EFFECT OF PLANT GROWTH-PROMOTING RHIZOBACTERIA ON CANOLA (*Brassica napus*. L) AND LENTIL (*Lens culinaris*. Medik) PLANTS

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by

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria that colonize the rhizosphere and, when applied to crops, enhance the growth of plants. Plant growth-promoting rhizobacteria may enhance plant growth either by direct or indirect mechanisms. The direct mechanisms of action include nitrogen fixation, production of phytohormones and lowering of ethylene concentrations. The objective of this study was to determine whether *Pseudomonas putida* strain 6-8 isolated from the rhizosphere of legume crops grown in Saskatchewan fields was able to promote the growth of canola cv. Smart and lentil cv. Milestone plants by direct mechanisms.

Initial studies determined the effect of strain 6-8 and other known phytohormone-producing PGPR strains on the growth of canola and lentil plants both in gnotobiotic and growth chamber conditions. Variations in the results were observed, as there were significant differences among trials. Strain 6-8 enhanced the growth of canola cv. Smart in growth pouches but not in pots in growth chamber studies. In the case of lentil cv. Milestone, strain 6-8 had no significant effect in growth pouches, but it significantly increased root dry weight, shoot dry weight and root surface area in pots in growth chamber studies. A similar effect was observed with wild-type strains GR12-2 and G20-18. Strain GR12-2 was consistent in promoting the growth of lentil cv. Milestone both in growth pouches and in pots in growth chambers when compared to other strains and the control.

The ability of the PGPR strains to produce auxin and cytokinin phytohomones in pure culture and in the canola rhizosphere was tested using the enzyme linked immunosorbent assay (ELISA). All the PGPR strains produced indole compounds and the concentration of the indoles produced increased with increasing concentrations of the

precursor tryptophan. There were no significant differences among PGPR strains in production of indole-3-acetic acid (IAA) when assayed using ELISA. The concentrations of IAA secreted by PGPR strains were extremely low (0.19 µg/ml – 9.80 µg/ml). Strain 6-8 produced the cytokinins, isopentenyl adenosine (IPA), zeatin riboside (ZR) and dihydroxyzeatin riboside (DHZR) in pure culture. Indole-3-acetic acid was detected in supernatants obtained from canola growth pouches inoculated with PGPR strains, but there were no significant differences in the concentrations of IAA secreted among PGPR strains. Significantly higher concentrations of IPA and ZR were observed in the rhizosphere of canola inoculated with strain 6-8 than in the non-inoculated control.

Strain 6-8 produced siderophores, solubilized inorganic phosphate and used 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, as sole nitrogen source. These traits are considered to be alternative mechanisms for direct plant growth promotion.

A qualitative and quantitative study of root colonization by strain 6-8 was conducted by tagging the strain with green fluorescent protein in conjunction with confocal laser scanning microscopy and by conventional plating. The populations of strain 6-8 were higher on canola roots than on lentil roots by conventional plating. Similar results were also observed in confocal laser scanning microscopy (CLSM) studies after 5, 7 and 9 days for canola and 3, 6 and 9 days for lentil.

Pseudomonas putida strain 6-8 produced cytokinins and also possessed other direct growth promoting characteristics. The ability of strain 6-8 to promote the growth of canola cv. Smart in growth pouches and lentil cv. Milestone in growth chamber studies

may be related to these direct growth promoting characteristics. Strain 6-8 may have potential for development as a plant growth-promoting rhizobacterial inoculant.

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LIST OF ABBREVIATONS

-	Negative
%	Percentage
°C	Degree centigrade
µg	Microgram
µl	Microlitre
µm	Micrometer
+	Positive
ACC	1-aminocyclopropane-1-carboxylic acid
ACES	N-(2-acetamido)-2-amino-ethanesulfonic acid
CAS	Chrome S azurol
CFU	Colony forming units
cm	Centimetre
d	Days
DHZR	Dihydroxyzeatin riboside
DMF	N,N-dimethylformamide
ELISA	Enzyme linked immunosorbent assay
FAME	Fatty acid methyl esterase
GFP	Green fluorescent protein
Gm	Gentamycin
h	Hours
HDTMA	Hexadecyltrimethyl-ammonium bromide
IAA	Indole-3-acetic acid

ICA	Indoel-3-carboxylic acid
IPA	Indole-3-propionic acid
IBA	Indole-3-butyric acid
IPA	Isopentenyl adenosine
Km	Kanamycin
l	Litre
LSD	Least significant difference
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
N	Nitrogen
nm	Nanometer
PGPR	Plant growth-promoting rhizobacteria
pmoles	Picomoles
Rif	Rifampicin
rpm	Revolutions per minute
RSM	Rhizosphere medium
s	Seconds
SIM	Similarity indices
Tn	Transposon
TNTC	Too numerous to count
TSA	Tryptic soy agar

TSB	Tryptic soy broth
v/v	Volume per volume
ZR	Zeatin riboside

1.0 INTRODUCTION

Preparations of live microorganisms (bacteria, fungi) utilized for improving plant growth and crop productivity are generally referred to as biofertilizers or microbial inoculants (Subba Rao and Dommergues 1998). *Rhizobium* spp. which fix nitrogen from the atmosphere and form root nodules on legumes, were the first biofertilizers identified and have been used commercially as inoculants for legumes for over 100 years (Kannaiyan 2002). Research in the field of biofertilizers has resulted in the development of different kinds of microbial inoculants or biofertilizers including nitrogen fixing bacteria, phosphate solubilizing microorganisms, vesicular – arbuscular mycorrhizae (VAM) and plant growth promoting rhizobacteria (PGPR).

During the late 19^{th} and early 20^{th} centuries inorganic compounds containing nitrogen, potassium and phosphorus (NPK) were synthesized and used as fertilizers. Due to the growth in human populations fertilizers were used to increase crop production and meet the rising demands for food. Increases in the production cost, and the hazardous nature of chemical fertilizers for the environment has led to a resurgence of interest in the use of biofertilizers for enhanced environmental sustainability, lower cost production and good crop yields.

Plant growth-promoting rhizobacteria (PGPR) are free-living soil-borne bacteria that colonize the rhizosphere, and when applied to seed or crops enhance the growth

of plants (Kloepper 1980). The rhizosphere is the soil found around the root and under the influence of the root. It is a site with complex interactions between the root and associated microorganisms (Sylvia *et al.* 1998). In the past 10-15 years close to 4,000 publications have appeared in the field of plant growth-promoting bacteria (Bashan and Holguin 1998). Plant growth-promoting rhizobacteria enhance plant growth either by direct or indirect mechanisms (Glick 1995). Plant growth promoting rhizobacteria that have been successful in promoting the growth of crops such as canola, soybean, lentil, pea, wheat and radish have been isolated (Kloepper *et al.* 1988; Chanway *et al*. 1989; Glick *et al*. 1997; Timmusk *et al*. 1999; Salamone 2000). The enhancement of plant growth by PGPR indicates their potential as biofertilizers in the field of agriculture. Bertrand *et al*. (2001) identified bacteria belonging to the genera *Pseudomonas, Varivorax*, *Agrobacterium* and *Phyllobacterium* as the most efficient PGPR associated with canola.

Screening of rhizosphere samples obtained from legume crops grown in Saskatchewan fields resulted in isolation of strains, which exhibit some characteristics of PGPR (Hynes and Nelson 2001). A *Pseudomonas putida* strain designated 6-8 was isolated from the rhizosphere of pea obtained from a field near North Battleford (Hynes and Nelson 2001). It was one of the strains selected from the culture collection based on its ability to utilize 1-aminocyclopropane-1-carboxylic acid (ACC) as sole source of nitrogen for growth, to produce siderophores and to enhance root length of canola in growth pouches. These are some of the characteristics exhibited by PGPR (Glick *et al*. 1999).

The hypothesis of the present study is:

Pseudomonas putida strain 6-8 enhances the growth of canola and lentil plants by direct mechanisms.

The hypothesis will be tested through experiments conducted in the laboratory. Strain 6-8 was initially screened for its effect on the growth of canola and lentil plants in growth pouches and in pots in the growth chamber and compared with other known PGPR strains (Table 1). The ability to produce the phytohormones, indole-3-acetic acid (IAA), isopentenyl adenosine (IPA), dihydroxy zeatin riboside (DHZR) and zeatin riboside (ZR) was assayed in strain 6-8 and the other known rhizobacterial strains. The pattern of root colonization by strain 6-8 was studied using a green fluorescent protein (GFP) marker.

1.1 Objectives

My research addressed the hypothesis using the following approaches:

1. Determination of the effect of strain 6-8 and other known rhizobacterial strains on growth of lentil and canola plants in gnotobiotic and growth chamber studies.
2. Determination of phytohormone production by strain 6-8 and other rhizobacterial strains using colorimetric and ELISA techniques.
3. Evaluation of other growth promoting mechanisms such as solubilization of inorganic phosphate and siderophore production, in strains 6-8, G20-18, GR12-2 and their mutants by *in vitro* screening.
4. Determination of the pattern of root colonization by strain 6-8 on canola and lentil plants using green fluorescent protein (GFP) as a molecular marker in conjunction with confocal laser microscopy.

2.0 LITERATURE REVIEW

2.1 Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria, isolated from the rhizosphere, which, when applied to seeds or crops, enhance the growth of the plant or reduce the damage from soil-borne plant pathogens (Kloepper *et al*. 1980). It has been estimated that more than 100 million tonnes of nitrogen, potash and phosphate-chemical fertilizers have been used annually in order to increase plant yield (Glick *et al*. 1999). The potential negative effect of chemical fertilizers on the global environment and the cost associated with production has led to research with the objective of replacing chemical fertilizers with bacterial inoculants.

Bacterial inoculants which help in plant growth are generally considered to be of two types a) symbiotic and b) free-living (Kloepper *et al*. 1988; Frommel *et al*. 1991). Beneficial free-living bacteria referred to as PGPR are found in the rhizosphere of the roots of many different plants (Kloepper *et al*. 1989). Breakthrough research in the field of PGPR occurred in the mid 1970s with studies demonstrating the ability of *Pseudomonas* strains capable of controlling soil-borne pathogens to indirectly enhance plant growth and increase the yield of potato and radish plants (Burr *et al*. 1978; Kloepper and Schroth 1981; Kloepper *et al*. 1980; Howie and Echandi 1983). The effect of PGPR on agricultural crops has been investigated and published by various authors in

in the last two decades with recent applications on trees (Bashan and Holguin, 1998; Enebak *et al*. 1998). During 1983 and 1984 more than 4,000 bacterial strains were isolated from the rhizosphere of plants grown in the Canadian High Arctic and screened for the ability to fix nitrogen. Some strains demonstrated the ability to reduce acetylene and colonize roots of canola when grown at low temperatures (Lifshitz *et al*. 1986). Strains which exhibited the potential to be PGPRs were identified as *Pseudomonas putida*, *P. putida* biovar B, *P. fluorescens*, *Arthobacter citreus* and *Serratia liquefaciens* (Lifshitz *et al*. 1986; Klopper *et al*. 1988). The ability of these strains to be used as bacterial inoculants in agriculture was tested in greenhouse and field trials with different formulations and they increased the yield of canola in both types of trial. Salamone (2000) reported the growth-promoting effect of *P. fluorescens* strain G20-18 on wheat and radish plants by production of cytokinin phytohormones. As the effect of PGPR on plants was demonstrated, the concept of PGPR began to gain importance and a large number of bacterial strains have been isolated, screened (Chanway and Holl 1993; Cattelan *et al*. 1999; Bertand *et al*. 2001) and evaluated for plant growth promotion (Lifshitz *et al*. 1987; Chanway *et al*. 1989; Abbas and Okon 1993; Glick *et al*. 1997; Zhang *et al*. 1997; Bashan 1998; Mayak *et al*. 1999; Bent *et al*. 2001; Salamone 2000).

Rhizosphere bacteria promote plant growth and yield either directly or indirectly (Kloepper *et al*. 1989; Glick 1995). The direct mechanisms of plant growth promotion may involve the synthesis of substances by the bacterium or facilitation of the uptake of nutrients from the environment (Glick *et al*. 1999). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of plant pathogens on plants by production of inhibitory substances or by increasing the natural resistance of the host (Handelsman and Stabb 1996; Nehl *et al*. 1996; Cartieaux *et al*. 2003).

