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Short Communication

Rhizobacterium *Bacillus cereus* induces root formation of pepper (*Piper nigrum* L.) stem cuttings

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Rhizobacteria have been widely reported with beneficial properties, able to promote growth and yield of various agricultural crops. In the present study, two strains of rhizobacterium *Bacillus cereus* were tested on its ability to induce and elongate roots of pepper stem cuttings after inoculation. Results showed that *B. cereus* UPMLH24 inoculation on fresh pepper stem cuttings stimulated root number (55% increase over control), length of longest root (25% increase over control), total root length (87% increase over control), root fresh weight (28% increase over control) and root dry weight (112% increase over control). Present study recommends *Bacillus cereus* UPMLH24 as a potential candidate in a formulation of a biostimulant for organic and sustainable nursery for pepper production.

Keywords: Inoculation, *Piper nigrum*, rhizobacteria, root induction, stem cutting

Pepper (*Piper nigrum* L.) or commonly known as black pepper is one of the important agricultural commodities in the world. It is conventionally propagated through stem cuttings of two to six nodes for field establishment (Abbasi *et al*., 2010). This technique is commonly practise due to cost effective and easy in planting material preparation. Pepper stem cuttings suffer the disadvantage of their being frequently infested with fungal, bacterial, viral and mycoplasmal pathogens. Such internal infections are difficult to control and are further spread by the vegetative propagation of pepper (Abbasi *et al*., 2010). Moreover, certain stem cuttings are hard to root without the aid of exogenous rooting hormones. Due to agronomic problems in pepper cultivation, plant growth-promoting rhizobacteria (PGPR) application can be propose as one of the alternative agricultural applications used to promote sustainability and health of pepper plant. PGPR exhibit plant growth-promoting properties via several beneficial mechanisms such as biological nitrogen fixation, phosphate solubilisation, and phytohormone production (Ali *et al*., 2009; Aziz *et al*., 2012; Zakry *et al*., 2012). In the present study, therefore, the effects of inoculation with two newly isolated plant growth-promoting rhizobacteria, *Bacillus cereus* strains UPMLH1 and UPMLH24 on root formation of pepper stem cuttings were evaluated under nursery conditions.

Materials and Methods

The Rhizobacteria

The selected rhizobacterial strains of *B. cereus* UPMLH1 and UPMLH24 used in the present study had been shown in earlier studies to stimulate early plant

development on shallot, mustard and mung bean (Zakry *et al*., 2010; Aziz *et al*., 2012). *B. cereus* UPMLH1 was characterised as a N_2-fixing and an IAA producing strain, while *B. cereus* UPMLH24 displayed only the former trait (Zakry *et al*., 2010). The strains are maintained at the Laboratory of Microbiology and Pathology at the Department of Crop Science, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus.

PGPR Bacillus cereus Inoculation on Root Formation of Pepper Cuttings

Efficiency of PGPR *Bacillus cereus* inoculation on root formation of five-node pepper stem cuttings of Kuching variety was evaluated under nursery conditions (50% shade). Five treatments (control, 1000 ppm indole-3-butyric acid (IBA), 2000 ppm IBA, *Bacillus cereus* UPMLH1 and *Bacillus cereus* UPMLH24) were assigned in a completely randomised design with 24 replicates cuttings per treatment. In the present study, the rate of 1000 ppm and 2000 ppm IBA were determined according to Singh (1990) and Sujatha *et al.* (2004) with some modification and are used to serve as positive controls. In the present study, IBA application was used to act as positive control of rooting stimulant. IBA is better root promoting compound than IAA (Wiesman *et al*., 1988) and it is widely used in agriculture as a commercial stimulant of root induction in cuttings (Dobbelaere *et al*., 2003). In addition, the chemical structure of IBA is nearly identical with indole-3-acetic acid (IAA) (Strader and Bartel, 2011), thus might gave a good comparison with microbial IAA (Idriss *et al*., 2007; Ali *et al*., 2009).

Surface sterilisation of five-node pepper stem cuttings was carried out with 70% ethanol, followed by 0.525% sodium hypochlorite (Clorox). Subsequently, sterilized pepper stem cuttings were treated by dipping them into overnight grown bacterial suspension (more than 10^8 cfu mL^{-1}) for 30 min and then air dried (Diby *et al*., 2005). Similar procedures were also employed on stem cuttings treated with IBA while the uninoculated controls were dipped into the sterilised nutrient broth medium only. The bottom three nodes of treated black pepper stem cuttings were placed in black polyethylene bags containing sterilised sand. The plants were irrigated twice a day. After 45 days, the following recordings were made on all the cuttings (Anandaraj and Sarma, 1994): percent survivability, rooting percentage, total number of root per cutting, length of longest root per cutting (cm), total length of roots per cutting (cm), root fresh weight per cutting (mg) and root dry weight per cutting (cm). The latter five traits were assessed only on cuttings that rooted. A cutting was considered rooted if a minimum of one root ≥ 1 mm in length was present. Percent survivability, indicating the percentage of pepper stem cuttings in a treatment group is alive (still fresh) for a given period of time (45 days observations) after recording, the formula is as follows:

$$\text{Percent survivability} = \frac{\text{initial number of fresh pepper stem cuttings - number of pepper stem cuttings died}}{\text{initial number of fresh pepper stem cuttings}} \times 100$$

