (Jain, 2009; Narsai et al., 2010). With the increasing number of analyses of large sets of microarray data, *Edf* and *TI* were selected as potential reference genes for rice gene expression analysis. In a recent article, the expression stabilities of *Edf, TI,* and other novel reference genes in rice were compared to those of the more traditional ones like *UBQ5* for 22 different experimental conditions (Wang et al., 2016). The results of these experiments showed that novel reference genes were globally more stable and *Edf* and *TI* were often among the most stable genes. Our study revealed similar results for the *Xoo* inoculation condition as *Edf* and *TI* were also found to be the best reference genes while the traditional housekeeping genes *18S rRNA* and *UBQ5* including *ARF* and *eEF1-α* were among the least stable genes. Though *18S rRNA* has probably been the most frequently used reference genes for gene expression analyses, increasing number of reports that it can have very low expression stability under blast infection and drought tolerance (Bevitori et al., 2014) and in different developmental and environmental conditions (Jain et al., 2006; Li et al., 2009). The present study for two different rice cultivars under *Xoo* inoculation also showed that *18S rRNA* was the least stable and therefore, should only be used as an internal control with the highest caution. Moreover, in the report of Wang et al. (2016), the stability of *Edf* and *TI* were found under hormone treatments including salicylic acid (SA), which plays an important signaling role in the activation of various plant defense responses following pathogen attacks as highlighted in the report of Dempsey et al. (2010). And since the expression of *Edf* and *TI* were found to be stable under SA treatment, these findings indicate why the expression of these two genes under *Xoo* inoculation were also stable as presented in this study.

5. Conclusion

In our study, *Edf* and *TI* were found to be the most stably expressed among all reference genes validated, thus should be suitable internal controls for the normalization of gene expression analysis in rice inoculated with *Xoo*. While the use of either of the two genes as internal control is adequate for gene expression analysis, using both genes as presented in this study, is suggested to produce more accurate and reliable results. Besides, due to the variability of the TAL effectors between *Xoo* strains, confirming the stability of these reference genes when using a new *Xoo* strain would also be advisable.

Declaration of Competing Interest

All authors declare that they have no conflict of interest and all ideas reflected in this manuscript have been agreed upon.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2019.100217.

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Application of wheat flour (*Triticum aestivum*) on spore density and sporulation efficiency of *Bacillus megaterium* isolated from *Litopenaeus vannamei* gastrointestinal tract

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Abstract. *Mahariawan IMD, Kusuma WE, Yuniarti A, Beltran MAG, Hariati AM. 2021. Application of wheat flour (*Triticum aestivum*) on spore density and sporulation efficiency of Bacillus megaterium isolated from* Litopenaeus vannamei *gastrointestinal tract. Biodiversitas 22: 3709-3715. Bacillus megaterium* is frequently used in fish farming, such as *white shrimp (*Litopenaeus vannamei*)* pond, which can produce spores with high stability in its implementation. Currently, spore production still requires the availability of high-cost carbon sources. The objective of this research was to evaluate the effect of different wheat flour doses on spore density and sporulation efficiency of *B. megaterium* BM1. In flasks, 50 mL of each test medium was treated with different doses of wheat (10, 20, 30 and 40 g. L^{-1}, respectively) and glucose was used as a control. Each treatment was inoculated with *B. megaterium* BM1 (2.6 x 10^8 CFU. mL^{-1}) and incubated in a shaker incubator (120 rpm) at 37 $^{\circ}$C for 120 hours. The results showed that the highest vegetative cell concentration (17 x 10^8 CFU. mL^{-1}), growth rate (0.8 $hour^{-1}$) and spore (14.7 x 10^8 spores. mL^{-1}) were found in the wheat flour dose of 30 g. L^{-1}. Furthermore, the highest sporulation efficiency was achieved at 20 g. L^{-1} of wheat (91.30%) and germination should be done at a dose less than 40 g. L^{-1}. The size of the spores was 1.35-1.39 μm. Thus, 30 g. L^{-1} of wheat flour is a potential dose to produce spore for probiotic candidates.

Keywords: Affordable carbon source, *Bacillus megaterium*, bacterial growth, spore density, sporulation efficiency

INTRODUCTION

Nowadays, the application of aquaculture biotechnology is one of the efforts to increase fish farming production (Ayoola and Idowu 2008; Danish et al. 2017; Rathore et al. 2019). Probiotics are potential biotechnology products from living microorganisms that are mostly used in the aquaculture industry (Sahu et al. 2008; Verma and Gupta 2015). The advantages of probiotic bacteria in extracellular enzyme secretion (Lee et al. 2012; Eshaghzadeh et al. 2014) and antibacterial compound production (Gozari et al. 2016; Prabhurajeshwar and Chandrakanth 2019; Silva et al. 2020) are important indicators to be continuously developed.

Bacillus is a genus of bacteria that is widely applied as a probiotic in fish and shrimp farming (Buruiana et al. 2014; Swapna et al. 2015; Won et al. 2020). One species of this genus that has been confirmed to have a positive impact on successful aquatic organisms in culture is *B. megaterium* (Li et al. 2009; Aftabuddin et al. 2013). This bacterial species has the ability to sporulate (Grage et al. 2017; Stancu 2020). Some of the benefits in spore as a probiotic candidate are surviving in acid pH at 2–2.5 (Ananthanarayanan and Dubhashi 2016), heat stability, and storage in dry form (Cutting 2011). From these benefits, bacteria in the form of spores show good stability when applied as probiotics (Bader et al. 2012; Lakshmi et al. 2017).

