Potential use of *Rhizobium* spp. to improve growth of non-nitrogen fixing plants

Hossain, Md. Shakhawat

Supervisor: Anna Mårtensson

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Department of Soil Sciences

P.O. Box 7014

SE-750 07 Uppsala

SWEDEN

http://www.mv.slu.se

ABSTRACT

Plant growth promoting rhizo-bacteria (PGPR) affect plant growth by producing and releasing secondary metabolites (plant growth regulators/phytohormones/biologically active substances), facilitating the availability and uptake of certain nutrients from the root environment and inhibiting plant pathogenic organisms in the rhizosphere. At the same time, plants produce root exudates containing e.g. sugars, amino acids, organic acids, vitamins, enzymes and organic or inorganic ions. Those substances in turn influence the rhizosphere microflora and also the behaviour of PGPR. In this work, I examined the potential use of legume bacteria, rhizobia as PGPRs since it has been shown that rhizobia (legume bacteria) can function as PGPR in non-nitrogen fixing plants.

In the present study, the interactions of nine different rhizobial strains with six different mixed non-nitrogen fixing plant species were examined in laboratory and greenhouse experiments. Mixed botanical plant's seeds were inoculated with same concentration of different rhizobial strains. Significantly increased plant biomasses indicate that rhizobia have naturally potential ability to promote the growth of non-nitrogen fixing plant. The concentration level of rhizobial inoculation is another important factor for seed germination and plant growth. In addition, linseed was inoculated with only one rhizobial strain of different concentrations. To complement experiments were conducted, one was for rhizobial growth and the other one was for interactions between rhizobia and pathogenic fungi. None of the strains tested prevented *in vitro* fungal growth towards bacterial colonies but after some days of contact between rhizobia and fungi, some strains showed a tendency to dissolve the fungal mycelium. The results showed that *Sinorhizobium meliloti* strains were the most effective and could be suggested to act as PGPR. The inoculation concentration of the rhizobial strain was crucial. A concentration of 10^4 cfu mL^{-1} of *Sinorhizobium meliloti* proved to be optimal for successful seed germination and growth of linseed.

CONTENTS

1. INTRODUCTION

1.1 Use of plant growth promoting rhizo-bacteria

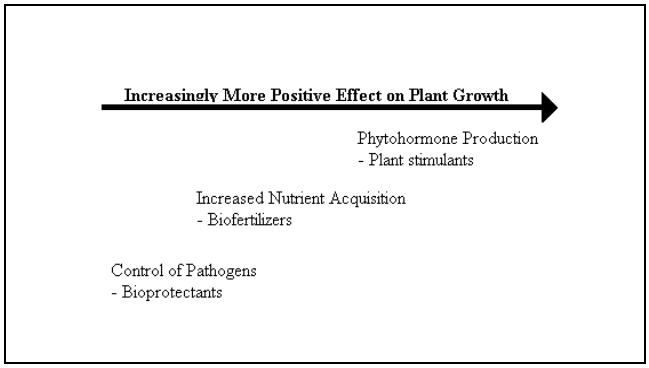
Soil-borne pathogens are well known for their devastating effects on plant health and yield. For successful disease management, it is important to find the most effective and economical ways to protect the plant from various pests or diseases. In recent years, the use of PGPR as inducers of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Wei *et al*., 1996). The use of natural PGPR strains in plant frontline defence may offer a practical way to deliver immunisation. PGPR have been reported to increase plant resistance to fungal, bacterial and viral diseases (Maurhofer *et al*., 1998), insects (Zehnder *et al*., 1997) and nematodes (Sikora, 1992). Mode of action studies have revealed that biological control by PGPR involves production of bacterial metabolites that reduce the population or activities of pathogens or deleterious rhizosphere microflora (Glick, 1995; Kloepper, 1996). These metabolites may include siderophores that bind Fe, making it less available to certain members of the native pathogenic microflora (Berthelin *et al*., 1991; Subba Rao, 1993).

1.2 Features of plant growth promoting rhizo-bacteria

A number of authors have reported that inoculation with plant growth promoting rhizo-bacteria (PGPR) can result in increased germination and seedling emergence and modify growth and yield of various cereal and non-cereal crops (Freitas & Germida, 1992; Chen *et al*., 1994; Javed & Arshad, 1997; Biswas *et al*., 2000; Dobbelaere *et al*., 2001; Matiru and Dakora, 2004; Wang *et al*., 2007). The growth stimulation in plants by PGPR can be a direct effect of production of secondary metabolites such as auxins, IAA, cytokinins, riboflavin and vitamins (Dakora, 2003). These stimulate growth of plant organs via cell division and expansion (Campanoni *et al*., 2003) or by improving nutrient availability (Glick, 1995; Chabot *et al*., 1996; Yanni *et al*., 1997). They also release organic acids, which help to make available forms of nutrients (Biswas *et al*., 2000) and often lead to increased plant growth through uptake of water and mineral nutrients or indirect when the rhizobia inhibits pathogens or deleterious microorganisms by producing siderophores,

HCN (Vidhayasekaran and Muthamilan, 1999; Wei *et al*., 1996) and antibiotics (Glick, 1995) in the rhizosphere (Fig 1).

Figure 1. Spectrum of mechanisms of plant growth promotion by PGPR.



1.3 Antibiotic production

Antibiotic production is one of the most intensively studied aspects of biocontrol, but in many cases it is difficult to distinguish between antibiosis and competition. Several studies have demonstrated that production of antibiotics (*e.g*. pyrrolnitrin, phycocyanin, 2,4-diacetylphloroglucinol) by microbial inocula can cause suppression of pathogens (Subba Rao, 1993; Glick, 1995). Glick (1995) was of the view that the most effective mechanism that a PGPR can employ to prevent proliferation of phytopathogens is the synthesis of antibiotics.

1.4 Siderophore production

Siderophores play an important role in the biocontrol of some soil-borne plant diseases and in plant iron nutrition (Loper and Buer, 1991). Siderophores are low molecular weight, high affinity iron (III) chelators that transport iron into bacterial cells (Leong, 1986). These systems are composed of ferric-specific ligands (siderophores) and their cognate membrane receptors as chelating agents in bacteria (Neilands, 1989). Subsequently, siderophores have been shown to be involved in the suppression of *Fusarium oxysporum* (Baker *et al*., 1986). Because siderophores sequester the limited

supply of iron (III) in the rhizosphere, they limit its availability to pathogens and ultimately suppress their growth (Schroth *et al*., 1984). There are two strategies for acquiring iron (Römheld, 1987). Strategy I is characterised by an increase in the activity of a NADPH-dependent 'reductase' and an increase in H^+ release. Strategy II is characterised by enhanced release of phytosiderophores and by a highly specific uptake system for Fe (III) phytosiderophores. Both activities are thought to enhance the solubilisation of Fe (III).

