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Effect of nitrogen fertilizer on hydrolytic enzyme production, root colonisation, N metabolism, leaf physiology and growth of rice inoculated with *Bacillus* sp. (Sb42).

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Abstract

A study was conducted at Universiti Putra Malaysia, Malaysia, glasshouse to determine the inoculation effect of Sb42 (locally isolated diazotroph) with different doses of nitrogen fertiliser (0, 30 and 60kg N ha^{-1}) on hydrolytic enzyme production and the growth of rice variety MR219. Using 16S rRNA partial gene sequencing, Sb42 was identified as *Bacillus* sp. Plants were harvested at 45 days after transplanting, and specific enzyme activities of endoglucanase (EG) and endopolymethylgalacturonase (EPMG) were determined. Results showed that the growth of rice and hydrolytic enzymes production were significantly ($P<0.05$) affected by bacterial inoculation and N application. The application of N at concentrations of 30 and 60kg ha^{-1} to inoculated plants reduced plant growth and specific enzyme activities. Inoculated plants without N application showed significantly higher ($P<0.05$) specific hydrolytic enzyme activity (88.9% EG and 20.1% EPMG), plant biomass (72.46%), rate of photosynthesis (9.38μmol$CO_2m^2s^{-1}$), leaf N content (3.16%) and N uptake (74.59mg $plant^{-1}$) compared to non-inoculated treatments. Root colonisation was observed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), which proved that Sb42 is able to colonise at the surface and interior of roots. The ability of Sb42 to produce hydrolytic enzymes and biological nitrogen fixation was affected by the application of N fertiliser.

Keywords: Biological nitrogen fixation; *Bacillus* sp.; Endoglucanase; Endopolymethylgalacturonase; Plant physiology; Plant biomass.
Abbreviation:NFb_Nitrogen free media; Amax_Net photosynthetic rates at light saturation points; ARA_Acetylene Reduction Assay; Cfu_Colony forming unit; IAA_Indoleacetic acid;PGPR_Plant Growth Promoting Rhizobacteria; SEM_Scanning Electron Microscope; SAS_Statistical Analyses System; TEM_Transmission Electron Microscope; OD_ Optical Density.

Introduction

Nitrogen fertiliser is one of the major inputs in agricultural systems for high crop production. However, high chemical N fertiliser applications lead to NO_3^- pollution and the formation of carcinogens (Saikia and Jain, 2007). Optimisation of biological nitrogen fixation (BNF) seems to be a suitable alternative to reduce the use of N fertilisers (Wu et al., 1995). Large amounts of nitrogen derived from biological fixation have been shown to be present in rice plants (Shenoy et al., 2001). The tropical soils of Malaysia harbor diverse groups of diazotrophic bacteria and nineteen potential *Bacillus* strains were shown to fix high N_2 levels using an acetylene reduction assay (ARA) (Naher et al., 2009). It is estimated that about 7.6 million tonnes of oil can be conserved annually if BNF replaces half of the N fertiliser applied to the 120 million hectares of lowland rice (Reddy et al., 1997). The colonisation of N_2-fixing bacteria on the root surface and interior is important for influencing positive responses of plant growth, and the association of plants with diazotrophs has been shown to be essential for plant growth improvement (Naher et al., 2011). In the non-symbiotic association for the N_2 fixation process, hydrolytic enzymes produced by bacteria can hydrolyse plant cell walls and play a crucial role in entering and colonising the plant roots. Verma et al. (2001) proved that the microbial activities of cellulase and pectinase enzymes showed the potential for inter- and intracellular colonisation. Several N_2-fixing bacterial strains isolated from rice roots were able to produce hydrolytic enzymes (Asilah et al., 2009). However, these hydrolytic enzymes are sensitive to some abiotic factors, in particular chemical fertilisers (Zakarauskaite et al., 2008).
The application of higher amounts of N fertiliser to lowland rice significantly inhibited the biological nitrogen fixation process (Henson & Heichel, 1984; Shrestha and Ladha, 1996). The present study was conducted to identify a locally isolated bacterial strain, Sb42, and to determine its inoculation effect on root morphology, the production and activity of hydrolytic enzymes (endoglucanase and endopolymethylgalacturonase), photosynthetic activity and tissue N accumulation in rice at different rates of N fertiliser.