The direct growth promoting mechanisms are as follows i) nitrogen fixation ii) solubilization of phosphorus iii) sequestering of iron by production of siderophores iv) production of phytohormones such as auxins, cytokinins, gibberellins and v) lowering of ethylene concentration (Kloepper *et al*. 1989; Glick 1995; Glick *et al*. 1999). For example strain GR12-2, *P. putida,* isolated from the rhizosphere of plants growing in the Canadian High Arctic, was found to promote growth of canola cv. Tobin by fixing nitrogen and enhancing the uptake of phosphate under gnotobitoic conditions (Lifshitz *et al*. 1986; Lifshitz *et al*. 1987), by synthesizing siderophores that can solubilize and sequester iron from the soil and supply it to the plants Glick (1995), by production of the phytohormone IAA (Xie *et al*. 1996) and by lowering of ethylene concentration via production of the enzyme ACC deaminase (Glick *et al*. 1994b).

The indirect mechanisms of plant growth promotion by PGPR include i) antibiotic production ii) depletion of iron from the rhizosphere iii) synthesis of antifungal metabolites iv) production of fungal cell wall lysing enzymes v) competition for sites on roots and vi) induced systemic resistance (Weller and Cook 1986; Dunne *et al*. 1993; Kloepper *et al*. 1988; Liu *et al*. 1995; Glick *et al*. 1999).

As this thesis is focussed on direct mechanisms of plant growth promotion, some of the direct mechanisms of action are reviewed in detail in the following sections.

2.2 Direct mechanisms of action

2.2.1 Nitrogen fixation

Nitrogen is one of the most common nutrients required for plant growth and productivity as it forms an integral part of proteins, nucleic acids and other essential biomolecules (Bøckman 1997). More than 80 % of nitrogen is present in the atmosphere, but is unavailable to plants. It needs to be converted into ammonia, a form available to

plants and other eukaryotes. Atmospheric nitrogen is converted into forms utilized by plants by three different processes a) conversion of atmospheric nitrogen into oxides of nitrogen in the atmosphere b) industrial nitrogen fixation uses catalysts and high temperature (300-500ºC) to convert nitrogen to ammonia and c) biological nitrogen fixation involves the conversion of nitrogen to ammonia by microorganisms using a complex enzyme system identified as nitrogenase (Kim and Rees, 1994). Biological nitrogen fixation fixes about 60% of the earth's available nitrogen and represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al*. 1997).

Plant growth-promoting rhizobacteria that fix nitrogen in non-leguminous plants are diazotrophs that form a non-obligate interaction with the host (Glick *et al*. 1999). The process of nitrogen fixation is carried out by the nitrogenase enzyme coded by *nif* genes (Masepohl and Klipp, 1996; Kim and Rees, 1994). The structural compositon of nitrogenase was elucidated by Dean and Jacobson (1992) as a two-component metalloenzyme consisting of a) dinitrogenase reductase, the iron protein and b) dinitrogenase which has a metal cofactor. Based on the metal cofactor three different nitrogen fixing systems have been discovered - Mo-nitrogenase, V-nitrogenase and Fe-only nitrogenase (Masepohl and Klipp, 1996). The existence of this nitrogen fixing system differs from one bacterium to another based on the conditions of growth (Bishop and Jorerger, 1990).

One of the best studied diazotrophs for nitrogen fixation is *Azospirillum* sp. isolated from nitrogen-poor soils by Beijernick in 1925 (Holguin *et al*. 1999). Members of this bacterial genus are capable of fixing atmospheric nitrogen and of promoting plant growth. A mixed inoculum of *Staphylococcous* and *Azospirillum* promoted the nitrogen

fixing activity of *Azospirillum* (Holguin and Bashan 1997). Combined inoculation of *A. brasilense* with *Pseudomonas striata* significantly increased grain yield, nitrogen and phosphorus uptake of sorghum (Alagawadi and Gaur, 1992). Oliveira *et al*. (1997) demonstrated that co-inoculation of clover with a mixture of *A. brasilense* Sp7 and *Rhizobium* sp. resulted in increased acetylene reduction, nodulation and shoot dry weight of plants.

Endophytic bacteria that colonize the interiors of plant tissues such as roots, stem and leaves and are able to fix nitrogen are also found to be beneficial for plant growth (James and Olivares, 1997). Some examples of endophytic diazotrophs of rice, maize and sugarcane are *Azotobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Azoarcus* spp., *Enterobacter asburiae* and some strains of *Burkholderia* spp. which serve as nitrogen fixers when other available sources of nitrogen are absent or at low levels (Reinhold *et al*. 1986, Döbereniner *et al*. 1993; Kirchhof *et al*. 1997).

Bacterial strains capable of reducing acetylene to ethylene were isolated from various native plants grown in the Canadian High Arctic by Lifshitz *et al*. (1987). All the strains belonged to the genus *Pseudomonas* and also demonstrated the ability to colonize roots of canola cv. Tobin under field conditions. However, the amount of nitrogen fixed by these bacteria was minimal and the positive plant growth response observed may be due to other factors such as phytohormone production and enhanced mineral uptake (James and Olivares, 1997).

2.2.2 Phytohormones

One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones (Glick 1995). Frankenberger

and Arshad, (1995) have discussed in detail the role of auxins, cytokinins, gibberellins, ethylene and absicisic acids (ABA) which, when applied to plants, help in increasing plant yield and growth. Microbial production of individual phytohormones such as auxins and cytokinins has been reviewed by various authors over the last 20 years (Pilet *et al*. 1979, Hartmann *et al*. 1983, Fallik and Okon 1989, Barbieri and Galli 1993, Patten and Glick 1996, Patten and Glick 2002).

2.2.2.1 Auxins

The production of an active substance by the fungi *Rhizopus suinus* and *Absidia ramosa* was identified to be auxin by Thimann (1935), following induction of curvature in *Avena* as demonstrated by Nielsen (Frankenberger and Arshad 1995). Various authors have identified the production of indole-3-acetic acid by microorganisms in the presence of the precursor tryptophan or peptone.

Some of the plant responses to auxin are as follows: a) cell enlargement b) cell division c) root initiation d) root growth inhibition e) increased growth rate f) phototropism g) geotropism h) apical dominance (Frankenberger and Arshad 1995).

Eighty percent of microorganisms isolated from the rhizosphere of various crops have the ability to produce auxins as secondary metabolites (Kampert *et al*. 1975; Loper and Schroth, 1986). Bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas*, and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus* and *Bradyrhizobium japonicum* have been shown to produce auxins which help in stimulating plant growth (Patten and Glick, 1996). Various metabolic pathways such as a) indole-3-acetamide pathway b) indole-3-pyruvic acid

pathway c) tryptophan side chain pathway d) tryptamine pathway and e) indole-3-acetonitrile pathway are involved in the production of IAA.

Phytopathogens such as *Agrobacterium tumefaciens*, *A. rhizogenes* and *P. syringae pv. savastanoi* synthesize IAA via the indole-3-acetamide pathway (Liu *et al*. 1982; Offringa *et al*. 1986). Koga *et al*. (1991b) suggested that *E. cloacae*, isolated from the rhizosphere of cucumber, synthesized IAA via the indolepyruvic acid pathway and promoted growth of various agricultural plants. *P. fluorescens* demonstrated the ability to convert L-tryptophan directly into indole-3-acetaldehyde (Narumiya *et al*. 1979). Strains such as *B. cereus* and *A. brasilense* produced IAA by the tryptamine pathway (Perley and Stowe, 1966; Hartmann *et al*. 1983).

Bacterial production of IAA suggests that the pathways involved in IAA production may play an important role in defining the effect of the bacterium on the plant (Patten and Glick 1996). Glick *et al* (1999) reported that most of the pathogenic strains of bacteria synthesized IAA via the indoleacetamide pathway while plants use the indolepyruvic acid pathway. This helps the bacteria to evade plant regulatory signals and thus the IAA produced induces uncontrolled growth in plant tissues. In contrast the benefical bacteria such as PGPR synthesize IAA via the indolepyruvic acid pathway and the IAA secreted is thought to be strictly regulated by the plant regulatory signals.

Differences in the production of IAA among bacterial strains can be attributed to the various biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes to convert active free IAA into conjugated forms. It is also dependent on environmental conditions (Patten and Glick 1996).

In 1979 Tien *et al* demonstrated that production of indole acetic acid and indole lactic acid by *Azospirillum brasilense* Sp13t SR2 increased with increasing

concentrations of tyrptophan (1 - 100 µg/ml). In contrast the production of indole acetic acid and indole-3-butyric acid by cultures of *A. brasilense* in the absence of tryptophan was identified using gas liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS) by Fallik and Okon (1989). Synthesis of IAA by *Rhizobium* spp. in the presence and absence of tryptophan has also been demonstrated (Kittel *et al*. 1989). Plant growth-promoting rhizobacteria strain G20-18 and two mutants produced IAA in pure culture (Salamone 2000). Bent *et al*. (2001) reported that the production of indole compounds by three different strains, *Paenibacillus polymyxa* L6, *P. poly*myxa Pw-2, and *Pseudomonas fluorescens* M20 increased in concentration with increasing concentrations of tryptophan (0-200 mg/ml) at different times. Reports by Asghar *et al*. (2002) showed that PGPR strains produced 24.6 µg/ml of auxins in the presence of the precursor L-tryptophan in the medium, which was 184-fold more than that without L-tryptophan.

Hartmann *et al*. (1983) showed that *Azospirillum brasiliense* Sp Cd resistant to 5-flurotryptophan excreted 30 times higher levels of auxins compared to the wild type. Mutants grown under nitrogen fixing conditions excreted 1 µg/ml of IAA, 10 times more than the wild type. There was no difference in levels of auxin production between the mutant and wild type strain of *A. lipoferum,* indicating differences in the tryptophan and IAA biosynthetic pathway between the two species (Hartmann *et al*. 1983). Following the identification of an efficient PGPR strain Xie *et al*. (1996) generated a mutant strain of *P. putida* GR12-2 that produced four times more indole acetic acid than the wild type in the presence of 0.1 mg/ml L-tryptophan. Recent studies by Patten and Glick (2002) showed IAA production by the wild-type and an IAA-deficient mutant of *P. putida*

GR12-2 in the presence of varying concentrations of tryptophan. The levels of IAA secreted by the wild type ranged from 0.5 – 32.7 µg/ml, while for the mutant it ranged from 0.5 – 2 µg/ml. Similar results were reported by Asghar *et al*. (2002) who found that PGPR produced 24.6 µg/ml of auxins in the presence of the precursor L-tryptophan, which was 184-fold more than that without L-tryptophan.

2.2.2.2 Cytokinins

Cytokinins are N^6-substituted aminopuriunes which, when applied to plants, influence their physiological and developmental processes (Salisbury and Ross, 1992). The history of cytokinins dates back to 1955 with the discovery of kinetin, a non-natural cytokinin by Miller *et al*. (1955) and the identification of a natural cytokinin, zeatin by Letham in 1963 (Frankenberger and Arshad, 1995). It has been reported that cytokinins produced by nonpathogenic microorganisms that live in close proximity to the roots also may influence plant growth and development (Arshad and Frankenberger, 1993).

Cytokinins represent another class of phytohormones produced by microorganisms (Cartieaux *et al*. 2003). There are fewer studies on cytokinin synthesis by microorganinsms than on microbial biosynthesis of auxins (Frankenberger and Arshad 1995). One of the common biosynthetic pathways utilizes adenine as the precursor of free cytokinin production in microorganisms such as *Corynebacterium fascians*, *Azotobacter* spp. and *Rhizopogon roseolus* (Murai *et al*. 1980; Nieto and Frankenberger, 1989; Miura and Miller, 1969).

Plant responses to exogenous applications of cytokinin result in either one of the following effects a) enhanced cell division b) enhanced root development c) enhanced

root hair formation d) inhibition of root elongation e) shoot initiation and certain other physiological responses (Frankenberger and Arshad, 1995).

Barea *et al*. (1976) reported that 90% of bacteria isolated from the rhizosphere of various crop plants demonstrated the ability to produce cytokinin-like compounds. Similarly Kampert and Strzelczyk (1980) showed that bacteria isolated from roots of pine seedlings secreted cytokinin-like substances in the growth medium. Early work by Tien *et al*. (1979) showed that a strain of *Azospirillum brasilense* had the ability to produce three cytokinin-like substances equivalent to about 0.001 µg of kinetin per ml. In 1991 Taller and Sturtevant identified the production of at least two cytokinin-active substances by rhizobial strains using a tobacco callus assay, HPLC and an enzyme-linked immunosorbent assay. The cytokinins produced ranged from 1 to several µg of kinetin equivalents and differed with the addition of the precursor adenine, soy seed extract and flavonoid inducers to the culture medium. The type of cytokinins produced was mostly zeatin. Upadhaya *et al*. (1991) reported the production of isopentenyladenine (iP) and zeatin (Z) in substantial amounts by two *Rhizobium* strains ANU 240 and IC 3342. Involvement of zeatin compounds in *Rhizobium* nodule formation was studied by Sturtevant and Taller, (1989).