1.5 Phytohormone production

Plant growth hormones are organic compounds that influence the physiological processes in plants at extremely low concentrations. Production of phytohormones by inocula has been suggested as one of the most plausible mechanisms of action affecting plant growth. There are five classes of well-known phytohormones, namely auxins, IAA, cytokinins, ethylene and abscisic acid. Soil microbiotas, particularly the rhizosphere microflora, are potential sources of these phytohormones (Frankenberger & Arshad, 1995; Costacurta & Vanderleyden, 1995; Patten & Glick, 1996; Arshad & Frankenberger, 1998). Plant growth regulators help to solublise nutrients so that they can easily be taken up by plant via activate the roots and stimulate cell division of root tissues. Solubilisation of nutrients such as phosphorus and iron by rhizobia makes them more readily available for plant uptake, as demonstrated by Belimov *et al*. (1995), Noel *et al*. (1996), Glick *et al*. (1998) and Biswas *et al*. (2000). They suggested that production of organic acids was the major mechanism of action by which insoluble phosphorus compounds were converted to more soluble forms. Other scientists report that rhizobia can create an acidic environment to promote mineral nutrient solubilisation (Alexander, 1977). The rhizobia influence crop growth and development by changing the physiological status (Glick & Bashan, 1997) and morphological characteristics of inoculated roots (Noel *et al*., 1996; Yanni *et al*., 1997), which favours improved nutrient uptake (Okon & Kapulnik, 1986). The ability of rhizobia to solubilise both inorganic and organic phosphate has been the subject of many investigations (Abd-Alla, 1994; Martin *et al*., 2002).

1.6 Other potential mechanisms

Other mechanisms for biological control of disease may include competition for infection sites and nutrients, parasitism on pathogens, *i.e*. destruction of fungal pathogens by the action of lytic enzymes (*e.g*. chitinase and β-1, 3-glucanase) that degrade fungal cell walls, and uncharacterised antifungal factors (Fridlender *et al*., 1993; Kloepper, 1996; Velazhahan *et al*., 1999). Buchenauer (1998) reported various mechanisms for biological control such as competition for space and nutrients in the rhizosphere and spermosphere, lytic enzymes, HCN and many other metabolites produced by rhizobia. A consortium of PGPR may often have more influence on biological control and plant growth than a single strain (Krishnamurthy & Gnanamanickam, 1998; Bapat & Shah, 2000). However, in some cases, mixtures of different strains had no synergistic effect. Recent work on the broad spectrum of PGPR-mediated induced systemic resistance against different pathogens in different crops has gained importance (Ramamoorthy *et al*., 2001).

The potential effect and successful contribution of PGPR strains on plant growth is strongly influenced by environmental factors including soil characteristics, plant species and even plant genotypes within a species, and other microflora indigenous to the rhizosphere (Nowak, 1998). Sub-optimal or unfavourable conditions may lead to little or no synthesis of biologically active substances in the root zone, resulting in the failure of PGPR to promote plant growth (Chanway & Holl, 1992). This hampers the practical use of PGPR, since effects are unpredictable due to varying environmental conditions.

1.7 Characteristics of rhizobia

Rhizobia (the fast-growing *Rhizobium* spp. and the slow-growing *Bradyrhizobium* spp.) or root nodule bacteria are medium-sized, rod-shaped cells, 0.5-0.9 μm in width and 1.2-3.0 μm in length. They do not form endospores, are Gram-negative, and are mobile by a single polar flagellum or two six peritrichous flagella. Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture. They grow well in the presence of oxygen and utilise relatively simple carbohydrates and amino compounds. Some strains of rhizobia require vitamins for growth. Rhizobia are likely to lose viability rapidly in water.

Optimal growth of most strains occurs at a temperature range of 25-30 ºC and a pH of 6.0-7.0 but despite their usual aerobic metabolism, many strains are able to grow well under microaerophilic conditions at oxygen tensions of less than 0.01 atm. Generally, most rhizobia produce white colonies. Fast-growing rhizobia produce an acid reaction in yeast mannitol medium containing bromthymol blue (pH 6.8) while slow growers produce an alkaline reaction.

1.8 Rhizobia as natural endophytes of legume plants

Rhizobia form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as signals by the legume host. These plant compounds induce the expression of nodulation (*nod*) genes in rhizobia, which in turn produce lipo-chito-oligosaccharide (LCO) signals that trigger mitotic cell division in roots, leading to nodule formation (Dakora 1995; Lhuissier *et al*., 2001).

1.9 Rhizobia as plant growth promoting rhizo-bacteria of non-legumes

During legume-cereal rotations and/or mixed intercropping, rhizobia are exposed to non-leguminous plants. A number of studies have shown that rhizobia naturally infect roots of rice (Yanni *et al*., 1997), wheat (Biederbeck *et al*., 2000) and oilseed rape (Lupwayi *et al*., 2000). Höflich *et al.* (1994) obtained significant shoot dry matter yield increases (7-8%) by inoculating maize, spring wheat and spring barley (*Hordeum vulgare* L.) with strain R39 of *R. leguminosarum* bv. *trifolii* in field experiments. Yanni *et al.* (1995) observed that certain effective wild-type strains of *R. leguminosarum* bv. *trifolii* are able to establish natural plant-bacterial associations that have the potential to promote growth of rice plants under both field and laboratory conditions, while Chabot *et al*. (1996) reported that field inoculation of maize and lettuce significantly increases shoot dry matter yield. Galleguillos *et al*. (2000) showed that the interaction between genetically modified (GM) *Sinorhizobium meliloti* and *G. mosseae* (AM) produced the highest growth effect (476% above the control) in *Lactuca sativa L.* (Table 1). A series of laboratory experiments conducted by Zahir *et al*. (2004) on two wheat cultivars under gnotobiotic (axenic) conditions demonstrated increases in root elongation (up to 17.3%), root dry weight (up to