Production of *B. megaterium* spores involves media composed of various components. One of the media components that presented an important role in initiating the sporulation is the carbon source (Mazmira et al. 2012; Gauvry et al. 2016; Rai and Tewari 2016). Commonly available commercial carbon sources are glucose, lactate (de Vries et al. 2005), and sucrose (Hassan et al. 2014) which provide a high price. The alternative of inexpensive carbon sources that could be used as a substrate to produce spore comes from agro-industrial products (Khardziani et al. 2017a). Wheat flour is an agricultural product that is relatively cheap and easy to find. The utilization of organic carbon from wheat flour is one way to reduce the spore production cost. A previous study underlined that the implementation of carbon sources with inappropriate doses can result in less spore production (<10^5 spores. mL^{-1}) (Senthilkumar et al. 2011). Thus, the use of wheat flour dosage needs to be further evaluated to obtain the optimal dose in producing vegetative cells and spores of *B. megaterium* BM1 isolated from GI tract of white shrimp.

Therefore, this study aimed to evaluate the effect of different wheat flour doses on spore density and sporulation efficiency of *B. megaterium* BM1.

MATERIALS AND METHODS

Preparation of *B. megaterium* BM1 culture

The isolate of *B. megaterium* BM1 which isolated from gastrointestinal tract of white shrimp was cultured in Nutrient Agar (NA) with adding sodium chloride 2% (w/v) at 30°C for 24 hours. The re-culture of *B. megaterium* BM1 isolates was conducted by inoculating the bacterial colonies that have grown on solid media using a loop needle into Nutrient Broth (NB) media. Furthermore, the incubation of the broth culture was in an incubator shaker (120 rpm) at 37°C for 18 hours as a stock culture.

Composition of tested media

The test media in this study consisted of wheat flour, ammonium chloride (NH_4Cl) and mineral salts. The doses of wheat flour used were 10, 20, 30 and 40 g. L^{-1}, respectively. In contrast, the NH_4Cl dose added was adjusted to each wheat flour (*T. aestivum*) dose to obtain a C:N ratio of 5:1. The composition of the test media that has been determined was dissolved by distilled water and enriched with several minerals such as $CaCO_3$ 0.3 g. L^{-1}, $MgSO_4$. $7H_2O$ 0.00033 g. L^{-1}, $MnSO_4$. H_2O 0.12 g. L^{-1}, $FeSO_4$. $7H_2O$ 0.084 g. L^{-1}, and $CaCl_2$. $2H_2O$ 0.09 g. L^{-1}. The control treatment used was glucose as a carbon source with the same C:N ratio and minerals as the wheat flour treatment. All of these treatments were repeated 3 times.

Cultivation condition

Fifty (50) ml of test media was put into each 100-ml-Erlenmeyer flask according to the treatment and sterilized using an autoclave. The stock culture of *B. megaterium* BM1 was inoculated into the test medium as much as 1% (v/v). The initial density obtained from the bacterial culture stock was 2.6 x 10^8 CFU. mL^{-1}. Fermentation of all treatments was carried out in a shaker incubator at a speed of 120 rpm for 24 hours for the vegetative phase and was continued to the sporulation phase for 120 hours.

Counting of vegetative cell, vegetative cell growth rate, spore production, sporulation efficiency and spore germination

The vegetative cells and spores were counted microscopically with the Neubauer chamber. Shape of the cells became a key to determine distinguish both of them. The vegetative cells are rod-shaped and spores are round-shaped. The special treatment was conducted before counting spores by heating them at 80°C for 15 minutes. To assist the calculation of vegetative cells and spores, samples were serially diluted. The measurement of sporulation efficiency was carried out using the highest spore and vegetative data in the predetermined period which referred to the formula from Monteiro et al. (2014). Furthermore, the ability of spores to germinate was done by planting the spore samples onto the germination medium (NB). Spore germination was observed in an incubation time of 24 hours.

Spore visualization on Scanning Electron Microscopy (SEM)

The highest spores that have been harvested from wheat flour with the optimal dose were visualized by morphology using a Scanning Electron Microscopy (SEM). This shape and size of the spore from the optimal dose of wheat will be compared to the spore that was grown in glucose media. The spore samples were separated between the supernatant and the pellets by centrifugation process at a rate of 1,000 x g using 4°C of temperature in 5 minutes. Afterward, the sample was conducted through incubation with 2.5% of glutaraldehyde in 1.5 hours. The pellets collected from the last stage of centrifugation were followed by gradual dehydration. Visualization of spores was observed in SEM TM 3,000. Spores were measured to determine the size of the spores produced from each treatment.

Statistical analysis test

The results obtained of whole parameters in this study were tested statistically by one-way ANOVA through SPSS 20 software. The differences of all treatments can be seen from DMRT Test.

RESULTS AND DISCUSSION

Vegetative cell density and growth rate of *B. megaterium* BM1

All doses of wheat showed a higher vegetative cell production than the control treatment (glucose) (Figure 1). Even at wheat flour doses of 20, 30, and 40 g. L^{-1} indicated that the vegetative cell density was two times greater than the control treatment. In this study, the highest of the vegetative cell was obtained at 30 g. L^{-1} of wheat flour with a density of 17 x 10^8 CFU. mL^{-1}. The growth rate of the vegetative cells of *B. megaterium* BM1 (Figure 2) in all wheat flour concentrations exhibited a higher value than that of the control treatment (glucose). The average growth rate of *B. megaterium* BM1 grown on wheat flour with a dose greater than 10 g. L^{-1} had a value above 0.75 $hour^{-1}$ and indicated no significant difference ($p>0.05$).

