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Potential of *Bacillus subtilis* for controlling bacterial leaf blight pathogen in rice

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Abstract

Bacterial leaf blight (BLB) of rice is an economically important disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) throughout the world. To control this disease, bacterial isolate of *Bacillus subtilis* UiTMB1 was screened for the antagonistic activity against the pathogen *in vitro* and *in vivo* studies. A bacterial assay and detached leaf technique were used to evaluate the potential of the bacterium against BLB pathogen in the laboratory. Meanwhile, the glasshouse study was conducted to further examine the aptitudes of the isolate on the disease control and growth-promoting of rice plants. The findings revealed that *B. subtilis* UiTMB1 is able to control the disease and enhance the growth of rice plants. Rice plants treated with *B. subtilis* UiTMB1 before being inoculated with BLB pathogen showed less severe disease symptoms with low disease severity index of 3.43 compared to rice plants without *B. subtilis* UiTMB1 with high disease severity index of 8.4. Besides controlling the disease, *B. subtilis* UiTMB1 was also promoting plant height, chlorophyll content, number of tillers and biomass of rice plants.

1. Introduction

Rice is considered a major crop and the main source of diet in Malaysia. The rice consumption pattern of the adult population in this country shows that Malaysians consume an average of two and a half plates of rice per day (Norimah, 2008). In 2014, approximately 679,239 ha were cultivated with rice and were planted twice a year (Department of Agriculture of Malaysia, 2015). According to Man and Sadiya (2009), there were approximately 116, 000 out of 296,000 rice growers in Malaysia which are full-time growers who depend on rice cultivation for their livelihood. With the improvement of living conditions, efficient new environmental genial agents are crucially needed to control rice diseases. Blast, sheath blight and sheath-rot, bacterial leaf blight, tungro virus and bakanae are considered as major diseases of rice (Gnanamanickam, 2009). Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is one of the oldest recognized diseases and it was first noticed by the farmers of Japan in 1884 (Takami, 1962). The disease is always causing great losses of rice production as it is widely spread across Malaysia including Perlis, Kedah, Pulau Pinang, Selangor, Melaka and Kelantan (Islam *et al*., 2007). A previous study by Saad (2000) stated that cultivation of susceptible rice variety MR84 attacked by BLB in 1988 to 1994 had caused a huge loss estimated about RM 50 million during those years. The disease infestation affects the reduction of grain yield up to 50% at various levels depending on the technique of rice planting, degree of rice cultivar susceptibility, stage of growing crops, and the conduciveness of environment which the disease can occur and spread among the rice plants (Gnanamanickam *et al*., 1999). Serious damages caused by BLB on rice have demanded the development of reliable strategies to manage the disease. Nowadays, the repeated and over applications of antibiotics and chemical pesticide compounds appear to be an affliction to the environment, human safety and also the emergence of pesticide resistance. Shifting to biological control agents as an alternative control method for the disease is gaining much attention among researchers to explore the potential of beneficial microbes against the BLB pathogen. For instance, Gangwar and Sinha (2010) and Dipankar *et al*. (2017) reported that *Trichoderma harzianum* and *Pseudomonas fluorescens* have potential as bio-control agents for BLB disease in India. Meanwhile, Sumera *et al*. (2016) found that the application of *Pseudomonas* spp. Rh323 is not only able to suppress BLB disease, but it also promotes plant growth of Basmati rice by producing secondary metabolites such as enzymes and hormones. The abilities

