

Germination, Seedling Growth, Amylase and Protease Activities in Malaysian Upland Rice Seed under Microbial Inoculation Condition

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(Received: 17 May 2014; accepted: 06 July 2014)

The author evaluated the effects of PPGR (plant promoting growth rhizobacteria) inoculation on seedling establishment and enzymatic activity in particular amylase and protease activity during germination of upland rice seeds. Through this study, we carried out an experiment by using three bacterial strains, *Nitrosomonas europaea*, *Rhodopseudomonas palustris* and *Acinetobacter sp.* on two landraces, SK1 and Panderas. We discovered that SK1 seedlings had the maximum increment of radicle length in response to *Rhodopseudomonas palustris* (Treatment B) with 4.50cm and gave significant result compared to other treatments. The vigor index of SK1 seeds also gave the same significant effect using the same strain, ($P<0.05$) compared to control. The vigor index of seedlings inoculated with treatment B was found to be 856.67 which was significantly different ($P<0.05$) compared to control treatment except for treatment A and B. The highest amylase released by seeds treated with AC. Single microbial inoculants (A, B and C) showed slow increment at the beginning of day 2 but increased up to 3 fold compared to multiple microbial inoculants (AB, AC, BC, ABC). However, control treatment gave a comparable result to multiple microbial inoculants at the 5th day. Thus, these results give a clear picture for the potential of PPGR for the upland rice germination and seedling establishment.

Key words: Germination; Amylase; Protease; Enzyme activity; Upland rice.

Rice (*Oryza sativa*) is the main staple food for Malaysian people. Malaysia is facing food security challenge where this country never met its self-sufficiency level in rice production and still relies in importing about 10 to 30% of imported rice from neighboring countries such as Thailand¹.

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Therefore, the government has targeted to achieve 70% of self-sufficiency level (SSL) of rice in 10th Malaysia plan with the major current focus is to increase the production of rice. Although facing land competition from more profitable crops like palm oil, there was slightly increase in land planted with paddy in Malaysia from 426, 226 hectares in 2007 to 426, 260 hectares in 2008. Breeding high yield, climatic change and disease resistance as well as high nutritious of rice variety is currently conducted by Malaysian researchers to enhance

yield production of rice stockpile for achieving rice surplus. Besides, planting upland rice is a new strategy to improve rice production and food security. In Malaysia, upland rice is mostly planted in Sabah and Sarawak. Upland rice farming is considered as an important initiative in achieving the national goal of self-sufficiency. It is usually grown in system where little water and fertilizer are applied.

To enhance the yield potential of local upland rice cultivar, there is a need to increase the production capacity per unit in which the seed quality is important input for higher production. Early and vigor seedling emergence growth is important for rice plant establishment and development². Through assistance of plant growth microbial inoculants, the development of rice will result in grain increment and fertilizer intake efficiency will be improved. Plant promoting growth rhizobacteria (PPGR) have been reported to enhance plant growth and thus improve plant productivity. There are several reports demonstrating PPGR effects on plant especially towards the seed. They facilitate the growth of root that plays a major role in nutrient uptake and trigger faster germination. Therefore, inoculation with microbial inoculants could help in rice seedling emergence and produce enzymes that could be beneficial in further studies. However, there is little information regarding microbial inoculation during the early rice seedling establishment. Hence, this study was initiated to investigate the efficiency of *Nitrosomonas europaea*, *Rhodopseudomonas palustris* and *Acinetobacter sp.* on rice seed germination and enzyme production. *Nitrosomonas europaea* is nitrifying bacteria that play a central role in the availability of nitrogen to plants and hence in limiting CO₂ fixation. *Rhodopseudomonas palustris* has been acknowledged by microbiologist to be one of the most metabolically versatile bacteria ever described. It is able to grow in the absence and presence of oxygen. *Acinetobacter sp.* is the bacteria known to be involved in biodegradation, leaching, removal of several organic and inorganic man-made hazardous wastes and is present naturally in paddy rice³. It is expected that the use of such microbes could enhance the nutritional value of rice notably in terms of its phytochemicals components including micronutrients and enzymes.