Table 1. Vegetative cell densities of germinated *B. megaterium* BM1 spores (x10^8 CFU. mL^{-1})

Incubation (hour)	Wheat Flour Doses (g. L^{-1})				
	10	20	30	40	Control
8	3.43±0.03^b	4.64±0.04^c	4.67±0.07^c	2.42±0.04^a	2.47±0.02^a
16	7.65±0.13^b	8.00±0.20^b	8.93±0.25^c	6.03±0.15^a	6.13±0.21^a
24	9.46±0.06^b	9.97±0.21^c	10.13±0.42^c	8.56±0.09^a	8.59±0.23^a

Note: The different notation between the numbers indicated significant difference ($p<0.05$)

Spore production of *B. megaterium* BM1

The emergence of spores in this study occurred at 12 hours of incubation for all wheat flour treatments. However, in the control (glucose) treatment, spores first appeared at 18 hours after inoculation (Figure 3). The density of spores continues to increase in line with the decreasing density of vegetative cells. After the initial spores were formed, the number of spores gradually increased up to 84 hours. For all dosages of wheat flour, the highest spore density was reached at hour 84. In contrast, the control treatment reached peak spore production at hour 96. Furthermore, the spores indicated the stationary phase until the end of the sporulation time. The highest spore density occurred at a dose of 30 g. L^{-1} of wheat flour with a value of 14.7 x 10^8 spores. mL^{-1}. This result revealed 233% greater than that of the control treatment (glucose). The trends as true for the vegetative cells in all wheat flour concentrations.

Sporulation efficiency and spore germination of *B. megaterium* BM1

In terms of the highest sporulation efficiency, can be seen from Figure 4 that there is no statistical difference in the dosage of wheat flour at 10 and 20 g. L^{-1} with a value of more than 90%.

In addition, the spores of *B. megaterium* BM1 that germinated in all treatments increased within 8 hours of the incubation period (Table. 1). The highest vegetative cell density from germination was obtained in the treatment of wheat at a dose of 30 g. L^{-1} (10.13 x 10^8 CFU. mL^{-1}). These results suggested that the spore regrowth from this dose had a value 117% higher than that of the control treatment (glucose).

Visualization of *B. megaterium* BM1 spores

Visualization of *B. megaterium* BM1 spores grown on optimal media (wheat flour at a dose of 30 g. L^{-1}) compared to commercial media (glucose) was aimed to determine the morphology and size of the spores obtained. The results revealed that there were no significant differences in the shape in each of the media tested (Figure 5). The results of spore size in this study were grown in wheat flour at a dose of 30 g. L^{-1} and glucose were 1.35 – 1.39 μm and glucose 1.09 – 1.47 μm) respectively.

Discussion

Microbes require a culture medium consisting of various components to grow, one of them is a carbon source. The results of this study recorded that the implementation of organic carbon sources derived from wheat flour under different doses had a significant effect on the production of vegetative cells. The availability of carbon as nutrition at the appropriate concentration can affect the maximum bacterial growth in the incubation period (Sarudu et al. 2015; Allen and Waclaw 2018). The distinction in time and density achieved by *B. megaterium* BM1 between the wheat flour and control (glucose) treatment was influenced by the different substrate types. Mikkelsen et al. (2009) and Nur et al. (2015) noted that the utilization of different carbon sources in culture media would affect bacterial growth. Moreover, another factor of carbon concentration in different carbon sources also influences vegetative cell growth (Molina-Ramírez et al. 2017). The carbon source available in the environment was absorbed by bacteria cells that function as the substrate of metabolic tissue, then was broken down to supply amino acids and several components that compose the cell (Wang et al. 2019). After reaching the maximum density, the vegetative cells of *B. megaterium* deceased at 12 hours or more. This condition was caused by the availability of nutrients in the culture media gradually decreased. In consequence, the nutrient uptake by vegetative cells would slow down and impacted starvation (Biselli et al. 2020).

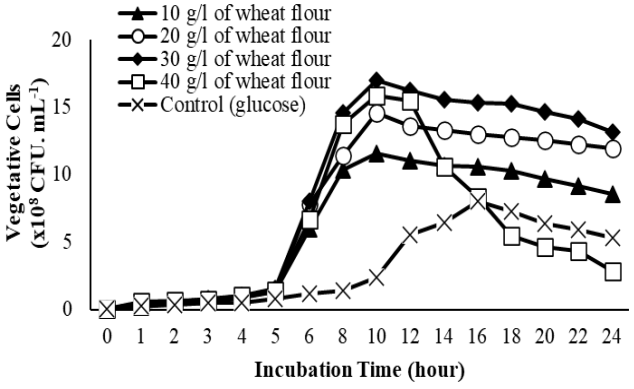

Figure 1. Vegetative cell density of *B. megaterium* BM1 under different doses of wheat flour

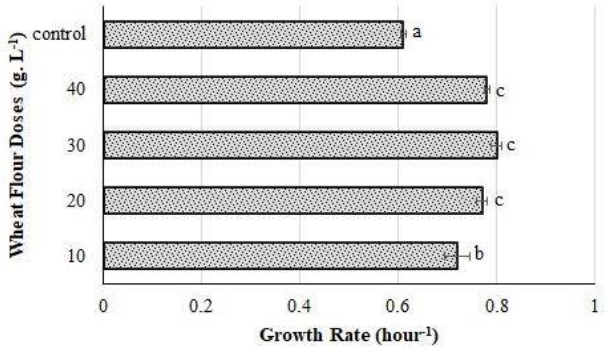

Figure 2. Maximum vegetative cell growth rate of *B. megaterium* BM1 under different doses of wheat flour. Mean data with different alphabets are significantly different at a level of $p<0.05$).

The maximum growth rate of vegetative cells was directly comparable to the highest density of vegetative cells obtained. The growth rate had different values depending on the growth phase of the bacteria. The bacterial growth rate actually began to decline when the culture conditions entered the stationary phase (Hall et al. 2013). Furthermore, physiologically, there was an increase in the size of *B. subtilis* cells in line with the growth rate especially in cell length, while the width was constant (Sauls et al. 2019). In this study, the dosage of wheat flour presented an important indicator in influencing the growth rate of vegetative cells. It can be seen that the doses of 30 g. L^{-1} and 40 g. L^{-1} of wheat are very close in value. The availability of nutrients in the environment at the right dose could modulate vegetative cells to carry out the macronutrient absorption process in the culture medium. Vadia and Levin (2015) explained that the nutrients available in the culture media would be catabolized by bacteria for biosynthetic process. Metabolites produced through central carbon metabolism could serve as intracellular signals that activated effector proteins in modulating cell cycle development.

