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African Journal of Microbiology Research

Full Length Research Paper

Abilities of *Achyla orion* and *Allomyces anomalus* to degrade petroleum and petroleum products as sole carbon sources

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Abilities of *Achyla orion* and *Allomyces anomalus* isolated from some crude oil polluted aquatic environments in Nigeria to biodegrade petroleum and petroleum products were determined. Baiting method using hemp and sesame seeds was used to isolate the two species of aquatic phycomycetes. The species were grown in liquid broth culture made of minimal mineral salts supplemented separately with petrol, diesel and kerosene and incubated at room temperature with agitation for two weeks. Biodegradation was monitored using spectrophotometer at 600 nm wavelength. Fat/lipid was extracted from pellets resulting from centrifugation of the final broth culture using selected fat extractor and quantified. *A. anomalus* gave highest mean growth values in broth medium supplemented with diesel (0.970) and kerosene (1.302) while that supplemented with petrol recorded the least mean growth value of 0.663. The mean growth values for *A. orion* showed a similar trend. Crude fat/lipid production was highest for both *isolates* grown in diesel supplemented broth culture medium and least for both isolates grown in petrol supplemented broth culture medium. These results imply that these two species of aquatic phycomycetes were able to degrade diesel and kerosene better than petrol with corresponding production/accumulation of fat/lipid as biodegradation product.

Key words: Aquatic phycomycetes, petroleum, fractions, mineral salt and supplement.

INTRODUCTION

Petroleum and petroleum products form one of the major pollutants of the aquatic environment especially in the

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Niger-Delta region of Nigeria and in freshwater bodies used for industrial and domestic purposes like washing of petrol and diesel engine automobiles. Microorganisms especially fungi have a higher tolerance to the toxicity of hydrocarbons due to their physiology and adaptation to such variations in the environment and have mechanisms for the elimination of spilled oil from the environment (Abatenh et al., 2017; Yuniati, 2018). The effect of oil on microbial populations depends upon the chemical composition of the oil and on the species of microorganisms present. Populations of some microbes increase; typically, such microbes use the petroleum hydrocarbons as nutrients according to Abatenh et al. (2017) and Mahjoubi et al. (2017). Okerentugba and Ezeronye (2003) showed that bacteria and fungi spp. (*Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp.) isolated from rivers and refinery effluents in the Niger-Delta region of Nigeria could degrade crude oil. They reported increase in biomass for fungal isolates after a 35-day growth period. Their result also showed changes in pH, optical density and total viable count for bacterial isolates after a 17-day period. Many researchers have studied the role of fungi in biodegradation process of petroleum products. The most common fungi, which have been recorded as biodegraders, belong to many genera. These genera include Alternaria, Aspergillus, *Candida*, *Cephalosporium*, *Fusarium*, *Gliocladium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Pleurotus*, *Polyporus*, *Rhizopus*, *Rhodotolura*, *Talaromyces* and *Torulopsis* (Gesinde et al., 2008; Obire and Anyanwu, 2009; Hadibarata and Tachibana, 2009; Romero et al., 2010). Furthermore, reviews on fungi in bioremediation and biodegradation of crude oil across the world (Das and Chandran, 2011; Jahangeer and Kumar, 2013) and in Nigeria, Obire and Putheti (2009) recorded yeasts and filamentous fungi as fungi biodegraders of crude oil. None of the reviewers encountered in this study reported aquatic phycomycetes as biodegraders of crude oil. Aquatic phycomycetes are the group of primitive fungi that have adapted to the aquatic environment by the possession of flagella for motility. Two groups based on possession of one or two flagella as *Chytridiomycetes* and *Oomycetes*, respectively are associated with aquatic phycomycetes (Khulbe, 2001). These fungi contribute to the energy flow and productivity of aquatic and semi aquatic ecosystem by their active role in the utilization and bio deterioration of a variety of organic and inorganic materials (Khulbe, 2001). Most research carried out on biodegradation of crude oil and its fractions have centered mainly on yeasts and filamentous fungi. There is a dearth of information on the ability of aquatic phycomycetes to degrade crude oil and its fractions especially in some water bodies in Jos, Plateau State and the oil rich Bayelsa State of Nigeria. Therefore, this study seeks to evaluate the biodegradation potentials of two species of aquatic phycomycetes by growing them in suitable broth culture medium that mimics their natural aquatic environment, using spectrophotometer to measure degradation of the substrates and finally confirming biodegradation by extraction of fatty acid/lipid as a metabolite produced from biodegradation.

MATERIALS AND METHODS

Sampling sites and sampling procedures

The sampling sites for the study were two sampling points on River Nuns at Nembe seaport (N4° 32' 32'', E6° 24' 02'') (Marine) and Ogbia waterside (Brackish water) in Bayelsa State and one freshwater body, Dorowa pond by College of Health and Technology, Zawan, in Jos, Plateau State of Nigeria (N9°46' 20'', E8° 52' 17''). Water samples collection for Plateau State were done in February 2013 while those from Bayelsa State were collected in the first week of March 2013. Water samples collection were done with the aid of sterile 500 ml bottles aseptically and transported to the Dermatophilosis laboratory of National Veterinary Research Institute (NVRI), Vom, Jos, Plateau State in coolers designed as hand refrigerator with icepacks maintained at 5°C. Water samples analyses were carried out as soon as practicable on the day of collection but not more than 24 h after collection especially water samples from Bayelsa State (Rankovic, 2005; Marano et al., 2008).

Isolation of aquatic phycomycetes for culture based studies

Aquatic phycomycetes were isolated using hemp seed (*Canabis sativum*) and sesame seed (*Sesamum indicum*) as baits according to the methods of de Almeida Nascimento et al. (2011) and Trifa and Adiba (2011). Direct observation and identification of isolated aquatic phycomycetes were made using the compound light microscope according to de Almeida Nascimento et al. (2011) and Trifa and Adiba (2011). Results as seen from the microscope under appropriate magnifications were then compared for similarity with pictures and descriptions found in manual for identification of aquatic fungi (Khulbe 2001) with the support of other standard references, which included Fuller and Jaworsky (1987) and Dick (1990). Two species of aquatic phycomycetes showing consistency in occurrence and presenting with clear identification properties both morphologically and microscopically were selected for the biodegradation experiment. The said species of aquatic phycomycetes isolates used for the study were *Achyla orion and Allomyces anomalus. A. orion* was isolated from Dorowa pond by College of Health and Technology, Zawan and Nembe seaport in Bayelsa State while *A. anomalus* was isolated from Ogbia waterside in Bayelsa State and Dorowa pond by College of Health and Technology, Zawan, Plateau State, Nigeria.

Abilities of test fungi (*A. orion and A. anomalus)* to degrade petroleum and petroleum products

Overnight cultures of the two test species (*A. orion* and *A. anomalus*) grown in Malt extract broth were introduced into aseptically prepared mineral salt medium supplemented with 1% v/v petroleum, diesel and kerosene separately in well corked 250 ml conical flasks. The biodegradation of petroleum and petroleum products was observed via optical density as measured

spectrophotometrically at 600 nm wavelength using broth culture of aquatic fungi isolated from experimental sites for a period of two weeks on minimal salt broth as used by Sebiomo et al. (2011) and Ekundayo et al. (2012).