The significant increase of growth and yield of important crops in response to application of PPGR have been well established and reported. Biological nitrogen fixation, solubilization of phosphate, production of phytohormone and suppression of plant disease are the most studied mechanism toward application of PPGR on crop field^{4,5,6,7}. Shoot and root biomass of rice when grown alone was significantly increased by 2–55% and 8–409%, respectively, when inoculated with *Acinetobacter sp.*⁸. Inoculation of *Pseudomonas fluorescens* in wheat and canola seeds could result in significant changes in root elongation for the effective nutrient absorption. Besides, *Azospirillum* strain could increase plant biomass, nutrient uptake and root length of crops⁹. Mia *et al*¹⁰ reported on the effect of rhizobia and PPGR inoculation on germination and seedling vigor of lowland rice. However, reports on the effects of combination microbial inoculants is still lacking in upland rice seeds. Hence, this study was conducted to investigate the efficiency of microbial inoculation in facilitating the upland rice seed germination and enzymatic production thus helps to provide energy for the growth of roots and shoots.

MATERIALS AND METHODS

Seeds Germination and Inoculation

Upland rice (*Oryza sativa L.*) seeds variety of Panderas and SK1 are collected from local farmers in Malaysia. Upland rice seeds (Panderas and SK1) were surface-sterilized with 5% sodium hypochlorite for 30 minutes and then rinsed with sterilized distilled water. Seeds were germinated on moist filter paper in Petri dishes at 28 °C for five days. The sterilized seeds were soaked in the respective different microbial inoculants for 45 minutes at room temperature. The seeds immersed in sterile water served as control. Ten seeds were placed in each petri dish contained each microbes treatment according to day of treatment. Three PPGR strains were selected for this study. Each strain was grown in specific media, *Nitrosomonas europaea* (A) in nitrogen-free broth, *Rhodopseudomonas palustris* (B) in MSSB broth and *Acinetobacter sp.* (C) in PSMS broth. Final concentrations of inoculums contain 3.3 x 10⁶ CFU/mL, 4.7 x 10⁶ CFU/mL and 8.5 x 10⁶ CFU/mL, for A,

B and C respectively. The mixture of the bacterial strains was prepared in sterile screw cap bottles before the experiment. Seven bacterial inoculants were designated as A, B, C, AB, AC, BC and ABC. The live bacterial cells were applied to each seed on the Petri dishes. The seed germination rate, vigor index including plumule and radicle length were determined after five days of germination using the formula below¹¹. The germinated seed were kept frozen in freezer for further analysis.

$$\text{Germination rate} = \frac{(\text{total seeds tested} - \text{number of germinated seeds})}{\text{Number of total seeds tested}} \times 100\%$$

$$\text{Vigor index} = (\text{mean plumule length} + \text{mean radicle length}) \times \text{germination rate} (\%)$$

Enzyme production in response to microbial efficacy

Enzymatic activity of germinated seeds was counted for day 0, 2, 4 and 5. Amylase activity assay was performed following method by Liu *et al.*¹² with minor modification. About 1g of germinated seeds were grounded and homogenized in 10mL of 0.05 M phosphate buffer (pH 6.0) (1:10). The resulting homogenate was filtered and centrifuged at 18 000 rpm for 10 min at 4 °C and then, filtered. The filtrate was immediately used as crude enzyme preparation. Samples were then incubated for 5 min at 30 °C. The reaction system consists of 0.5mL of substrate solution (1% soluble starch in 0.05M phosphate buffer, pH 7.2) and 0.5mL enzyme solution. 1mL of DNSA was added and the tubes were heated at 90 °C for 5 min and

cooled at room temperature. Then, samples were added with distilled water until volume reached 10ml before read at 540nm.

The protease was assayed according to the method described by Li *et al.*¹³. 1 g of germinated seeds homogenized in 10 mL of 0.1M Tris HCl (pH8.0) in ice bath (1:10). The homogenate were centrifuged at 10 000 rpm for 30 min at 4 °C. The supernatant was used for protease assay. Enzyme solution (1mL) was added to 1mL substrate solution (0.6% casein in 0.1M Tris HCl buffer pH 8.0). The reaction was carried out at 40°C for 10 min and stopped by incubating at 90 °C for 5min and adding 2mL of 0.4M TCA. Each test tube was allowed to stand for 15 min at room temperature. The mixture was centrifuged at 12000 rpm for 10 min. Protease activity was measured as the increment in absorbance at 280nm.