The process of vegetative transformation into spores is known as sporulation. The difference in time of spore formation was initiated by nutritional factors in the culture media which began to decline after passing through the stationary phase. The stress condition of vegetative cells formed self-defense against nutrient depletion in the environment. The carbon source of wheat exhibited a higher spore density compared to the control treatment. The utilization of wheat flour in this study showed a good substrate for producing *B. megaterium* BM1 spores. Other studies that utilize local materials such as molasses and soy flour could produce spores at 5.90 x 10^8 spores. mL^{-1} (Salazar-Magallon et al. 2015). Apart from being influenced by the type of carbon sources, the concentration of the carbon sources used as a growth medium also played important role in the expression of the capacity for spore formation in the genus *Bacillus* (Khardziani et al. 2017b).

Certainly, the concentration of the various carbon sources showed a different number of spores obtained. For instance, the results of this study explained that the highest dosage of wheat flour (40 g/L) resulted in a lower spore production of *B. megaterium* BM1 than the doses of 30 g. L^{-1} and 20 g. L^{-1}. This result was in line with the research by Posada-Uribe et al. (2015) who showed that the use of a carbon dose (2 g. L^{-1}) could produce spores of 5.1 x 10^8 CFU. mL^{-1} while at a carbon dose (>11 g. L^{-1}) did not generate spores of *B. subtilis*. Jackson and Bothast (1990) stated that giving high carbon concentration up to 40.8 g. L^{-1} inhibited sporulation. According to Kang et al. (1992), at 200 g. L^{-1} of carbon, the bacteria unable to generate the spores. In terms of the results of this study, the application of lower (10 g. L^{-1}) and higher (40 g. L^{-1}) concentrations of wheat were revealed at the same time resulted in close spore production values. This condition was suspected by the uptake of available nutrients by bacteria at different carbon concentrations. At the lowest carbon dose, it has not been able to provide high vegetative cells density that would be spore candidates. In addition, the administration of the highest carbon dose also impacted the sporulation. The implementation of high glucose levels could inhibit the sporulation by repressing the *spo0A* gene transcription (Monteiro et al. 2005). The inhibition of the sporulation was known as the pathway of carbon metabolism (Gauvry et al. 2016). Thus, the carbon concentration should be employed in optimal conditions. In consequence, the environment did not experience a shortage or excess carbon source that could prevent the number of spore production.

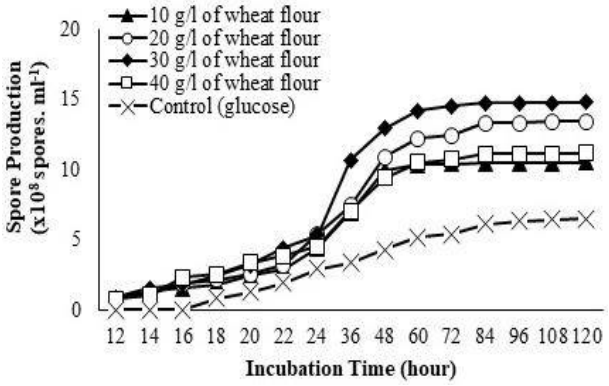

Figure 3. Spore production of *B. megaterium* BM1 under different doses of wheat flour

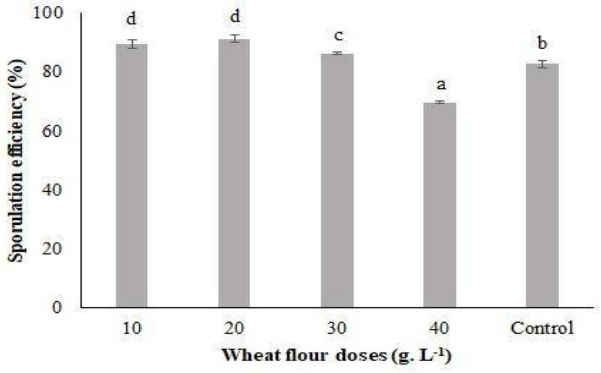

Figure 4. Sporulation efficiency of *B. megaterium* BM1 under different doses of wheat flour. Mean data with different alphabets are significantly different at a level of $p<0.05$).

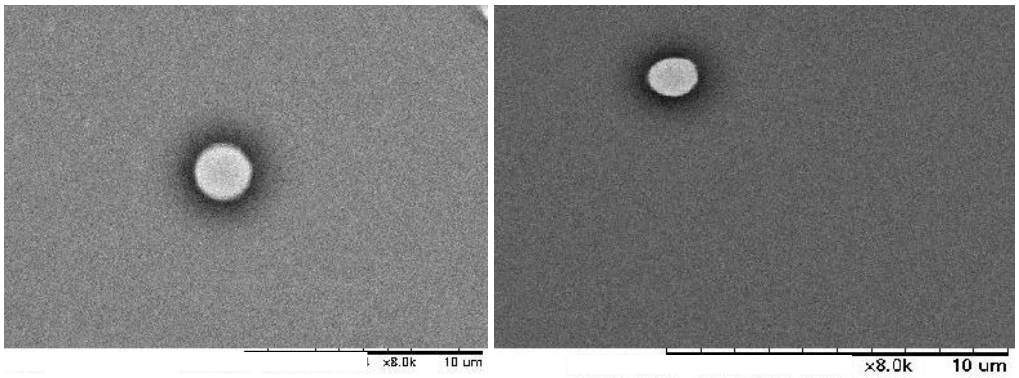

Figure 5. Morphological of *B. megaterium* spores under different carbon sources; A) control (glucose); B) Wheat flour at a dose of 30 g. L^{-1} (x8,000 magnification)