Rooting percentage was calculated by counting the number of rooted pepper stem cuttings, and dividing the initial number of fresh pepper stem cuttings, the formula is as follows:

$$\text{Rooting percentage} = \frac{\text{number of rooted pepper stem cuttings}}{\text{initial number of fresh pepper stem cuttings}} \text{ x } 100$$

Total number of root per cutting was determined by counting the individual number of primary roots emerged from cutting node and the number was divided by total number of rooted cuttings to obtain average number of roots per cutting. Length of longest primary root in cm per cutting was calculated by selecting and measuring the length of individual root present on each cutting with the longest length then divided with number of rooted cuttings to obtain the average number per cutting. Total lengths of roots per cutting was determined by measuring length of individual root present on each pepper stem cutting and then sum the length of individual root to obtain total of root length in cm. Root fresh weight per cutting was evaluated by weighing total root sample recovered from each cutting. Subsequently, similar fresh roots samples were oven dried at 70°C for 48 h to determine dry weight. All measurements were compared by an analysis of variance followed by least significant different test (IBM SPSS version 21) at probability level of 5%.

Bacterial Population of Inoculated Pepper Stem Cuttings

Populations of PGPR *Bacillus cereus* strains UPMLH1 and UPMLH24 in sand and root surface were assessed after 45 days of treatment. Each plant has triplicate root samples and three plants per treatment were randomly selected. Treated plants were uprooted gradually and the lower 2 cm portion of root tips was selected to proceed with serial dilution with 0.02M phosphate buffer after removing adhering sand particles. The fresh weight of root samples were recorded before proceed with serial dilution. The serially diluted suspension was spread on nutrient agar plates and number of colony recorded after 24 hours of incubation. Population was expressed as log of colony forming unit (CFU) per 1 g fresh weight of root and sand. All measurements were compared by an analysis of variance followed by least significant different test (IBM SPSS version 21) at probability level of 5%.

Results

Rooting responses of pepper cuttings to PGPR and IBA applications is presented in Table 1. The treatments did not significantly affect percent survivability and rooting percentage. However, relative to inoculation with *Bacillus cereus* strain UPMLH24 produced higher percent survivability at 96 %, and also rooting percentage at 96 %, and this was seen as comparable with hormonal treatment with 1000 ppm IBA (100 % survivability and 88 % rooting). *B. cereus* UPMLH24 inoculation had significantly stimulated the emergence of roots (113) of pepper stem cutting as compared to 73 number of roots in the control. *B. cereus* UPMLH1 inoculation did not alter root number (84) significantly as compared to control (73). The effectiveness of *B. cereus* UPMLH24 inoculation on inducing root numbers was similar with hormonal IBA treatment at 1000 ppm which was recorded at 131 number of roots (no significant different at $p>0.05$). Besides that, pepper stem cuttings treated with *B. cereus* UPMLH24 showed significantly increased length of the longest root in each cutting at 11.06 cm as compared to control at 8.83 cm. However, pepper stem cutting treatment with *B. cereus* UPMLH1 (9.50 cm) was not significantly different from the control (8.83 cm). The lengthy of longest root of pepper stem cutting treated with UPMLH24 strain was seen similar with hormonal IBA treatment at 2000 ppm (11.63 cm).Total root length of pepper cuttings increased significantly after treatment with *B. cereus* UPMLH24 which was recorded at 280.99 cm. In comparison, control cuttings had a mean total root length of 150.32 cm. Treatment with *B. cereus* UPMLH1 (166.87 cm) did not give results that were significantly different from the control (150.32 cm).

Table 1: The effects of PGPR *Bacillus cereus* inoculation on root formation of pepper stem cuttings 45 days after treatments.

Treatment	Survivability (%)*	Rooting (%)*	Total root number /cutting	Longest root length (cm) /cutting	Total root length (cm) /cutting	Root fresh weight (mg) /cutting	Root dry weight (mg) /cutting
Control	88	79	73 c	8.83 cd	150.32 c	781.33 c	154.25 de
1000 ppm IBA	100	88	131 a	18.88 a	480.28 a	3920.33 a	873.00 a
2000 ppm IBA	83	83	27 d	11.63 bc	93.99 c	963.33 b	195.00 cd
B. cereus UPMLH1	92	79	84 bc	9.50 cd	166.87 bc	936.33 b	237.59 c
B. cereus UPMLH24	96	96	113 ab	11.06 b	280.99 b	1003.00 b	327.50 b

Measurements shown are the means of 24 readings* and 12 readings. Means in a column bearing the same letter are not significant at p>0.05.