Statistical analysis

All experiments were conducted in three replications. All data were reported as mean ± standard deviation (SD). All data were subjected to analysis of variance (ANOVA) using SPSS statistical package. Significance was accepted at P <0.05.

RESULTS

As reported in Table 1, the study has shown no significant different (P<0.05) in germination rate for both upland rice seeds. In case of seedling vigor index, the most efficient inoculant was *Rhodopseudomonas palustris* (treatment B)

Table 1. Effect of microbial inoculant on seed germination rate and vigor index¹.

| | | Treatments | | | | | | | |
|----------|----------------------|-----------------|------------------|----------------|-----------------|-----------------|-----------------|------------------|----------------|
| | | CT | A | B | C | AB | AC | BC | ABC |
| SK1 | Germination rate (%) | 100.00± 0.00a | 96.67±5 .77a | 100.00± 0.00a | 100.00± 0.00a | 100.00± 0.00a | 100.00±0 .00a | 100.00±0 .00a | 96.67±5 .73a |
| | Vigor index (%) | 603.33± 20.82ab | 708.00± 20.78bc | 856.67± 5.77c | 720.00± 96.44bc | 633.33± 66.58b | 606.67±5 1.32ab | 440.00±4 3.58a | 614.67± 97.90b |
| Panderas | Germination rate (%) | 80.00±1 0.00a | 90.00±5 .77a | 93.33±3 .33a | 93.33±3 .33a | 93.3±3 .33a | 96.00±4. 00a | 85.67±4. 33a | 77.33±5 .67a |
| | Vigor index (%) | 434.00± 56.32ab | 565.67± 17.79abc | 623.33± 38.68c | 602.67± 8.67bc | 585.00± 68.74bc | 462.93±9 .13abc | 397.47±2 0.84abc | 388.90± 35.49a |

¹Values are means of three replicates ± SD. Means on the same row with the different superscripts are significantly different (P<0.05); CT: Control; A: *Nitrosomonas europaea*; B: *Rhodopseudomonas palustris*; C: *Acetinobacter* sp.; AB: *Nitrosomonas europaea* + *Rhodopseudomonas palustris*; AC: *Nitrosomonas europaea* + *Acetinobacter* sp.; BC: *Rhodopseudomonas palustris* + *Acetinobacter* sp.; ABC: *Nitrosomonas europaea* + *Rhodopseudomonas palustris* + *Acetinobacter* sp.

which significantly contributed ($P<0.05$) to higher vigor index compared to control. The vigor index of seedlings inoculated with this microbe was 856.67 which differ significantly ($P<0.05$) as compared to the other treatments except for treatment A and B.

Figure 1 and 2 clearly exhibited the plumule and radicle length of upland rice. Panderas seeds treated with *Rhodopseudomonas palustris* (treatment B) has higher values of radicle and plumule length compared to all those microbial inoculants. However, the plumule length of rice seedlings in all treatments was not significantly different ($P>0.05$). Furthermore, inoculation of *Nitrosomonas europaea* (Treatment A) resulted in minimum increase in plumule length which was

up to 0.13 cm longer than control treatment. The radicle length has the maximum increase recorded for SK1 seeds treated with *Rhodopseudomonas palustris* (Treatment B) with 5.9 cm and gave significant higher ($P<0.05$) compared to other treatments. The lowest increase of radicle length was observed in treatment AB (2.83±0.17), deduced that the strain was less efficient in stimulating radicle growth. The overall vigor index was ranged from 388.90 to 623.33. Rice seeds inoculated with *Rhodopseudomonas palustris* and *Acetinobacter* sp. inoculants gave significantly high vigor index of 623.33% and 602.67% respectively compared to control (434.00%). In general, this result indicated that *Rhodopseudomonas palustris* could exhibit good growth promoting effects in both upland rice