Sporulation efficiency at doses of flour more than 20 g. L^{-1} showed a decreasing value. Even the highest dose of wheat (40 g. L^{-1}) showed a lower sporulation efficiency than the control treatment. This condition explained that the high production of vegetative cells and spores was not supported by the high sporulation efficiency obtained. Sporulation efficiency could be achieved with a high percentage when supported by spore availability (Stojanović et al. 2019) and high growth rates (Carvalho et al. 2010). Several factors that influence spore production in increasing efficiency were carbon, nitrogen, and other macronutrients. The carbon and nitrogen sources played an important role in metabolic synthesis by bacteria to initiate sporulation (Anderson and Jayaraman, 2003). The highest sporulation efficiency of *B. megaterium* BM1 in this study had a value of 15% greater than the sporulation efficiency of *B. subtilis* AWS133 grown on DSM media that researched by Serio et al. (2006). In addition, the concentration of carbon sources (glucose) of 0.8–2 g. L^{-1} in the culture media resulted in the sporulation efficiency of *B. subtilis* at 50–90% (Posada-Uribe et al. 2015). Hence, it could be concluded that the sporulation efficiency really depended on the *Bacillus* strain used and the nutritional components in the culture media (Roy et al. 2015).

In general, the higher the carbon doses given, the higher the vegetative cell production obtained. On the other hand, in this study, the dose of wheat flour at 30 g. L^{-1} exhibited a bacterial density higher than at the dose of 40 g. L^{-1}. This condition explained that the high dose of wheat as a carbon source impacted vegetative cells that could not absorb the total of carbon available in culture media optimally. In line with these results, Thomas et al. (2014) also revealed that bacterial growth on excess carbon sources could reduce the survival of *S. aureus* when it entered the stationary phase. Moreover, the availability of excess carbon in bacteria caused metabolic stress which can inhibit bacterial growth (Zhang et al. 2020). Interestingly, excess carbon (glucose) levels not only inhibited the respiration process but also encouraged the transfer of electron to alternate acceptors (Thomas et al. 2014).

The number of spores germinated into vegetative cells showed that wheat flour with a dose of less than 40 g. L^{-1} was the best treatment. If the dose of wheat flour given was more than that, it would show lower germination than the control treatment. Germination is a process of returning spores to vegetative cells induced by the availability of new nutrients (carbon, protein, and nucleosides) (Setlow 2003; Krawczyk et al. 2017). Apart from being influenced by nutrient availability, the germination process was also initiated by the presence of non-nutritional factors such as Ca^{2+}-DPA, cationic surfactants, salts, and lysozymes (Setlow 2003). The occurrence of spore germination through releasing of DPA depended on the degradation of the spore cortex layer (Francis and Sorg 2016). Some of the variable conditions that affected sporulation were nutritional composition, temperature, spore preparation method, salt concentration, and oxygen availability (Bressuire-Isoard et al. 2018). Thus, the results showed that the spore quality from different doses of wheat flour at the time of sporulation led to differences in the density of vegetative cells from the germinated spores.

The spore size in this study had a larger size than the size of *B. subtilis* spores (0.8 to 1.2 μm) studied by Ricca and Cutting (2003). The difference in size can be caused by the different *Bacillus* species used. In addition, the cause of differences in spore size can be tested by sequencing analysis and gene expression of the strains used (Carrera et al. 2007). Although not many studies have shown the effect of differences in spore size and structure on the quality of *Bacillus* spores, this study showed that lower spore size grown in wheat flour at a dose of 30 g. L^{-1} resulted in a higher spore count than glucose.

In conclusion, the application of wheat flour can be a potential carbon source to produce *B. megaterium* BM1 spores with the highest production at the dosage of 30 g. L^{-1} and sporulation efficiency established less than 40 g. L^{-1}.

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Effect of different carbon doses of tapioca (*Manihot esculenta*) flour on vegetative cells and spore production of *Bacillus megaterium*

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Abstract. *Bacillus megaterium* is a spore forming bacteria that is mostly used as a probiotic in aquaculture. Spore formation for probiotic production using carbon source is costly. This research evaluated the effect of different carbon doses of tapioca (*Manihot esculenta)* flour on vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium*. Experiments were carried out in Aquaculture Laboratory, University of Brawijaya. In flasks, 50 ml of growth media were used and were inoculated with 1% of *B. megaterium* (2.6 x 10^8 cells/ml). The cultures were mixed (120 rpm) and incubated at 37 ^{0}C for 120 hours with 4 different treatments of carbon doses (5, 10, 15 and 20 g/L, respectively). The results showed that the highest vegetative cells (8.4 x 10^7 cell/ml) and spore production (4.1 x 10^7 spore/ml) of *B. megaterium* were found at 15 g/L carbon dose. However, it was not followed by high sporulation efficiency (only 49.01%). The high spore germination was observed in more than 5 g/L carbon dose. Thus, 5-15 g/L carbon doses of tapioca flour could positively enhance vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium* and could be used as a potential source of probiotics in aquaculture.

1. Introduction

Bacillus is a genus that commonly used as a probiotic in the aquaculture industry [1,2]. Several advantages of using it for the aquatic animal are supporting digestive fish function through an essential enzyme production [3], reducing ammonia and nitrite concentration [4] and improving immune system and health status of fish [5]. On the other hand, in aquaculture practice, there are some problems of probiotic in the vegetative cell such as inactivated bacteria in an acid environment (stomach) [6] and stressed in limited of nutrition [7]. The impact of this case is bacterial cell damage [8] consequently it will not work optimally in the digestive tract of an organism. One of the solutions to repair the problems is by using spore bacillus as probiotic [9]. The spore is dormant, resistant to lack of nutrition, heat temperature and organic chemicals [10,11]. The spores can survive in a long time even hundreds of years [12]. One of *Bacillus* species that produce spore is *B. megaterium* which has exosporium as a special characteristic in the outer membrane [13].