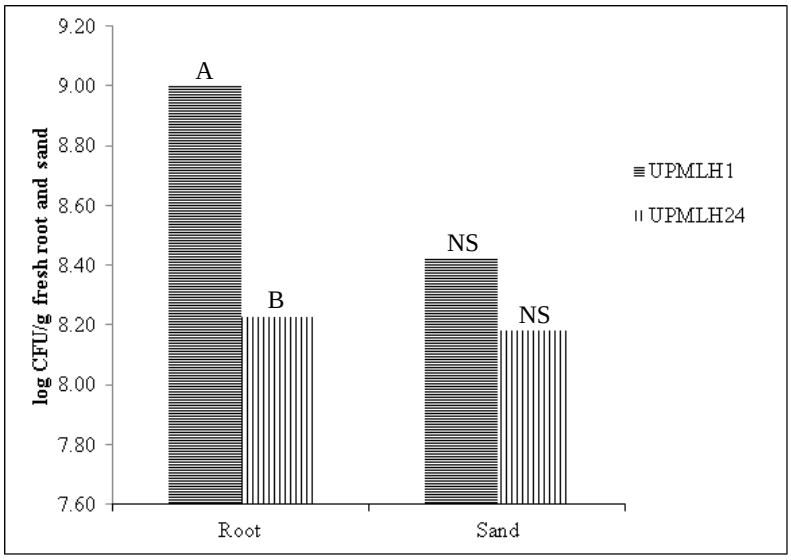

Figure 1: Colonization pattern of the two tests isolates UPMLH1 and UPMLH24 in the root and sand medium of pepper stem cuttings after 45 days. Observation means of triplicate plants; means followed by different letters are significantly different at 0.05 level. NS = no significant different.

All the experimental treatments resulted in increased fresh weight of the roots as compared with the controls. PGPR *B. cereus* UPMLH1 (936.33 mg) and *B. cereus* UPMLH24 (1003.00 mg) inoculation were performed better than the control (781.33 mg) in terms of total root fresh weight. Most of the treatments significantly increased root dry weight of pepper cuttings as compared to control (154.25 mg). *B. cereus* UPMLH24 inoculation (327.50 mg) and *B. cereus* UPMLH1 inoculation (237.59 mg) were performed better than control at 154.25 mg (significant at $p<0.05$). Total bacterial populations associated with roots and in sand medium were shown a positive cells survivability after 45 days of inoculation (Figure 1). Inoculation treatments for 45 days encountered 8.2 to 8.4 log CFU/g of sand medium and 8.2 to 9.0 log CFU/g of fresh root. No significant different of inoculant populations ($p>0.05$) between *B. cereus* strains UPMLH1 and UPMLH24 was found

in sand medium. However, *B. cereus* strain UPMLH1 was significantly recorded higher population in root of pepper stem cuttings at 9.0 log CFU/g of fresh root than *B. cereus* strain UPMLH24 at 8.2 log CFU/g of fresh root.

Discussion

Plant growth-promoting rhizo-bacteria (PGPR) have been used in conjunction with the cultivation of many important agricultural crops. They are commonly introduced through seed and soil inoculation. In this regard, the inoculation with PGPR on stem cuttings is a less common practice. In the present study, the effects of inoculations of two strains of PGPR *Bacillus cereus* on to the stem cuttings of pepper (*Piper nigrum* L.) were evaluated on root induction 45 days after inoculation. The 45 days period of observation was considerable sufficient for variation in root induction which recorded some rooting parameters such as total number of roots in each cutting and maximum length of roots (Anandaraj and Sarma, 1994). Root-ability of pepper stem cutting is also a critical event in the nursery production due to low stem cutting viability and difficulty to obtain healthy runner shoots. The successful treatment application in the present study would benefit pepper nursery growers.