Table 2. Changes in amylase and protease activity during upland rice seed germination¹

| Assay series Day 2 | Amylase activity (U/mL) | | Protease activity (U/mL) | |
|-----------------------|--------------------------|---------------------------|--------------------------|-------------------------|
| | SK 1 | Pandera | SK 1 | Pandera |
| Control | 14.09±2.32 ^a | 12.15±0.43 ^{bc} | 0.68±0.00 ^a | 0.44±0.01 ^a |
| A | 14.49±2.70 ^a | 6.26±0.94 ^a | 0.96±0.01 ^c | 0.42±0.00 ^a |
| B | 11.52±0.64 ^a | 8.78±0.39 ^{ab} | 1.09±0.00 ^g | 0.44±0.00 ^a |
| C | 12.82±0.34 ^a | 9.97±0.09 ^{ab} | 0.99±0.00 ^f | 0.43±0.01 ^a |
| AB | 24.38±1.67 ^b | 30.35±3.69 ^e | 0.94±0.01 ^d | 1.13±0.02 ^b |
| AC | 30.05±3.17 ^b | 26.11±0.00 ^{de} | 0.83±0.00 ^c | 0.88±0.30 ^c |
| BC | 30.09±3.04 ^b | 24.71±0.09 ^d | 0.82±0.00 ^b | 1.58±0.09 ^b |
| ABC | 31.45±1.71 ^b | 16.55±0.73 ^c | 0.82±0.08 ^b | 1.11±0.05 ^b |
| Day 4 | | | | |
| Control | 27.41±3.82 ^{ab} | 20.71±1.28 ^{abc} | 0.88±0.04 ^{bc} | 0.92±0.00 ^b |
| A | 23.10±0.56 ^a | 19.92±0.60 ^{ab} | 0.85±0.00 ^{bc} | 0.74±0.00 ^a |
| B | 26.96±3.86 ^{ab} | 12.82±0.77 ^a | 0.91±0.00 ^{cd} | 0.73±0.00 ^a |
| C | 29.78±6.56 ^{ab} | 24.44±0.81 ^{bcd} | 0.91±0.00 ^{cd} | 1.01±0.01 ^c |
| AB | 21.92±1.54 ^a | 33.84±0.04 ^d | 0.83±0.00 ^b | 1.06±0.01 ^c |
| AC | 38.29±4.12 ^b | 22.89±4.72 ^{bc} | 0.89±0.00 ^{bcd} | 1.21±0.00 ^f |
| BC | 27.50±4.11 ^{ab} | 30.08±4.24 ^{cd} | 0.95±0.00 ^d | 1.03±0.01 ^{cd} |
| ABC | 26.18±0.96 ^{ab} | 22.31±2.27 ^{abc} | 0.74±0.00 ^a | 1.06±0.00 ^{de} |
| Day 5 | | | | |
| Control | 35.99±0.77 ^a | 41.45±2.74 ^b | 0.82±0.00 ^{bcd} | 0.88±0.05 ^b |
| A | 26.59±0.25 ^a | 28.19±3.13 ^{ab} | 0.84±0.01 ^d | 0.74±0.00 ^a |
| B | 26.56±2.62 ^a | 30.78±2.96 ^{ab} | 0.76±0.00 ^a | 0.77±0.00 ^a |
| C | 34.81±1.84 ^a | 31.20±0.25 ^{ab} | 0.83±0.01 ^{cd} | 0.73±0.00 ^a |
| AB | 35.21±1.56 ^a | 37.97±5.70 ^{ab} | 0.81±0.00 ^{bc} | 1.15±0.01 ^d |
| AC | 40.18±4.89 ^a | 41.52±3.00 ^b | 0.82±0.01 ^{bc} | 0.78±0.00 ^a |
| BC | 36.48±6.86 ^a | 22.89±6.69 ^a | 0.81±0.00 ^b | 0.79±0.00 ^{ab} |
| ABC | 30.38±6.99 ^a | 34.63±2.96 ^{ab} | 0.82±0.01 ^{bcd} | 1.01±0.04 ^c |

¹ Values are means of three replicates ± SD. Means on the same column with the different superscripts are significantly different ($P<0.05$) ; CT: Control; A: *Nitrosomonas europaea*; B: *Rhodopseudomonas palustris*; C: *Acetinobacter* sp.; AB: *Nitrosomonas europaea* + *Rhodopseudomonas palustris*; AC: *Nitrosomonas europaea* + *Acetinobacter* sp.; BC: *Rhodopseudomonas palustris* + *Acetinobacter* sp.; ABC: *Nitrosomonas europaea* + *Rhodopseudomonas palustris* + *Acetinobacter* sp.

tested.