Petroleum and petroleum products namely diesel and kerosene were purchased from NNPC mega filling station at Secretariat junction in Jos, Plateau State and were dispensed into 200 ml sterile bottles and transported to the laboratory. A sterile inoculating needle was used to pick aquatic phycomycetes mycelium or pinhead of hyphae and inoculated into sterile malt extract broth (35 ml quantity prepared with drops of antibiotics, streptomycin sulphate, to suppress bacteria growth). The broth cultures of the two test isolates were then incubated at room temperature (25°C) and left to stand for 24 h. These served as the overnight broth cultures used in the biodegradation experiment. The broth culture medium used was prepared according to the pioneering method of preparing mineral salt medium by Mills et al. (1978) as modified by Okpokwasili and Okorie (1988) and further used by Sebiomo et al. (2011). The composition of the medium was Nacl=10.0 g, $MgSO_4.H_2O$ =0.42 g, Kcl=0.29 g, KH_2PO_4=0.83 g, Na_2HPO_4=1.25 g, $NaNO_3$=0.42 g, distilled water=1000 ml.The mineral salt medium prepared as above was sterilized by autoclaving at 121°C for 15 min and allowed to cool to 45°C. Simultaneously petroleum, diesel and kerosene were filtered using Millipore filter core paper (AP2029300) made in Bedford, Massachusette, USA.

A known volume (150 ml) of the sterilized and cooled mineral salt medium was dispensed into seven 250 ml conical flasks. Two three sets of the conical flasks were separated for the introduction of overnight broth culture of each of the test isolates of aquatic phycomycetes after supplementing with petroleum and petroleum products. The seventh flask that served as control was not supplemented but had one of the isolates inoculated. 1% v/v of each of the filtered petroleum and petroleum products were introduced into each of the flasks containing 150 ml MSM except the seventh flask. 35 ml of overnight broth cultures (using malt extract broth) of each isolate of aquatic phycomycetes were then separately seeded into different flasks supplemented with petrol, diesel and kerosene, resulting into six flasks, three per test organism.

A. anomalus was seeded into the seventh flask without supplement and used as control. The resulting flasks with test organisms were incubated at room temperature with constant agitation by clipping flasks to a laboratory gyratory shaker to mix the contents in order to enhance biodegradation. Biodegradation of petrol, diesel and kerosene by *A. orion and A. anomalus* were then monitored at two days interval for 14 days by measuring the optical density of the content of each of the appropriately labeled flasks using Jenway-6405 uv/vis spectrophotometer at 600 nm wavelength (Sebiomo et al., 2011).

Changes in pH were also determined in the course of the experiment using a portable pH meter. At the end of the 14 days period, the aquatic phycomycetes isolates in each of the flask that have visibly increased in biomass as a result of growth were harvested by centrifugation in a rotary centrifuge at standard setting of the centrifuge. The solid residues (increased biomass of aquatic phycomycetes isolates) were each dried on sterile whatman No1 filter paper as pellets and the weights were taken with a digital weighing balance. The supernatant was decanted. The dried residues were then wrapped in separate filter papers and used for the extraction of accumulated fat/lipid.

Fat/lipid extraction from biodegradation experiment

The seven resulting pellets from the biodegradation experiment above were subjected to fat/lipid extraction using the Soxhlet method of AOAC (1980). Weights of the resulting pellets were less than 1 g and the extraction of fat/lipid was carried out using selecta fat extractor at the Biochemistry laboratory of National Veterinary Research Institute, Vom, Plateau State, Nigeria.

RESULTS

The results of growth measurement using spectrophotometer readings for *Achyla orion* in Figure 1 were high on the first day (start day, 0) for growth on all the media supplemented with petrol, diesel and kerosene with 0.632, 0.450 and 0.451 readings respectively. These readings lowered for day 2 and day 4, after which there were progressive, increases on day 6 and day 8. This continued on day 12 for only the medium supplemented with kerosene. The media supplemented with petrol and diesel showed a decline on the day 12 of measurement. It was also observed as shown in Figure 1, that *A. orion* recorded the highest reading on medium supplemented with diesel which peaked at 1.306 on day 10. The medium supplemented with petrol conversely showed the lowest peaks.

The result of growth measurement with *A. anomalus* in Figure 2 shows that the readings on the 1st days (0 day) were slightly higher than that of day 2. Growth continued to increase from day 2 to day 10 and slightly decline on day 12 for growth on media supplemented with diesel and kerosene but increased on day 12 for media supplemented with petrol. *A. anomalus* recorded the highest growth readings on media supplemented with kerosene, which peaked also on day 10 at 2.150. Another observation from the result in Figure 2 shows growth readings on media without any supplement presented with lower values throughout the duration of the study when compared with growth on media supplemented with diesel, kerosene and even petrol. The growth on medium without supplement showed a progressive increase in growth from the start day 0 to the last day, day 12 representing a somehow normal pattern.

The mean values of the spectrophotometer readings with respect to *A. anomalus* in Figure 3 shows lowest mean value of 0.622 for medium without supplement followed by that supplemented with petrol (0.663) and diesel (0.970).

In comparison, the growth of *A. orion* and *A. anomalus* on media supplemented with petrol showed the least readings for both test isolates with respect to Mean measurements as can be seen in Figure 3. This tends to suggest that petrol is the least utilized supplement as compared with diesel and kerosene.

The mean pH of experimental culture media for the two test isolates fell within the acidic range as shown in Figure 4.

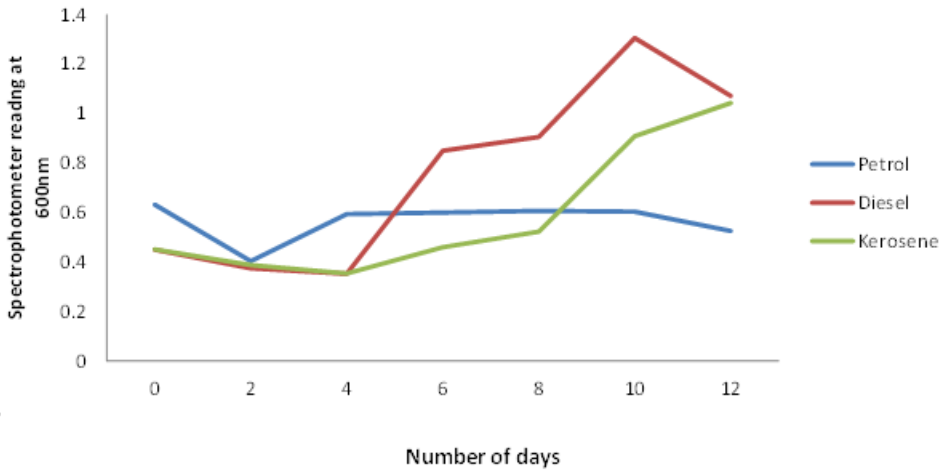

Figure 1. Biodegradation/Utilisation of Petroleum and Petroleum products by *Achyla orion.*