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Production of *B. megaterium* spore requires medium that consisted of carbon source. Glucose and lactate as commonly commercial carbon source but they have costly [14,15]. Alternative carbon source that could be used in medium to produce spore comes from local farming product such as tubers and beans. Tapioca (*Manihot esculenta*) flour is a carbon source with the composition of organic carbon more than 40% based on experimental study (data unpublished). In Indonesia, tapioca flour is cheap, easily found and contained high carbon content. Development of low-cost medium is needed to reduce the cost of spore production which can be used as a probiotic in fish farming. Thus, this study evaluated the effect of different carbon levels of tapioca flour on vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium*.

2. Materials and methods

2.1 Strain of bacteria

B. megaterium used in this study was isolated from shrimp pond in Tuban East Java which had confirmed by molecular analysis 16SrRNA.

2.2 Culture Media

Tapioca flour was used as the carbon source and ammonium chloride (NH_4Cl) served as nitrogen source. The doses of the carbon were divided by four treatments (5, 10, 15 and 20 grams per liter of distilled water (Aquadest®), respectively), while the nitrogen doses were adjusted to the carbon doses to achieve C:N ratio 5:1. Culture media consisted of tapioca flour and ammonium chloride based on treatments were dissolved with Aquadest® and enhanced with some minerals such as $CaCO_3$ (0.3 g), $MgSO_4$ (0.00033), $MnSO_4$ (0.12 g), $FeSO_4$. (0.084 g), $CaCl_2$ (0.09 g) [16], with little modifications. All treatments were replicated three times.

2.3 Inoculum preparation

Bacteria from the frozen stock were inoculated by a loop needle to a 100 mL shake flask containing 20 mL of nutrient broth as an inoculum medium. The culture was incubated in rotary shaker at 37 ^{0}C and 120 rpm for 18 hours. Bacterial cells were counted as an initial density that would be the stock culture.

2.4 Cultivation condition

The research used 100-ml-erlenmeyer flasks with 50 ml medium and were inoculated 1% (v/v) stock culture of *B. megaterium* (2.6×10^8 cells. ml^{-1}) in each flask. The culture conditions were similar to inoculum preparation in rotary shaker (at 37 ^{0}C, 120 rpm) for 24 hours of vegetative cells and continued to 120 hours for sporulation. In continuum, the spore germinations were carried out in the 100-ml-erlenmeyer flasks with 50 ml nutrient broth medium and were inoculated for high spore production in all treatments. The desired density of spore germination in each of treatments was 10^6 cfu/ml.

2.5 Determination of vegetative cells, spore count, sporulation efficiency and spore germination

Characteristics of vegetative cells and spore were observed and determined by their shapes. Vegetative cell has a rod-shaped and spore has a circle one. They were diluted with Aquadest® for sterilization to simplify counting before counting in the Neubauer chamber. Sporulation efficiency (%) was determined based on the ratio of maximum spore production and vegetative cells [17]. Spore germination was calculated by the number of spores that have changed to the vegetative cell.

2.6 Statistical analysis

All data were analyzed by one-way ANOVA in statistical software. The differences of all treatments were tested using Duncan's Multiple Range Test ($P<0.05$).

3. Result and discussion

3.1 Vegetative Cells of B. megaterium

Vegetative cells production of *B. megaterium* cultured at different carbon (tapioca flour) levels are presented in Figure 1.

The highest vegetative cells were obtained at carbon dose 15 g/L with a density of 8.4 x 10^7 cells/ml. Overall, result shows that the higher carbon doses, the higher vegetative cells produced. However, at 20 g/L carbon dose, the peak of production was reached of vegetative cells and were found lower than carbon dose 15 g/L. This condition is caused by the amount of carbon available in the environment exceeds the limits of the bacterial ability to use carbon as a nutrient that supports their lives. In giving too much carbon, the bacteria could not utilize and absorb all the carbon available optimally, although incubation time before 10 hours has the highest density when given 20 g/L carbon doses. Carbon is a limiting factor for bacterial growth [18,19]. The main principle of carbon as a limiting factor occurs when the bacterial growth rate increases with an increasing C/N ratio [20]. Excessive of acetate concentration as the carbon source in the environment causes unbalanced carbon metabolism, therefore, it can inhibit the bacterial cellular growth [21].

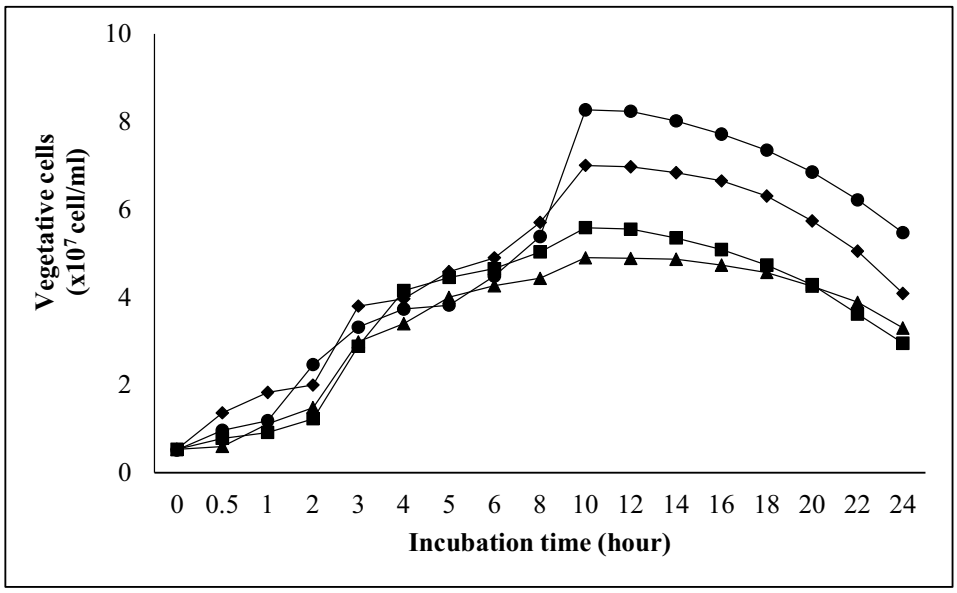

Figure 1. Time Series of vegetative cells production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■: 10 g/L; ●: 15 g/L; ◆: 20 g/L)