Cytokinin production by *A. brasilense*, *Azotobacter chroococcum* and *A. vinelandii* was identified by Horemans *et al*. (1986), Nieto and Frankenberger (1989) and Taller and Wong (1989). Addition of precursors such as adenine and isopentyl alcohol to the culture medium of *A. chroococcum* resulted in optimal synthesis of cytokinins such as Z, iP and diH[9r]Z and resulted in enhanced growth of *Raphanus sativus* under gnotobiotic and green house conditions (Nieto and Frankenberger 1989).

Similar results were observed when *A. chroococcum* was applied to *Zea mays* L. and increased plant growth compared to the non-inoculated control (Nieto and Frankenberger, 1991). Mixed cultures of *Azospirillum brasilense* and *Arthrobacter giacomelloi* produced higher level of cytokinins than single cultures (Cacciari *et al*. 1989).

Timmusk *et al*. (1999) reported the production of cytokinins by a free-living soil bacterium *P. polymyxa* using immunoaffinity chromatography (IAC). The cytokinins were further characterized using HPLC with on-line ultraviolet detection and GC-MS which resulted in the identification of iP in the culture medium. Recent studies by Salamone *et al*. (2001) have shown higher production of the cytokinins isopentenyl adenosine (IPA), zeatin riboside (ZR) and dihydroxyzeatin riboside (DHZR) by the wild type strain *P. fluorescens* G20-18 compared to two mutants, CNT1 and CNT2. Further it was noted that addition of the precursor adenine increased cytokinin production in cultures of G20-18. Previous studies by Salamone (2000) have also shown that the ability of mutant strains CNT1 and CNT2 to promote the growth of radish plants was impaired compared to G20-18.

2.2.3 Lowering of ethylene concentration, phosphate solubilization and siderophore production

Apart from production of phytohormones various other direct mechanisms of action have been associated with PGPR for enhancing plant growth. These include a) lowering of ethylene concentration by production of ACC deaminase, b) phosphate solubilization and c) production of siderophores (Glick 1995).

Honma and Shimomura (1978) isolated microorganisms capable of utilizing 1-aminocyclopropane-1-carboxylate (ACC) from the soil. The metabolism of ACC by a bacterium belonging to the genus *Pseudomonas* was identified due to the presence of the enzyme ACC deaminase. In 1991 Klee *et al*. reported that some soil pseudomonads possess the enzyme ACC deaminase which hydrolyzes ACC, the immediate precursor of ethylene, to ammonia and α-ketobutyrate. Strain GR12-2, a plant growth promoting rhizobacterium, which stimulates the growth of canola under gnotobiotic conditions (Lifshitz *et al*. 1986), was found to carry the functional ACC deaminase gene (Glick *et al*. 1994a). Further, in order to ascertain whether the enzyme plays a role in gnotobiotic stimulation of canola seedling root elongation, the bacterium *P. putida* GR12-2 was chemically mutagenized (Glick *et al*. 1994b). None of the ACC deaminase mutants was able to promote the elongation of canola seedling roots under gnotobiotic conditions when compared to the wild-type strain.

Jacobson *et al*. (1994) characterized the enzyme ACC deaminase as a trimer with a molecular mass of 105 kDa. The enzyme activity was found in the cytoplasm of the bacterium and the enzyme had a temperature optimum of 30ºC and a pH optimum of 8.5. Based on the presence of the enzyme ACC deaminase Glick *et al*. (1995) described a novel procedure for rapid isolation of PGPR which have the ability to utilize ACC as sole nitrogen source.

Glick *et al*. (1997) proposed a model for lowering of plant ethylene concentrations by plant growth-promoting rhizobacteria based on the presence of the enzyme ACC deaminase. The model suggests that uptake and subsequent hydrolysis of ACC by the PGPR decreases the quantity of ACC outside the plant, which must exude higher amounts of ACC to maintain a balance between the internal and external ACC levels.

Lowering of ACC concentrations within the plant also reduces the amount of ethylene produced in the plant, decreasing the inhibitory effect of ethylene on root elongation and leading to longer roots (Patten and Glick 1996). In order to test the model canola seeds were imbibed in the presence of a chemical ethylene inhibitor, various PGPR and a strain with ACC deaminase (Penrose *et al*. 2001). The results demonstrated that, in the presence of the ethylene inhibitor or ACC deaminase-expressing strains, the growth of canola seedling roots was enhanced and the levels of ACC in the roots were lowered. The bacterium utilizes ACC as a nitrogen source, thereby lowering the internal plant ethylene concentration, which leads to increased root elongation (Cartieaux *et al*. 2003)

Bacteria isolated from the rhizosphere are capable of increasing availability of phosphorus to plants either by mineralization of organic phosphate or by solubilization of inorganic phosphate by production of acids (Lifshitz *et al*. 1987). These bacteria are referred to as phosphobacteria and have been considered to have potential use as inoculants. Barber *et al*. (1976) showed that mixed populations of rhizosphere bacteria enhanced uptake of phosphate by young barley seedlings, while causing a decrease in the uptake of phosphorus in older plants. The increase in dry weight was directly correlated to phosphorus uptake by barley seedlings. A similar result was seen by Lifshitz *et al*. (1987). When 1mM phosphorus was added to the growth medium, root elongation of inoculated and non-inoculated canola seedlings in sterile growth pouches was stimulated. The combined effects of bacterial inoculation (*P. putida* GR12-2) and addition of phosphate on root and shoot elongation and on root and shoot weight were significant. Seed inoculation with *P. putida* GR12-2 increased the uptake of labeled phosphorus (^{32}P) and also enhanced shoot elongation of seedlings grown in sterile soil. Kucey *et al*. (1989) reviewed microbially-mediated increases in plant-available

phosphorus. Cattelan *et al*. (1999) performed an *in vitro* screening of 116 isolates obtained from soil for various PGPR traits including the ability of bacteria to solubilize phosphorus. The study indicated that isolates, which have the ability to produce ACC deaminase or siderophores or those able to solubilize phosphorus, might increase early soybean growth in nonsterile soil.

Siderophores are low-molecular weight iron-binding molecules that are synthesized by many microorganisms under low-iron conditions (Neilands, 1981a). Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots (Kloepper *et al*. 1980). Marschner and Römheld (1994) reported that plants may also utilize siderophores synthesized by microorganisms colonizing the rhizosphere; this would be a source of soluble iron for the host plant. Plants such as sorghum, oats, peanut, cotton, cucumber and sunflower demonstrated the ability to use radiolabelled microbial siderophores as a sole source of iron (Crowley *et al*. 1988; Bar-ness *et al*. 1991; Cline *et al*. 1984; Jurkevitch *et al*. 1986; Wang *et al*. 1993). Growth of cucumber in the presence of microbial siderophores resulted in increased plant biomass and chlorophyll content (Ismande 1998). Uptake of microbial siderophores by plants has been attributed to microorganisms living within plant tissues or closely associated with the plant surface (Bar-ness *et al*. 1992).

One of the suggested modes of growth promotion of nodulated legumes under field conditions is by microbial production of siderophores, which facilitate the uptake of iron from the environment (Kloepper *et al*. 1980, Schippers *et al*. 1987). A nodulated legume has been found to have an increased demand for iron compared to that of a non-nodulated plant (Deryto and Skorupska, 1992*). Pseudomonas* sp. strain 267 enhanced symbiotic nitrogen fixation in clover under gnotobitoic conditions, produced fluorescent

siderophores under low-iron conditions and secreted B group vitamins (Kozaczuk *et al*. 1996). Tn5 insertion mutants of strain 267 defective in siderophore production did not differ from the wild-type in promoting the growth of clover, suggesting that the siderophore production had no effect on stimulating nodulation. In contrast Gill *et al*. (1991) demonstrated that mutants of *Rhizobium melioti* that were unable to produce siderophores were able to nodulate the plants, but the efficiency of nitrogen fixation was less compared to the wild-type, indicating the importance of iron in nitrogen fixation. *Kluyvera ascorbata*, a siderophore-producing PGPR, was able to protect plants from heavy metal toxicity (Burd *et al*. 1998).

2.3 Effect of PGPR on plant growth

The physiological effect of microbial IAA on plant growth depends ultimately on the amount of hormone that is available to the plant, which is based on the interaction between the plant and the bacterium (Patten and Glick 1996). Three general types of possible association between the plant and the bacterium are important in order to exert a positive effect. These are a) transfer of IAA genes directly into the host genome as is the case in *Agrobacterium* species b) infection of internal regions of the plant and secretion of IAA into the surrounding tissue and c) colonization of the external surface and secretion of IAA as an exogenous source to plants. In the case of beneficial rhizobacteria the effect is primarily thought to be advantageous when the bacteria are colonizing the external surface of the plant (Del Gallo and Fendrik 1994).

Early work showed that PGPR such as *Azotobacter paspali* secreted IAA into culture media and significantly increased the dry weight of leaves and roots of several plant species following root treatment (Barea and Brown 1974). *Azospirillum brasilense*,

which had the ability to produce plant growth-promoting substances such as indole acetic acid, indole lactic acid, gibberellin and cytokinin when applied to pearl millet (*Pennisetum americanum* L.), increased the number of lateral roots which were densely covered by root hairs (Tien *et al*. 1979)

Experiments with pure plant hormones showed that gibberellin increased production of lateral roots and cytokinins stimulated root hair formation, but reduced lateral root formation and main root elongation. A combination of all three phytohormones was found to produce changes in the root morphology of pearl millet similar to those produced when inoculated with *A. brasilense* (Tien *et al*. 1979).

When *P. putida* GR12-2, a nitrogen fixing strain isolated from the rhizosphere of an arctic grass on Ellesmere Island, was applied to seeds of canola (*Brassica campestris*) it increased root length in sterile growth pouches (Lifshitz *et al*. 1986). Barbieri *et al*. (1986) found that inoculation of wheat seedlings with *A. brasilense* increased the number and length of lateral roots. In 1989 Fallik and Okon showed that when maize seedlings were inoculated with 10^7 cfu of *A. brasilense,* root surface area was significantly increased two weeks after sowing compared to that of the control. The increase in root surface area was attributed to the synthesis of IAA, identified in culture medium using GLC and GC-MS, in the range of 32-40 ng/ml. Roots of maize seedling inoculated with *A. brasilense* also were found to have higher amounts of free and bound IAA as compared to the control. Inoculation of seedling hypocotyls and roots of canola, wheat, tomato and sunflower with *A. paspali* altered plant growth and development and significantly increased shoot and root weight and root surface area (Abbas and Okon 1993). The results also indicated that plant growth promotion was dependent on the inoculum concentration.

The ability of *Pseudomonas fluorescens* M.3.1 to produce auxins in the presence of maize root exudates in the culture medium indicated that the strain can convert exudates into secondary metabolites which can play an important role in plant development and yield. (Benizri *et al*. 1998). Twelve weeks after inoculation with *P. polymyxa* L6, Pw-2 and *Pseudomonas fluorescens* M 20 IAA was identified in lodgepole pine root tissues (Bent *et al*. 2001). Similarly, the production of IAA in the rhizosphere of wheat and radish inoculated with PGPR strains G20-18, and the mutants CNT1 and CNT2 has been reported by Salamone (2000). Rhizobacteria isolated from the rhizosphere of *Brassica* species were found to produce auxins in the presence of L-tryptophan (Asghar *et al*. 2002). Seed inoculation with different rhizobacteria significantly increased plant height, number of branches, number of pods per plant, grain yield and oil content. A highly significant correlation between L-tryptophan-derived auxin production by plant growth-promoting rhizobacteria S54 *in vitro* and grain yield, number of pods and number of branches per plant was also demonstrated.

The importance of the concentration of IAA in promoting root development was demonstrated by Barbieri and Galli (1993) by inoculation of wheat seedlings with *A. brasilense* SpM7918. This strain produced 70% less IAA and showed reduced ability to promote root system development compared to the wild-type strain, Sp6. In another case strain GR12-2 which produces low levels of IAA (Xie *et al*. 1996) was found to increase root length of canola seedlings two to three fold (Glick 1995) when compared to the mutant GR12-2/*aux1*, which overproduced IAA and significantly inhibited the growth of canola roots (Xie *et al*. 1996). Dubeikoysky *et al*. (1993) inoculated two different species of plants with a recombinant strain of *Pseudomonas fluorescens* that produced high levels of IAA and found that the root development of black current

cuttings was stimulated while root development of sour cherry cuttings was suppressed, suggesting that the concentration of IAA produced was optimal for black current and inhibitory for sour cherry.

It should be emphasized that phytohormones such as auxin do not act alone, but may interact with other known phytohormones (Barendse and Peeters 1995). Addition of cytokinin with auxin was found to stimulate mature root cells to undergo polyploid mitosis, a characteristic of early nodule development (Frankenberger and Arshad 1995).