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of *Bacillus* spp. to inhibit the growth of BLB and enhance plant growth have been widely reported, for example, a study conducted in China revealed that *Bacillus amyloliquefaciens* has the ability to reduce BLB infestation and significantly increase plant fresh weight from 50.03% to 73.11% and dry weight from 64.11% to 86.65% in the treated rice plants (El-shakh *et al*., 2015). Meanwhile He *et al*., (2019) applied *B. subtilis* strain BJ-1 for controlling blast disease of rice caused by fungal pathogen *Magnaporthe oryzae* in China. Besides rice, *Bacillus* spp. has been reported as a biological control agent for many other crop diseases such as *B. Subtilis* for anthracnose disease of red pepper (Lee *et al*., 2020) and chilli (Ashwini and Srividya, 2014), tomato diseases (Cucu *et al*., 2020), controlling aflatoxigenic *Aspergillus parasiticus* (Siahmoshteh *et al*., 2018), maize disease (Douriet-Gámez *et al*., 2017) and many other diseases. Although a few studies have investigated the bio-control efficacy of *B. subtilis* on rice such as Yang *et al*., (2009) for controlling blast disease, there is no research involving *B. subtilis* against BLB of rice under Malaysia climatic condition. Therefore, the main objective of this present study was to evaluate the potential of *B. subtilis* (UiTMB1) isolated from asymptomatic rice leaves as a biological control agent for controlling BLB and growth-promoting capabilities to rice plants in the glasshouse. This will provide the potential strain for developing the effective bio-control product of managing this disease.

2. Materials and methods

2.1 Pathogen and Bacillus subtilis

The pathogen *X. oryzae* pv. *oryzae* was obtained from a ready stock at the laboratory of Plant Pathology, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM). Meanwhile, *B. subtilis* UiTMB1 was isolated from asymptomatic rice leaves collected from Jitra, Kedah, Malaysia. The isolate was identified and confirmed as *B. subtilis* by using a Biolog System® and based on cultural and morphological characteristics. The bacterial isolate coded as *B. subtilis* UiTMB1 can be described as an aerobic, spore-producing, Gram-positive, rod-shaped bacterium (0.7–0.8 × 2.0–3.0 μm). The catalase reaction, gelatin liquefaction, starch hydrolysis, casein hydrolysis, sugar fermentation, and Voges-Proskauer tests were all positive, while the oxidase reaction and indole test were negative. These data indicated that strain UiTMB1 was a member of the genus *Bacillus*.

2.2 Bioassay of B. subtilis UiTMB1 against the pathogen

Bacterial activity assay and detached leaf technique were used to evaluate the efficacy of *B. subtilis* UiTMB1 against the BLB pathogen *in vitro* and *in vivo* experiments. A method of bacterial activity assay proposed by Hastuti *et al*. (2012) was used to observe and measure the formation of the clear zone between the pathogen and *B. subtilis* UiTMB1. In this bacterial assay, 1 mL of BLB pathogen suspension (10^8 CFU/mL) was spread uniformly on Nutrient agar in the Petri plate and incubated at 28±2°C for 24 hrs prior to inoculation with *B. subtilis* UiTMB1. A fresh culture of 24 hr-old *B. subtilis* UiTMB1 on Nutrient agar was taken out as a disc with a diameter of 5 mm by a cork borer and then placed on the BLB pathogen. The plate was then allowed to incubate for another seven days before observing and measuring the formation of a clear zone surrounding the disc. Nutrient agar disc without pathogen was also placed on BLB pathogen to serve as controls. This plate treatment was carried out in five replicates. Meanwhile, the detached leaf technique was conducted using healthy rice leaf segment as suggested by Akhtar *et al*. (2008). Rice leaf segments (variety MR263) obtained from rice plants grown in the glasshouse for 60 days were in this experiment. The leaf segments with a size of 5 cm in length were surface sterilized with 10% of sodium hypochlorite solution for 30 s to eliminate unwanted microbes and then rinsed with sterile distilled water twice. The leaf segments were dipped in the BLB suspension (10^8 CFU/mL) and then placed on moist sterilized filter paper in the Petri plate. Then, 1 mL of *B. subtilis* UiTMB1 was dispensed uniformly on the inoculated leaf segments and kept in the incubator at 30±2°C for 14 days. The leaf segments inoculated with pathogen alone served as a positive control.

2.3 Preparation of rice seedlings and experimental design

Susceptible rice variety MR263 seeds were soaked in sterile water overnight in order to enhance germination (Hastuti *et al*., 2012). The seeds were then grown on a moist sterilized filter paper in the Petri plates and maintained in a growth chamber at 30±2°C and 100% relative humidity (RH) for five days. Thus, the germinated seeds were then transferred into plastic growth chambers with a diameter of 28 cm containing clay loam soils. For each plastic growth chamber, five germinated seeds were transplanted and grown in a month prior to the experiment. This experiment consists of five treatments as shown in Table 1. Each treatment consisted of 10 replicates and all replicates were assigned in completely randomized design in the glasshouse.