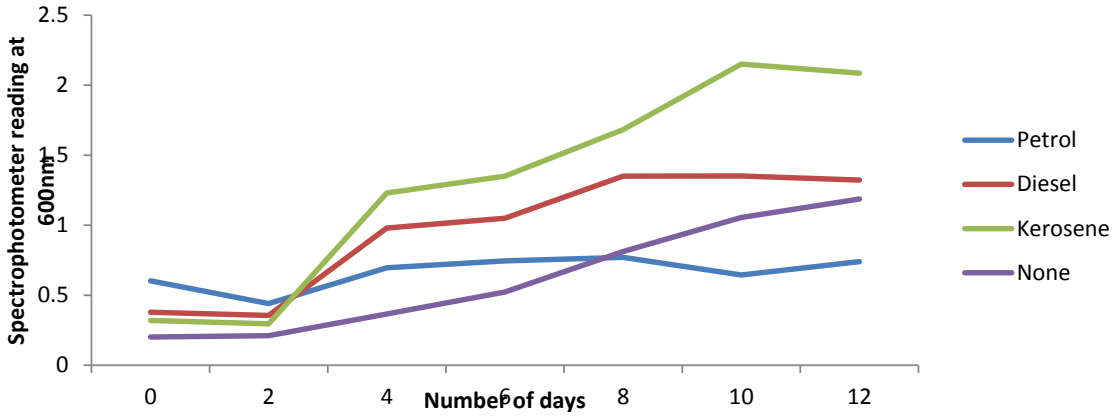

Figure 2. Biodegradation/Utilisation of Petroleum and Petroleum products by *Allomyces anomalus.*

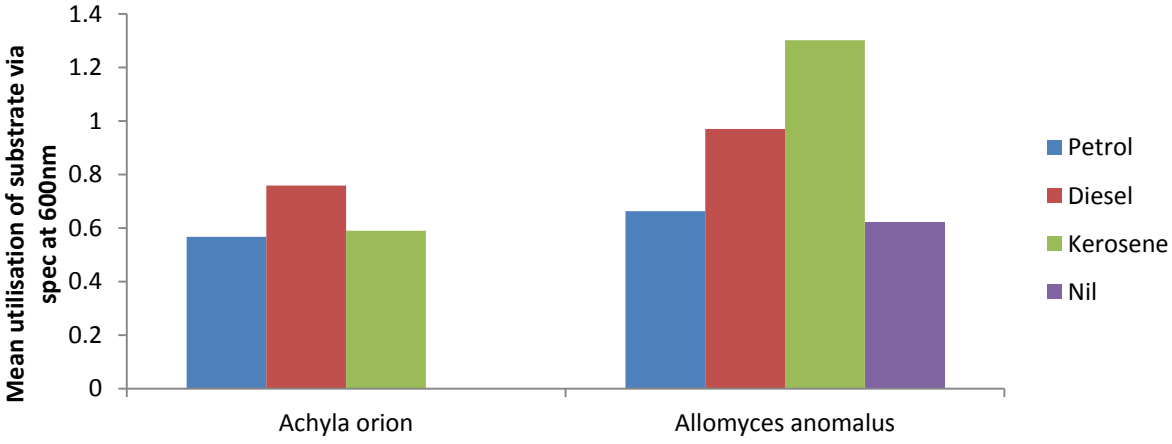

Figure 3. Mean utilization of Petroleum and Petroleum products by test isolates.

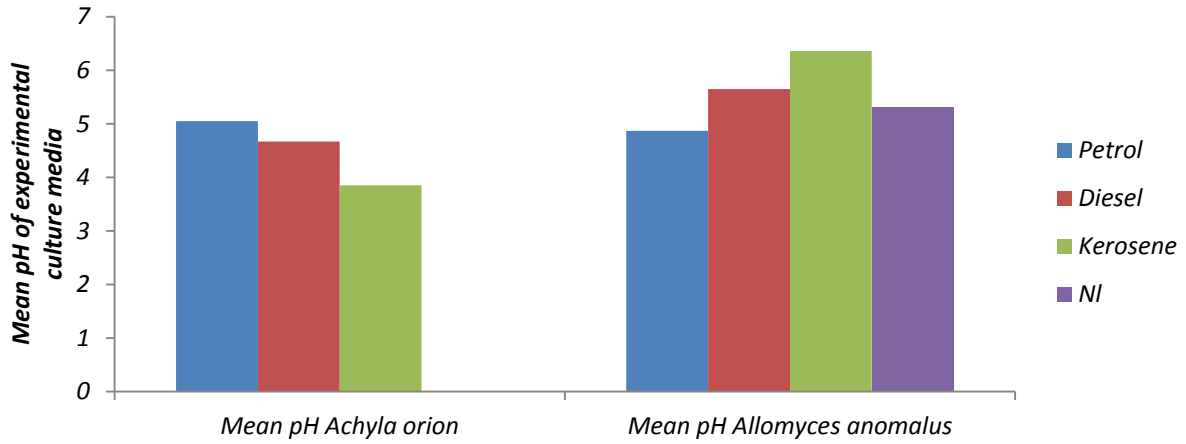

Figure 4. Mean pH of experimental culture media.

Table 1. Crude fat/lipid from biodegradation experiment.

Sample name	pH of final broth culture	Weight of pellet from fbc (g)	Weight of extracted fat/lipid	% crude fat /lipid g/100 g
Achyla +petrol	5.08	0.028	0.00	0.00
Achyla+kerosene	4.26	0.0140	0.00	0.00
Achyla+diesel	5.89	0.1260	0.0390	30.95
Allomyces+petrol	4.92	0.0120g	0.00	0.00
Allomyces+kerosene	7.16	0.0800	0.0050	6.25
Allomyces+diesiel	6.38	0.1880	0.0240	12.77
Allomyces+nil	6.15	0.0890	0.0060	6.74

Determination of by-product of petroleum and petroleum products degradation by test fungi (crude fat/lipid)

The by-product of the petroleum and petroleum products determined was crude fat/lipid produced at the end of the experiment as an accumulated product. The result of crude fat/lipid extraction from aquatic phycomycetes pellets from the biodegradation experiment in Table 1 shows the highest crude fat/lipid extract from pellets of *A. orion* and *A. anomalus* grown in mineral salt broth culture medium supplemented with diesel producing 30.95 and 12.77 g/100 g crude fat respectively. *A. anomalus* grown on kerosene and without supplement also produced 6.25 and 6.74 g/100 g crude fat respectively

DISCUSSION

The pattern of rise in the spectrophotometer reading at 600 nm for *A. orion* from day 0 to day 10 for petrol and diesel and decline in day 12 and rise from day 0 to day 12 for kerosene enriched broth culture media shows that the inoculated aquatic phycomycetes species was actively growing and metabolizing the substrate. With respect to *A. orion* the growth on diesel and kerosene enriched broth culture media gave the highest mean spectrophotometer readings of 0.759 and 0.590 respectively. Petrol enriched broth culture medium recorded the least mean of 0.567. This seems to suggest that the isolate, *A. orion* utilized or degraded diesel and kerosene better than petrol as supplements. For *A .anomalus*, the growth on kerosene and diesel enriched broth culture media gave the highest mean spectrophotometer readings of 1.302 and 0.970 respectively. Petrol enriched broth culture medium again recorded the least mean of 0.663. The pattern of rise and fall in spectrophotometer readings for *A. anomalus* signifying growth was similar to that of *A. orion*. The pH values of the growth media of both test aquatic phycomycetes isolates fell within the acidic range. This seems to be in line with Davis and Westlake (1979) who stated that fungi could grow in environmentally stressed condition like low pH and poor nutrient status while

bacteria cannot. Sebiomo et al. (2011) work on utilization of crude oil and gasoline by ten bacteria and five fungi isolates also reported reduction in pH of the culture fluid in flasks within their 14 days of incubation with pH readings also falling within the acidic range. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products (Nwachukwu and Ugoji, 1995; Okpokwasili and James, 1995). Thus organic acids probably produced account for the reduction in pH levels (Oboh et al., 2006).