Few studies have been published on microbial production of cytokinins. In 1980 Kampert and Strzelczyk reported that seven of ten strains of coryneform bacteria isolated from roots of pine seedlings released compounds in culture medium which exhibited cytokinin-like activity. The exogenous production of cytokinins by these organisms was found to have a profound effect on plant growth. Further, the production of exogenous cytokinins was also hypothesized to supplement the plant's endogenous level or alter the plant's synthesis and metabolism of other plant regulators. Production of cytokinins by *Rhizobium* species was found to have ecological and physiological effects on nodulation (Sturtevant and Taller 1989). Exogenous application of Z and [9R] Z was shown to stimulate nitrogenase activity in root nodules of *Vigna mungo* while iP and [9R]iP had negative effects (Jaiswal *et al*. 1982). Exogenous application of cytokinins induced cortical cell division in the roots of soybean, cowpea and alfalfa (Bauer *et al*. 1985).

Apart from *Rhizobium* spp., cytokinin-producing bacteria such as *Azotobacter* and *Azospirillum* were also shown to produce cytokinin-like substances on addition of precursors to the culture medium (Nieto and Frankenberger 1989). Salmeron *et al*. (1990) reported that *A. chroococcum* isolated from the roots of maize has the ability to

produce cytokinins. Nieto and Frankenberger (1990, 1991) demonstrated that the addition of a cytokinin precursor and inoculum to radish and corn significantly increased plant yield and growth compared to the control. A similar result was observed by Salamone (2000) where inoculation of radish with strain G20-18 increased the growth and yield of the plants compared to the mutants CNT1 and CNT2, which had reduced ability to produce cytokinins IPA, DHZR and ZR. Production of cytokinins by wild-type strain G20-18 and the mutants in the rhizosphere of radish inoculated with the PGPR strains was also estimated and compared with the levels of cytokinins produced by non-inoculated roots. Bent *et al*. (2001) studied the production of DHZR in the root tissue of lodgepole pine when inoculated with *Paenibacillus polymyxa* L6, Pw-2 and *Pseudomonas fluorescens* M20. The concentration of DHZR was found to be elevated in roots inoculated with *P. fluorescens* M20 compared to that of the other two strains.

2.4 Root colonization

Successful colonization and persistence in the plant rhizosphere are required for PGPR to exert their beneficial effect on plants (Elliot and Lynch 1984). Rhizosphere colonization is also considered to be a crucial step in the application of microorganisms for beneficial purposes such as biofertilization, phytostimulation, bicontrol and phytoremediation (Lugtenberg *et al*. 2001). Root colonization, which is a complex process, is under the influence of various parameters such as bacterial traits, root exudates, biotic and abiotic factors (Benizri *et al*. 2001). The method chosen for studying the traits associated with root colonization depends on the objective and different approaches and techniques have been used to quantify and identify inoculated strains on the host plant (Lugtenberg *et al*. 2001).

The most common method utilized to identify bacterial traits associated with root colonization is to produce mutants and compare their colonization ability with that of the parental strain (Benizri *et al*. 2001). A mutant strain *P. fluorescens* WCS365 with Tn5*lacZ* mutation colonized the roots to a lesser extent than the wild-type (de Weger *et al*. 1987; Dekkers *et al*. 1998c; Simons *et al*. 1997; Kozaczuk and Skorupska, 2001). Dekkers *et al*. (1998b) showed that the gene encoding NADH dehydrogenase I plays an important role in root colonization. Another gene required for efficient colonization is the *sss* gene, encoding a site-specific recombinase of the lambda integrase family which helps in adapting cells to rhizosphere conditions (Dekkers *et al*. 1998a). Further, it was hypothesized that a two-component system involving genes *colR* and *colS* plays an important role in the root colonizing ability of *P. fluorescens* strain WCS365 (Dekkers *et al*. 1998c). A recent study by Miller *et al*. (2001) has shown that the gene *rpoS* is essential for plant root colonization by *P. putida* in a competitive environment. Rainey (1999) identified as many as twenty genes that were induced during root colonization using a novel promoter trapping technology.

Previous studies have shown that rhizobacteria are attracted to seed and root exudates by chemotaxis which might be the first step in the seed and root colonization process (Currier and Stobel, 1977; Heinrich and Hess, 1984; Scher *et al*. 1985). De Weger *et al*. (1987) observed that mutant strains devoid of flagella had reduced ability to colonize potato roots showing that motility plays an essential role in the process of root colonization. A similar approach by Bashan and Holguin (1995) led to the comparison of a non-motile *A. brasilense* mutant with the parental strain. The results indicated that the wild-type was more effective at colonizing roots near the area of inoculation

compared to the mutant. The role of chemosensory pathways and flagellar motility has been reviewed by Blair (1995).

The effect of two associative PGPR on root colonization of lupin and pea was investigated by Wiehe *et al*. (1994). The two strains colonized the lupin rhizoplane and root tip more intensively than the pea rhizoplane. The sparse colonization of root tip in the case of pea may be attributed to the fast growth of the tip and may be related to nitrogen-limitation in the root zone (Jagnow *et al*. 1991). The lack of consistency in colonization may be related to changes in exudation pattern of the root during the life cycle of the plant, leading to lower numbers of pseudomonads in the later phase of plant growth (Miller *et al*. 1989).

The effect of abiotic factors such as pH and phosphorus on root colonization has been studied by Bauske *et al*. (1997) and Chabot *et al*. (1998). The former reported the effect of botanical aromatic compounds and seed surface pH on growth and colonization of cotton plant by PGPR, while the latter described the effect of phosphorus on root colonization by phosphate-solubilzing *R. leguminosarum biovar phaseoli* and on growth promotion of maize. The effect of temperature on root colonization has been studied by various authors and the results indicated that rhizobacteria were able to colonize roots at lower soil temperatures (5° C) more effectively than at higher temperatures (25° C) (Benizri *et al*. 2001).

Immunological and molecular techniques have been used to quantify and identify inoculated strains on plant roots (Benizri *et al*. 2001). Immunological techniques such as ELISA and immunofluorescence colony staining have helped in quantification and visualization of the strain on the plant roots. Molecular techniques also aid in quantification and visualization. Tagging bacteria with marker or reporter genes

facilitates identification of the strain on the plant roots. Colorimetry is used for detection of gene products of *lacZ* and *xylE* (Benizri *et al*. 2001). Other markers, which are observed with the help of charge-couple device cameras and confocal laser scanning microscope, are the *Lux* (luciferase) and GFP (green fluorescent protein) gene products. Roberts *et al*. (1999) developed an image analysis method using the lux gene cassette for determination of the spatial colonization pattern of bacteria in the rhizosphere. Following seed application the majority of E6 bacterial populations were found on the upper regions of the cucumber tap root and they were not able to colonize other regions of the root system. The colonization of wheat roots by *A. brasilense* was studied by using fluorescently-labeled rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy (Assmus *et al*. 1995). Enhanced green fluorescent protein in conjunction with CSLM has been used in studying the pattern of root colonization by strain WCS365 on tomato roots (Lugtenberg *et al*. 2001).

Apart from marker genes the classical electron microscope has also been used in root colonization studies. In 1991 Hong *et al*. utilized a scanning electron microscope to investigate plant-microbe interactions under gnotobiotic conditions. Following treatment of canola seed with *P. putida* GR12-2 the bacteria were located in the depressions and on the surface of the seed. Seeds treated with *P. putida* GR12-2 showed the presence of bacteria 4 days after introduction into growth pouches. In another study Chin-A-Woeng *et al*. (1998) observed that bacteria were present below the mucigel layer using scanning electron microscopy (SEM).

2.5 Green fluorescent protein (GFP)

Green fluorescent protein (GFP) is a fluorescent protein obtained from the marine invertebrates *Aequorea victoria* (Pacific Northwest jellyfish) and *Renilla reniformis* (Sea pansy) (Cubitt *et al*. 1995). Studies on bioluminescent marine hydroid Obelia led to the discovery of GFP by Jim Morin (Hasting, W.J, 1996). The fluorescent protein, which emits light on addition of calcium, is refered to as aequorin (Chalfie and Kain, 1998). This primary photoprotein transmutes blue chemiluminescence into green fluorescence, which imparts the green color to *A. victoria* and *R. reniformis* (Cubitt *et al*. 1995).

Aequorin consists of 238 amino acid residues and transmutes blue chemiluminescence from a primary photoprotein into a green fluorescence with a highest absorbance peak at 395 nm and the lowest at 475 nm (Cubitt *et al*. 1995). The fluorescent protein is stable up to a temperature of 65°C, pH 11, 1% SDS, or 6M-guanidium chloride and resists the action of protease for many hours. Studies by Prasher *et al*. (1992) have demonstrated that the GFP chromophore is derived from the primary amino acid sequence through the cyclization of Ser65-Tyr66 and Gly67 within the hexapeptide. The molecular structure of GFP was elucidated by Yang *et al*. (1996). The protein was found to be in the shape of a cylinder comprised of 11 strands of β-sheet with an α-helix on the inside and short helical segments on the ends of the cylinder. Due to the unique folding of the protein the structure was named a β can. Mutants of GFP are obtained by altering one of the amino acids and most of the studies attempting to generate mutants have resulted in the partial or complete loss of fluorescence (Heim *et al*. 1994). Mutation studies incroporating Thr, Cys, Leu, Val and Ala in the place of Ser65 have generated fluorescence of GFP with six-fold greater brightness compared to wild type and the oxidation rate was fourfold faster (Heim *et al*. 1994).

The advantage of using GFP as a marker for gene expression was well demonstrated by Chalfie *et al*. (1994) where the GFP expressed in *Escherichia coli* (prokaryote) under the control of a T7 promoter and in *Caenorhabditis elegans* (eukaryote) transformed with GFP were able to produce a strong fluorescence under blue light. This indicated that the fluorescence does not require any additional gene products and that chromophore formation is not species-specific and uses ubiquitous cellular components or autocatalysis. Utilization of GFP as a molecular marker enables identification of cells under illumination of ultraviolet light and helps in continuous monitoring of reactions. Further, due to the small size of the GFP protein incorporation into cells is very easy, which provided justification for use of GFP as a molecular marker in various studies (Chalfie *et al*. 1994; Cubitt *et al*. 1995).

Due to the advantage of monitoring gene expression and protein localization in living cells, GFP has been used in monitoring the interaction between bacteria and the plant host (Chalfie and Kain, 1998). Intial studies began with the use of GFP in analyzing the symbiotic interaction between the plant host and *Rhizobium*. Studies by Gage and Margolin (2000) illustrated the use of bacteria expressing mutated *gfp* (S65T) for examining the early stages of nodule formation in *R. meloliti*. Tombolini *et al*. (1997) investigated the colonization of GFP-tagged *P. fluorescens* on the roots of Japanese lotus plant. *Pseudomonas fluorescens* was chromosomally tagged with *gfp* fused to a strong constitutive promoter present in a Tn5 delivery vector. The interaction of PGPR strains such as *Pseudomonas*, *Azospirillum* and *Rhizobium* spp. with their respective hosts was studied by using bi-functional *gfp* and *gus*A containing a mini-Tn5 transposon derivative for localization and simultaneous monitoring of marked cells (Xi *et al*. 1999). Studies by Stuurman *et al*. (2000) used GFP color variants expressed on broad host range vectors

for visualizing interaction of rhizobia with plants. Finer and Finer (2000) studied the colonization and infection of soybean cotyledons by *A. tumifaciens* tranformed with plasmid containing GFP. In a recent study GFP was used as a marker for studying the process of root colonization and infection by *Fusarium oxysporum f.sp.radicis-lycopersici* on roots of tomato (Lagopodi *et al*. 2002).

3.0 MATERIALS AND METHODS

3.1 Bacterial strains

The bacterial strains used in this study are summarized in Table 3.1. Stock cultures of PGPR strains were maintained in 20% glycerol at -70°C. All the strains were cultured in half-strength tryptic soy broth (TSB) (Difco – Becton Dickinson Microbiology Systems, Sparks, MD, USA) at room temperature for 48 h with continuous agitation at 150 rpm to an optical density of 1.39, 1.46, 1.44, 1.32, and 1.52 for strains G20-18, CNT2, GR12-2, GR12-2/*aux1* and 6-8 respectively at 600 nm.

Identification of strain 6-8 was performed by using fatty acid methyl esterase (FAME) analysis and comparing the results with other known strains in the database (Misko, 2002). The method utilizes the technique described by Paisley (1994). Strain 6-8 was grown on half-strength TSA plates and provided to the Department of Soil Science at the University of Saskatchewan for identification using FAME analysis and the assay was carried out in duplicate. Bacterial isolates were identified using MIDI Microbial Identification Software (MIS) (Sherlock TSBA Library Version 3.80, Microbial ID, Inc., Newark, DE). Isolates with similarity indices (SIM) of 0.3 or greater were considered positively identified, whereas isolates with a SIM of less than 0.3 were considered tentatively identified (Siciliano and Germida, 1999).