2.4 Preparation and inoculation of B. subtilis *UiTMB1 inoculum on rice seedlings*

Inoculum of *B. subtilis* UiTMB1 was prepared by

transferring 10 loops of 24 hr-old culture into 100 mL nutrient broth and grown for 48 hrs in the incubator shaker at the setting temperature of 28±2°C and shaken at 150 rpm. The broth culture was then diluted in sterile distilled water and adjusted the colony forming unit (CFU) to 10^8 - 10^9 CFU/mL according to a method by Krishanti *et al*. (2015). Suspension of BLB pathogen was also prepared following the same procedure and adjusted to 10^8 - 10^9 CFU/mL. Both suspensions of pathogen and *B. subtilis* UiTMB1 were applied to rice seedlings by spraying using different sets of hand sprayer with 50 mL for each growth chamber. Rice plants in the treatments of T1C, T2P and T3C were inoculated with BLB pathogen by cutting off the leaf tips and dipping for 2 mins in the pathogen suspension to provide a high chance for the disease infection then followed by spraying the pathogen suspension to the plants. After seven days, the pre-inoculated plants with a BLB pathogen in the treatment T1C were sprayed with *B. subtilis* UiTMB1 suspension and this treatment served as a curative treatment to assess the ability of the agent to combat the established pathogen on the rice plants. In contrast, rice plants in the treatment of T2P were served as a preventive treatment to evaluate the ability of *B. subtilis* UiTMB1 to prevent the plants from the pathogen attacks. Meanwhile, rice plants inoculated with pathogen alone in the treatments of T3C were used as a negative control and rice plants inoculated with *B. subtilis* UiTMB1 alone (T4C) were used as a positive control (Table 1).

Table 1. Five treatments used to evaluate the efficacy of *B. subtilis* (UiTMB1) to inhibit BLB pathogen and enhance the growth performance of rice plants

Treatment	Description
1. Curative (T1C)	Inoculation with BLB pathogen for seven days then followed by *B. subtilis*
2. Preventive (T2P)	Inoculation with *B. subtilis seven days then followed by BLB pathogen*
3. Negative Control (T3C)	Inoculation with BLB pathogen alone
4. Positive control (T4C)	Inoculation with *B. subtilis alone*
5. Healthy Control (T5C)	Without any inoculation

2.5 Disease severity and plant growth assessments

Biological control aptitudes of *B. subtilis* UiTMB1 against *X. oryzae* pv. *oryzae* on rice plants were assessed based on disease severity index (DSI) that was previously developed by IRRI (1996) according to a formula of DSI as below:

$$DSI = \frac{n(1) + n(3) + n(5) + n(7) + n(9)}{tn}$$

Where: n (1), n (3), n (5), n (7) and n (9) = Number of leaves showing severity score of 1, 3, 5, 7 and 9. *tn* = Total number of leaves scored.

Disease severity was recorded as a percentage of the leaf tissue area infected out of a total leaf area examined. The following scale as shown in Table 2 (Chaudhary, 1996) was used for scoring of BLB severity on rice plants. The growth performance of the treated rice plants with *B. subtilis* UiTMB1 was assessed weekly by collecting the data of plant height, the number of tillers, length of roots, chlorophyll content, plant dry weight (biomass) and grain yield. The collected data were then analysed statistically by ANOVA and Least Significant Difference (LSD) at $P \leq 0.05$.