The aspect of the experiment that may further buttress the utilization of petroleum and petroleum products is probably the result gotten when *A. anomalus* was grown in MSM broth culture medium without supplements of petrol or petroleum products. The growth on these MSM broth medium without supplement was a normal growth of consistent increase in the spectrophotometer reading from day 0 to day 12 while the spectrophotometer reading for MSM broth culture medium supplemented with petrol and petroleum products declined on the day 12 after an initial increase from day 0 to day 10.

Another good evidence of utilization of petroleum and petroleum products was that *A. anomalus* grown on MSM broth culture without any supplement recorded the least mean as signified by the spectrophotometer reading when compared with its growth on the MSM broth medium supplemented with petrol, kerosene and diesel. The higher spectrophotometer reading signifying growth activity for the MSM broth culture supplemented with petrol and petroleum products must have arisen from the metabolism of these added supplements by the aquatic phycomycetes species.

Comparing the growth patterns of *A. orion* and *A. anomalus* on MSM broth culture supplemented with petrol and petroleum products, one finds that both species shows the least growth values on broth culture supplemented with petrol and the highest spectrophotometer readings from the broth cultures supplemented with diesel and kerosene for *A. orion* and *A. anomalus* respectively. This somehow presupposes that these two aquatic phycomycetes species have abilities of utilizing these supplements as carbon sources, most importantly that they also have the least preference for utilization of petrol than diesel and kerosene. Therefore, there must be a structural reason that facilitates better growth values on MSM broth culture supplemented with diesel and kerosene than that supplemented with petrol. It is therefore important to note that diesel fuel is commonly heavier and more powerful than gasoline (petrol) engines. Martinez (2014) gave the average densities of gasoline (petrol), kerosene and diesel as 750, 780 and 830 kg/m^3, respectively. Diesel and kerosene were utilized better than petrol by the two aquatic phycomycetes species in this research work. Most of the earlier reported works on biodegradation of petrol and petroleum products by aquatic fungi have not involved aquatic phycomyctes, this seems to be one of such reports of biodegradation of petrol and petroleum products by species of aquatic phycomycetes.

Looking at the crude fat/lipid extraction from the final broth culture (FBC) at the end of the biodegradation experiment, one finds interesting results that supports the growth rate experiments. For instance, looking at the weight of the pellets from the final broth culture, one finds the least final weight values for the two species on broth culture supplemented with petrol. This may imply that the two species did not metabolize or accumulate the supplemented petrol to the extent of causing any appreciable increase in the final weight of the species as shown from the weight of the pellets from the final broth culture. In the same vein, since the final weight of aquatic phycomycetes grown in broth medium supplemented with petrol was the least, crude fat/lipid was not extracted from the pellets gotten from the final broth culture supplemented with petrol. The support of the fact that the aquatic phycomycetes species may have better abilities of utilizing diesel and kerosene more than petrol may also be buttressed from the final weight of the pellets resulting from the species grown on broth culture supplemented with diesel and kerosene. For instance, the weight of the pellets of Achyla *orion and Allomyces anomalus* grown on diesel supplemented broth culture were the highest at 0.1260 and 0.1880 g/100 kg respectively and equally produced the highest quantities of crudefat/lipid of 30.95 and 12.77 g/100 g respectively. For the kerosene supplemented final broth culture, *A. orion* did not yield any crude fat/lipid but *A. anomalus* yielded some 6.25 g/100 g of crude fat/lipid. *A. anomalus* grown on broth culture without supplement also yielded some 6.74 g/100 g of crude fat/lipid, which possibly shows that under normal circumstances, these species of aquatic phycomycetes are characterized by the possession of oil globules in their life cycle, which may be equivalent to the crude fat/lipid that was extracted. Hence, the highest quantity of crude fat/lipid produced by both species of aquatic phycomycetes grown on broth culture supplemented with diesel may therefore suggest better utilization and accumulation of diesel than both kerosene and petrol. In line with the results of this work where population of the species increased as evidenced by the increase in biomass as reflected by the weight of the pellets from the final broth culture, Abatenh et al. (2017) and Mahjoubi et al. (2017) also stated that population of some microbes increases and that such microbes use petroleum hydrocarbon as nutrients.

Conclusion

The study shows that both *A. orion* and *A. anomalus*

metabolized or utilized diesel and kerosene better than petrol as supplements in mineral salt broth culture medium. The study further concludes that the aquatic phycomycetes isolates after degrading the substrates probably also accumulated crude fat/lipid as a bye product of the degradation experiment since crude fat/lipid was extracted from the pellets resulting from the final broth culture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effects of inoculation of plant growth promoting rhizobacteria to minimize panicle grain shattering habit for increased yield of rice (*Oryza sativa* L.)

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Locally isolated *Bacillus subtilis* (UPMB10) as nitrogen fixer and *B. pumillus* (GM118) as phosphate solubilizer act as plant growth promoting rhizobacteria (PGPR). The objective of this study is to determine the response of single and co-inoculation of these PGPR on the panicle breaking strength to minimize panicle grain shattering to increase rice yield. The ability to fix nitrogen and solubilize phosphate, production of indole-3-acetic acid (IAA) and grain yield production of single and co-inoculants of PGPR were studied. The result demonstrated that single and combined inoculations were able to fix nitrogen and solubilize phosphate. Besides, the ability of UPMB10 and mix inoculation to produce high concentration of IAA enables them to provide a high breaking force needed to detach grain from panicle compared to GM118 and in turn increase the rice grain yield. Hence, this study showed the potential single and combined inoculations as a biofertilizer to increase rice productivity in the granary areas of Malaysia.

Key words: Plant growth promoting rhizobacteria (PGPR), co-inoculation, *Bacillus*, indole-3-acetic acid (IAA), grain shattering, yield, *Oryza sativa* L.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) is a free living soil born bacteria that colonize the rhizosphere. The interaction between plant root exudates and bacteria to support each other in term of symbiosis interaction for growth and development also enhanced the plant growth when applied to the seed crops (Yu et al., 2012). Besides, it can increase the nutrient status of host plants through the mechanism of biological N_2 fixation, phosphate (P) and potassium (K) solubilization.