The amylase activity of SK1 cultivar reached a peak point at the 5th day in all the treatments and showed increments except for treatment B (Figure 3 and Table 2). The highest amylase released by both SK1 and Panderas seeds

inoculated with inoculants AC (40.18 U/mL and 41.52 U/mL). Single microbial inoculants (Treatment A, B and C) have shown a pattern of slow increment at the beginning of day 2 but increased up to 3 fold compared to multiple microbial inoculants (Treatment AB, AC, BC, ABC). Even control gave

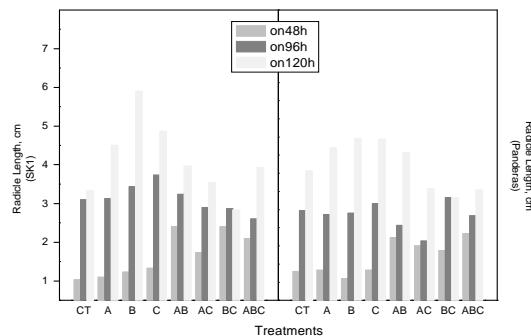


Fig. 1. Effects of microbes on radical length during seed germination.

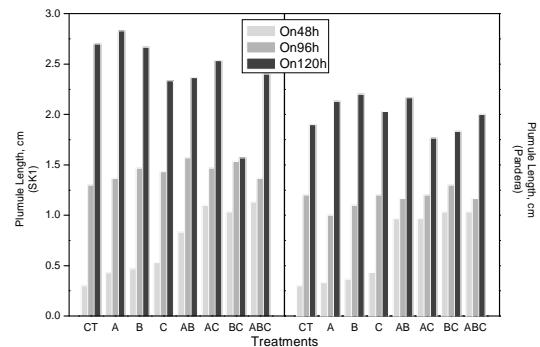


Fig. 2. Effects of microbes on plumule length during seed germination.