Results

Strain identification

The 16S rRNA partial gene sequence analysis proved that the isolated diazotrophic bacteria belongs to *Bacillus* sp. and is

similar to *Bacillus subtilis* (98%). The gene sequence is provided in Gene Bank (accession no. JQ820260).

Soluble protein content and specific enzyme activity in root tissue

Plants inoculated with bacteria significantly increased the soluble protein content in root tissue. However, the production of soluble protein decreased with an increased urea-N application (Fig. 1). Higher soluble protein levels (10.36mg ml^{-1} extract) were observed in plants inoculated with the bacterial isolate at 0kg N, in comparison to the non-inoculated plants. Differences in hydrolytic enzyme activity (EG and EPMG) of roots inoculated with N_2-fixing bacteria and urea application are shown in Figs. 2 & 3. Bacterial inoculation increased the specific activity of both the EG and EPMG enzymes in root tissue. However, the specific activity for EG decreased with increased urea application. The specific activity of the EPMG enzyme in inoculated and non-inoculated plants decreased with an increased urea application up to 30 to 60kg N ha^{-1} (Fig. 3). Plants inoculated with Sb42 and without urea application showed the highest specific activity for EG (54.59 units mg^{-1} protein) and EPMG (62.01 units mg^{-1} protein) compared with other treatments. There was an 88.9% increase of EG and a 20.1% increase of EPMG in the inoculated plants over the control.

Root morphology and plant biomass production

Bacterial inoculation and urea application significantly affected the root morphology and plant biomass (Fig. 4 & 5). At 0kg N-urea, inoculated plants showed the highest root length, root volume and root surface area compared with other treatments. A significant effect was found for bacterial inoculation and N fertiliser application on plant biomass production. Inoculated plants, at 0kg N, showed a higher biomass than non-inoculated plants. However, plant biomass in inoculated plants started to decrease with 30kg N ha^{-1} urea application and the biomass of non-inoculated plants increased with an increased urea application (Fig. 5). Inoculated plants and those without the application of urea showed the highest plant biomass (72.46%) compared to non-inoculated control plants.

Plant-tissue nitrogen concentration, uptake and plant photosynthetic activity

Significant differences in the concentration and uptake of N in plant tissue were observed among the treatments. The tissue N concentration of inoculated plants decreased with increased urea application. However, in non-inoculated plants, the N concentration increased with increased urea application (Table 1). The highest tissue N concentration (3.16%) was found in inoculated plants without the application of urea. Subsequently, N uptake in inoculated plants also decreased with an increased urea application. The N uptake for non-inoculated plants was low, even at 60kg N. Inoculated plants at 0kg of urea showed the significantly ($P<0.05$) highest N uptake (74.59mg $plant^{-1}$) compared to other treatments. There was 53.8% increase in tissue N content and 162.2% in inoculated plants over the controls.

The photosynthetic rate of the youngest expanded leaf was significantly influenced by bacterial inoculation and urea application (Table 1). Inoculated plants without the application of N showed significantly ($P<0.05$) higher leaf photosynthesis rates and leaf chlorophyll contents (SPAD chlorophyll meter) compared to other treatments. In inoculated treatments, the SPAD chlorophyll value increased by 35.78% over the non-inoculated control.

Visual observation of colonisation using of SEM and TEM

The inoculated diazotrophs colonised and proliferated in the root. Applied bacteria (Sb42) were colonised on the surfaces of primary and secondary roots, root hair zones (SEM micrograph) and the interior of roots (TEM micrograph) (Figs. 6 & 7).