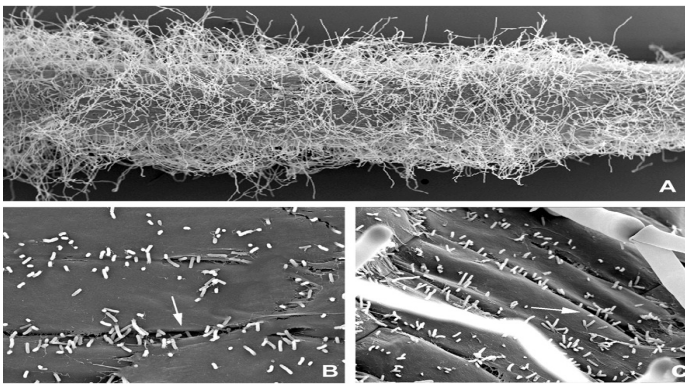
13.5%), shoot elongation (up to 37.7%) and shoot dry weight (up to 36.3%) of inoculated wheat seedlings. In addition, inoculation of PGPRs (*Bacillus licheniformis* CECT 5106 and *Bacillus pumilus* CECT 5105) in forestry nurseries has proved to be crucial in enhancing the survival of young tree seedlings when transplanted to the field. Inoculated seedlings with a more developed root system achieve better nutrition and survival after transplanting (Probanza *et al*., 2001). Various workers have emphasised that plant growth promotion by rhizobia in the field is likely to require the coordination of a cascade of events affecting plant growth promoting activity by a variety of different mechanisms (Bayliss *et al*., 1993; Frankenberger & Arshad, 1995; Glick, 1995; Arshad & Frankenberger, 1998). However, the precise mechanism by which rhizobial strains stimulates plant growth need to be known in order to optimise each mechanism.

Table 1. Summary of available data on the response of different corps to PGPR inoculation

Crop	PGPR species	Parameter	% increase over non-inoculated control	Reference
Brassica campestris	*Azotobacter*	Yield	34.4	Lifshitz *et al*. (1987)
Brassica napus	*Pseudomonas brassicacearum*	Shoot weight	21.2	Belimov *et al*. (2001)
Helianthus annuus L.	*Rhizobium* sp. Strain YAS34	Shoot dry weight	50.0	Alami *et al*. (2000)
Lactuca sativa	*Sinorhizobium meliloti* + AM	Biomass	476.0	Galleguillos *et al*. (2000)
Oryza sativa	*R. leguminosarum* bv *trifolii*	Shoot weight	30.0	Biswas *et al*. (2000)
Solanum tuberosum	*Azotobacter*	Yield	45.3	Zahir & Arshad (1996)
Triticum aestivum	*Azospirillum brassilense*	Plant dry weight	62.0 (no added N)	Dobbelaere *et al*. (2001)
Zea mays	*Pseudomonas*	Yield	18.9	Javed *et al*. (1998)
Zea mays	*Azospirillum irakense*	Plant dry weight	16.0	Dobbelaere *et al*. (2001)

In addition, a number of reports have demonstrated the ability of rhizobia to colonise roots of non nitrogen fixing plant and localise themselves internally in tissues, including the xylem (Spencer *et al*., 1994) (Figure 2). Chabot *et al*. (1996) showed that *Rhizobium leguminosarum* bv. *phaseoli* colonised roots of lettuce and maize plants. The co-application of *Azorhizobium caulinodans* and flavonoids such as naringenin and daidzein, even at very low concentrations ($5x10^{-5}$ M) significantly enhanced microsymbiont colonisation of roots and promoted localisation in the xylem of *A. thaliana* (Stone, 2001). The same flavonone was shown to enhance the colonisation of rice roots and internal localisation in xylem by *A. caulinodans* strain ORS571 (Gopalaswamy *et al*., 2000).

Figure 2. Scanning electron microscopy of Sakha 102 rice roots colonised by *Rhizobium leguminosarum* bv. *trifolii* strain E11. (*A*) Low-magnification micrograph showing a 4-mm rootlet segment with numerous root hairs and 'windows' between them providing the opportunity to view the rhizoplane surface. (*B*, *C*) Higher magnification micrographs showing the colonisation of the epidermal surface by the bacteria. Arrows point to localised sites where the bacteria have entered small crevices at junctions between epidermal cells. (Source: Indicate origin of figure as Name, YEAR).



1.10 Prerequisites for rhizobia to function as successful plant growth promoting bacteria

It is very important to have a good interaction between rhizobia and non-nitrogen fixing plants as otherwise the positive effect is not exploited. To act as PGPR with non-nitrogen fixing plants, rhizobia should be able to colonise and survive in the rhizosphere of these plants; the colonisation of roots by inoculated bacteria is an important step in the interaction between beneficial bacteria and the host plant. However, it is a complex phenomenon influenced by many biotic and abiotic parameters. The rhizobia may also have one or several of the characters attributed to PGPR, such as secondary metabolite production, siderophores, HCN (cyanide), antibiotic production etc (Table 2). Screening can be used identify strains with the maximum survival rate. The present study sought to identify positive relationships between some strains of *Rhizobium* and some species of agricultural importance through N, P, Mg and dry matter (DM) analyses.

Table 2. Reported effects of plant growth promoting rhizobia *in vitro*, data from Antoun H. *et al*. (1998).

Rhizobial species	No. of strains tested	Cyanogens[a] (%)	Sidero-phore producers[b] (%)	3-Indoleacetic acid (IAA) prod-ucers[c] (%)	P-solubilisers[d] (%)
Arctic rhizobia	47	0	49	96	2
B.japonicum	18	0	67	33	5
R.leguminosarum bv. *phaseoli*	30	13	93	50	67
R.leguminosarum bv. *trifolii*	22	9	86	45	4
R.leguminosarum bv. *viciae*	82	2	91	51	71
Sinorhizobium meliloti	62	0	95	56	84

[a]A change in colour from yellow to orange-brown of filter paper impregnated with 0.5% picric acid-2% sodium carbonate indicates the production of cyanide (Bakker & Schippers, 1987).

[b]Rhizobia forming an orange halo on chrome azurol S agar plates or growing on TSA (10%) agar plates containing 50 mg L^{-1} of 8-hydroxyquinoline are considered positive siderophore producers (Alexander & Zuberer 1991).

[c]IAA producing bacteria can be separated from organisms producing other indoles (yellow to yellow-brown pigment) by their characteristic pink to red colour produced after exposure to Salkowski reagent for 0.5-3 h (de Britto Alvarez, 1995).