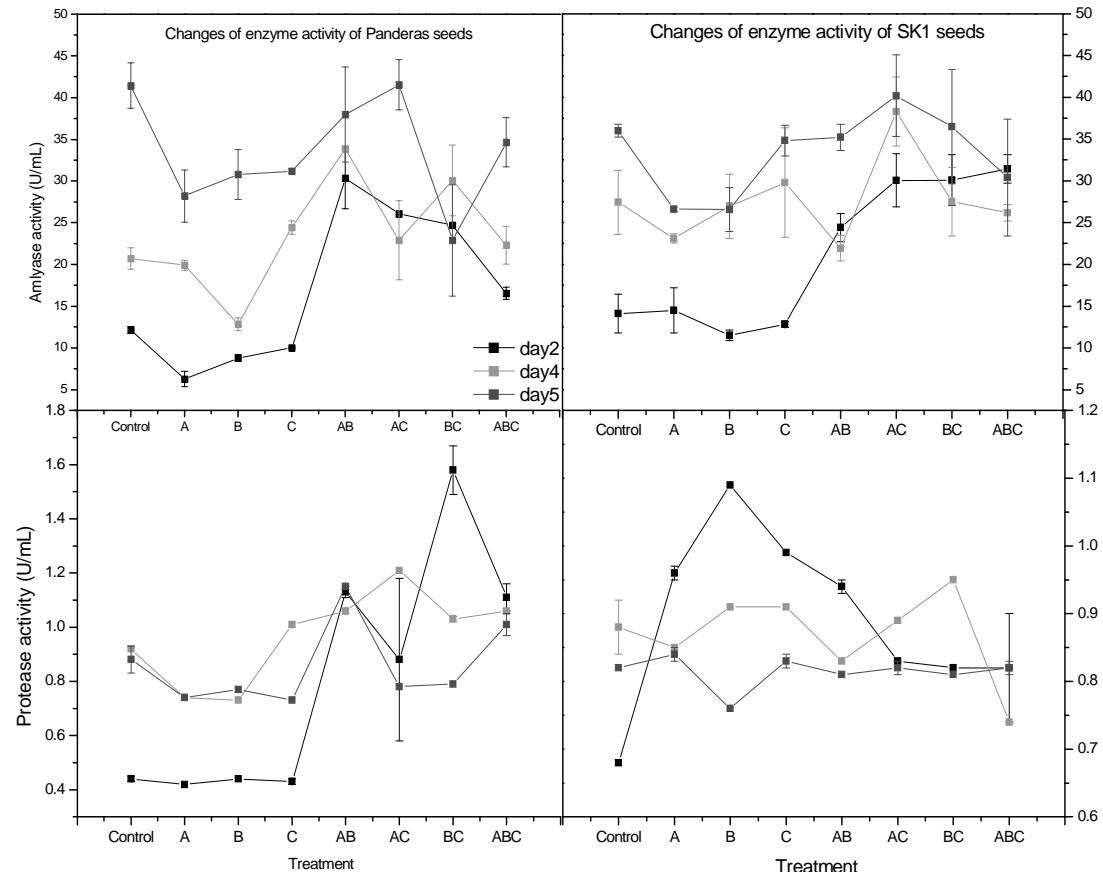


Fig. 3. Changes in amylase and protease activity of upland rice seeds treated with different microbes during germination

a comparable result to multiple microbial inoculants at the 5th day. Treatment AC gave the highest amylase activity (41.52 U/mL) on the fifth day of germination. Other microbial inoculants did not display remarkable changes but were lower than control in amylase activity during germination.

The maximum increase of protease activity was observed in SK1 seeds inoculated with treatment BC (0.95 U/mL) at 96 hours of germination, followed by B (0.91 U/mL). Treatment AC gave the highest protease activity (1.21 U/mL) on the fourth days of Panderas seed germination. However, the protease activity decreased gradually at the following 24 hours later. Both seeds displayed a rise and fall pattern of protease activity between treatments at 96 hours and 120 hours of germination. Negative effect of protease on fifth day of germination was recorded for treatment A, B, C, AC and BC as they exhibited reduction of 0.74, 0.77, 0.73, 0.78 and 0.79 U/mL respectively compared to control.

DISCUSSION

Microbial effect on seed germination

In the present investigation, two different upland rice landraces having different ranges of microbial inoculation were evaluated for the effects on seed germination, seedling vigor and enzymatic activity. This study revealed that germinated upland rice seeds treated with *Rhodopseudomonas palustris* (Treatment B) have greater value in root growth. However, the combination of microbial inoculants (Treatment AB) in SK1 seeds gave a better seedling enhancement in terms of shoot elongation. This result was in agreement with the findings of Lee *et al.*¹⁴ who discovered the inoculation effect of *Rhodopseudomonas* on growth of tomato. They observed that inoculated seeds resulted in better germination and lycopene content of tomato. *Rhodopseudomonas* is also known to secrete phytohormones and metabolites such as IAA (indole acetic acid), which is known to promote cell elongation in plants^{6,15}. It has been shown that inoculations of plants with *Rhodopseudomonas* could result in significant changes in growth parameters. Significant increases in yield and agronomical outputs in response to microbial inoculants have been reported by Koh and Song¹⁶ and Gravel *et al.*¹⁷.

Rhodopseudomonas sp. is known as of the phototropic purple nonsulphur bacteria (PPNSB) that could contribute to reduce N fertilizer in rice cultivation by increasing biological nitrogen fixation to raise crop yield. Gamal-Eldin and Elbanna¹⁸ found that inoculation of flooded rice with *Rhodobacter capsulatus* increased rice grain yield by 1.65 tha⁻¹ in the field application. Numerous studies have demonstrated that there were substantial improvements of rice yield under laboratory condition and field using application of PPNSB inoculation^{19,20,21}. In fact, Doni *et al.*²² has listed 70 article researches regarding on microbial involvement in paddy rice growth and yield. Mia *et al.*¹⁰ also showed the inoculation of PPGR increased seed emergence, seed vigor and seedling root attributes of lowland rice. Ashrafuzzaman *et al.*²³ reported that application of bacteria isolate on rice seedlings showed better performances due to induction of IAA production and phosphorus solubilization. Thus, from this study revealed that the significant improvement of seed performance might be due to IAA production.