Discussion

The partial gene sequences analysis using 16S rRNA proved that the applied bacterial strain, Sb42, is *Bacillus subtilis* sub sp. subtilis. *Bacillus subtilis* contains a central domain of *NifA* and *NtrC* family of regulators, which is an equivalent of σ^{54} Gram-negative bacteria (Dtbarbouillo et al., 1991). The *NifA* and *NtrC* gene factors are known to control biological nitrogen fixation of the protobacteria (Merrick, 1983). In the present study, an increased tissue nitrogen content of the inoculated, un-fertilised plant indicated the ability of the applied strain for biological nitrogen fixation. The reductions of cell growth and enzyme activities, as well as N fixation activity, of inoculated treatment with higher doses of N fertiliser were observed. In a state of an abundance of nutrients, microbes use their preferred sources for optimum growth rate and, unlike many bacteria, the nitrogen metabolism regulatory genes of *Bacillus subtilis* are regulated by the availability of rapidly metabolised nitrogen sources (Fisher, 1999). The increment of nitrogen fertiliser in the present study reduced the activity of the applied inoculum and the resultant effect was reflected in reduced plant growth rates, photosynthesis rates, leaf chlorophyll contents and significant differences in chlorophyll contents and the rate of photosynthesis found between treatments. Plants inoculated with *Bacillus* sp. and without the application of chemical N showed the highest photosynthetic activity. These results proved that N_2 fixation by *Bacillus* sp. can support plant growth without the application of N fertiliser at the vegetative stage of rice. Triplett (1996) reported that N_2 fixation in sugarcane produced high yields without the use of any N fertiliser. However, N fixation affected by nitrogen fertiliser application was observed in the present study. Rivera et al. (1991) also pointed out that a high concentration of mineral-N decreased the population of N_2-fixing bacteria in sugarcane plants. Roesch et al. (2006) supported this agreement by proving that the colonisation of N_2-fixing bacteria in maize plants was inhibited during the early stages of growth by high doses of N fertiliser. The applied N_2-fixing bacterial strain has the ability to produce hydrolytic enzymes (EG and EPMG), which is a possible mechanism for this bacteria to enter into the plant roots and be sustained as endophytes. The TEM photograph confirms the inoculated bacterial colonisation inside the root tissue. The contribution of endophytes is more prominent, as the inside of tissue provides a low oxygen pressure, which is suitable for nitrogen fixation. Results of several studies showed that endophytes contribute a prominent yield by BNF, which ranged from 31% to 54%, with *Herbaspirillum seropedicae* and 28% to 31% in *Burkholderia* spp. inoculation (Baldani et al., 2000). The plant biomass yield increased by approximately 245% in rice inoculated with *Stenotrophomonas maltophilia* (Naher et al., 2011). The specific activity of the hydrolytic enzymes and soluble protein content produced by plant roots was significantly affected by bacterial inoculation and N application. The

Table 1. Effect of *Bacillus* sp. (Sb42) inoculation on rice tissue N content, N uptake, photosynthesis rate and leaf chlorophyll content at 45 days of plant growth.

N application (kg ha^{-1})	Tissue N content (%)		N uptake (mg plant^{-1})		Leaf chlorophyll content		Photosynthesis rate (µmol CO_2 m^{-2}-s^{-1})	
	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated
0	3.10^{c}	2.0^{a**}	74.59^{c}	28.45^{a}	37.12^{c}	27.33^{a}	9.38^{d}	3.40^{a}
30	2.98^{c}	2.4^{ab}	37.40^{ab}	37.40^{ab}	35.72^{bc}	33.12^{b}	7.69^{c}	4.85^{ab}
60	2.83^{b}	2.6^{b}	41.54^{b}	44.31^{b}	33.96^{b}	33.36^{b}	5.87^{bc}	7.25^{c}

* ANOVA (P<0.05)**Means within the same column followed by the same letters are not significantly different at P<0.05

specific activity for the enzymes of inoculated plant decreased with N fertiliser application. This might be due to the sensitivity of inoculated bacteria to the presence of N fertilisers that can limit the activity of hydrolytic enzymes (Zakarauskaite et al., 2008). The plant growth decreased with increased N fertiliser levels in inoculated plants. An increased N uptake, plant growth and root development seen without the application of N fertiliser, proved that this strain is able to fix N_2 and produce growth hormones. Similar studies revealed the capability of free-living N_2-fixing bacteria to increase rice plant heights and dry biomass production (Naher et al., 2009; Islam et al., 2009). A previous study with *Azospirillum* sp., which is classified as N_2-fixing bacteria, showed a positive effect on root biomass and surface area production (Bashan et al., 2004), and an earlier study with free-living bacterial inoculation showed increases in root elongation (20%), shoot elongation (38%), root dry weight (13%) and shoot dry-weight (36%) in wheat (Zahir et al., 2004). In greenhouse controlled conditions with axenic sugarcane plants, it was shown that inoculation with *Gluconacetobacter* sp. could lead to an increase of N content and weight of both shoots and roots (Sevilla et al., 2001). The results of this study also indicated that the addition of N fertiliser affected plant growth. The excessive N depressed the growth of plants. This might be due to the toxic accumulation of nutrients in tissue leading to the decline of plant growth (Landis et al., 2005). However, inoculations with *Bacilllus* sp. from the natural association in rice roots contributed significantly to the increase tissue N content, plant biomass, and hydrolytic enzyme activities without supplement of a nitrogen fertiliser.