Present study found that inoculation of PGPR *B. cereus* strain UPMLH24 promoted root induction and stimulation of pepper (*P. nigrum* L.) stem cuttings. The contribution of PGPR inoculation was seen comparable to that of indole-3-butyric acid (IBA), applied at 1000 ppm. IBA at 1000 ppm has been reported as optimum rate in enhancing rooting and sprouting of pepper stem cuttings (Singh, 1990; Sujatha *et al*., 2004). Inoculation with *B. cereus* UPMLH1 did not improve root initiation of fresh pepper stem cuttings, although it gave better total fresh weight (20 % increase over control) and root dry weight (54 % increase over control). An earlier report found that *B. cereus* UPMLH1 was an IAA producer (Zakry *et al*., 2010), and the availability of this auxin was expected to benefit the development of adventitious roots in the cuttings (Jarvis, 1986). Nevertheless, over-concentration of IAA might inhibit rooting since, according to Jarvis (1986), root elongation requires low concentrations of IAA. Moreover, adventitious rooting in non-woody cuttings is controlled mainly by endogenous IAA (Jarvis, 1986), and so exogenous auxins might not be as useful. The inhibitory effects of excessively high auxin on root development were evident from the responses to two concentrations of IBA, viz. 1000 ppm and 2000 ppm. The former stimulated root induction and proliferation whereas an application of the higher IBA concentration (2000 ppm) appeared to have harmed the rooting process of pepper cuttings. Application of 1000 ppm IBA showed improvements in all rooting parameters viz. root number (80 % increase over control), length of longest root (114 % increase over control), total root length (220 % increase over control), root fresh weight (402 % increase over control) and root dry weight (466 % increase over control). This finding was similar as reported by Singh (1990) and Sujatha *et al*. (2004). The benefit of inoculation with *B. cereus* UPMLH24 believed to have been due to the release of tryptophan from newly emergence and proliferation of pepper roots that concurrently stimulates the secretion of IAA and subsequently promoted root growth (Baca and Elmerich, 2007) and the ability of UPMLH24 stimulates root growth may be involved tryptophan dependent pathway (Idriss *et al*., 2007). According to Kravchenko *et al*. (2003), IAA could be synthesised by multiple pathways in a bacterial cell from the precursor tryptophan, which can be obtained from the degradation of roots and microbial cells and from root exudates. It should be noted that the concentration of

bacterial auxin produced depends on the response and physiological development of the plant due to endogenous hormone levels of its host, which may vary according to the genotype and age of the plant (Ahmad *et al*., 2005). Moreover, it has also been proposed that the biosynthesis of auxins in bacteria would be a strategy to detoxify tryptophan excesses in the rhizosphere (Bar and Okon, 1993). The role of *B. cereus* UPMLH1 might be different with strain UPMLH24, though both are from similar species. It could be suggested that there exists a synthesis pathway independent of tryptophan in *B. cereus* UPMLH1, which produces IAA in the absence of tryptophan as demonstrated in Zakry *et al*. (2010). According to Martnez-Morales *et al*. (2003), higher rates of tryptophan assimilation increase the production of auxins in bacteria. Surplus amount of IAA in pool which produced exogenously from UPMLH1 cells and endogenously from pepper stem cutting may inhibit rooting induction in pepper stem cutting.

Both *B. cereus* strains used in the present study exhibited nitrogen-fixing-like activity as demonstrated in the earlier work (Zakry *et al*., 2010) with *B. cereus* strain UPMLH1 relatively providing more nitrogen than *B. cereus* strain UPMLH24. However, *B. cereus* UPMLH24 which provided lesser amount of nitrogen exhibited impressive rooting performance in pepper cuttings, suggesting that other mechanisms of action may be involved in the process of root formation. Probably *B. cereus* UPMLH24 stimulates rooting, leading to a physiological changes in the pepper cutting by synthetizing low IAA concentrations and by protecting the plant from important pathogenic micro-organisms, producing an antagonistic effect as reported by Gilbert *et al*. (1990) and Handelsman *et al*. (1990). Present study suggest that the ability of *B. cereus* inoculation to increase rooting of *P. nigrum* cuttings may depend on the endogenous levels of IAA in the stem cutting and those produced by the respective bacteria. Unfortunately, present study did not analyse IAA levels in cutting during the experiment.

The bacterial population ranged between 10^8 and 10^9 cells/g of fresh root and sand medium in *P. nigrum* cuttings, similar and even better to what has been obtained in other studies in which rhizospheric populations ranged from 10^6 to 10^8 cells/g (Ruppel *et al*., 2006; Peralta *et al*., 2012). Although *B. cereus* UPMLH24 had significantly lower population than UPMLH1, it may be sufficient to generate significant improvement on root growth better than UPMLH1. Moreover, this phenomenon indicates that UPMLH24 cells may have higher per cell efficiency and productivity over UPMLH1 cells, thus exhibited uniqueness in capability though came from similar species and rhizosphere of host plant, *P. nigrum*.

Conclusion

Bacillus cereus UPMLH24 inoculation was the most promising inoculant for root induction in pepper stem cuttings and this holds promise for its use in the formulation of a biostimulant compatible with sustainable and organic agriculture. The present study also showed that although pepper stem cuttings were easy to root, supplementation with exogenous auxin (1000 ppm IBA) further enhanced rooting in pepper.

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