Table 2. Disease severity scale for evaluation of bacterial leaf blight of rice in the field

Disease Rating	Percentage of infected leaf area by BLB
0	0
1	1 >1-10%
2	3 >11-30%
3	5 >31-50%
4	7 >51-75%
5	9 >76-100%

2.6 Colonization of B. subtilis (UiTMB1) in Rice leaf Tissues

Colonization of inoculated *B. subtilis* UiTMB1 in rice leaf tissues was assessed based on a method of Mattos *et al*. (2008). At the final stage of the glasshouse experiment, destructive sampling of rice leaves from the treatments of TC1, T2P and T4C were conducted to evaluate the colonization of *B. subtilis* UiTMB1 in the leaf tissues. The rice leaves were collected from four pots of rice plants for each treatment and brought to the laboratory. The rice leaves were washed under running tap water and cut into small segments of 1 cm in length before its surface was sterilized with 1% of sodium hypochlorite for 10 mins and 70% ethanol for 1 min, then followed by rinsing with sterile distilled water twice. The rice leaf segments were weighed to about 1 g and then crushed in 5 mL of sterile saline with mortar and pestle. The suspensions were then refined using filter papers and diluted into 10-fold serial dilutions before 100 µL was spread onto NA plates. The NA plates were incubated at 28±2°C for 2-4 days and three replicates of the NA plates were used for each treatment. Bacterial colonization in the rice leaf tissues was counted and expressed as colony-forming unit (CFU) per fresh weight plant tissues (g).

3. Results and discussion

3.1 Bioassay of B. subtilis UiTMB1 against the pathogen

Results of bacterial activity assay and detached leaf technique proved that *B. subtilis* UiTMB1 was able to inhibit the growth of BLB pathogen. A 42 mm of clear inhibition zone formed surrounding the disc of *B. subtilis*

UiTMB1 indicated that the biological agent inhibited the growth of pathogen which was not observed on the control disc. This finding was further supported by the results of a detached leaf experiment that revealed *B. subtilis* UiTMB1 is able to combat the attack of BLB pathogen on rice leaf segments as shown in Figure 1. The leaf segments inoculated with BLB pathogen and *B. subtilis* UiTMB1 showed fewer symptoms of BLB, however, the disease symptoms have appeared on the leaf segments inoculated with BLB pathogen alone. The ability to inhibit the growth pathogen could be associated with secretion of antimicrobial substances such as antibiotics, enzyme, toxin and other proteins. According to Hussain *et al*. (2017), bacitracin A and polymyxin B which are peptides families of antibiotic separated from crude lyophilized extracts of *B. subtilis* have strong inhibition ability against some pathogens. There is a high possibility the clear inhibition zone produced by *B. subtilis* UiTMB1 was due to the antibiotic action.

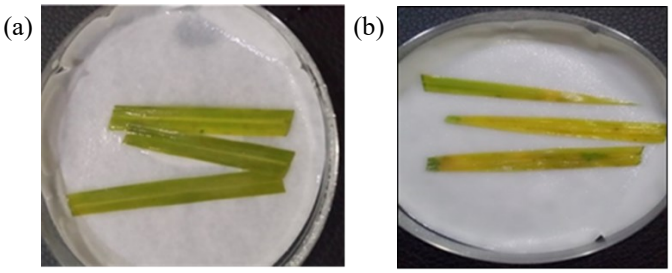

Figure 1. Inoculated rice leaf segments with BLB pathogen and *B. subtilis* UiTMB1. (a) The rice leaf segments inoculated with BLB pathogen and *B. subtilis* UiTMB1 showed less symptoms of the disease as compared to (b) the control treatment with BLB pathogen alone.

3.2 Disease severity index and plant growth performance

The effectiveness of *B. subtilis* UiTMB1 against BLB pathogen within the first three weeks of observation indicated that the lowest disease severity was recorded for the T2P treatment with DSI value of 2.94% and then followed by the treatment of T1C with 4.10% (Table 3). In contrast, the highest DSI was observed in the treatment of T3C (negative control) with 5.58%. Furthermore, the findings showed that the rice plants treated with *B. subtilis* UiTMB1 prior to inoculation with the BLB pathogen (T2P- preventive) recorded only 3.43% DSI at six weeks of observation as compared to the rice plants treated with the BLB first, then *B. subtilis* UiTMB1 (4.50% DSI) (T1C- curative). These results suggested that *B. subtilis* UiTMB1 has potential to protect rice plants from a severe infection of the BLB pathogen (Figure 2). The isolate of *B. subtilis* UiTMB1 also has the ability to prevent and cure the plants of BLB disease. In contrast, rice plants in the treatment of negative control (T3C) with BLB pathogen alone recorded the highest DSI after six weeks of observation