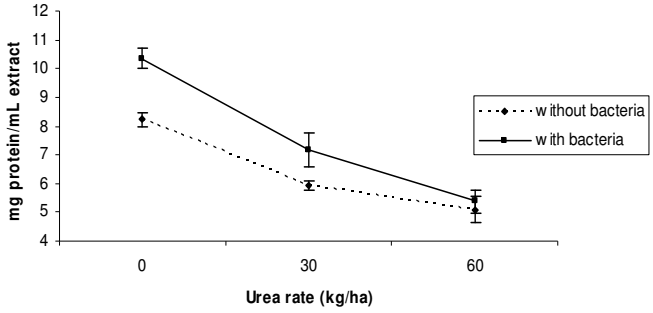

Fig 1. Effect of N2-fixing bacteria and urea on soluble protein content of rice roots. Bars in lines indicate standard error of means (n = 5).

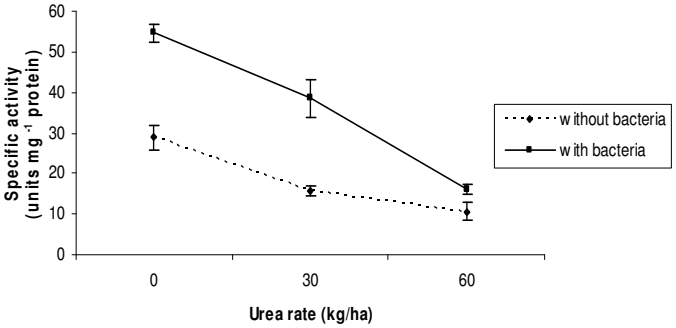

Fig 2. Effect of N2-fixing bacteria and urea on specific activity of EG enzyme. Bars in lines indicate standard error of means (n = 5).

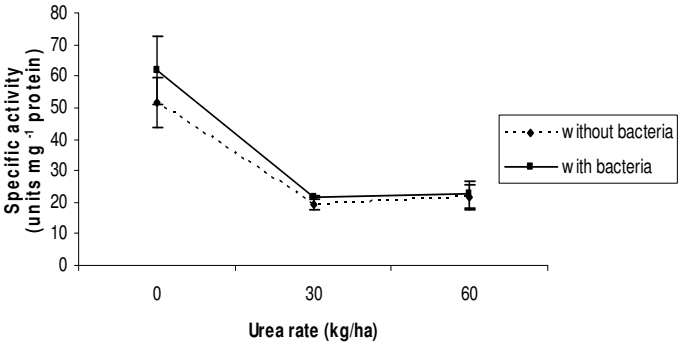

Fig 3. Effect of N_2-fixing bacteria and urea on specific activity of EPMG enzyme. Bars in lines indicate standard error (n = 5).

Materials and methods

Identification of strain

DNA extraction from pure isolates was carried out using the Genomic DNA Mini Kit UK AS. The Rep PCR was done using primer RH1820: 5′ -CCGACGGCTAACATTC-3′. The PCR product was purified using the GeneJET PCR purification kit, Fermentas, Thermo scientific, EU, and was sent for partial sequence analysis to the First BASE laboratories in Malaysia.

Soil and seedling preparation

The soil (0-15cm depth) belonged to Bernam soil series, which was clay-loam in texture (*Fluvaquentic* or *Typic Endoaquepts*) (Soil Survey Staff, 1998), and was collected from the Tanjong Karang rice irrigation project area, Selangor, Malaysia. The soil contained total N (0.27%), available P (44.57ppm), potassium (312ppm), total C (2.94%) and had a pH of 5.32. The soil was sterilised and