[d]Rhizobial colonies forming clarification halos on dicalcium phosphate agar plates are considered phosphate solubilisers (Goldstein, 1986).

2. OBJECTIVES

The objective of the study was to identify the rhizobial strains with the greatest and widest range of capacity for use as a potential growth promoter for non-nitrogen fixing plants.

3. MATERIALS AND METHODS

3.1 Plant material

The seeds of various non-nitrogen fixing plants were obtained from the seed company Impecta AB, Julita, Sweden. Some data and characteristics of the species selected are presented in Table 3.

Table 3. Plant species used in the study

Common name	Scientific Name	Variety	Family	Germination rate (%)	Common uses
Chinese cabbage	*Brassica rapa subsp. pekinensis*	Narinosa	*Cruciferae*	70	Leaf vegetable
Lemongrass	*Cymbopogon flexuosus*		*Poaceae*	25-30	Vegetable and ornamental
Sunflower	*Helianthus annuus*	Pacino	*Compositae*	70	Oil, protein and ornamental
Linseed	*Linum usitatissimum*	Blue flowering	*Linaceae*	85	Oil, tannin and medicinal
Common poppy	*Papaver rhoeas*		*Papaveraceae*		Medicine and ornamental
Maize	*Zea mays*	Harlequin	*Gramineae*		Starch

All these plant species were selected randomly as non-nitrogen fixing plants. They are of different size, shape, structure, colour, taste, odour, growth rate, germination rate, germination time, lifecycle, ecology and occurrence in nature.

3.2 Rhizobial strains

Eight different rhizobial strains were obtained from Elomestari Ltd, Finland, and one strain (E-11) was obtained from Italy. A summary of the different strains is presented in Table 4.

Table 4. Description of the nine different rhizobacterial strains selected for use in the composite screening experiments

Expt. code	HAMBI code	Species	Other name	Source
E-11	-	*R.leguminosarum* bv.*trifolii*		Italy
PAR-804	1148	*Rhizobium loti*	*Lotus corniculatus*	St.Petersburg
PAR-803	1126	*Rhizobium loti*	*Lotus corniculatus*	
PAR-601	540	*Rhizobium galegae*	HAMBI	Finland
PAR-401	714	*R leguminosarum* bv.*viciae* 38	HAMBI	Finland
PAR-307	-	*R leguminosarum* bv.*viciae* 16HSa	MTT	
PAR-207	-	*Sinorhizobium meliloti* CXMI-105	Pushkin	St.Petersburg
PAR-201	-	*Sinorhizobium meliloti* Mel2	HAMBI	Finland
PAR-102	461	*R leguminosarum* bv.*trifolii* 503	173C HAMBI	Finland

Most of these strains have already been tested or examined regarding their potential for both leguminous and non nitrogen fixing plants. The important characteristics of the *Rhizobium* strains are described below.

3.3 Growth medium

Growth medium was obtained from the company Askania AB, Sweden. The medium is completely sterilised sandy soil and the commercial product name is ‘Silversand 55’. The particle size distribution is: 40% of particles are 0.35 mm, 30% 0.50 mm, 16% 0.25 mm,

10% 0.707 mm. The chemical composition is: 99.2% SiO_2, 0.09% Al_2O_3, 0.058% Fe_2O_3 and 0.1% loss on ignition.

3.4 Growth and development of rhizobia

Yeast Mannitol Broth (YMA) growth medium for rhizobia was prepared according to Somasegaran (1994). Briefly, 0.1 g NaCl, 0.5 g K_2HPO_4, 0.10 g $MgSO_4.7H_2O$, 10 g mannitol and 0.5 g yeast extract were added to 1 L distilled water in a 2-L flask, dissolved under continuous stirring and the pH adjusted to 6.8 with 0.1 N NaOH. Then 15 g malt agar were added and the mixture was shaken to create an even suspension. This was heated by microwave oven at 90°C until fully boiled and then 5 mL portions of malt YMA solution were transferred into each of 72 test-tubes, which were autoclaved at 121°C for 20 min. All sterilised test-tubes were placed in a sloping stand to cool and to create a sloping layer of YMA.

The nine different rhizobial strains were taken from their source tube using sterilised plastic loops, streaked in zigzags onto the YMA slopes (8 replicates per strain) and allowed to grow and develop in the dark at room temperature (25°C).

3.5 Preparation of rhizobial inoculant

After one week, successfully growing and developing rhizobial colonies on the YMA media were prepared as a bacterial suspension. For this purpose, 4.5 g K_2HPO_4 (equivalent to 0.02 M K_2HPO_4) was dissolved in 1 L distilled water in a volumetric flask and the pH adjusted to 7 by adding HCL carefully before being used as cell dilution medium. Five mL portions of this K_2HPO_4 solution were transferred into sterilised glass tubes in which the nine different rhizobial colonies were diluted by streaking with sterilised plastic loopfuls collected from the pure bacterial culture in aseptic conditions. All tubes were shaken by electric shaker, an additional 25 mL K_2HPO_4 solution were added to each tube and they were shaken again until the bacterial cells were uniformly dispersed.

The concentration of rhizobial suspension of all different strains was determined and adjusted to 10^5 cfu (colony forming units) mL^{-1} using a spectrophotometer. The concentration of the solution was adjusted to 0.1 OD (Optical Density), which is equivalent to 10^5 cfu mL^{-1}, by adding K_2HPO_4 or rhizobial cfu as needed using the calculation $C_1V_1=C_2V_2$. The spectrophotometer wavelength was 540 nm and pure K_2HPO_4 solution was used as a blank and reference sample. An additional three glass tubes were included, one containing only 25 mL solution K_2HPO_4 and the other two containing 2.5 mL from each of the nine prepared strains. One of these was used as a mixed strain treatment and the other as a sterilisation treatment by autoclaving at 121°C for 30 min. Thus there were 12 treatments in total (9 single strains, a mixture of all strains, a sterilisation treatment and the control).

3.6 Plant inoculation

Seed bacterisation was carried out according to the supervisor's instructions. The design of the main experiment involved mixing all six types of crop plant seeds in one pot (2 seeds per plant species) and with 6 replicates for each treatment. Thus there were 2 (seeds each type of plant) * 6 (replications) * 12 (treatments), equivalent to 144 seeds for each type of crop plant.

