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Effects of Free-Living Diazotrophs on Plant Growth and Root Colonisation of Pak Choi

A.M. Asilah

Department of Agricultural Science, Faculty of Technical and Vocational, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia

ABSTRACT

An experiment was conducted to determine the effects of free-living diazotrophs on the growth and root colonisation of pak choi. Free-living diazotrophs were isolated from soil samples obtained from the selected areas of the university campus. The isolation method used Jensen's liquid (N-free medium) and a spectrophotometer to determine diazotrophic bacterial growth and check the level of turbidity, respectively. Bacteria that grew in the N-free liquid medium were assumed to be diazotrophs. Three diazotrophic strains were selected from the isolation in the N-free liquid medium for a pot experiment. Growth of the strains in the Jensen's liquid medium was an indicator for selection of strains in this study. The level of turbidity was compared with that of the control. Results showed that shoot biomass was significantly affected by the diazotrophs. Plants inoculated with Bacterium 3 produced significantly larger shoots ($F_{2.35} = 2.10$, $P < 0.001$) than those grown with Bacterium 1, 2 and 0 (uninoculated). However, the fresh weight of root and root colonisation analyses were unaffected by treatment. The plants did not grow well and were stressed for most of the experiment. The combined effects of autoclaving and nutrient limitation possibly adversely affected plant health. Nevertheless, plants grew sufficiently well for the experiment to be continued and for testing the efficacy of the bacteria.

Key words: Pak choi, diazotrophs, N-free liquid medium.

INTRODUCTION

The dramatic increase in the level of global warming and climate change since the last century has led to a huge strain on the environment (Alburquerque *et al.* 2013). The agriculture sector contributes a large amount of greenhouse gases which have an effect on climatic change (Montzka *et al*. 2011; Vermeulen *et al.* 2012; Pittelkow *et al.* 2014). At times, agricultural practices may have harmful effects on the environment such as the process of anthropogenic emissions of nitrous oxide (N_2O) and methane (CH_4) (Pittelkow *et al*. 2013). Attempts are been made to meet world demand for food by raising food production in a sustainable manner. Fertiliser usage plays a vital role in protecting food security and reducing environmental impacts. Therefore, several methods must be adopted to increase crop production whilst keeping environmental impact low. There is a

*Corresponding author : E-mail: *asilah@ftv.upsi.edu.my*

potential approach to implement the method which is the utilisation of beneficial microorganisms to reduce usage of chemical fertilisers. Therefore, in this study, the effects of plant growth-promoting bacteria (PGPB) such as diazotrophs, on growth and root colonisation on pak choi were studied. Diazotrophs are beneficial microorganisms that play a crucial role in sustainable agriculture. Examples of bacteria under the PGPB category are nitrogen fixers and phosphate solubilisers. These bacteria have beneficial effects on growth enhancement of plants and eliminate their dependence on fertilisers (Prakamhang *et al*. 2009; Weyens *et al.* 2009; Yang *et al.* 2009). Phosphate solubilisation and nitrogen fixation are processes which can increase N and P uptake by PGPB such as nitrogen fixers and phosphate solubilisers (Çakmakçi *et al*. 2006; Pineda *et al*. 2010). Root colonisation is a considerable factor in successful interactions between PGPB and plants that lead to beneficial responses in plant growth enhancement (Bashan 1986). Exceptional colonisation may lead to reduced use of chemical fertilisers for plant production (Bloemberg and Lugtenberg 2001; Çakmakçi *et al*. 2006). Therefore, this study aims to isolate free-living diazotrophs from various sources and determine their root colonisation rates and effects on plant growth.

MATERIALS AND METHODS

A pot experiment was conducted to quantify the effects of three bacterial isolates which are potential nitrogen fixers (based on their ability to grow in an N-free medium) on pak choi. Prior to the experiment, bacteria were isolated from soil samples as described in the method below.