Table 3.1 Characteristics of bacterial strains used to study direct growth promotion of canola and lentil plants

Bacterial Strains	Genus and Species	Phenotype	Characteristics	Origin	Reference
G20-18	*Pseudomonas fluorescens*	Wild type	Cytokinins and IAA produced	Arctic grass Ellesmere Island	Salamone *et al.* (2001)
CNT2	*Pseudomonas fluorescens*	Tn*phoA* mutant of G20-18	Reduced production of cytokinins		
GR12-2	*Pseudomonas putida*	Wild type	IAA produced	Arctic grass Ellesmere Island	Lifshitz *et al*. (1986)
GR12-2/*aux1*	*Pseudomonas putida*	Tn5 mutant of GR12-2	Over producer of IAA		Xie *et al*. (1996)
6-8	*Pseudomonas putida*	Wild type		Pea rhizosphere North Battleford	Hynes and Nelson (2001)
6-8-Rif$^+$	*Pseudomonas putida*	Spontaneous rif-resistant mutant of 6-8	Rif$^+$		Present study
6-8-GFP-Rif$^+$	*Pseudomonas putida*	Strain 6-8-Rif$^+$ tagged with GFP	Km$^+$, Rif$^+$ and Gm$^+$		Present study
S-17-1(λ pir)	*Escherichia coli*	Suicide mini-transposon delivery plasmid pAG408	Km$^+$, Gm$^+$		Suarez *et al*. 1997

3.2 Plant cultivars

In order to study the direct effects of the PGPR strains on plant growth two different plant systems were selected. Seeds of canola (*Brassica napus* L. cv Smart 45A71) obtained from Dr. John Balsevich, Plant Biotechnology Institute, Saskatoon, SK, Canada and lentil (*Lens culinaris* Medik.) cv Milestone from the Crop Development Centre at University of Saskatchewan were utilized.

3.3 Gnotobiotic assay

The gnotobiotic assay was performed as described by Lifshitz *et al*. (1987). Initially the bacterial strains were cultured as described in section 3.1. The purity of the strains was checked by plating onto half-strength tryptic soy agar (TSA) ((Difco – Becton Dickinson Microbiology Systems, Sparks, MD, USA) plates and incubating at 27°C for 48 h. Seeds were surface sterilized by treatment with 95% ethanol (v/v) for 10 to 20 s, followed by soaking in 20% bleach (v/v) for 10 min in the case of canola seeds and for 20 min for lentil seeds. Seeds were washed with sterile distilled water 5-7 times in order to remove excess bleach. The seeds were then air dried by placing them in the laminar flow hood for 24 h. Surface-sterilized seeds were picked at random and placed onto half- strength TSA plates and incubated at 27°C for 24 h to further check for any contamination. Seeds were considered to be free from contamination if there was no bacterial or fungal growth on plates incubated with surface-sterilized seeds.

Seed growth pouches (GP) (Mega International, Minneapolis, MN, U.S.A) sterilized at 121°C for 15 to 20 min were filled with 10 ml of sterile half-strength N-

free Hoagland's nutrient solution (Appendix 8.2.1). A 100-fold dilution of the bacterial cells grown in half strength TSB was performed using 0.1 M $MgSO_4$. Surface-sterilized seeds were soaked in 10 ml of bacterial suspension for 10-15 min with gentle agitation. Seeds treated with bacterial suspension were aseptically sown in the growth pouches. Replicates of each treatment were performed (6 seeds per pouch and 7 pouches per treatment). The pouches were wrapped with Saran plastic wrap in order to prevent the loss of moisture and incubated at 18°C with a 16/8-h light/dark cycle in a growth cabinet (Certomat® CS-1, Bethlehem, PA, USA) for 7 d for canola and 10 d for lentil. At the end of the incubation period the pouches were opened and the root length, root dry weight and number of lateral roots were determined. The assay was repeated three times and the data obtained were analyzed.

The second set of growth pouch experiments was performed in order to determine the ability of PGPR strains to produce the phytohormones, indole-3-acetic acid (IAA), isopentenyladenosine (IPA), dihydroxyzeatin riboside (DHZR) and zeatin riboside (ZR) in the presence of canola roots. The effect of inoculation of canola with PGPR strains G20-18, CNT2, GR12-2, GR12-2/*aux1*, and 6-8 on the concentration of these phytohormones in the rhizosphere was measured. Bacterial culture conditions and inoculation of canola seeds were performed as described above. The growth pouches were filled with 25 ml of Hoagland's N-free nutrient solution and seeds treated with bacterial strains were transferred aseptically to growth pouches (6 seeds per pouch and 3 pouches per treatment). Seeds treated with 0.1 M $MgSO_4$ were considered as controls. The growth pouches were incubated in a growth cabinet with gentle shaking at 100 rpm to create aerobic conditions for growth of PGPR strains. Supernatants from growth pouches (10 ml) were obtained by centrifugation at 4000 rpm for 20 min at 4°C and

filtration using 0.22 µm membrane filters. Filtrates were stored at -70°C until further used. IAA and the cytokinins IPA, DHZR and ZR were estimated using ELISA as described in section 3.5.

3.4 Growth chamber study

Bacterial culture conditions, surface sterilization of seeds and inoculation of seeds with bacterial cultures were performed as described in section 3.2. The growth chamber study was conducted under non-sterile conditions and utilized pots and soil mixture as described by Chanway *et al*. (1989). Pots of size 10 cms X 13 cms (approximately 5 inches in diameter) were filled with Turface (montmorillonite clay, Applied Industrial Materials Corp., Deerfield, U.S.A) and Terra-Lite Redi-Earth (W.R. Grace and Co., Ontario, Canada) in a ratio of 1:1. Seeds soaked in bacterial culture were sown into the pots. Replicates were performed (5 seeds per pot and 5 pots per treatment). The pots were arranged in a completely randomized manner in the growth chamber. The plants were grown in a growth chamber with day and night temperatures of 19°C and 16°C, respectively, and with a 16/8-h light/dark cycle. Plants were watered with half strength N-free Hoagland's nutrient medium and water alternately. One week after planting seedlings were thinned down to two seeds per pot and the seedlings removed were used as the first week samples. Sampling was done every 7 d up to 28 d for canola. In the case of lentil the first sampling was performed 10 d after seed germination and subsequent samplings were done every 7 d up to 31 d. Root length, root dry weight, shoot dry weight and root surface area were determined. Root surface area was determined using the calcium nitrate method (Carley and Watson 1966). In the case of

lentil the first sampling was performed 10 d after planting. The experiment was repeated three times and the data obtained were analyzed.

3.5 Identification and quantification of phytohormones

Production of indoles by PGPR strains was assayed based on the method described by Patten and Glick (2002). Wild type (6-8, GR12-2, G20-18) and mutant strains (CNT2, GR12-2/*aux1*) were propagated in DF salts minimal medium (Appendix 8.2.3) for 48 h at room temperature. Twenty microlitres of each bacterial inoculum were transferred to 10 ml of DF salts supplemented with various concentrations of L-tryptophan (0, 50, 100, 200 and 500 µg/ml) obtained from a filter-sterilized 2-mg/ml stock prepared in warm water. After incubation for 48 h the density of the culture was measured at 600 nm and 10 ml of culture were sampled. The bacterial cells were removed by centrifugation at 4,000 rpm for 20 min at 4ºC. One millilitre of the supernatant was mixed with 4 ml of Salkowski's reagent (Appendix 8.2.2) in the ratio of 1:4 and incubated at room temperature for 20 min. The absorbance was measured at 535 nm using a colorimeter (Spectronic 20D, Rochester, NY, USA). The quantity of indoles was determined by comparison with a standard curve using IAA in the concentration range of 0-15 µg/ml.

Indole-3-acetic acid production by strains G20-18, CNT2, GR12-2, GR12-2/*aux1* and 6-8 was estimated using the ELISA technique. Supernatants from cultures grown in DF salts minimal medium amended with 0, 100 and 500 µg/ml of L-tryptophan were assayed. Three-millilitre aliquots of supernatant were methylated by adding four to five drops of 2.0 M (Trimethylsilyl) diazo-methane in diethyl ether, then samples were

vortexed at high speed for one minute and placed in a fume hood to evaporate excess ether from the samples (Nelson, K, NRC Plant Biotechnology Institute, Personal Communication).

The cytokinin phytohormones synthesized by the PGPR strains were identified and quantified as described by Salamone *et al.* (2001). Single colonies of G20-18, CNT2 and 6-8 were inoculated into 50 ml of minimal medium (Appendix 8.2.4) supplemented with glucose and cultured at room temperature on a shaker at 150 rpm. Three milliliters of the culture were drawn out at 0, 12, 24, 48, 72, 96, 168, 240 and 336 h, and centrifuged at 4,000 rpm for 20 min at 4°C. The supernatant was filter sterilized and transferred to centrifuge tubes and stored at -70°C until further used for detection of phytohormones.

The phytohormones IAA, IPA, DHZR and ZR were assayed using ELISA kits (Phytodetek, Agdia Inc, Elkhart, IN, USA). Stock solutions of the cytokinins IPA, ZR, DHZR (0.1 µmole/ml) and IAA (10 µmole/ml) were prepared in absolute methanol for IAA, IPA, ZR and N,N-dimethylformamide (DMF) for DHZR. Standard concentrations of 0.2-50 pmoles/ml (IPA, DHZR), 0.2-100 pmoles/ml (ZR) and 78-2500 pmoles/ml (IAA) were used. One hundred microlitres of standard or the sample were used for each assay.

The immunological principle of competitive antibody binding was used to measure concentrations of phytohormone in supernatants of the bacterial cultures. A competitive binding reaction is set up between a constant amount of alkaline phosphate (tracer), a limited amount of the antibody and the unknown sample containing phytohormone. The color produced on addition of substrate is inversely proportional to the amount of phytohormone in the sample. The intensity of the color was read at 405nm using an

ELISA plate reader (Packard SpectraCount^{TM}, Meriden, CT, USA) and related to phytohormone concentrations by means of a standard curve.

3.6 Detection of siderophores

Siderophore production by strains 6-8, G20-18, GR12-2 and their mutants was detected as described by Schwyn and Neilands (1987) with several modifications. The assay was performed in 96-well microtitre plates and utilized the ternary complex chrome azurol S/iron (III)/hexadecyltrimethyammonium bromide as an indicator (Appendix 8.2.6). Change in the dye color from blue to orange indicated production of siderophore. A loopfull of frozen culture was transferred to 3 ml of rhizosphere medium (Appendix 8.2.5) and the strains were cultured for 48 h at room temperature with shaking at 150 rpm. Seventy-five microlitres of the culture, 75 µl of CAS and 30 µl of RSM were added to each well and mixed with gentle tapping. The entire plate was incubated at room temperature for 30 min. Appearance of an orange color in the wells was scored as positive for siderophore production. Two replications of each strain were used and the experiment was performed twice. *Pseudomonas syringae* R55 and strain 6-10 from the culture collection were used as positive and negative controls, respectively.

3.7 Solubilization of inorganic phosphate

Bacterial strains were evaluated for their ability to solublize inorganic phosphate. Agar medium containing calcium phosphate as the inorganic form of phosphate was utilized in this assay (Yang, G., Becker Underwood, Personal Communication, Appendix 8.2.7). Bacterial strains were cultured as described in section 3.1. A loopfull of each culture was placed on the plates; five per plate, and the plates were incubated at

27°C for 7 d. A zone of clearing around the colonies after 7 d was scored as positive for phosphate solubilization. Strain 2-9 was used as a positive control. The experiment was performed twice with five replicates for each bacterial strain.

3.8 Characterization of PGPR strains for ability to use ACC as sole nitrogen source

The ability of PGPR strains to utilize ACC as sole nitrogen source was assayed as described by Glick *et al*. (1995) with some modifications. The assay was performed in 96-well microtitre plates. All PGPR strains were grown as described in section 3.1 and a ten-fold dilution was performed in 0.1M $MgSO_4$. Stock solutions of ammonium sulphate, 0.1M magnesium sulphate and DF salts minimal medium (Appendix 8.2.8) were prepared and sterilized by autoclaving. A 3.0 mM solution of ACC (Appendix 8.2.8) as the source of nitrogen was also prepared and filters sterilized as ACC is heat labile. One hundred and fifty microlitres of DF salts minimal medium and 30 µl of each bacterial culture were added to the wells in microtitre plate. Twenty microlitres of ACC, $(NH_4)_2SO_4$ or 0.1M $MgSO_4$ were added to the wells. The plates were incubated at 27°C for 120 h. Growth of strains in each well supplemented with ACC, $(NH_4)_2SO_4$ or 0.1M $MgSO_4$ was observed at 0, 24, 48, 72, 96 and 120 h by measuring the optical density at 405nm. Strain GR12-2, previously shown to utilize ACC as sole nitrogen source (Glick *et al*. 1995), served as the positive control.