For seed surface sterilisation, the seeds of each individual crop species were placed in an Erlenmeyer flask and treated with 1% sodium hypochlorite. The contents were gently swirled to bring the seeds and sterilant into contact for 2 min. The sterilant was then drained off and the seeds were rinsed six times with sterile water and dried under sterile conditions. Finally, the seeds placed in an Erlenmeyer flask (144 seeds per flask).

After that, the seeds were steeped with treatments or different rhizobial suspensions and the flasks marked with the name of treatments. Seeds treated only with 0.02 M K_2HPO_4 solution were used as the control. All flasks swirled gently to bring the seeds and rhizo-bacteria into close contact for 30 min to obtain a uniform inoculum of 10^5 cfu $seed^{-1}$.

3.7 Greenhouse experiment

The main greenhouse experiment was conducted in mid-August 2006. The treated and control seeds (2 per crop species) were sown immediately into plastic pots (previously prepared) using sterilised plastic spoons and placed in a greenhouse where the air temperature was 27^{o}C and the soil temperature 30^{o}C. Seeds were covered immediately with 1 cm soil after sowing.

Seed germination started within three days and continued over the next couple of days. In the second week after germination, normal cultivation practices were begun. These included watering twice a week at an initial rate of 100 mL per pot, which was increased to 200 mL according to the growth and development of plants. One plant per pot of *Zea mays* and *Helianthus annuus* was pruned to produce a well-balanced distribution of foliage. Liquid plant nutrient solution (Bayer Company) containing 7.0% nitrogen, 2.2% phosphorus, 5.0% potassium, 0.04% sulphur, 0.01% boron, 0.02% iron, 0.01% copper, 0.01% manganese, 0.005% molybdenum, 0.005% zinc and 0.006% magnesium was introduced after two weeks at an initial rate of 1 mL nutrient solution in 2 L distilled water. The nutrient concentration was then doubled every week for four weeks to a constant 4 mL nutrient solution in 2 L water, applied during watering. All pots were moved after three weeks to a greenhouse with average day temperature 25^{o}C, night temperature 22^{o}C, light intensity 30 kLx and average relative humidity 85%. All plant roots were re-inoculated twice with the same treatments at the same concentration using a sterilised glass dropper applying the liquid to the soil surface. The first re-inoculation was two weeks after sowing and after the second five weeks after sowing. The position of the pots was rearranged every week to achieve uniform light distribution in all treatments. All plants ware harvested with their roots intact after 58 days (Figure 3) and soil was carefully removed from the plants with running water. All cleaned plants were then labelled and placed in a dryer at 60^{o}C for 48 h for determination of dry weight of biomass. Plant roots and shoots were separated and weighed separately by digital balance and the data were recorded.

Figure 3. Plants in the greenhouse at 58 days.

3.8 Impact of inoculant density on germination and development of *Linum usitatissimum*

In the first experiment, *Sinorhizobium meliloti* (PAR-207) strain was found to be the best growth promoting strain tested and had a wide range of growth promotion activity. Therefore a complementary greenhouse experiment was carried out to test its performance in winter. For this purpose, one type of plant (*Linum usitatissimum*) was selected as the host plant and strain *Sinorhizobium meliloti* (PAR-207) was used as the growth promoter. In this second experiment, there were some changes to the procedure. A total of 60 viable seeds of *Linum usitatissimum* sterilised with 1% sodium hypochloride (as described above) were used.

These seeds were inoculated with increasing concentrations (CFU 10^3, CFU 10^4 and CFU 10^5) of PAR-207, which were considered as different treatments. The sterilised treatment involved autoclaving strain PAR-207 at 121°C for 20 min and 0.02 M K_2HPO_4 solution was used as the control. The seed inoculation procedure was the same as before and inoculated seeds were immediately sown in plastic pots (4 seeds per pot, 3 replicates per treatment and 5 treatments) previously prepared in the greenhouse and covered with 1 cm soil. The average temperature of the air was 20.3°C, soil temperature (5 cm depth) was 20.6°C, light intensity was 30 kLx and relative humidity 85%. Seed germination began after three days. After two weeks, seedlings were again treated with previously applied

concentration of strain PAR-207 suspension using sterilised glass droppers. Cultivation operations such as watering, applying plant nutrients, rearranging the position of pots were carried out as before but there was no pruning. All plants were harvested at 56 days (Figure 4) by carefully removing soil from the root zone through running water, dried at 60^{o}C for 48 h and dry weight of root and shoot biomass were determined separately using a digital balance.

Figure 4. *Linum usitatissimum* (linseed) in the greenhouse at 56 days.

3.9 Rhizobial growth

The growth rate of three different rhizobial strains was determined, namely PAR-207, PAR-804 and E-11. Yeast Mannitol Broth was prepared as before (section 3.4) and added to 12 Erlenmayer flasks (4 treatments including control x 3 replicates). All flasks with YMB were then autoclaved at 121^{o}C for 20 min.

Five mL of 0.02 M K_2HPO_4 stock were added to each of three test-tubes and colonies of the three strains tested were streaked into the solution by sterilised plastic loops collected from pure exponential cultures. All test-tubes were shaken on an electric shaker for uniform dilution. A further 20 mL K_2HPO_4 stock solution were added to each test-tube and they were shaken well again. Sterilised YMB (300 mL) was then added to each of 12 sterilised flasks and 5 mL strain suspension from the test-tubes was added to each of the nine replicate treatment flasks (the three control flasks received no inoculant). All 12

flasks were shaken on a mechanical shaker at a rotation rate of 112 rpm. The initial (hour 0) bacterial concentration of each strain (3 replicates) was measured by spectrophotometer (wavelength 540 µm, temperature 25°C, sterilised YMB solution as reference sample). The concentration of the three strains was measured in the same way at 12 h, 24 h and 48 h of growth. The last 24 h marked the exponential phase.

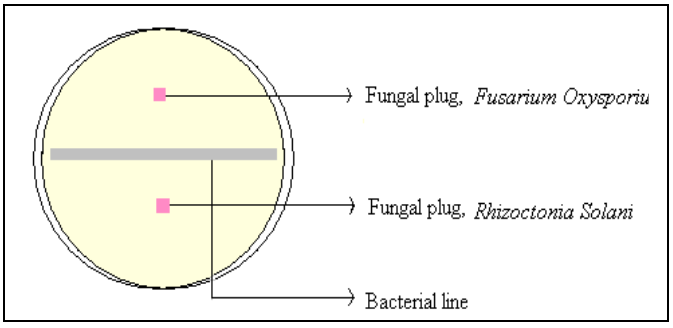
3.10 Interaction between rhizobia and pathogenic fungi

Nine different rhizobial strains and two types of fungi (*Fusarium oxysporium* and *Rhizotania solani*) were used for this purpose. The experiment was performed on YMA nutrient medium. The procedure is shown in Figure 5.