Procedure of Soil Sampling for Diazotrophic Isolation

Soil samples were collected from under pine trees, field edge, grass and nettle areas of the arboretum in the Sutton Bonington Campus of the University of Nottingham. The samples were randomly collected and taken to the laboratory for isolation and estimation of PGPB populations.

Diazotrophic Isolation from Soil

One gram (fresh weight) of soil sample was placed in a sterile universal tube. A series of 10-fold dilutions was conducted for each collected soil sample and replicated three times. The universal tubes containing soil samples were shaken, and 0.1 mL aliquot was plated onto a spread plate containing Jensen's agar to determine diazotrophic bacterial growth. Jensen's agar is nominally N-free. Jensen's agar medium comprised 20 g sucrose, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g NaCl, 0.1 g $FeSO_4$, 0.005 g Na_2MoO_4 and 20 g agar in 1 L. Bacterial colonies that grew were isolated into single species cultures and streaked onto the Jensen's medium. Given that the agar present in the medium is likely to contain nitrogen, each bacterium was inoculated into a 50 mL Falcon tube containing a Jensen's liquid medium (i.e. the agar was absent). All the tubes were shaken for three days at room temperature prior to determining the turbidity level by using spectrophotometer (500 nm wavelength). All the samples were compared with

Jensen's liquid medium (without bacteria) as a medium of control. Bacteria that showed growth in the N-free liquid medium were assumed to be N-fixers and were maintained on Jensen's agar medium.

Growth Medium Preparation and Seed Germination

Sand was washed a few times by using deionised water. The washed sand was air-dried and mixed with top soil (3:1). Subsequently, the mixture of sand and soil was sterilised using an autoclave (121°C for 20 min on two consecutive days) and placed in pots (120 g mixture of soil and sand per pot). Five pak choi seeds were sown in each pot. The plant pots were watered daily with sterilised deionised water until the first harvest. Approximately 20 mL of Hoagland N-free nutrient solution was applied on alternate days to the remaining plant pots. After two weeks, the Hoagland solution was changed to low nutrient fertiliser NPK compound. Approximately 30 mL of the nutrient fertiliser was applied daily to the plant pots. The pots were located in a growth room with 12 h light-dark cycle with daytime and night-time temperatures of 20°C and 18°C, respectively. After seed germination, the plants were thinned to one plant per pot. The plants were also left to recover for two days before inoculating bacteria. This experiment of four treatments (three bacterial isolates and one control) × three sampling times with four replications was established in factorial randomised complete block design.

Preparation and Application of Inocula

Three diazotrophic strains isolated from N-free liquid medium were selected for this experiment. Three sterile falcon tubes were used by filling each with 35 mL sterile deionised water. For each bacterium, a sterile loop was used to scrap cells from the surface of the agar plates. The sterilised loop of bacteria was added to the falcon tubes containing sterilised deionised water. This method was continued until the water was cloudy with the cells. Each plant was inoculated with approximately 2×10^2 cfu/mL of live bacterial cells. The control pots (non-inoculated treated) were applied with the same amount of sterilised deionised water (2 mL). The number of cells applied to each plant for each bacterium was determined using a dilution series and plating onto Jensen's agar medium. A spectrophotometer (500 nm wavelength) was also used to determine the diazotrophic population by measuring the level of turbidity.

Procedure for Sampling Plants

Plants were harvested on three occasions, namely, 13, 34 and 47 days, after inoculating with the bacteria.

Determination of Shoot Biomass

Shoots were cut with a scalpel just above the soil line, and each shoot was put into an individual envelope. Each envelope was labelled according to the treatments. All these envelopes were dried in an oven at 57°C to 60°C for three days until constant weight was achieved.