3.9 Root colonization of canola and lentil plants by strain 6-8

3.9.1 Bacterial strains and culture conditions

Strains 6-8-Rif^{+} and *Escherichia coli* S-17 (λ pir) (Suarez *et al*. 1997), harboring the mini-transposon suicide delivery plasmid pAG408 which contains the green fluorescent protein (gfp), were propagated on Luria Bertani medium (Appendix 8.2.9) for 24 h at room temperature with appropriate concentrations of antibiotics (100 µg/ml of rifampicin for spontaneous rif-resistant strain of 6-8, 50 µg/ml of kanamycin and 30 µg/ml of gentamycin for *E.coli* S-17 λ pir). Spontaneous rif-resistant strains were obtained by initially streaking wild type strain 6-8 onto half-strength TSA wedge plates amended with a concentration gradient of rifampicin from 0-100 µg/ml and incubating at 27 º C for 72 h. Rifampicin mutant colonies of strain 6-8 were restreaked on to TSA plates ammended with 100 µg/ml of rifampcin inorder to check for antibitotic resistance. No differences were found between strain 6-8-rif^{+} and the wild-type strain 6-8 with respect to growth characterisitics in pure culture in half-strength TSB , production of siderophores and ability to solubilize inorganic phosphate.

3.9.2 Transfer of *gfp* gene present in pAG408 into strain 6-8 by conjugation

Transfer of the *gfp* gene from plasmid pAG408 into strain 6-8 was performed as described by Goldberg and Ohman (1984) and D. Korber (University of Saskatchewan, personal communication). The recipient (strain 6-8) and the donor *(E.coli* S-17 λ pir) were grown as described in section 3.9.1. One millilitre of each culture was centrifuged

at 10,000 rpm for 20 min at 4ºC. The supernatant was removed and the bacterial pellets were washed twice in 1 ml of 0.9% sodium chloride (NaCl) and finally re-suspended in 50 µl of NaCl. Fifty microlitres of the recipient were transferred to the donor and mixed vigorously by vortexing. One hundred microlitres of the culture were spotted on 0.22 µm sterile membrane filters on the surface of LB agar plates. The plates were incubated at 27ºC for a period of 24 h to allow bacterial conjugation. Following incubation the membrane filter from the plates was removed and washed in 1mL of NaCl to remove the cells adhering onto the membrane. One hundred microlitres of the culture were plated directly onto LB agar plates supplemented with appropriate concentrations of antibiotics (100 µg/ml rifampicin, 50 µg/ml kanamycin and 30 µg/ml gentamycin) for selective isolation. The plates were incubated at 27ºC for 48 h. After incubation colonies were screened for green fluorescence with a 366 nm UV lamp. Positive GFP transconjugants were transferred to selective citrate agar plates (Appendix 8.2.10) supplemented with citrate as carbon source for selective isolation as *E.coli* lacks the ability to utilize citrate. Transconjugants (6-8-GFP-Rif^{+}) and the mutant (6-8-Rif^{+}) were checked for their growth rates and ability to produce siderophores (as described in section 3.6), a trait that was positive in the wild type strain 6-8. Positive strains were further used for root colonization studies.

3.9.3 Gnotobiotic root colonization studies

Strain 6-8-GFP-Rif^{+} was cultured on half-strength TSB for 48 h at room temperature. Seeds of canola cv. Smart and lentil cv. Milestone were surface sterilized and inoculated with strain 6-8-GFP-Rif^{+} as described in section 3.3. Seeds treated with 0.1M $MgSO_4$ served as a control. The seeds were transferred to sterilized growth

pouches with half-strength Hoagland's N-free nutrient solution as described in section 3.3. The growth pouches (4 seeds per growth pouch and 4 growth pouches for each harvest) were incubated in a growth cabinet at 18ºC with a 16/8-h light/dark cycle. Growth pouches were harvested at 5, 7 and 9 days for canola and 3, 6 and 9 days for lentil. The pattern of root colonization by strain 6-8 at each time interval was observed on three different roots at different locations on the root (top - adjacent to the seed, mid region and region near the root tip) by using confocal laser scanning microscopy.

A second set of experiments was performed in order to enumerate the number of colony forming units (CFU) of bacteria colonizing the roots of canola and lentil using the dilution plating technique. Roots obtained at three different time intervals were divided into the three segments as indicated earlier and suspended in 10 ml of 0.1M $MgSO_4$. Each root segment was vortexed vigorously for 30 s in order to remove bacteria adhering to the root surface. Ten-fold dilutions were performed and plated onto half-strength TSA and incubated at 27ºC for 48 h in order to determine the CFU per segment of the root. The initial density of bacteria present on the seeds at the time of inoculation was also calculated for canola and lentil by dilution plating.

3.9.4 Confocal laser scanning microscopy (CLSM)

An MRC 600 Lasersharp system (Bio-Rad Microsciences, Toronto, Ontario Canada) equipped with an Ar ion laser source (excitation wavelength, 488 and 514 nm) and mounted on a Nikon model FXA microscope equipped with a 40 X objective (0.55 numerical aperture) was used to obtain optical images of strain 6-8-GFP on root surfaces of canola and lentil. Monochrome sequences of images were taken along the optical Z-axis with increments of 5 µm. Three different locations on the root were analyzed

starting from the top region near to the seed, middle segment of the root and the root tip and five different random scans were performed on each segment of the root. At each location a triplicate analysis was performed with five scans for each replicate. The total number of bacteria present in each root segment was calculated manually by counting the number of fluorescent bacteria identified in the image analysis.

3.10 Statistical analyses

Gnotobiotic assay and growth chamber data were tested for homogeneity of variance, using Bartlett's test prior to performing analysis of variance. Analysis of variance was performed in a one way randomized block and one way randomized design for gnotobiotic assays or two way randomized block and two way randomized design for growth chamber studies of canola and lentil. The means were compared using an LSD test ($p=0.05$) to detect significant differences among treatments. CoStat^{R} 6.204 (CoHort Software, Monterey, CA, 93940, U.S.A.) was used for statistical analysis. Blocks represented the number of trials of each experiment (3 trials).

4.0 RESULTS

4.1 Strain identification

Strain 6-8 was isolated from the rhizosphere of pea obtained from North Battleford, Saskatchewan as part of a larger study to isolate and characterize plant growth-promoting rhizobacteria from local soils (Hynes and Nelson 2001). Initial screening demonstrated that strain 6-8 produced siderophores, used ACC as sole nitrogen source, grew at 10 and 15^{O}C, increased root length of canola grown under gnotobiotic conditions, did not suppress the growth of *Pythium, Rhizoctonia* or *Fusarium* on *in vitro* plate assays and did not produce indoles. The identification of strain 6-8 was performed using FAME analysis and compared with known strains in the database using percentage of similarity indices (SIM). Duplicates of strain 6-8 were analyzed, confirming that the highest SIM value (0.87) was with *P. putida* biotype A.

4.2 Gnotobiotic studies

4.2.1 Canola gnotobiotic study

The ability of strains G20-18, CNT2, GR12-2, GR12-2/*aux1* and 6-8 to promote the growth of canola cv. Smart in sterile growth pouches was studied. The experiment was repeated three times. There was a significant effect of trials when the data were combined for statistical analysis (Appendix Table 8.1.1). Therefore, the data could not

be analyzed together. Each trial was analyzed individually and the treatments were compared within each trial (Appendix Table 8.1.2). There was a significant effect of

inoculation on root length and number of lateral roots in two out of three trials. There was no significant effect of inoculation on root dry weight in any of the three trials.

Strains G20-18, CNT2 and 6-8 significantly increased root length compared to the control during the first trial (Fig 4.1). Plants inoculated with strains G20-18 and 6-8 also had a greater root length than those inoculated with the mutant GR12-2/*aux1*. There was no effect of the PGPR strains on root length during the second trial. In the third trial strains GR12-2, GR12-2/*aux1* and 6-8 increased root length compared to the mutant CNT2, but not the control.

There was no significant positive effect on number of lateral roots by any of the PGPR strains when compared to the control in all three trials (Fig 4.2). Strains GR12-2 and 6-8 decreased the number of lateral roots compared to the control in trials one and two, respectively. In the third trial strains G20-18, CNT2, GR12-2, GR12-2/*aux1* and 6-8 decreased the number of lateral roots compared to the control.

4.2.2 Lentil gnotobiotic study

A similar study was also performed with lentil cv. Milestone to evaluate the effect of PGPR strains on promoting the growth of lentil under gnotobiotic conditions. There was a significant effect of trials when all three trials were combined for statistical analysis (Appendix Table 8.1.3). Therefore data obtained for each trial were analyzed separately (Appendix Table 8.1.4). There was a significant effect of inoculation on root length and root dry weight in one out of three trials. In the case of lateral root formation the effect of inoculation was significant in all three trials.

The root length of lentil was significantly increased by inoculation with strain GR12-2/*aux1* compared to that of strain 6-8 but not that of the control during the first

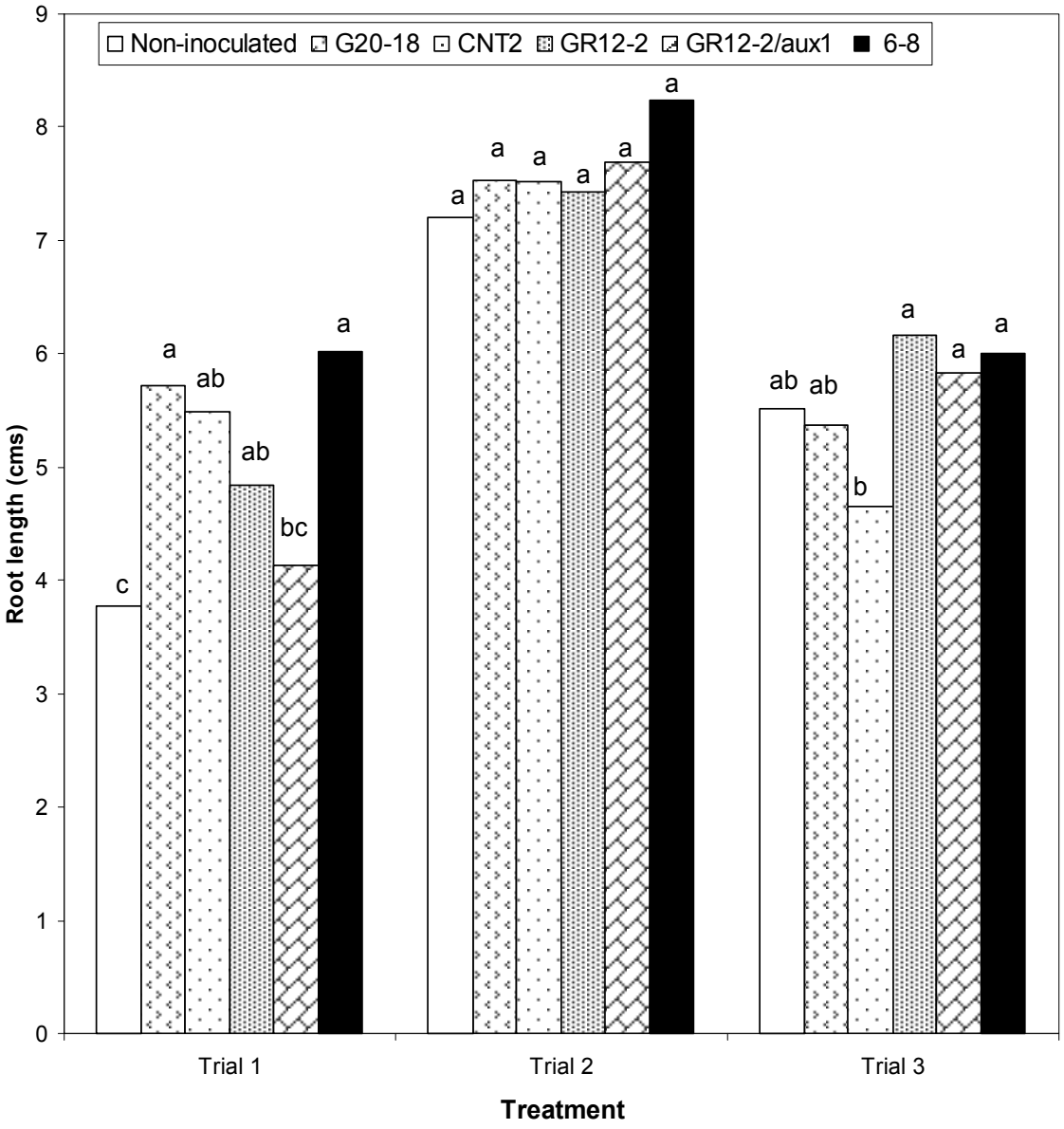


Figure 4.1 Effect of PGPR strains on the root length of canola grown under gnotobiotic conditions. Bars are means of each treatment with seven replicates per treatment. Bars with the same letters for a parameter within an experiment indicate no significant differences between means as determined by LSD test (P = 0.05).