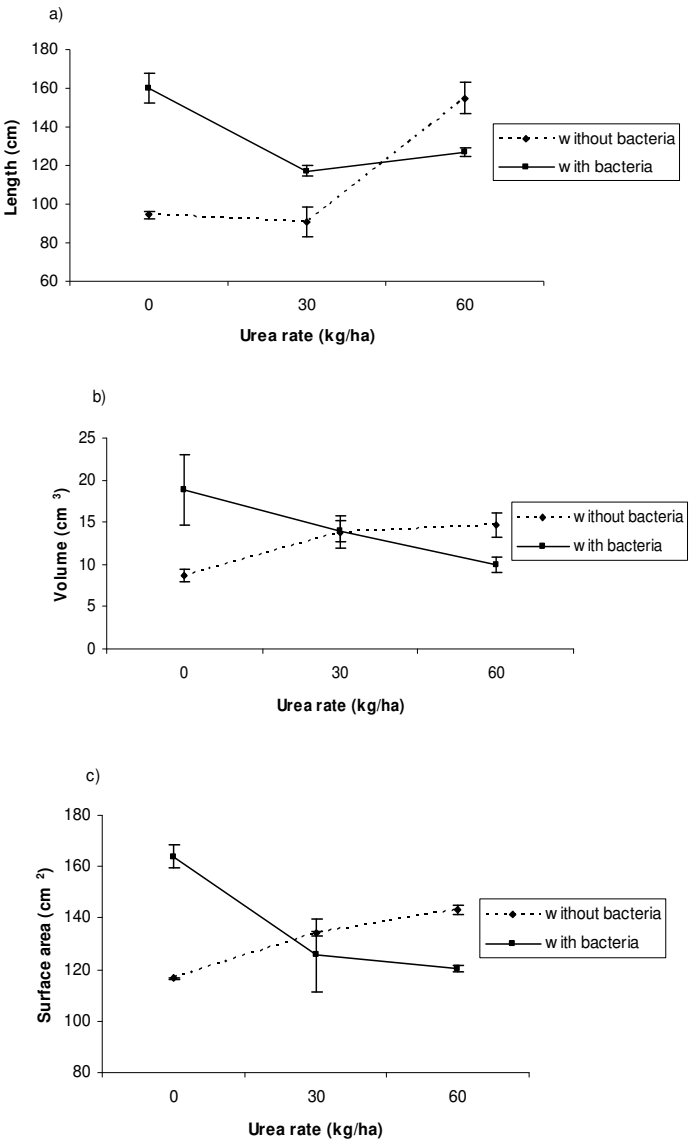

Fig 4. Effect of N2-fixing bacteria and urea on root morphology (5a=root length, 5b= root volume and 5c root surface). Bars in lines indicate standard error of means (n = 3).

placed in undrained pots (14.5cm x 12.5cm) and flooded with water up to 2.5cm above the soil surface. Blanket doses of P_2O_5 and K_2O at the rates of 60 and 40kg ha^{-1}, respectively, (equivalent with 26.22 P and 33.2 K kg ha^{-1}) were applied to each pot. Rice seeds (*Oryza sativa*, variety MR 219) were surface-sterilised with 70% ethanol (AR Grade) and 3% Clorox™. Seven day old seedlings were soaked in the bacterial inoculum for two hours before planting in the respective pots.

Application of inocula and N fertiliser (urea)

Bacillus sp. (Sb42) was shaken in nutrient broth for 72 hours. The bacterial cells were harvested by centrifugation and washed with 0.85% sterilised phosphate buffer solution (PBS). Approximately 10^8cfu ml^{-1} of live washed bacterial cells were directly inoculated into the plants in each pot**.** Before inoculation, the optical density (OD) measurements were checked and the populations were confirmed using the drop plate count method in an Nfb media plate. The same amount of dead cells (cells autoclaved for 30 min at 121°C) was applied to the non-inoculated pots. Three levels of N fertiliser in the form of urea were applied at 0, 30 and 60kg ha^{-1}. Plants were grown in glasshouse for 45 days. The air temperature in the glasshouse ranged from 27-35°C during the day and 25-28°C during the night, while the air humidity was recorded at 70-80% during the study period.

Extraction of enzymes from the root tissue

The EG and EPMG activity of rice roots (45 days) were determined as described by Adriano-Anaya *et al*. (2006) with some modifications. Roots were pulverized in a chilled mortar. The resulting powder was homogenised in 100mM Tris-HCl buffer (pH 7) containing 0.02g polyvinylpyrrolidone, 10mM $MgCl_2$, 10mM $NaHCO_3$, 10mM ß-mercaptoethanol, 0.15mM phenylmethyl sulphonyl fluoride (PMSF) and 0.3% (w/v) Triton X-100. Sodium azide (0.03%) was added to all solutions to inhibit microbial growth. The resultant liquid was filtered through filter paper (Whatman No.1) and centrifuged at 20,000 *g* for 20 min at 4°C. The ratio of root weight to volume of solution used was 1:5. The supernatant fraction was dialysed (Snakeskin Pleated Dialysis Tubing: Pierce, MWCO: 7000) against several hundred volumes of the same diluted extracting solutions (1:9, v/v) for 18 h at 4°C. The samples were frozen until required.