Determination of Rhizosphere Population

The root system was removed from the soil and shaken to remove the soil. Soil remaining on the roots after shaking was considered as the rhizosphere fraction. The entire root system was then placed into 10 mL of sterilised deionised water in a sterile tube (universal tube). Subsequently, the tube was vortexed for 10 s to remove the root system. The roots were then placed into a clean and dry universal tube which was kept to one side in the fridge. The 'rhizosphere solution' was serially diluted to a 10^{-2} dilution. Approximately, 0.1 mL of each dilution was plated onto a plate with Jensen's medium, and the plates were incubated at 20°C.

Determination of Endosphere Population

The root system was taken from the fridge (which was maintained after washing to remove the rhizosphere soil) and rinsed three times thoroughly in sterilised deionised water, to remove any residual soil particles. The roots were then surface sterilised in 6% sodium hypochlorite for 20 min. The roots were dipped into 70% (v/v) for 30 sec (the method generally used for secondary surface sterilisation). The roots were then rinsed in sterile deionised water twice and macerated separately in universal tubes in 9 mL of sterilised deionised water. Lastly, approximately 0.1 mL of the solution (which contained root fragments) was placed on plates containing Jensen's medium and were incubated at 20°C.

RESULTS

Root Fresh Weight

Root fresh weight was unaffected by treatment, but significantly large roots were found at harvest 3 ($F_{1,23} = 0.17$, $P < 0.001$) (Figure 1). Therefore, expressing the data by using root weight as a covariate is important when observing the bacteria (log CFU/mL).

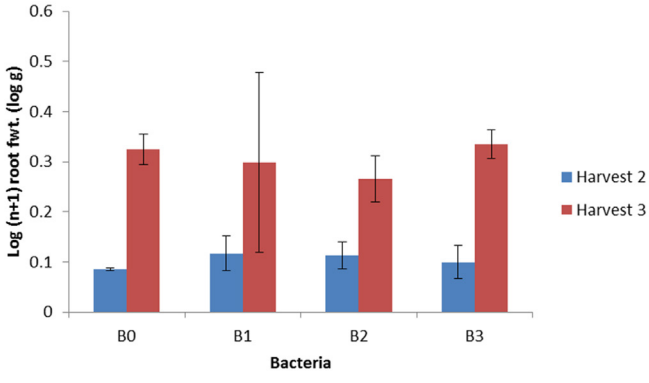

Figure 1. Effect of bacterial inoculation at different sampling times in inoculated and uninoculated plants on root fresh weight production.

Note: The data were log-transformed to satisfy the requirements for ANOVA (the distribution should be normal). Data are mean ± standard error (n = 4).

Shoot Biomass

Shoot biomass was significantly affected by bacteria ($F_{3.35} = 4.37$, $P = 0.01$) (Figure 2). Furthermore, plants inoculated with Bacterium 3 produced significantly larger shoots ($F_{2.35} = 2.10$, $P < 0.001$) than those grown with Bacterium 1, 2 or 0 (uninoculated).

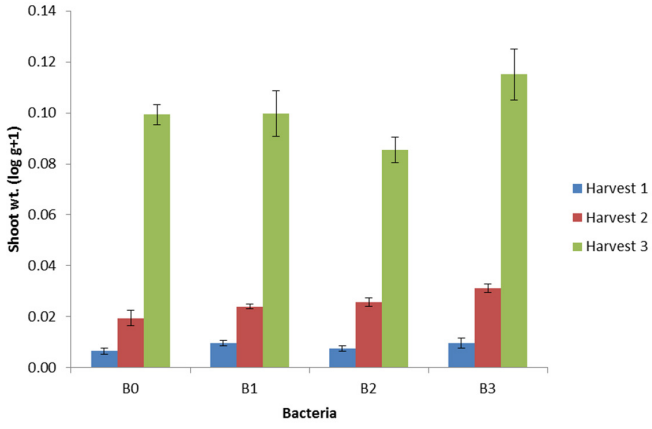

Figure 2. Effect of bacterial inoculation at different sampling times in inoculated and uninoculated plants on shoot biomass production.