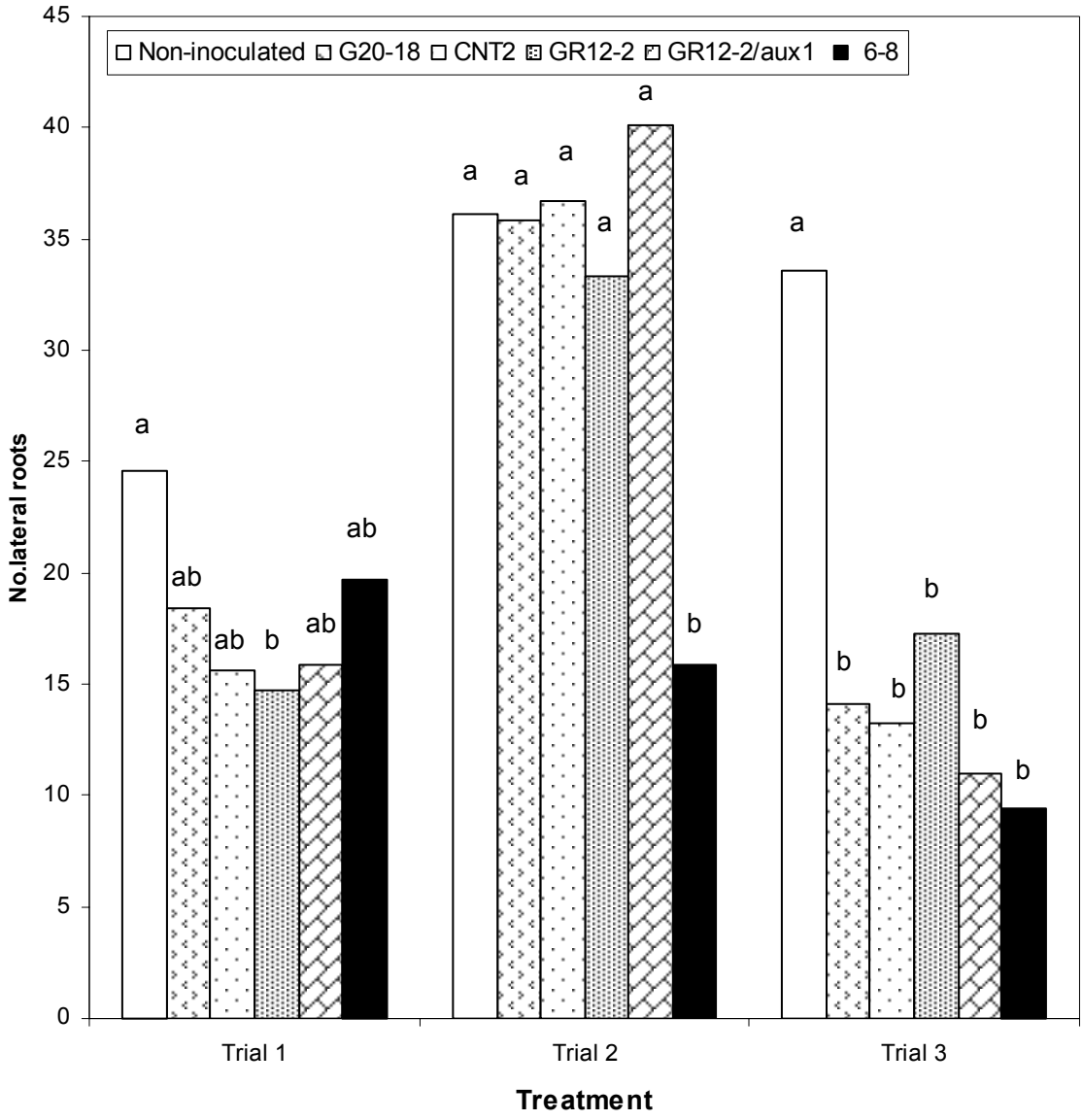


Figure 4.2 Effect of PGPR strains on lateral root formation of canola grown under gnotobiotic conditions. Bars are means of each treatment with seven replicates per treatment. Bars with the same letters for a parameter within an experiment indicate no significant differences between means as determined by LSD test (P = 0.05).

trial (Fig 4.3). In the second trial there was no significant effect of any of the PGPR strains on root length. Strains GR12-2 and GR12-2/*aux1* increased the root length of lentil compared to the control, in the third trial. Strains GR12-2 and G20-18 significantly increased root dry weight compared to CNT2 and 6-8 but not the control during the first trial (Fig 4.4). In the second trial strains G20-18 and GR12-2 significantly decreased the root dry weight compared to the control. Strain GR12-2/*aux1* decreased root dry weight compared to the control in the third trial.

Strain G20-18 significantly increased lateral root formation when compared to CNT2, GR12-2/*aux1* and 6-8 but not the control in the first trial and in the third trial compared to the control, GR12-2, GR12-2/*aux1* and 6-8 (Fig 4.5). In the second trial strain 6-8 increased lateral root formation compared to the strains G20-18, CNT2 and GR12-2 but not the control.

4.3 Growth chamber studies

4.3.1 Canola growth chamber studies

Growth chamber studies were performed for four weeks and the effect of PGPR strains on plant growth was monitored by determining root length, shoot dry weight, root dry weight and root surface area.

A significant effect of trial (block) and time was observed in growth chamber studies of canola cv. Smart when all three trials were analysed together (Appendix Table 8.1.5) for all parameters measured except root dry weight. Therefore the data from each trial were analyzed individually (Appendix Table 8.1.6). Root length, root dry weight and root surface area were not affected by the treatments in any of the three trials

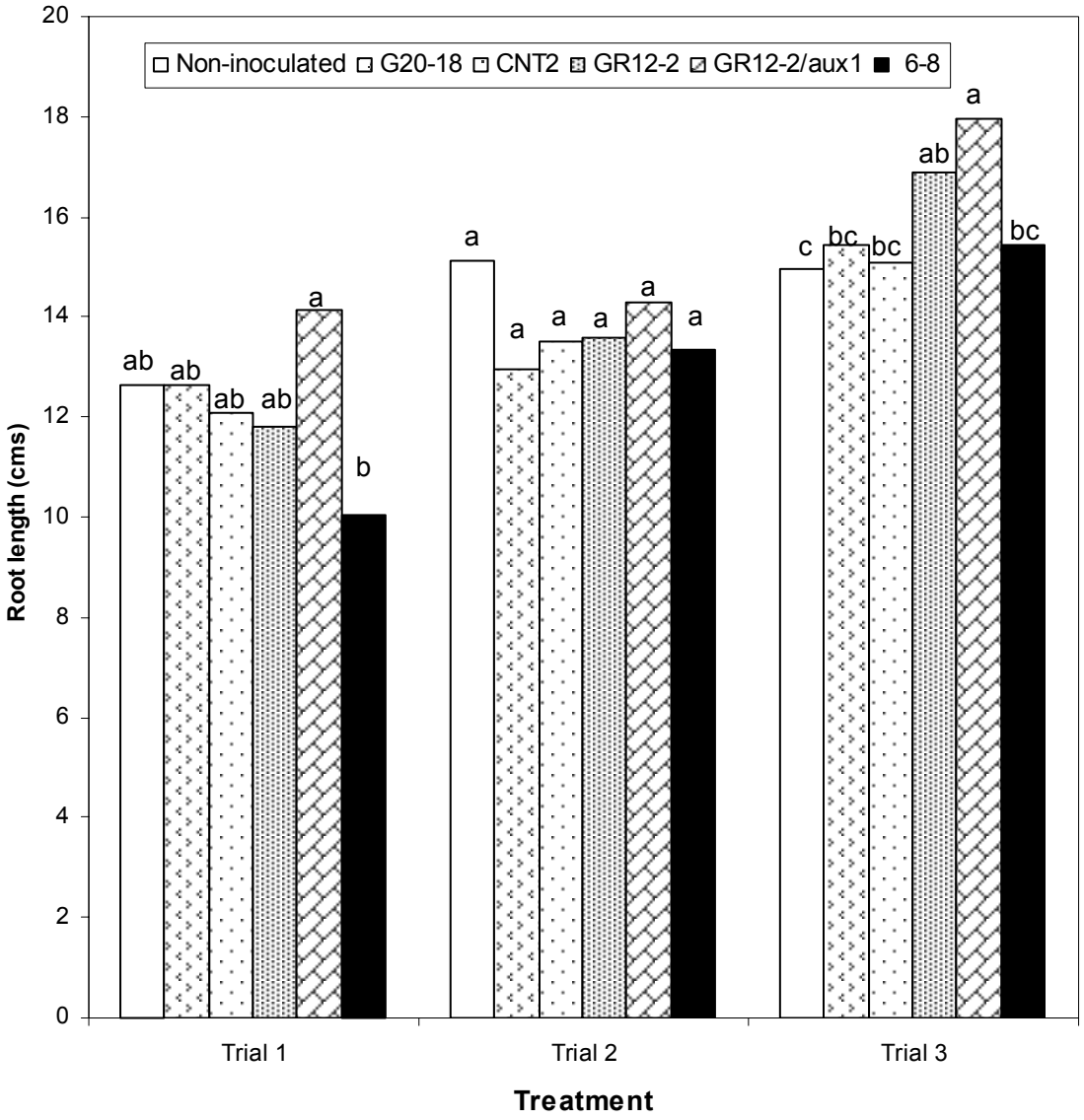


Figure 4.3 Effect of PGPR strains on root length of lentil grown under gnotobiotic conditions. Bars are means of each treatment with seven replicates per treatment. Bars with the same letters for a parameter within an experiment indicate no significant differences between means as determined by LSD test (P = 0.05).

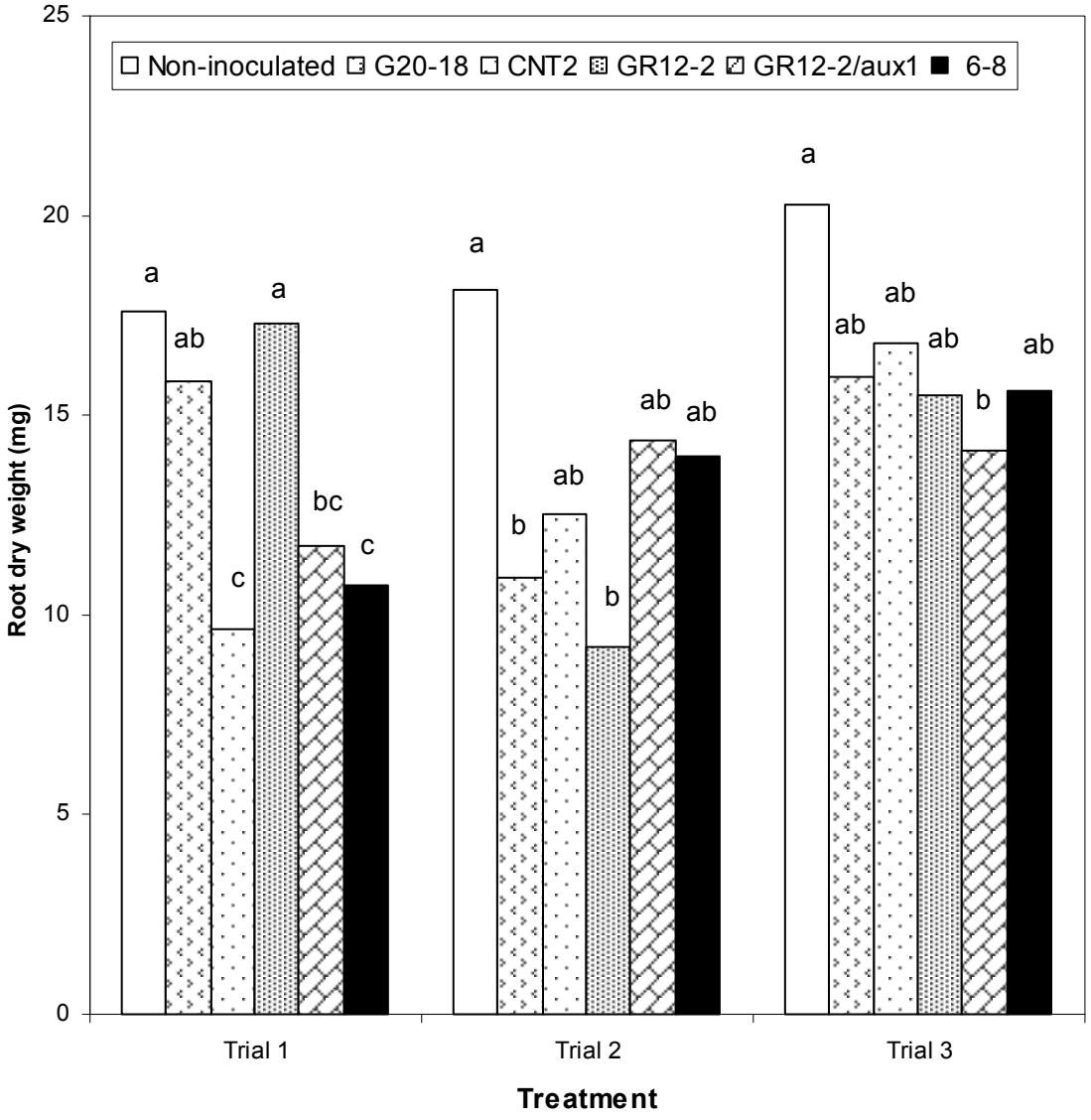


Figure 4.4 Effect of PGPR strains on root dry weight of lentil grown under gnotobiotic conditions. Bars are means of each treatment with seven replicates per treatment. Bars with the same letters for a parameter within an experiment indicate no significant differences between means as determined by LSD test (P = 0.05).