Determination of soluble protein and enzyme activity in root tissue and growth culture

Total soluble protein for the supernatants was quantitatively determined using a Bradford assay with BSA as the standard (Bradford, 1976). The activities of EG and EPMG were assayed by the viscosity method (Adriano-Anaya et al., 2006) using CMC and citrus pectin as substrates, with some modifications. The reduction in viscosity was determined at 0-30 min intervals. Approximately 0.5ml of the reaction mixture was sucked from a 2ml tube into a 1ml syringe, and then allowed to flow down to the 2ml tube. The time taken for the meniscus to flow down from the 0.60 to 0.10ml mark (between 1 and 3 min) was recorded. The reaction mixture in the 2ml tube contained 1ml of 0.5% substrate in 50mM citrate-phosphate buffer (pH 5) and 0.2ml supernatant. Viscosity reduction was determined at 37°C. One unit of enzyme activity was expressed as the specific activity, U (where U=RA mg^{-1} protein), and RA is the relative activity calculated by applying the following formula (Bateman, 1963):

$$\%\ V = (T_0 - T_A) \times 100 \times T_0\ ;\ T_{50} = 50 T_A\ \%\ V^{-1};$$

$$RA = T_{50} \times 10^3\ \text{RA}$$

Where, RA = the reciprocal of time in hour for 50% viscosity loss, T_0 = the viscosity of the reaction mixture at 0 time, T_A = the viscosity of the reaction mixture at 30 min, V = the viscosity loss of the reaction mixture at 30 min, and T_{50} = the time necessary to reach a 50% viscosity loss of the reaction mixture at 30 min. Controls for the enzyme assays consist of autoclaved extracts of the enzymes.

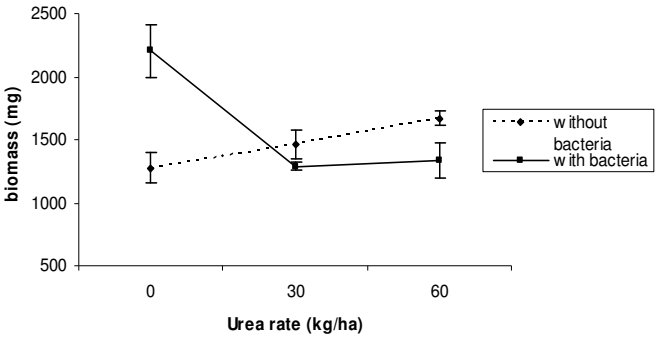

Fig 5. Effect of N_2-fixing bacteria and urea on plant growth. Bars in lines indicate standard error of means (n = 5).

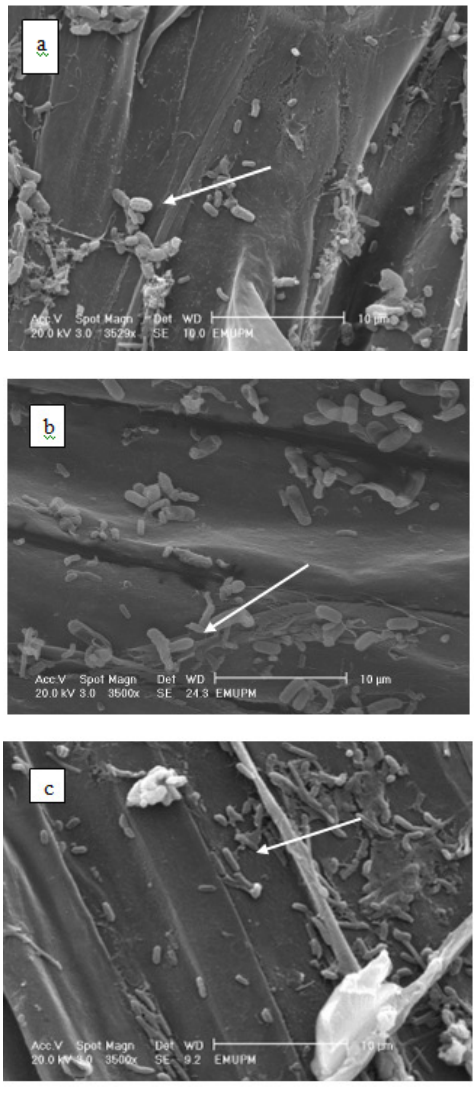

Fig 6 a, b, c. Bacterial colonization on the roots surface. White arrows show the bacterial cells.