Note: The data were log-transformed to satisfy the requirements for ANOVA (the distribution should be normal). Data are mean ± standard error (n = 4).

Rhizosphere Population

The rhizosphere population (log CFU g^{-1} root) was unaffected by treatment (Figure 3), but fewer bacteria were observed at harvest 3 than that at harvest 2 ($F_{1.23} = 0.01$, $P = 0.002$). When data were expressed on per mL of extraction liquid basis, the rhizosphere population also remained unaffected by treatment ($F_{3.22} = 2.49$, $P = 0.087$). However, the P value might indicate a trend. The CFU in the rhizosphere, whether expressed as CFU/mL or as per gram, was insignificant. Root weight was used as a covariate in the analysis of these data to account for the different root sizes associated with each plant. Expressing the data on a per weight basis did not account for any treatment-related changes in root architecture (e.g. altered length, root hair or branching), which may have affected bacterial colonisation but not necessarily root biomass. Therefore, expressing the data on the basis of the amount of extraction fluid was arguably appropriate, particularly because root weight was used as a covariate. The difference highlights the importance of expressing the data correctly. Future studies will quantify root architectural changes, particularly root length. Furthermore, understanding which portion of the root is colonised by the N_2 fixers is important.

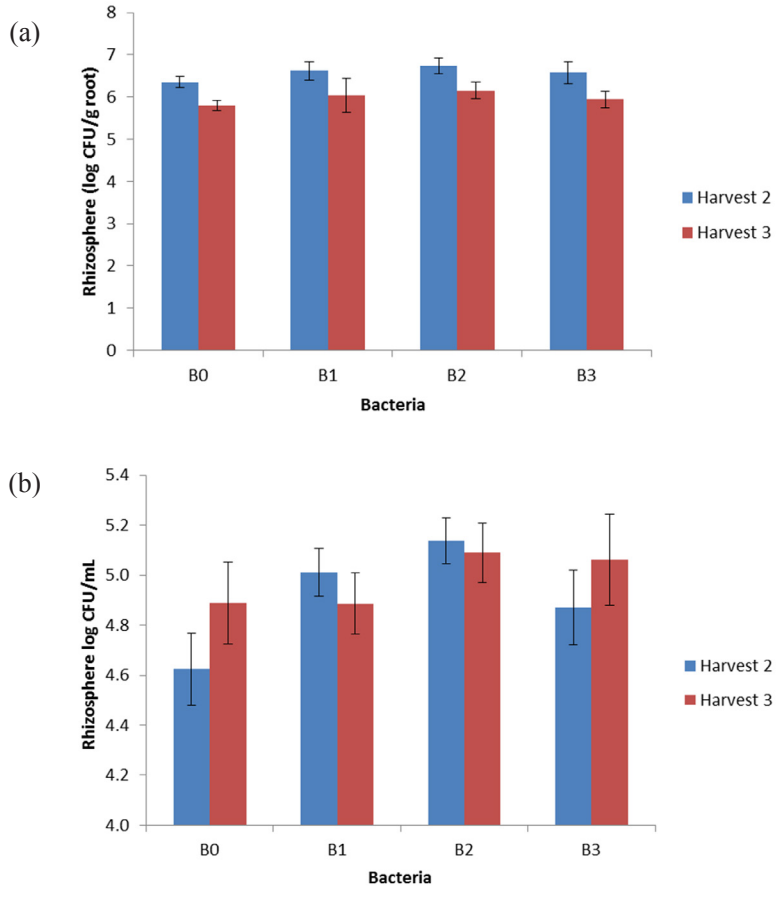

Figure 3. Changes in number of rhizosphere bacteria at different sampling times in inoculated and uninoculated plants, expressed on a per unit weight of root basis (a) and per mL of solution used for root preparation (b).

Note: The data were log-transformed to satisfy the requirements for ANOVA (the distribution should be normal). Data are means ± standard error (n = 4).