Nevertheless, the combination of microbes did not produce significant improvement hence growth enhancement could be retarded. Felici *et al.*²⁴ reported the combination of *B. subtilis* and *A. brasilense* gave deleterious effect on plant growth. Similarly, low effectiveness was shown on establishment of arbuscular mycorrhizas when inoculants of *Pseudomonas*, *Gliocladium* and *Trichoderma* species were combined²⁵. Thus, rice seedlings treated with *Rhodopseudomonas palustris* inoculants improved seed germination consequently promote better vigor index of both upland rice landraces.

Changes of amylase and protease activities during seed germination

During seedling growth establishment, storage proteins were degraded to supply the embryo with amino acids essential nutrients. This process involved the production of proteolytic enzymes from cells of the aleurone layer²⁶. During the first two days of germination, protease activity was high and thereafter declined in some treatments. This result was similar to Rahman *et al.*²⁷ findings that reported that all enzymatic activity including amylase and protease was increased during first 1-2 day before had declined after 48 hour onwards. No important changes were

observed in all treatments. However, protease assay of SK1 seeds treated with *Rhodopseudomonas palustris* and *Acetinobacter* sp. obtained the highest expression of enzymatic activity on the beginning of germination and then decreased during the following days. This may be caused by the degradation of soluble protein to free amino acids to establish early germination for upland rice seeds associated with microbial interaction. Recent study by Li *et al.*¹³ has reported that protease activity in germinating brown rice has increased sevenfold during 6 days of germination. They discovered that protease activity was optimum at acidic pH and at 40°C under acidic and alkaline state. Several reports have remarked the role of aminopeptidase and carboxypeptidase in the storage of protein mobilization during early germination^{28, 29}. Typically, if all these endoprotease, edopeptidases, carboxypeptidase were investigated well, this could be understood better to clarify this reduction pattern. We are still not clear of the rise and fall pattern shown by the protease activity. The decrease may be due to the hydrolyzed proteins has migrated from the endosperm to embryo with some molecules found through roots and shoot. Nielsen *et al.*³⁰ has conceived that proteinase inhibitors are found in cereal grains that would limit their access to proteinase regulation in specific seed compartments tissue such as aleurone, embryo and endosperm.

Rice seeds possessed high amounts of starch. The amylase activity was initiated after 2-4 days of germination due to hydrolysis of starch. This process provided the energy for growth of shoots and roots³¹. Okamoto and Akazawa³² discovered that amylase activity was almost localized in the epithelium and continually progressively to the endosperm. Duarah *et al.*³³ also observed that the amylase activity in the rice seeds were significantly higher than in the leaves with 58.4 to 94.4 compared to 2.2 to 6.65 $\mu\text{g g}^{-1}\text{h}^{-1}$. Therefore, it can be concluded that inoculation of PPGR helps in triggering seed germination as more starches are available in seeds.

The protease detected in germinated upland rice showed complex relationship with different properties of acid edopeptidase which played a major role in early seed of protein mobilization³⁴. The protease activity may present for the breakdown of storage protein into soluble

protein, peptides and free amino acids³⁵. In this study, we did not performed the free amino acid and soluble protein assay although Hough *et al.*³⁶ said they were more hydrolyzed products (peptide and free amino acid) released in the shoots and roots. Correlation between soluble peptides with release of protease activity is also needed to confirm this study whether the reduction of protease assay is due to specialized substrate used (casein). This study suggested that *Rhodopseudomonas palustris* is very promising and performed better than other microbes in growth performance and this could be utilized as PPGR to help the growth and development of upland rice. Thus, the application of single microbial inoculants may give much beneficial effects other than co-inoculation of microbial treatments for use as a good candidate for biofertilizer.

ACKNOWLEDGEMENTS

The authors wish to express their greatest gratitude and appreciations to the UTM for providing financial support through a research grant (Q.J130000.2444.00G065) under Fundamental Research Grant Scheme (FRGS) Fund.

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