In all treatments, the highest vegetative cell was found at the incubation time of 10 hours, then the stationary phase occurred until the 12 hours. Afterwards, a phase of decreased vegetative cells occurred until 24 hours of incubation time. Vegetative cells decline is caused by the longer incubation time affect the availability of the nutrients in the culture media decreases. Nutrients derived from carbon source (tapioca flour) and nitrogen source (ammonium chloride) are used as food sources for the lives of these bacteria. Bacteria require carbon as constituents of cells and energy source while the nitrogen as the main element in amino acids and protein [22]. Decreasing of vegetative cells is influenced by inappropriate culture media such as lack of nutrients, pH, oxygen and temperature [23]. Apart from cell death, this research reveals that a declining vegetative cell population is also indicated by the formation of *Bacillus* spores to survive in the environmental conditions that are less supportive of life.

3.2 Spore production of B. megaterium

Spore-forming bacteria can change themselves from vegetative cells to spores when an unfavourable environmental condition (nutrients decline) [24]. The production of *B. megaterium* spores in the culture media with different carbon doses are presented in Figure 2.

B. megaterium spore initially appeared 14 hours of incubation in almost all treatments of carbon doses of tapioca flour. The beginning of the spore formation was after the growth of vegetative cell of *B. megaterium* reached a peak. Initial spores continued to increase in line with a decrease of vegetative cell density [25]. At 14 – 24 hours of incubation, the spores in all treatments increased gradually and reached a stationary phase at 120 hours. The highest spore production was with dose level of 15 g/L dose (4.1 x 10^7 spore/ml) and showed almost similar results when given lower dose of 5-10 g/L carbon dose. On the other hand, 20 g/L carbon dose showed a lower number of spores compared to other treatments (3.5 x 10^7 spore/ml).

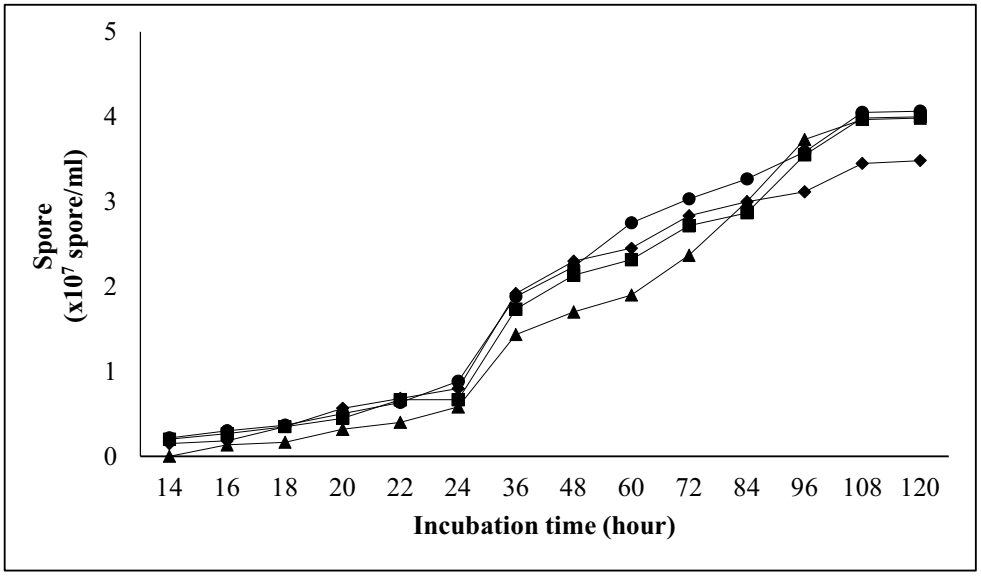

Figure 2. Time series of spores production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■ : 10 g/L; ● : 15 g/L; ♦ : 20 g/L)

The high vegetative cell was not an indicator of the high spore production. Given a carbon dose of 15 g/L, the vegetative cells count was 8.4 x 10^7 cell/ml but the spore production was recorded only at 4.1 x 10^7 spore/ml. Increasing amount of carbon doses affects the amount of carbon available in the culture media that impacted to bacterial lives. Other studies concurred with the recent result that the higher carbon doses given, the lower the spores produced. Glucose concentration as a carbon source showed that glucose with a dose of 3.5 g/L produces 4.3 x 10^9 spores/ml. Contrastingly, a dose of 20 g/L carbon produced 3.4 x 10^9 spores/ml. This condition inhibited the induction of several enzymes involved in the sporulation process [26]. Likewise, *Trichoderma harzianum* when given carbon source (glucose) 30 g/L, smaller spores count was recorded at 1.6 x 10^9 spore [27]. This case indicated that the dose of carbon depends on bacterial strain and carbon source that used in spore production.

3.3 Sporulation efficiency of B. megaterium

Sporulation efficiency is the percentage maximum vegetative cell that converts the highest spore production [17]. The sporulation efficiency of *B. megaterium* was seen in Figure 3.

The sporulation efficiency of *B. megaterium* was affected by carbon dose of tapioca flour. The highest of sporulation efficiency of *B. megaterium* was showed in the media with the carbon dose of 5 g/L with 80.97% sporulation efficiency. However, results showed no significant differences when

given with a higher dose of 15 and 20 g/L carbon levels. The high counts of vegetative cells and spores did not follow a high sporulation efficiency. Increasing the sporulation efficiency of *B. coagulans* can be expected through the addition of carbohydrates, mineral salts, pH in culture media [28]. In addition, the concentration of glucose as a carbon source should be reduced because it can decrease the results of sporulation efficiency [17]. In fact, the usage of low glucose content (2.0 g/L) increases spore production between 0.51 x 10^9 cfu/ml and 1.87 x 10^9 cfu/ml with sporulation efficiency 50.7% and 93.2%, respectively [29]. Another carbon source (acetate) only requires ≤ 0.04% to increase the sporulation efficiency of *Saccharomyces cerevisiae* [30]. The results shown by some of these researches indicated that each carbon source has an optimal dose to achieve the highest sporulation efficiency.