Endosphere Population

The endosphere population (log CFU g^{-1} root) was unaffected by treatment (Figure 4), but significantly fewer bacteria were observed at harvest 3 than that at harvest 2 ($F_{1.23}$ = 1.07, P < 0.001). In contrast, when data were expressed on per mL of extraction liquid basis, a large number of cells were isolated from roots inoculated with bacterium B3 and few from the uninoculated control roots (Treatment as a single factor, $F_{3.22}$ = 22.92, P < 0.001). A similar number of bacteria were isolated from all treatments that received bacterial inoculum at harvest 2 and 3, but numbers extracted from the uninoculated roots were lower at harvest 3 compared with those at harvest 2 ($F_{3.22}$ = 3.14, P = 0.046).

Root weight was used as a covariate in the analysis of these data to account for the different root sizes associated with each plant. Expressing the data on a per weight basis did not account for any treatment-related changes in root architecture (e.g. altered length, root hair or branching), which may have affected bacterial colonisation but not necessarily root biomass.

Therefore, expressing the data on the basis of the amount of extraction fluid is arguably appropriate, particularly because root weight was used as a covariate. The difference highlights the importance of expressing the data correctly. Future studies will quantify root architectural changes, particularly root length. Furthermore, understanding which portion of the root is colonised by the endophytes is important.

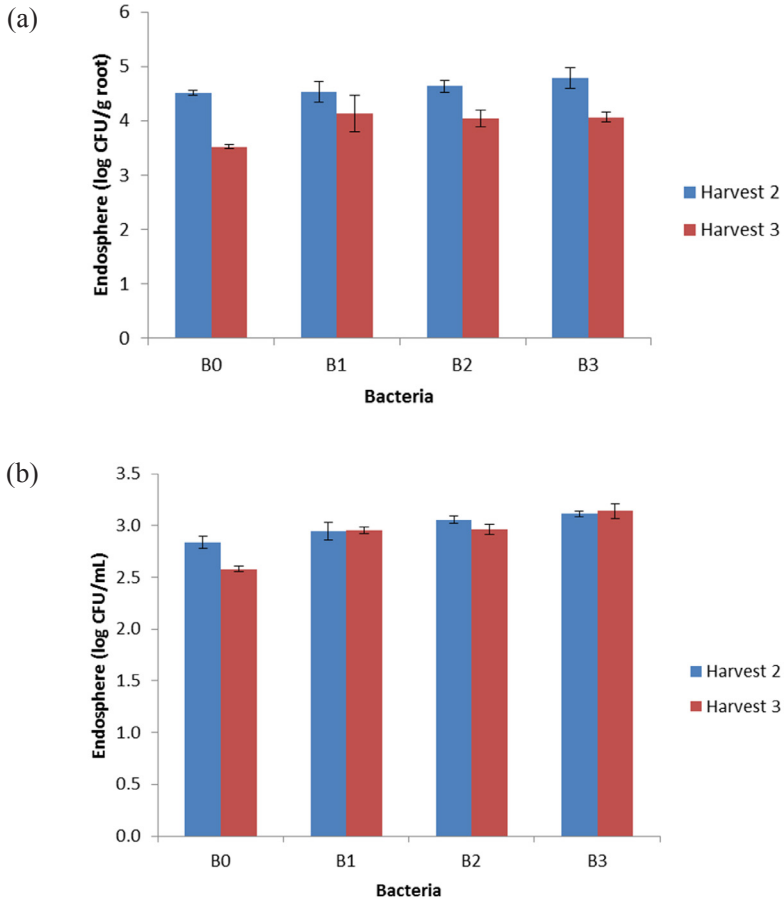

Figure 4. Changes in number of endosphere bacteria at different sampling times in inoculated and uninoculated plants expressed on a per unit weight of root basis (a) and per mL of solution used for root preparation (b).

Note: The data were log-transformed to satisfy the requirements for ANOVA (the distribution should be normal). Data are mean ± standard error (n = 4).