When single PGPR inoculation of rhizobacteria were applied, they showed potential ability to enhance shoot growth, root density and yield of rice. However, recent studies showed that co-inoculation of PGPR are better than single inoculation as they provide more balanced

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nitrogen, phosphorus, and mineral nutrients (Mohammadi and Sohrabi, 2012). These improvements in growth attributes of plants caused by PGPR are due to the potential of fixing and solubilizing mineral fertilizer and nutrition for the plant and improved the absorption of production of phytohormones resulting in the increased availability of nutrients to plants and root permeability (Glick, 2012). Phytohormones produced by PGPR such as IAA is crucial as they are capable of increasing shoot growth, root hair density and length, enhance rice seed germination and improve growth (Saharan and Nehra, 2011).

Normally in wild species of rice, grain shattering habit is an adaptive trait for seed dispersal at maturity to prevent seeds from being eaten by predator and guaranteed the continuous propagation of rice seed (Lin et al*.,* 2007). However, easy shattering causes considerable yield loss in cultivated grain rice. This opinion was supported by Sadeghi et al*.* (2010), which stated that the threshing force had significant difference in different rice varieties. Breaking tensile strength force is the force required to detach a grain from the panicle which describes the grain shattering habit, assumed to be one of the effective loss-evaluating parameters during harvesting (Alizadeh and Allameh, 2011). It is an important criterion for evaluating rice variety in the interactive effect with PGPR inoculation programs. Hence, one way to combat this problem is by promoting the use plant growth promoting rhizobacteria (PGPR).

This approach of using PGPR to reduce grain shattering should be conducted in Malaysia to determine the effectiveness of locally isolated PGPR toward the increment of grain quality of local rice varieties and in turn increase the national rice yield production. Here, we report the characteristic and locally isolated effects of single and co-inoculation of PGPR namely *Bacillus subtilis* (UPMB10) and *Bacillus pumillus* (GM118) on the panicle breaking strength to minimize panicle grain shattering under controlled and field conditions.

MATERIALS AND METHODS

Source of inoculation, culture conditions and treatments

The two PGPR strains, *B. subtilis* (UPMB10) and *B. pumillus* (GM118) were obtained from the culture collection of the Department of Agriculture Technology Faculty of Agriculture, Universiti Putra Malaysia, Serdang. *B. subtilis* was initially isolated from oil palm rhizosphere and *B. pumillus* from a paddy soil. Bacterial single colonies from stock were prepared by touching and streaking on nutrient agar plates in a third streak pattern. The plates were then incubated for 24-48 h at 30ºC. A single colony was inoculated into 700 ml nutrient broth in an Erlenmeyer flask. Inoculated flasks were shaken (200 rpm) in a rotary shaker for 10 h at 30ºC. The final concentration of the bacterial cultures was adjusted to about 10^8 cfu/ml (Kausar et al., 2011). Four treatments were conducted for the rice plant experiment which: [1] Control (without inoculation), [2] *B. pumillus sp.* (GM118), [3] *B. subtilis sp*. (UPMB10) and [4] *B. pumillus sp*. + *B. subtilis sp*. (Mixture).

Phytohormones production

Phytohormones production or Indole-3-acetic acid (IAA) was determined by colorimetric method (Gordon and Weber, 1951). Fully grown bacterial culture were incubated in 100 mL tryptic soy broth (TSB) and shaken for 24 h. One mL of the bacteria culture was transferred into new 100 mL TSB with addition of 5 mL L-tryptophan as the pre-cursor of Indole-3-acetic Acid. TSB without bacterial inoculation served as control. 1.5 mL of the bacterial culture were transferred into sterile Eppendorf tube and centrifuged at 7000 rpm for 7 min. One mL of the supernatant was mixed with two mL of Salkowsky reagent (2% of 0.5 M $FeCl_3$ in 35% prechloric acid) and allowed to settle for 25 min for development of pink color which indicates IAA production. The absorbance values of each isolate was determined by using spectrophotometer at 535 nm and compared using the standard curve. The IAA standard curved was prepared using pure IAA stock as 0, 5, 10, 15, 20, 25, 30, 35, 50 and 45 µg/mL of IAA ($Y=0.0186x$; $R^2= 0.9802$). Supernatants of un-inoculated test tubes were used as control, where no visible color was observed.

Evaluation of PGPR application on rice

The experiment was conducted in a glasshouse, at Universiti Putra Malaysia and field study in IADA Kemasin Semerak, Kelantan, Malaysia to evaluate the effects of UPMB10 and M118 on rice grain yield MR263. For glasshouse experiment, seeds were sown in trays of 3.2 × 3.2 × 4.5 cm cell size in a medium of peat and sandy (1:1) soil. When the seedling reached 5 cm height (after two weeks), they were transplanted into buckets (4 plants per pot) 40 cm diameter (20 kg soil pot^{-1}) with four replications. The soil used in this study was collected from Kemasin Semerak, Kelantan Malaysia. The used nitrogen fertilizer was urea (46% N), potassium fertilizer was muriate of potash (60% K_2O) and triple super phosphate (50% P_2O_5) at application rate 0.76 g N $plant^{-1}$, 0.71 g K $plant^{-1}$ and 0.42 g P $plant^{-1}$. For field study, they were transplanted into micro plot (4 m × 4 m) with three replications. Total of fertilizers per hectare per season applied were: 121.1 kg nitrogen, 67.3 kg phosphorus and 45.5 kg potassium. The fertilizers were applied manually three times per season after transplant. The plants were harvested at days 105 after transplant and air dried and stored in the laboratory for analysis. Three replicates with two single inoculations treatments plus one combination inoculation and control were studied in field experiments.

Plant inoculation with *Bacillus sp*.

An appropriate dilution of 10^8/mL bacterial cell suspension of *B. pumillus* or/and *B. subtillis* were used to inoculate onto the plants by spraying with pump sprayer (ratio 1:100 ml H_2O) in all experiments at 20, 45 and 65 days after sowing.

Grains physiological analysis

The rice yield parameters which include total grain number per panicle, fill grains per panicle, 1000 g weight, total spikelet per panicle, panicle length and number of primary branch per panicle were determined after harvest. The filled grains were separated from unfilled grains by using salt solution of 1.06 specific gravity (Seizo, 1980).

Grain panicle strength analysis

Determination of panicle strength was done according to Thurber et al*.* (2011) and Alizadeh and Allameh (2011) methods. Six panicles

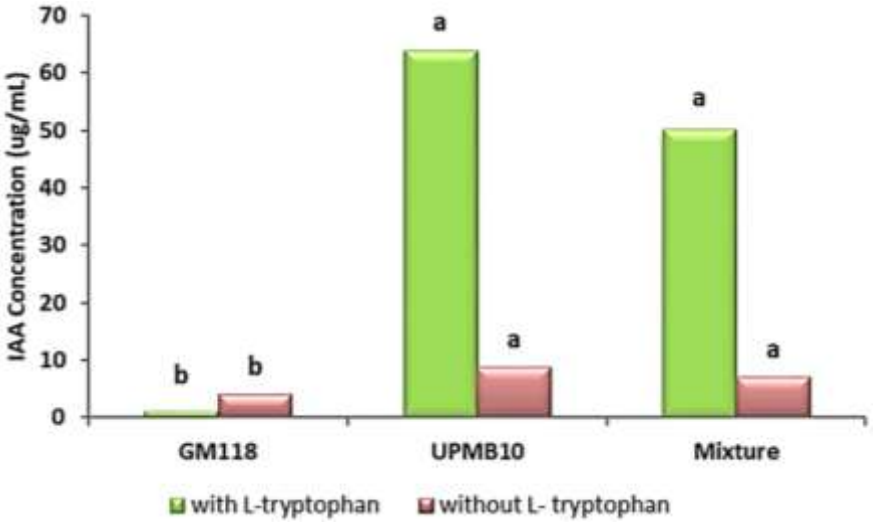

Figure 1. IAA production of single and combine inoculation with and without addition of L- tryptophan.