Table 3. Disease severity index recorded for the treatments of rice plants inoculated with *B. subtilis* UiTMB1 and BLB pathogen for six weeks

Observation week	Disease severity index (DSI)		
	T1C (Curative)	T2P (Preventive)	T3C (Negative Control)
1	0.57±0.31[a]	0	1.39±0.09[b]
2	1.90±0.21[b]	0.59±0.04[a]	2.43±0.04[c]
3	4.10±0.11[b]	2.94±0.19[a]	5.58±0.14[c]
4	4.10±0.11[a]	4.13±0.19[a]	6.48±0.09[b]
5	5.11±0.22[a]	4.97±0.12[a]	6.66±0.11[b]
6	4.50±0.38[a]	3.43±0.16[a]	8.36±0.12[b]
LSD(P≤0.05)	0.003	0.001	0.132

Values are expressed as mean± standard error (SE). Values with the different superscripts within the row are significantly different according to the t-test at P<0.05.

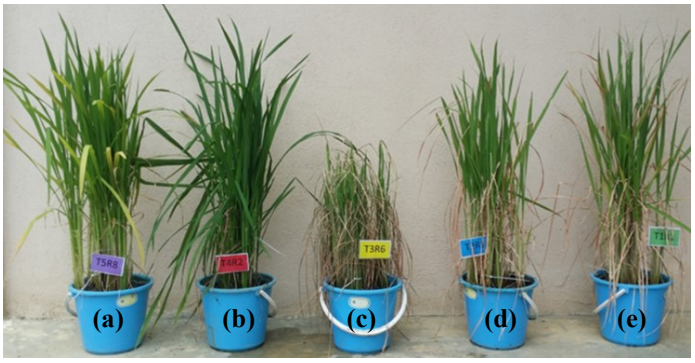

Figure 2. Rice plants treated with and without BLB pathogen and *B. subtilis* UiTMB1 displayed the different levels of DSI. (a) Plant without any inoculations (T5C), (b) plant inoculated with *B. subtilis* UiTM alone, (c) pathogen alone, (d) preventive and (e) curative treatment.

on the disease symptom development. The disease progress with time displayed that the BLB severity recorded in the treatment T3C (negative control) was constantly increasing consecutively. A similar trend for the BLB severity was recorded in the treatments of T1C and T2P, however, the progress was slow and the signs of disease recovery on rice leaves can be spotted on week six and this recovery can be related to the decreasing the DSI value (Table 3). Rice plants applied with *B. subtilis* UiTMB1 (T4C) were observed to be growing healthier than the rest as shown in Figure 2. The growth performance of rice plants due to *B. subtilis* UiTMB1 was evaluated based on plant height, chlorophyll content, number of tiller and length of roots as shown in Table 4. The findings showed that rice plants with *B. subtilis* UiTMB1 (T4C) to be growing taller than rice plants without the bacterial inoculum in the control treatment (T5C). A similar trend was observed for the chlorophyll content, the number of tillers and root length between rice plants with and without *B. subtilis* UiTMB1. This result proved that the biological control agent *B. subtilis* UiTMB1 is able to promote plant growth. Potential of *B. subtilis* as plant growth promoters has been reported by many researchers

Table 4. Effect of application *B. subtilis* UiTMB1 on growth performance of rice plants

Treatment	Plant Height (cm)	Chlorophyll Content	No. of Tiller	Length of Root (cm)
Curative (T1C)	93.50±1.80[a]	33.79±0.63[c]	11±0.58[d]	43.33±3.38[a]
Preventive (T2P)	92.33±0.88[a]	33.91±1.68[c]	15±0.67[c]	40.00±1.00[a]
Negative Control (T3C)	-	-	-	-
Positive control (T4C)	93.67±0.88[a]	42.78±0.47[a]	24±0.33[a]	51.00±2.08[a]
Healthy control (T5C)	88.83±0.44[b]	38.46±0.74[b]	20±0.67[b]	40.66±5.20[a]

Values are expressed as mean± standard error (SE). Values with the different superscripts within the row are significantly different according to the t-test at P<0.05.