A small amount of pure exponential rhizobacterial colony of each of the nine strains was picked up with a sterile plastic loop from the pure bacterial culture tube and streaked in a single 8 cm line longitudinally on the upper surface of separate YMA plates. These plates were then kept for seven days at 25°C for growth and development.

After seven days, two types of fungal plugs of 14-day-old PDA cultures were placed at a distance of 2 cm from each rhizobial line (Figure 5). The plates were incubated at 25°C. Horizontal fungal growth towards the bacterial line was recorded daily.

Figure 5. Inoculation of rhizobacteria by two types of fungal plugs.



3.11 Statistical analysis of data

All plant dry biomass data were arranged by a combination process ($^{12}C_2$= 12*11/2*1=66) for the 6 replicates of each plant species and used as input in the MINITAB-14 statistical software. The means and standard deviation were calculated for root and shoot dry biomass. The analysis of variance (ANOVA) on these was determined by a basic two-sample T-test. Significance levels were expressed at the 95% confidence limit ($P<0.05$).

4. RESULTS

4.1 Seed/plant inoculation studies

Inoculation of crop seed or plant with all rhizobial strains except PAR-803 and PAR-307 improved shoot and root dry biomass, as did the sterilisation treatment (Table 5). However, no single rhizobial strain caused significant ($P<0.05$) increases in all six types of crop plant tested. Some strains showed a broad range of ability for biomass production and host range specificity, while others showed a narrow range.

Strain PAR-207 was the best potential performer compared with other strains for biomass production and it also had the potential to promote growth and development of a broad range of host plant species, such as shoot growth of *Papaver rhoeas,* root and shoot growth of *Brassica rapa,* root and shoot growth of *Linum usitatissimum* and root and shoot growth of *Helianthus annuus,* compared with the control and the other treatments.

Strain PAR-201 also promoted significant root growth of *Brassica rapa,* root growth of *Cymbopogon flexuosus,* root and shoot growth of *Linum usitatissimum* and shoot growth of *Papaver rhoeas.* Strain PAR-401 caused significant root growth of *Linum usitatissimum,* root growth of *Papaver rhoeas* and root and shoot growth of *Zea mays.* Strain PAR-102 showed significant root growth of *Linum usitatissimum*, root and shoot growth of *Brassica rapa* and root and shoot growth of *Papaver rhoeas.* The mixed strain inoculum led to significant root growth of *Papaver rhoeas*, shoot growth of *Linum usitatissimum* and root and shoot growth of *Zea mays* (Figure 6). Strain PAR-601

produced significant effects for root growth of *Brassica rapa*, root growth of *Papaver rhoeas* and root and shoot growth of *Linum usitatissimum.* Strain E-11 caused significant shoot growth of *Papaver rhoeas* and root growth of *Cymbopogon flexuosus,* while PAR-804 brought about significant root growth of *Papaver rhoeas* (Table 5).

Figure 6. Corn root appearance after inoculation with mixed rhizobia (right) and uninoculated control (left).

Overall, strain PAR-201 was the best for *Papaver rhoeas* and *Cymbopogon flexuosus*, whereas PAR-207 was best for *Linum usitatissimum, Brassica rapa* and *Helianthus annuus*. PAR-401 was the best for *Zea mays,* considered on an individual plant basis (Table 6).

Table 5. Effect of inoculation with various rhizobial strains on shoot and root dry weight (g) of six types of crop plants after 58 days of growth

Rhizobial strain	*Brassica rapa*		*Cymbopogon flexuosus*		*Helianthus annuus*		*Linum usitatissimum*		*Papaver rhoeas*		*Zea mays*	
	Shoot	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**
E-11	0.33	0.05	0.08	0.04[a]	0.85	0.21	0.93	0.13	0.09[a]	0.01	2.68	1.12
PAR-804	0.35	0.15	0.07	0.03	0.91	0.27	0.83	0.19	0.08	0.07[a,b]	2.55	1.05
PAR-803	0.29	0.04	0.05	0.02	0.80	0.21	0.74	0.10	0.04	0.01	2.95	0.90
PAR-601	0.38	0.07[a,b]	0.12	0.07	0.55 [c]	0.18	1.00[a,b]	0.15[a]	0.08	0.02	2.71	1.00
PAR-401	0.40	0.09	0.10[b]	0.06	0.82	0.28	0.95	0.17[a]	0.08	0.01[a]	4.87[a]	1.59[a]
PAR-307	0.29	0.08	0.07	0.05	1.28	0.37	0.81	0.13	0.08	0.01	2.38	0.83
PAR-207	0.58[a,b]	0.09[a,b]	0.05	0.03	2.49[a,b]	0.82[a,b]	1.26[a,b]	0.22[a]	0.15[a,b]	0.02	5.41	1.85
PAR-201	0.41	0.07[a,b]	0.09[b]	0.05[a,b]	1.33	0.47[b]	1.03[a]	0.17[a,b]	0.18[a,b]	0.03	3.38[b]	1.44
PAR-102	0.52[a,b]	0.08[a,b]	0.08	0.03	0.41 [c]	0.11	0.89	0.16[a,b]	0.07[a,b]	0.02[a,b]	1.64	0.83
Control	0.27	0.04	0.06	0.03	1.64	0.41	0.71	0.09	0.04	0.00	2.18	0.69
Sterilisation	0.30	0.06	0.04	0.02	1.31	0.32	0.91	0.15	0.13	0.02	2.51	0.77
Mixed strains	0.34	0.06	0.06	0.03	1.27	0.38	1.02[a]	0.14	0.13	0.02[a,b]	4.25[a]	1.57[a]

[N.B: [a]Values significantly higher than the control; [b]Values significantly different from other strains; [c]Values significantly lower than the control $P<0.05$ in all cases.]