Table 1. Total grain number per panicle, fill grains per panicle, 1000 g weight, total spikelets per panicle, panicle length and number of primary branch per panicle at 100 DAT.

Treatments	Total Grain Panicle^{-1} (No.)	Filled Grain Panicle^{-1} (No.)	1000 Grain Weight (g)	Total Spikelets Panicle^{-1} (No.)	Panicle Length (cm)	Primary Branch (No.)
Control	79.58^{b}	79.58^{b}	16.68^{b}	102.54^{b}	22.4^{b}	11.5^{b}
GM118	**95.04^{a}**	**95.04^{a}**	18.75^{b}	**119.46^{a}**	**23.7^{a}**	**12.9^{a}**
UPMB10	**98.83^{a}**	**97.92^{a}**	18.16^{b}	**129.04^{a}**	**23.7^{a}**	12.2^{ab}
Mixture	89.71^{ab}	90.63^{ab}	**22.53^{a}**	**119.58^{a}**	23.6^{ab}	11.7^{b}
CV	25.7	23.1	32.7	17.2	7.1	8.7

Reported data are the mean of three replications and values in each column with different letters are significantly different according to Duncan`s Multiple Range Test at $p \leq 0.05$.

were randomly selected and analyzed for breaking tensile strength (BTS), or shattering level. BTS is a measure of the maximum amount of weight, in grams; a single grain can hold or attached to pedicle before being detached (Thurber et al., 2011). The shattering level was measure for each panicle by separating the panicle into three portions; upper, middle and lower. An electronic force device (FGP-1 Nidec SHIMPON) with the resolution of ± 0.01 N was used for measurement of the shattering force. The measurement of the maximum force was recorded when the applied force breaks the grain from the panicle. Average BTS values for the measurements were recorded for each sample.

Statistical analysis

All data were statistically analyzed using the SAS Software Program (Version 9.3), and treatment means were compared using Duncan Multiple Range Test ($P < 0.05$).

RESULTS

The interaction effects of single inoculation, *B. subtilis* (UPMB10) and *B. pumillus* (GM118) and also combined inoculations (Mixture) on production of IAA is given in Figure 1. In the presence of L-tryptophan, application of UPMB10 and Mixture indicated the highest IAA production which were 63.8 and 50.1 µg/mL, respectively, while GM118 produced only 1.3 µg/mL (Figure 1a). Besides, UPMB10 and Mixture still showed the highest IAA production which were 8.74 and 7.04 µg/mL, respectively even without the addition of L-tryptophan as compared to GM118 (4.07 µg/mL) (Figure 1b). The result showed that IAA production by bacteria inocula were significantly increased by the single inoculation of UPMB10 and Mixture compared to single inoculation of GM118.

Generally, all of the single inoculations, GM118 and UPMB10 and combined inoculation (Mixture) treatments indicated significant higher result in total number of grain per panicle, fill grains per panicle, 1000 grains weight, total spikelets per panicle, panicle length and number of primary branch per panicle at 100 DAT compared to control as represented in Table 1. The application of

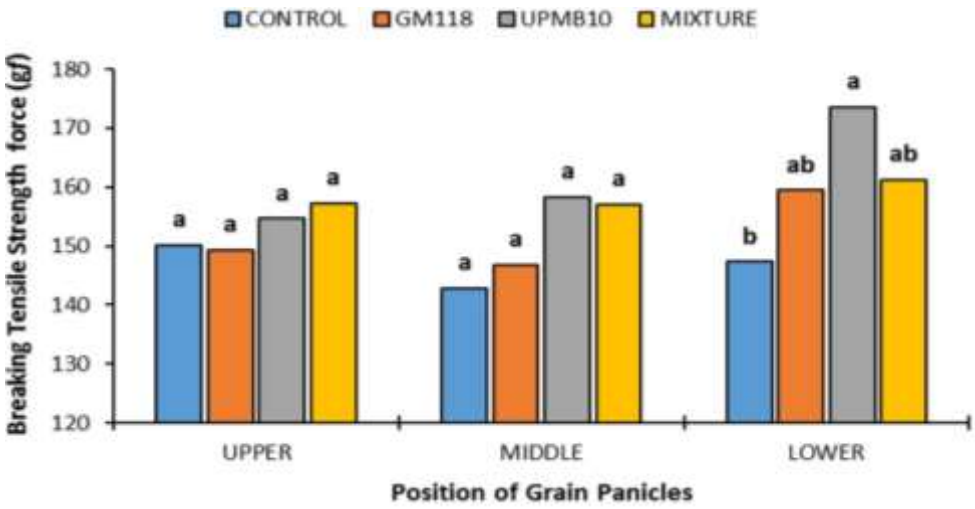

Figure 2. The average shattering force value measured after application single inoculation of GM118 and UPMB10, and Mixture (GM118 + UPMB10) inoculation with different position of grain panicles. Bar graphs with different letters are significantly different according to Duncan`s Multiple Range Test at $p \leq 0.05$

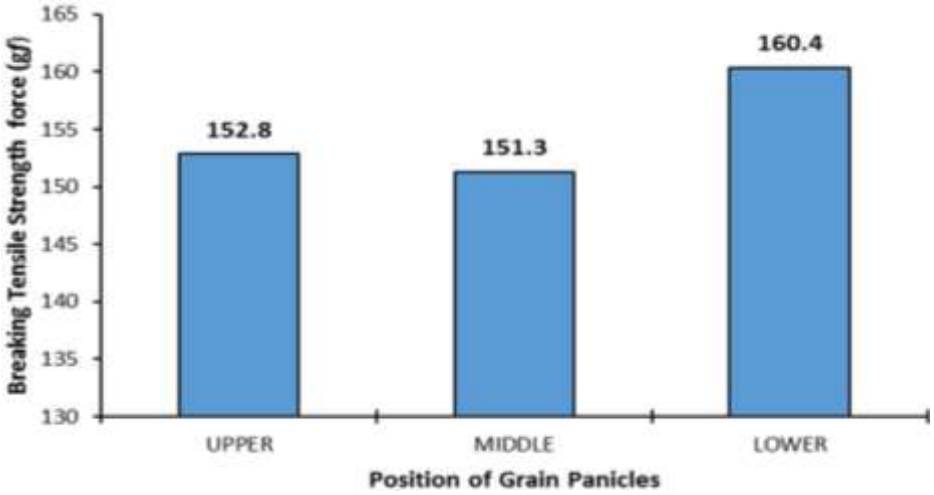

Figure 3. Comparison between shattering force values at three positions of grain on the panicle.