Table 5. Effect of application *B. subtilis* UiTMB1 towards biomass of rice plants

Treatment	Leaves (g)	Stem (g)	Roots (g)	Panicles (g)
Curative (T1C)	4.24±0.49[b]	9.57±1.21[b]	9.63±1.09[b]	14.74±1.64[b]
Preventive (T2P)	3.98±0.37[b]	9.34±0.57[b]	6.97±0.67[b]	13.11±1.73[b]
Negative Control (T3C)	-	-	-	-
Positive control (T4C)	12.62±0.28[a]	13.21±1.08[a]	23.67±2.92[a]	22.15±0.32[a]
Healthy control (T5C)	4.98±0.10[b]	11.40±0.90[ab]	7.69±0.67[b]	15.15±2.27[b]

Values are expressed as mean± standard error (SE). Values with the different superscripts within the row are significantly different according to the t-test at P<0.05.

such as Rekha *et al*. (2020) in rice, Lee *et al*. (2020) in wheat and Jamily *et al*. (2019) in barley and many other studies. Previous studies by Chen *et al*. (2007) and Harman (2011) discovered that *Bacillus* species could enhance the biosynthesis of plant growth-promoting hormones such as Gibberellic acid (GA3) and Indole-3-acetic acid (IAA). Meanwhile, the similar trend observed for the chlorophyll content, the results showed that the highest chlorophyll content recorded by SPAD in the treatment of T4C and compared to other treatments. The lowest chlorophyll content recorded in both treatments of T1C and T2P could be explained by chlorosis symptoms due to BLB disease. The highest number of tillers (24 tillers) and the lengthiest of roots (51 cm) were also recorded in the treatment T4C. This finding proved that *B. subtilis* UiTMB1 is promoting the plant growth of rice. This finding could be related with the findings of Rekha *et al*. (2020) mentioned that *B. subtilis* RR4 AF1 able to promote rice growth by enhancing the production of primary metabolite malic acid and the secondary metabolite salicylic acid in roots. Furthermore, the results of the biomass of leaves, stem, roots and panicles showed that there was a significant difference among the treatments at $P \leq 0.05$ (Table 5). Biomass of rice leaves in the treatment T4C showed the highest weight with 12.62 g and then followed by treatment T5C (4.98 g), but the lowest weight was observed in the treatment T2P (3.98 g) (Table 5). A similar result was obtained for the biomass of stems, roots and panicles where the highest value was recorded in the treatment T4C and the lowest value in the treatment T2P.

3.3 Colonization of B. subtilis UITMB1 in rice leaf tissues

Leaf tissues of rice colonized by bacterial *B. subtilis* UiTMB1 were examined and the findings proved that *B. subtilis* UiTMB1 in the treatments T1C and T2P has an ability to colonize the plant tissues and at the same time suppress the BLB pathogen. This is reflected by the high population density of *B. subtilis* obtained in the treatment T4C with 2.7×10^9 CFU g^{-1} and then followed by T1C and T2P as shown in Table 6. This result indicated that the biological control agent is able to maintain its population within leaf tissues.

Table 2. Disease severity scale for evaluation of bacterial leaf blight of rice in the field

Treatment	Population Density ($\times10^9$ CFU/g)
Curative (T1C)	2.1±0.67[a]
Preventive (T2P)	2.3±0.51[a]
Positive control (T4C)	2.7±0.77[a]

Values are expressed as mean± standard error (SE). Values with the different superscripts within the column are significantly different according to the t-test at P<0.05.

4. Conclusion

Our data indicate that *B. subtilis* UiTMB1 has great potential as a bio-control agent and also as plant growth-promoting bacterium. However, this study would be more interesting with the mechanisms used by the isolate to inhibit the pathogen of BLB which is not covered by the study. The production of antimicrobial compounds either enzymes, toxins or other proteins will need to be identified in the future. Great potential is shown by the isolate and could be explored further to improve the efficacy of the bacterium under the real conditions in the rice fields by developing a reliable and stable formulation of a bio-control agent using recent technology.

Conflict of interest

The authors declare no conflict of interest.

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