Table 6. Best strain for individual crop species

Plant Name	Best Strain
Brassica rapa	PAR-207
Cymbopogon flexuosus	PAR-201
Helianthus annuus	PAR-207
Linum usitatissimum	PAR-207
Papaver rhoeas	PAR-201
Zea mays	PAR-401

4.2 Impact of inoculant density on germination and development of *Linum usitatissimum*

Two concentrations of bacterial suspension (10^5 cfu mL^{-1} and 10^4 cfu mL^{-1}) were found to significantly increase root dry biomass of *Linum usitatissimum* compared with the control (Figures 7 and 8), the sterilisation treatment and the 10^3 cfu mL^{-1} concentration (Table 7). The germination rate also differed with different concentrations of rhizobial inoculant, with the 10^4 cfu mL^{-1} concentration giving 100% germination, 10^5 cfu mL^{-1} giving 60% and 10^3 cfu mL^{-1} giving 25%. These rates can be compared with the sterilisation treatment, which gave 50% germination, and the control, which gave 25%.

Figure 7. Inoculation of linseed with strain PAR-207 at 10^4 cfu mL^{-1}, (left) and uninoculated control (left).

Fig 8. Inoculation of linseed with strain PAR-207 at 10^4 cfu mL^{-1} on shoot and root biomass of *Linum usitatissimum* at harvest (right), uninoculated control (left).

Table 7. Effect of rhizobial inoculation of *Linum usitatissimum* on shoot and root dry weight (g) after 56 days of growth

Linum usitatissimum		
Treatment	**Shoot DM, g**	**Root DM, g**
Control	0.29	0.03
Sterilisation	0.23	0.03
CFU 10^3 cells mL^{-1}	0.37	0.04
CFU 10^4 cells mL^{-1}	0.45	0.09^{ab}
CFU 10^5 cells mL^{-1}	0.411	0.09^{ab}

[a]Values significantly higher than the control; [b]Values significantly different from the other strains, $P < 0.05$ in both cases

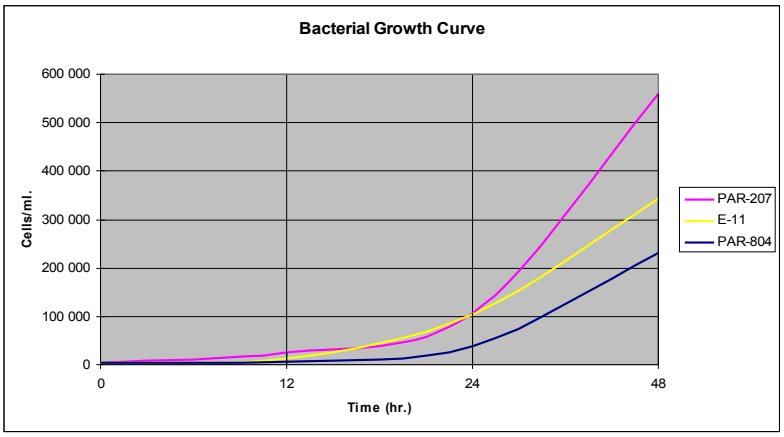
4.3 Bacterial growth

The generation time or doubling time of the rhizobia differed in the different strains tested. The mean generation time of PAR-804, PAR-207 and E-11 was 9.4, 10.1 and 14.1 hours respectively in the exponential phase (Table 8 and Figure 9).

Table 8. Change in rhizobial counts (cfu mL^{-1}) with time and mean generation time (Gt) and generation number of some of the different rhizobial strains used

Strain	0 hr.	12 hr.	24 hr.	48 hr.	Gn	Gt
PAR-804	$3.4x10^3$	$0.7x10^4$	$0.3x10^5$	$2.3x10^5$	2.5	9.4
PAR-207	$4.3x10^3$	$2.4x10^4$	$1.0x10^5$	$5.6x10^5$	2.4	10.1
E-11	$4.0x10^3$	$1.2x10^4$	$1.0x10^5$	$3.4x10^5$	1.7	14.1

Figure 9. Growth curves for the rhizobial strains PAR-207, E-11 and PAR-804.



4.4 Interaction between rhizobia and pathogenic fungi

There was no direct inhibition of fungal growth and development close to the rhizobial line. In actual fact the fungal mycelium touched the bacterial line easily and successfully (Figure 10) but after few days of contact between rhizobia and fungi, some strains (PAE-401, PAR-307, PAR-804, PAR-803, PAR-601, PAR-102 and E-11) showed a tendency to dissolve the fungal mycelium towards the fungal plug (Figure 11).

Figure 10. Fungal mycelium touching the bacterial line.

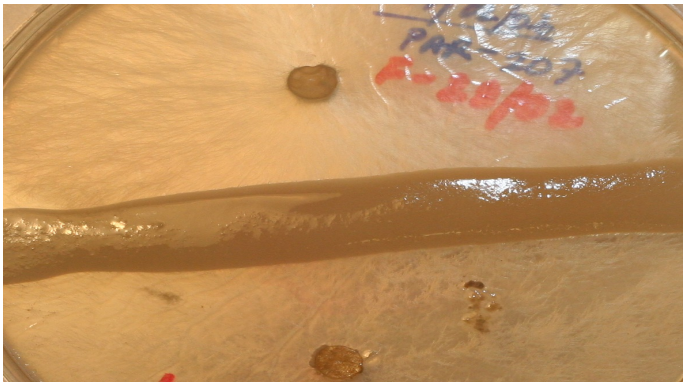
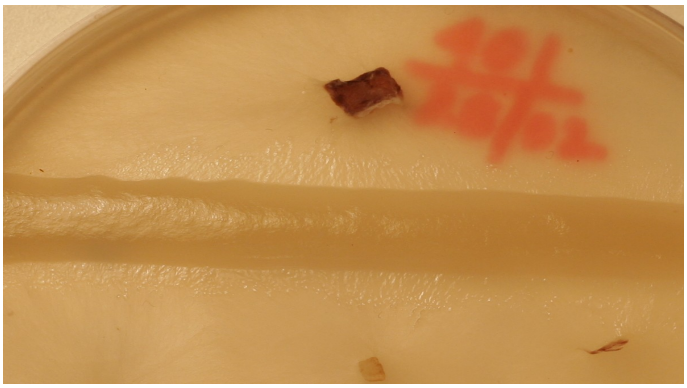


Figure 11. Strain PAR 401 merged with the fungal mycelium.