UPMB10 showed the highest total number of grain per panicle (98.83), number of filled grain per panicle (97.92), total number of spikelets per panicle (129.04) and panicle length (23.7 cm). However, GM118 indicated the highest number of primary branch (12.9) and showed same panicle length (23.7 cm) result with UPMB10, while Mixture showed the highest 1000 grains weight which was 22.53 g. Besides, most of parameter data showed insignificant difference between inoculations treatment except on data of 1000 grains weight and number of primary branch.

Figure 2 represent the average shattering force value measured after application of single inoculation, GM118 and UPMB10 and combined inoculation (Mixture) with different positions of grain panicle; upper, middle and lower. A significant difference was shown in lower position of grain panicle. Detaching the grain from the lower position of the panicle required a force of 160.4 g*f* but the forces required by those of the upper and middle positions were 152.8 and 151.3 g*f,* respectively (Figure 3). Meanwhile, single inoculation of UPMB10 indicated the highest breaking force to detach grain from pedicle (162.2 g*f*), followed by mixture (158.6 g*f*) and GM118 (151.9 g*f*) and control treatments required only 146.7 g*f* to detach grain from pedicle (Figure 4). The effect of single and combine inoculations on the rice grain yield in

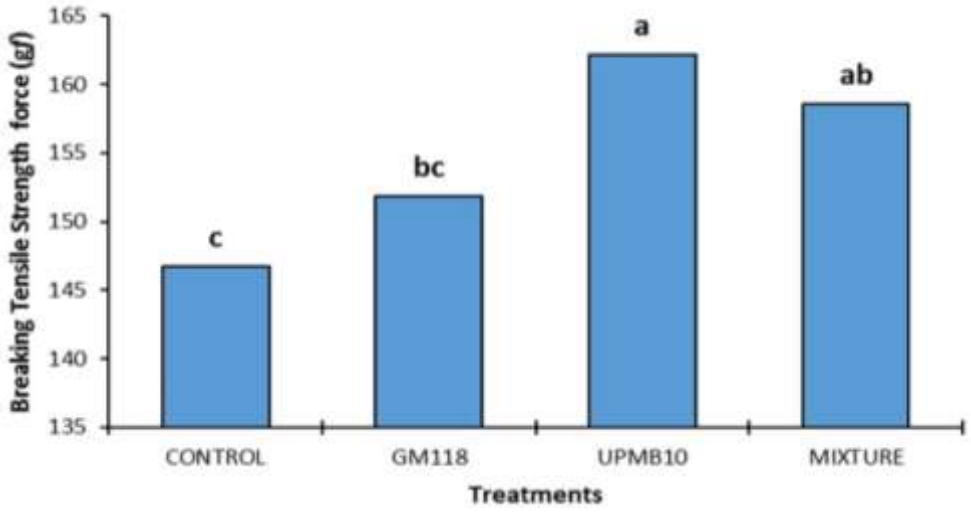

Figure 4. The average shattering force value measured after application single inoculation of GM118 and UPMB10, and Mixture (GM118 + UPMB10) inoculation of the grain panicle; Bar graphs with different letters are significantly different according to Duncan`s Multiple Range Test at $p \leq 0.05$.

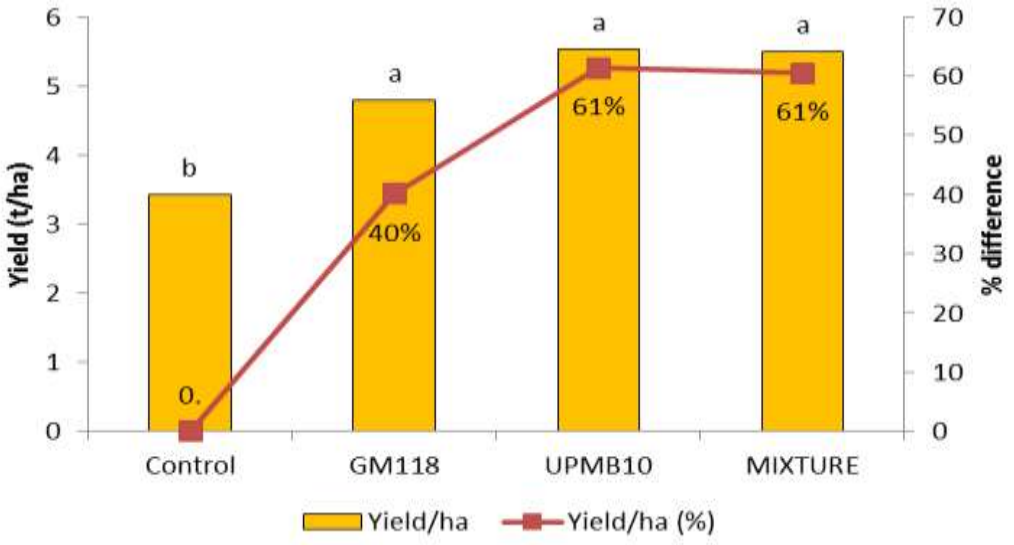

Figure 5. Grains yield (t/ha) in the field of experiment in IADA Kemasin Semerak granary measured after application of single inoculation of GM118 and UPMB10, and Mixture (GM118 + UPMB10) inoculation. Bar graphs with different letters are significantly different according to Duncan`s Multiple Range Test at $p \leq 0.05$

IADA Kemasin Semerak granary is given in Figure 5. The highest grain yield (t/ha) was shown by single inoculation, UPMB10 and combined inoculation (mixture), which 5.53 and 5.50 t/ha, respectively, and followed by single inoculation, GM118 (4.80 t/ha) as compared to control treatment. Both of UPMB10 and mixture indicated 61% difference yield/ha from control, meanwhile, GM118 indicated only 40% difference yield/ha from control. both

DISCUSSION

Plant growth promoting rhizobacteria (PGPR) used in this study were single inoculation; *Bacillus subtilis* (UPMB10) and *B. pumillus* (GM118) and also combine inoculation of UPMB10 and GM118 (Mixture). UPMB10 act as nitrogen fixer while GM118 act as phosphate solubilizer (Supplementary result: Plates 1 and 2). The presence of