DISCUSSION

Diazotrophs were isolated from the rhizosphere and endosphere of all plant roots regardless of the treatment. Previous literature also proved that several species of PGPB can colonise the rhizosphere of plant and simultaneously grow endophytically (Darbyshire and Greaves 1973; Quadt-Hallman *et al*. 1997; Sturz and Nowak 2000). These bacteria are facultative endophytes and include genera such as *Azospirillum*. The free-living nitrogen-fixing bacteria or diazotrophs, including the *Azotobacter* spp., are usually associated with the rhizosphere or the rhizoplane where they generally live epiphytically. Obligate endophytes are only found within the plant and are not free-living. Endophytes can access plant roots, either through active or passive mechanisms and colonise the host tissue without damaging the host, internally (Hallman *et al.* 1997; Reinhold-Hurek and Hurek 1998; Hardoim *et al*. 2008). Previous literature suggests that this kind of bacteria can be isolated from surface-disinfected plant tissues or that some can be extracted from internal plant tissues. Besides, the aforementioned bacteria are capable of invading inner tissues such as xylem vessels and spread systemically (James and Olivares 1998).

Fewer bacteria were isolated from the control treatments, but their presence in the uninoculated soil reflected the limited effectiveness of the autoclaving procedure. However, another possible effective sterilisation method, such as gamma (γ) irradiation, should be used after autoclaving to confirm the absence of microorganisms in the uninoculated soil. Previous researchers also showed that sterilisation procedures used for soil were autoclaved (121°C, 1 h) or underwent gamma (γ) irradiation (50 kGy) (Mahmood *et al*. 2014). The plants did not grow well and were stressed for most of the experiment.

Consequently, nitrogen was given to the plants after the first harvest, but their continued lack of growth may be due to the autoclaving process which has previously been reported to release toxic compounds. The combined effects of autoclaving and nutrient limitation may have adversely affected plant health. Nevertheless, plants grew sufficiently well for the experiment to be continued and for testing the efficacy of the bacteria.

Endosphere population results showed that plants were unaffected by treatment. However, numerous existing works showed that endophytes benefit plants. Most endophytic microbes offer benefits to the plants (Compant *et al*. 2005; Sessitsch *et al*. 2005; Sun *et al*. 2009; Rashid *et al.* 2012). Moreover, bacterial endophytes could colonise the plant interior and enhance plant growth through different mechanisms (Glick 1995; Hallman *et al.* 1997; Reiter and Sessitsch 2006; Rashid *et al.* 2012). The mechanisms involved include nitrogen fixation (Compant *et al.* 2005), induced systemic resistance (ISR) (Ait Barka *et al*. 2002), antibiotic production (Ezra *et al*. 2004) and phytohormone production (Lee *et al.* 2004). In addition, these mechanisms also increase phosphate solubilisation (Wakelin *et al*. 2004) and nutrient availability (Puente *et al*. 2009), in the case of facultative endophytes. Previous researchers also showed that plant-growth promoting rhizobacteria, such as diazotrophs, are important in plant growth

enhancement through one or more mechanisms, including enhancement of nutrient uptake, direct stimulation of plant growth, elimination of plant pathogens and/or induction of pathogen resistance in plant hosts (Tailor and Joshi 2014). However, in this study, diazotrophic population was reduced towards the end of the experiment. This phenomenon could be due to diazotrophs not maintaining their viability over time.

CONCLUSION

Results of this study showed that root colonisation of these diazotrophs on the surface (rhizosphere) and interior (endosphere) of pak choi roots significantly affected plant growth enhancement, particularly shoot biomass. However, the fresh weight of root and root colonisation analyses were unaffected by treatment. Future research should consider the potential effects of the diazotrophs on other analyses, such as plant biomass, enzyme extraction, hormone determination and bacterial identification. Therefore, the plant roots must be reinoculated to increase population, biological activity and diazotroph viability.

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