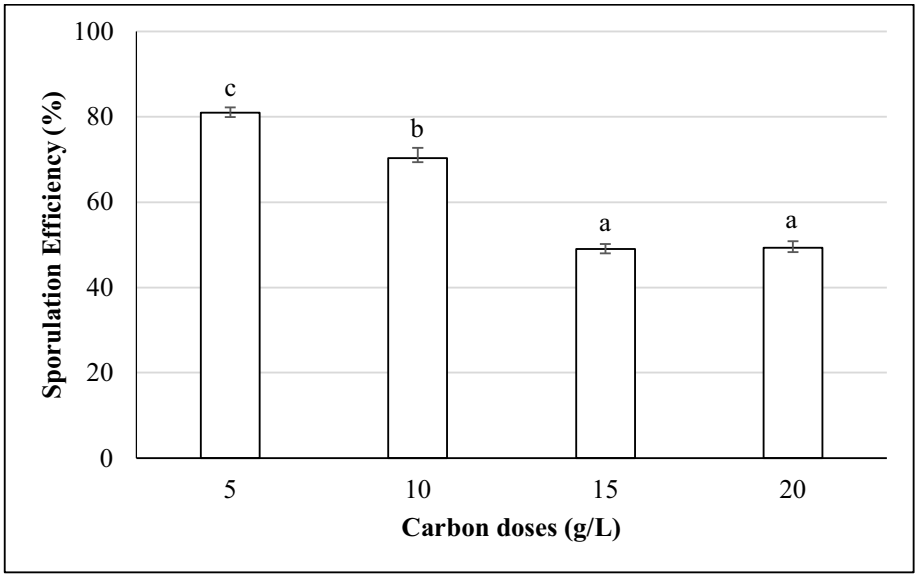

Figure 3. Sporulation efficiency of *B. megaterium* under different carbon doses of tapioca flour

3.4 Germination of B. megaterium

Germination process is the proliferation of spore to vegetative cell when the environmental supporting condition ensues [31]. The spore germination of *B. megaterium* was presented in Figure 4.

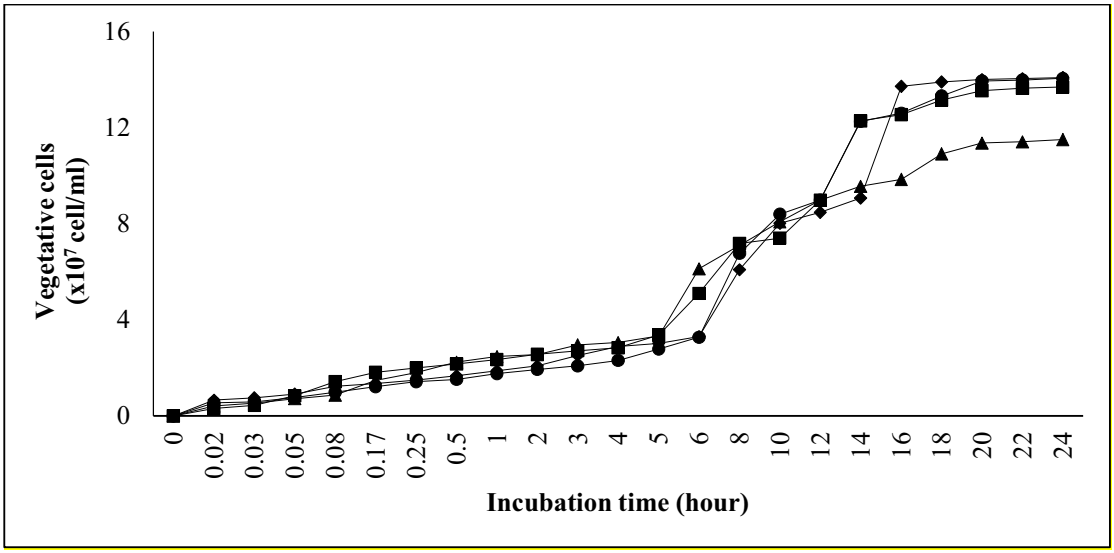

Figure 4. Time series of spore germination of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■ : 10 g/L; ● : 15 g/L; ♦ : 20 g/L)

In this study, the spore germination of *B. megaterium* was recorded at five (5) hours since there was no significant difference between the different treatment means. The highest vegetative cell from spore germination was found more than 5 g/L of carbon dose. The entire treatments achieved 100% spore germination before 0.5 hours in the nutrient broth medium due to the presence of new nutrients that stimulated bacteria cell division. Other reasons from some studies showed that spore germination influenced by the heat activation of spore. The percentage spore germination reached 100% after 0.5 hours when heat activation of spore at 65 ^{0}C [32] different to this study did not use heat activation. Another study showed that *B. subtilis* produce lower spore germination at 52.9%, than when spore was not heated treatment with 94% spore germination rate [31]. The difference of this germination percentage was influenced by concentration, density of spore, incubation temperature, heat activation and oxygen [33].

4. Conclusion

Carbon doses of tapioca flour affected the vegetative cells, spore production, sporulation efficiency and germination of *B. megaterium*. The highest vegetative cells (8.4 x 10^7 cell/ml) and spore production (4.1 x 10^7 spore/ml) of *B. megaterium* were found in the carbon dose of 15 g/L with sporulation efficiency 49.01%. The spore germination optimally was observed in more than 5 g/L of carbon dose.

5. References

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