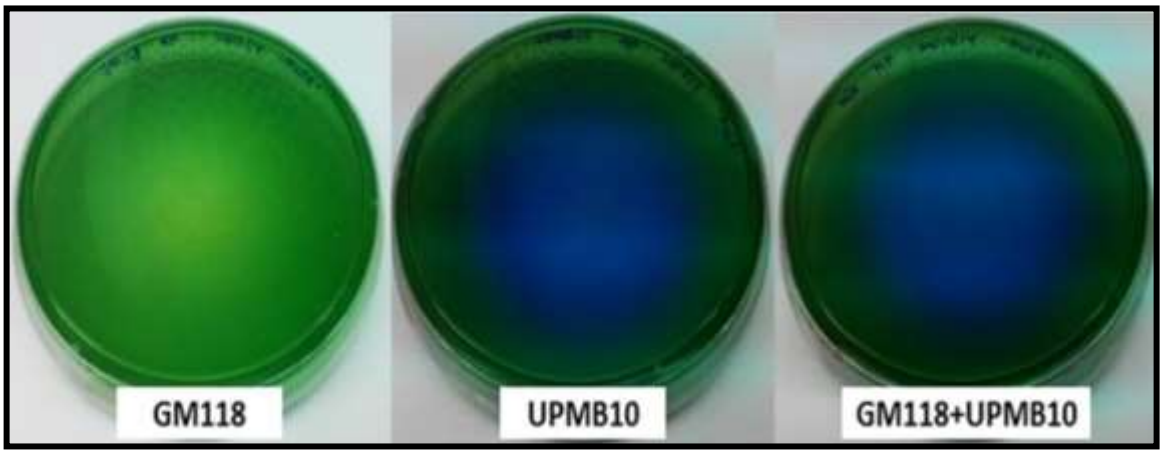

Plate 1. Nitrogen fixing ability of single and combine inoculation on N-free solid malate (Nfb) medium agar plates. Source: Day and Döberenier (1976).

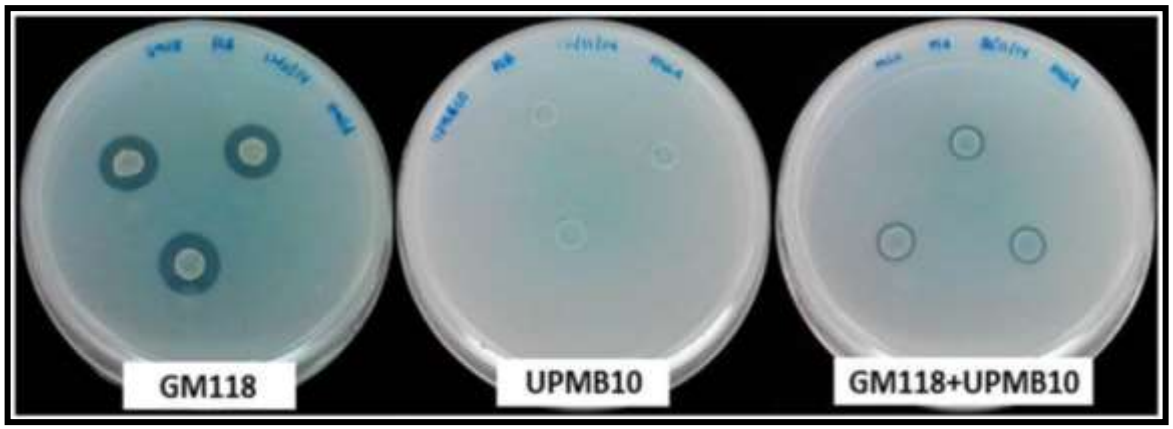

Plate 2. P solubilization of single and combine inoculation on Pikovskaya agar media plates. Source: Pikovskay (1948).

UPMB10 and GM118 in Mixture that caused it has the potential to fix nitrogen and solubilize phosphate. Both characteristics are very useful in ensuring that these isolates have potential to promote plant growth.

From Table 1, total number of grain per panicle, fill grains per panicle, 1000 grains weight, total spikelets per panicle, panicle length and number of primary branch per panicle were significantly different between inoculated treatments and control. With presence of bacteria inoculation its improvement grain physiological quality and showed positive symbiosis interaction between plant and bacteria. Rice growth performance is exposed to environmental factors which affect the physiological processes inside rice plant cells. Improving rice physiological characteristic is considered to be desirable because of its agronomic importance towards the high rice yield achievement (Li et al*.,* 2012). This finding is in agreement with Alam et al. (2008) and Islam et al. (2012) who obtained increased rice grain yield in bacteria inoculation over the uninoculated control.

UPMB10 produced the highest breaking force to detach grain from panicle and this finding was complementary to the in-vitro assay, with showed that UPMB10 produce the highest concentration of IAA either with or without L-tryptophan as represented in Figure 1. The presence of UPMB10 in Mixture who contributed to the higher concentration of IAA compared to GM118 and in turn requires high breaking force to detach grain from panicle. IAA, one of the phytohormones, can be considered as the most physiologically active auxin in plants that influences root and shoot dry matter partitioning, root and stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants (Cleland, 1990; Subba, 1999). Biswas et al. (2000) suggested a possibility of interrelation between increased tiller production and yield of rice with a change in

hormonal equilibrium, particularly IAA, due to rhizobial inoculation. Hence, it can be assumed that IAA inhibits or prolongs abscission process in the panicle via reduction of sensitivity of abscission zone (AZ). This abscission process is triggered by ethylene hormone and interplay with IAA. Meir et al. (2010), showed the role of IAA in early inhibition of organ abscission. A basipetal through the AZ inhibited abscission by rendering the AZ insensitive to ethylene. Several researchers have reported that in abscission process, the interplay between IAA and ethylene is well established (Roberts et al., 2002). Furthermore, it has been reported that elevated levels of IAA can "protect" AZ cell from abscission inducing stimulate on Arabidopsis (Basu et al., 2013). This finding can be assumed that the UPMB10 was the key trigger of hard detachment of grain panicle.

Besides, previous studies also indicated that IAA production could be a practical characteristic to choose endophytic and rhizosphere competent bacteria for rice growth promoting agents (Etesami and Alikhani, 2016; Etesami et al., 2015). The dynamic responsive pattern of IAA distribution within PGPR is a key factor to support plant growth, its reaction toward root rhizosphere, and specifically for stimulating development of plant organs.

It is clearly shown that the application of both single and combined inoculations of PGPR significantly increased the non-shattering of grain panicle (Figure 2). The result also indicated a significant difference between the lower position of grain panicle but not in the upper and middle positions and in turn showed that the lower position requires a higher force to detach grain from the panicle (Figure 3). Besides, only lower grain position of the panicle showed significant effect toward non-shattering habit. This may be due to non-uniform grain maturity across the panicle, which affects the required detaching force of a grain from the panicle (Lee and Huh, 1984; Szot et al., 1998).

This shattering habit result was also supplementary with the enhancement of total grain number and total spikelet number per panicle pattern in a time. So, the number of grains remain on the panicle can be preserved until harvesting time and reduce post-harvest losses. Hence, the national SSL percentage target to achieve yield in average 6 ton/ha can be done by applying the bacteria PGPR.

Conclusion

The data in this study showed that the positive effect of applying single inoculation; *B. subtilis* (UPMB10) and *B. pumillus* (GM118) along with combined inoculations (Mixture) of PGPR give significant increase in yield production compared to control. Besides that, the ability of UPMB10 and Mixture inoculation produce indole-3-acetic acid (IAA) and gives dissimilar result in grain shattering habit compared to GM118 inoculation. Again, single inoculation of UPMB10 and Mixture showed that both of them produced high breaking force energy to prevent detachment of grain from panicle. Moreover, increased number of total spikelet per panicle and total grain per panicle data also can be observed. Hence, PGPR that produce IAA can increase non-shattering of grain on panicle and in turn increase rice yield production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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