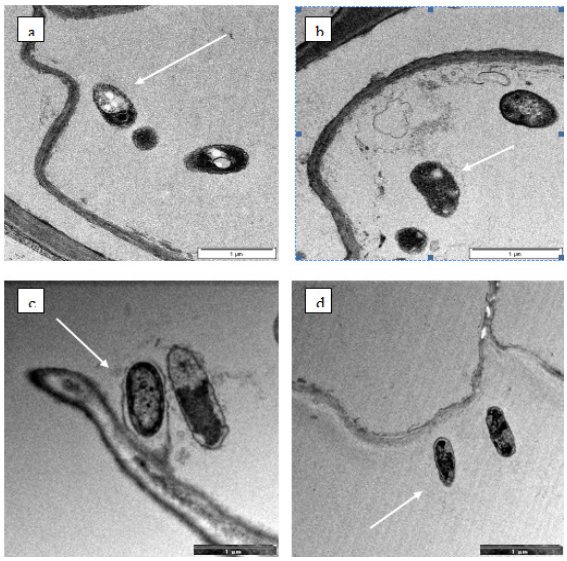

Fig 7 : a, b, c, d. Bacterial colonization of interior roots. White arrows show bacterial cells.

Determination of plant-tissue nitrogen concentration and uptake

Plant-tissue nitrogen concentration was determined according to the semi-micro Kjeldahl method (Bremner, 1996) and tissue nitrogen uptake was calculated.

Determination of root morphology, photosynthetic activity and plant biomass

At each sampling date, root length, root volume and root surface area was observed by using the root scanner model Win Rhizo STD1600 WIA - EPSON EXPRESSION 1680. Single-leaf net photosynthesis rate (A_{max}) was determined (45 days after transplanting) from a fully expanded leaf of each treatment by using LT-6400XT portable photosynthesis system, LI-COR Inc. Lincoln, Nebraska, USA. Leaf chlorophyll content was determined using a portable chlorophyll meter (MINOLTA^{TM} SPAD-502). Biomass production was determined at harvest (45 days after transplanting) by washing the roots and dried in oven at 70°C for three days until a constant weight was achieved.

Study of root colonisation using SEM and TEM

Seven day old inoculated seedlings were selected for the SEM study. Roots were cut into 1cm^3 sections, pre-fixed with 4% glutaraldehyde, and washed with 0.1M sodium cacodylate buffer. For post-fixation, the samples were fixed in 1% osmium tetroxide. The samples were dehydrated in series of acetone (30, 60 and 90%) and then dried in a critical point dryer, mounted onto the stub, coated with gold and observed under the SEM (Philips XL 30 ESEM). Samples for TEM analysis of the inoculated roots were cut into 1mm^3 sections, pre-fixed with 4% glutaraldehyde, and washed with 0.1M sodium cacodylate buffer. Post-fixation, the samples were fixed in 1% osmium tetroxide. The samples were then dehydrated in series of acetone (30, 60 and 90%) and then infiltrated with resin, embedded into beam capsules, polymerised in the oven, cut into thick and ultra-thin sections and stained. They were observed under the TEM (Leo 912AB EFTEM).

Statistical analysis

The experiment was laid out in a factorial completely randomised design with 7 replications. The data were analysed using SAS (9.1 version) and treatments means were compared using Tukey's test ($P<0.05$).

Conclusion

The locally isolated strain *Bacillus* sp. Sb42 that colonised on the surface and interior of rice roots improves plant growth and contributes in the nitrogen fixation process without the addition of nitrogen fertiliser. The association of these bacteria with the plants significantly increased the production of hydrolytic enzymes in the root system, which is an important mechanism for endophytic colonisation and the subsequent nitrogen fixation. The capability of *Bacillus* sp. Sb42 for nitrogen fixation and hydrolytic enzyme production was affected by the excessive amount of nitrogen fertiliser application (30-60kg ha^{-1}).

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