5. DISCUSSION

In this study, composite screening approaches were simultaneously employed to select the most effective potential rhizobia for non-nitrogen fixing plants. Some of the results presented were not statistically significant due to missing data, where some seeds did not germinate or germinated late. For example, PAR-207 did not show statistically significance differences for both root and shoot of *Zea mays* or PAR-201 for root growth of *Papaver rhoeas* (Table 5) although the mean values obtained appeared to be substantially different. However, PAR-207 had the best overall band score and it demonstrated a wide range of capability to improve all the types of non-nitrogen fixing plants tested compared with other treatments or the control. PAR-207 is a strain of *Sinorhizobium meliloti*. PAR-201 is another strain of the same species and also proved to significantly improve plant growth (Table 5). In our study, it appears that *S. meliloti* is the best strain for supporting growth and development of non-nitrogen fixing plants. Most of *Sinorhizobium meliloti* based strains produces IAA (Kittell *et al*., 1989), sidero-phore, soluble P and no produce HCN (Table-2, Antoun *et al*. (1998). However, the chemical compositions of exopolysaccharides (EPS) and lipopolysaccharides (LPS) of *Sinorhizobium meliloti* are different than other rhizobial stains (Bauer, 1981**).** Galleguillos (2000) has also reported the beneficial effects of *Shinoribium meliloti* on lettuce. The PGPR capacity of the other bacteria used in the present experiment is not very clear, and according to the results, it seems not to be high.

It has already been demonstrated that *Rhizobium leguminosarum* bv *trifolii* strain E11 has a good effect in rice (Yanni, 2001) due to production of indole-3-acetic acid (Dazzo *et al*., 2000). Hilali *et al*. (2000) showed that *Rhizobium leguminosarum* bv *trifolii* increased grain yield of wheat by 18%. This bacterium is a native root endophyte of the rice from Egyptian Nile delta and there is a natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots, the potential of which to promote rice growth has been assessed by Yanni & Rizk (1997).

However in the present study, crop species grown alone rather than in mixtures were stimulated most highly by individual strains, showing highest growth and development of biomass (Table 6). The reason may be that individual strains ecologically prefer individual crop species due to different root exudates, which highly attract particular rhizobial strains. However, mixed plant species may affect rhizobial strains due to mixed or combined and complex overlapping root exudates that influence their growth, development, activities and diversity in the new ecological root zone. Most rhizobia are attracted toward amino acids, organic acids and sugars by a chemotactic response (Bowra & Dilworth, 1981). However, *Rhizobium loti* strains are not attracted by these compounds but by a non-agglutinating glycoprotein (chemotactin) present in the root exudate of *Lotus corniculatus* (Troch & Vanderleyden 1996). Thus strains PAR-803 and PAR-804 are not suitable for non-nitrogen fixing plants. Generally, different microbial strains have different characteristics as regards being attracted and attached to the niche non-nitrogen fixing plant and thus differing ability as growth promoters.

The complementary studies performed showed significant rhizobial growth and plant development even in adverse environmental conditions. Interestingly, the first complementary study showed that rhizobia also influenced seed germination and plant growth depending on their concentration (Table 7). A concentration of 10^4 cfu mL^{-1} of PAR-207 gave the highest seed germination rate and growth of *Linum usitatissimum* compared with other concentrations and strains tested or the control. However, this experiment was unable to demonstrate significant shoot growth due to lack of light and

temperature in the greenhouse in winter. The growth promotion mechanism could thus be developed or enhanced by an inoculation regime based on the appropriate rhizobial population density.

Rhizobial release of nodulation signals such as lipo-chito-oligosaccharides (LOCs) is known to stimulate seed germination in a wide range of plant species by a still unknown mechanism (Matiru & Dakora, 2004). For example, recent findings show that lumichrome and LCOs released by rhizobia stimulate seed germination and growth of crop plants (Zhang *et al*., 2002). Rhizobia influence seeds at the time of seed germination, when dormancy is broken and seeds ultimately produce and exude solutes or low molecular weight metabolites by biological and physiological processes (Crowe & Crowe, 1992). These compounds subsequently attract potential concentrations of rhizobia. The diversity and the numbers in which they are present depend very much on the composition and concentration of nutrients exuded by plant roots (Hale & Moore, 1979).

Rhizobacterial growth rate cannot indicate precisely which plant growth promoting strain gives rise to the highest growth rate. No correlation was found between bacterial growth rate and plant growth rate, but a moderate growth rate may be better for non-nitrogen fixing plants (not proven by the data). The second complementary experiment (rhizobacterial growth) showed that strain PAR-804 had a higher growth rate in the exponential phase but was not as good a plant growth promoter as strain PAR-207 (unique finding for this study). Rhizobial growth depends on nutritional status of the growth medium, chemical reactions and enzymatic activates. Flavonoids also enhance the growth rate of certain rhizobia at the time of root colonisation (Hartwing *et al*., 1991). In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in nature. However, a fast growth rate may be of benefit when colonising the root and also e.g. to counteract plant pathogens.

In the study on the interaction between rhizobia and plant pathogenic fungi, the *in vitro* experiments performed showed that none of the nine rhizobial strains tested was able to prevent direct growth of fungi towards the bacterial line and successful contact between

the two. However, after a couple of days some strains such as PAE-401, PAR-307, PAR-804, PAR-803, PAR-601, PAR-102 and E-11 were observed to dissolve and merge the fungal mycelium towards the old dense mycelium (Figure 11). The severity of this effect varied from strain to strain and with nutrient availability in the medium. The possible reason and mechanism is that after contact with the fungus, rhizobia start to use the nutritional contents of fungal mycelium which they hydrolise by enzymatic reaction for their growth and development and thus the rhizobial line moves towards the fungal mycelium line (Fridlender *et al*., 1993; Kloepper, 1996). Exo-polysaccharide (EPS) of rhizobia binds the fungal mycelium, which protects fungal cells against desiccation (no previous data).

6. CONCLUSIONS

Significantly increased plant biomasses indicate that rhizobia have naturally potential ability to promote the growth of non-nitrogen fixing plant. But the ability of performance depends on the proper association between rhizobial strains and non-nitrogen fixing plant species. The concentration level of rhizobial inoculation is another important factor for seed germination and plant growth. The results confirm earlier studies indicating that certain strains of rhizobia can promote growth of non-nitrogen fixing plant, possibly through mechanisms that involve changes in growth physiology and root morphology. More rhizobial strains should be screened through laboratory and field experiments to exploit their potential as PGPR for sustainable plant production.

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