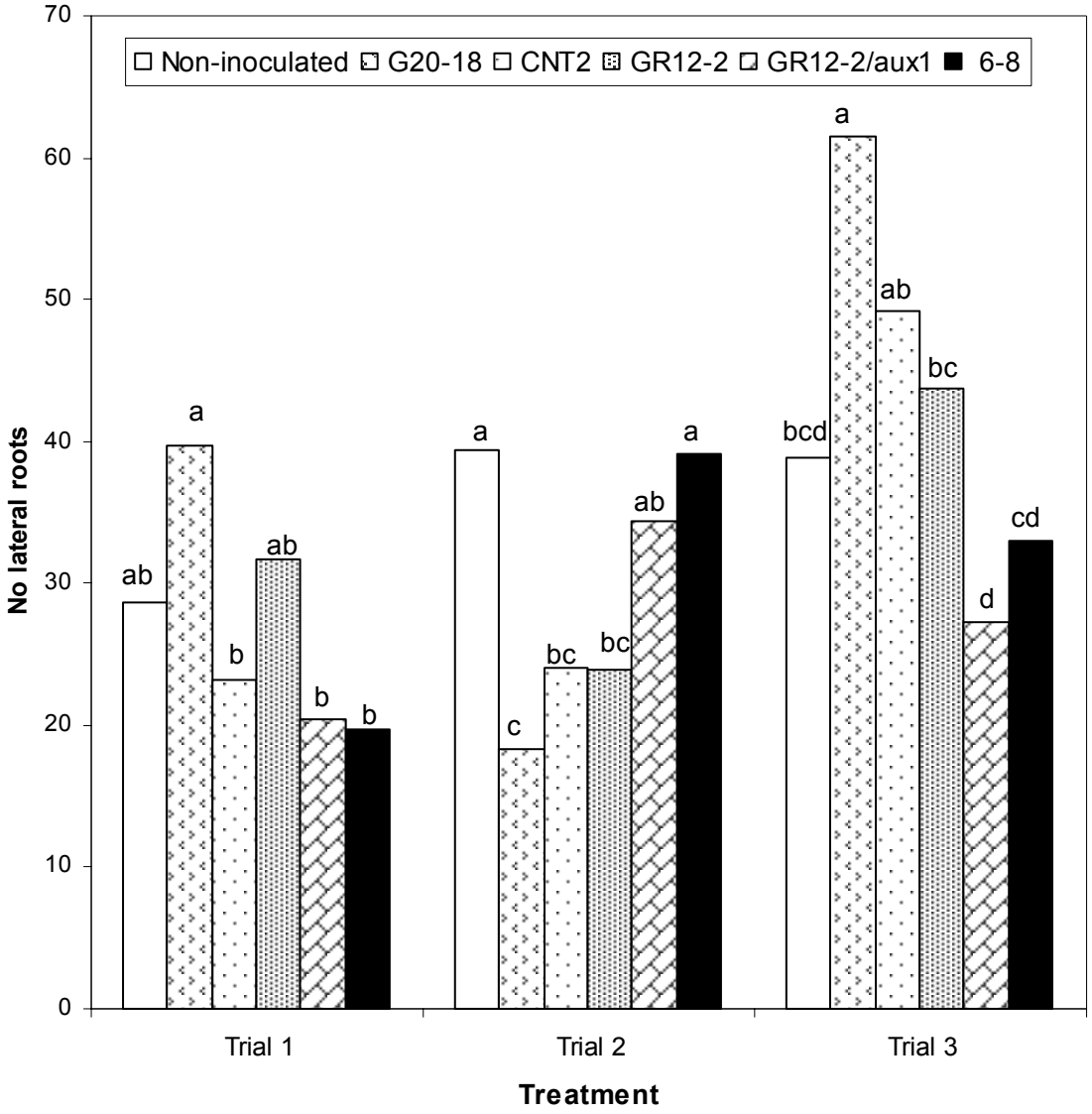


Figure 4.5 Effect of PGPR strains on lateral root formation of lentil grown under gnotobiotic conditions. Bars are means of each treatment with seven replicates per treatment. Bars with the same letters for a parameter within an experiment indicate no significant differences between means as determined by LSD test (P = 0.05).

(Appendix Table 8.1.6). However shoot dry weight was significantly affected by treatment in two of the three trials. A significant effect of time of harvest was observed in each of the trials. There was no significant interaction of treatment with time in the case of root length, while there was significant treatment with time interactions in one of the three trials for root dry weight and root surface area and for shoot dry weight in two of the three trials.

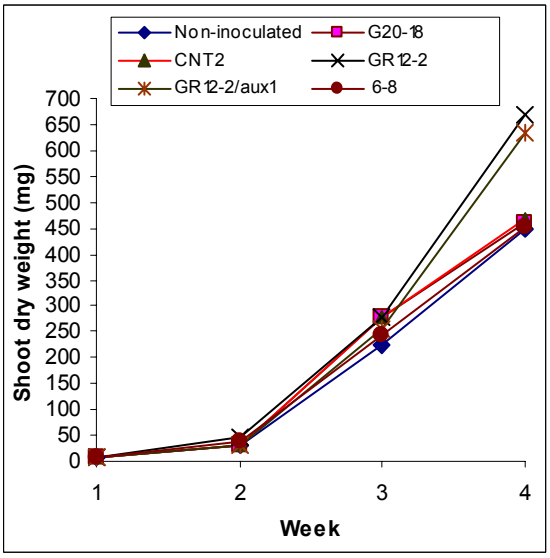
There was no significant effect of any of the treatments on shoot dry weight of 7- and 21-day old canola plants during the first trial (Table 4.1). In the second week of plant growth strain GR12-2 significantly increased shoot dry weight when compared to the control, G20-18, CNT2 and GR12-2/*aux1*. Shoot dry weight of 28-day-old canola plants was significantly increased by strains GR12-2 and GR12-2/*aux1* when compared to the control, G20-18, CNT2 and 6-8 (Table 4.1 and Fig 4.6 A). In the second trial strain GR12-2 significantly increased the shoot dry weight of 7-day-old canola plants when compared to the control (Table 4.1). Strain G20-18 significantly increased shoot dry weight of 14-day-old canola plants when compared to the mutant CNT2 and GR12-2 but not the control in trial two (Table 4.1). Strain G20-18 also increased the shoot dry weight of three-week-old canola plants as did strain GR12-2 when compared with the control, GR12-2/*aux1* and 6-8. Strain GR12-2 increased shoot dry weight of 28-day-old canola plants when compared with the mutant GR12-2/*aux1* but not with the control (Table 4.1 and Fig 4.6 B). The results of the third trial were different from the first two trials as there was no significant effect of any PGPR strains on shoot dry weight of canola plants (Table 4.1 and Fig 4.6 C).

Table 4.1 Effect of PGPR strains on shoot dry weight of canola cv. Smart grown in pots in a growth chamber. Means obtained from an individual trial, with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

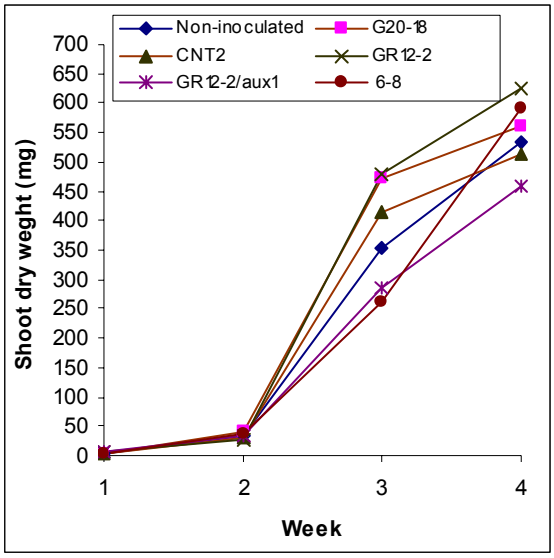
Mean Shoot Dry Weight (mg)

Trials	Non-inoculated	G20-18	CNT2	GR12-2	GR12-2/*aux1*	6-8
Trial 1						
Week 1	6.73 a*	6.25 a	6.96 a	7.42 a	8.05 a	7.94 a
Week 2	30.93 b	32.18 b	30.94 b	46.58 a	31.34 b	39.39 ab
Week 3	223.06 a	276.94 a	277.95 a	277.37 a	253.76 ab	245.06 a
Week 4	447.35 b	458.51 b	468.30 b	668.43 a	634.21 a	454.80 b
Trial 2						
Week 1	4.46 b	4.69 ab	4.52 b	5.64 a	5.20 ab	4.92 ab
Week 2	34.49 ab	42.00 a	29.51 b	26.27 b	33.56 ab	35.98 ab
Week 3	354.63 bc	471.09 a	414.1 ab	478.76 a	285.43 cd	260.70 d
Week 4	533.87 ab	561.10 ab	512.01 ab	626.10 a	457.57 b	591.26 ab
Trial 3	A.N.S					

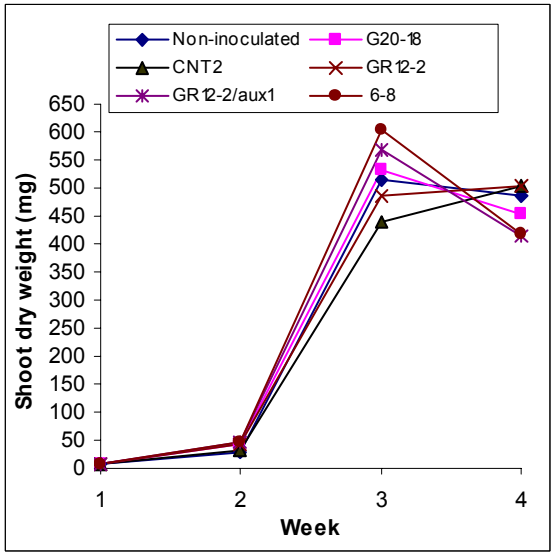
A.N.S ANOVA not significant
* Means with the same letters for a single sample within each trial indicate no significant difference between means (P=0.05)



A- Trial 1



B – Trial 2



C – Trial 3

Figure 4.6 Effect of PGPR strains on shoot dry weight of canola grown under growth chamber conditions. A, B and C indicate the results obtained in each individual trial during four weeks of growth. Values are means at each harvest with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

4.3.2 Lentil growth chamber studies

The ability of PGPR strains to promote the growth of lentil cv. Milestone was studied under growth chamber conditions. There was a significant effect of trial (block) for root dry weight, shoot dry weight and root surface area when data from the three trials were analysed together (Appendix Table 8.1.7). Therefore the data from each trial were analyzed individually (Appendix Table 8.1.8).

There were significant effects of treatment for root dry weight of lentil in all three trials and for shoot dry weight in two out of three trials (Appendix Table 8.1.8). For root length and root surface area the effects of treatment were significant only in the first trial. A significant effect of time of harvest was observed in each of the trials. A significant interaction of treatment with time of harvest was observed for root dry weight and root surface in all three trials. In the case of root length and shoot dry weight two out of three trials had significant interactions of treatment with time of harvest.

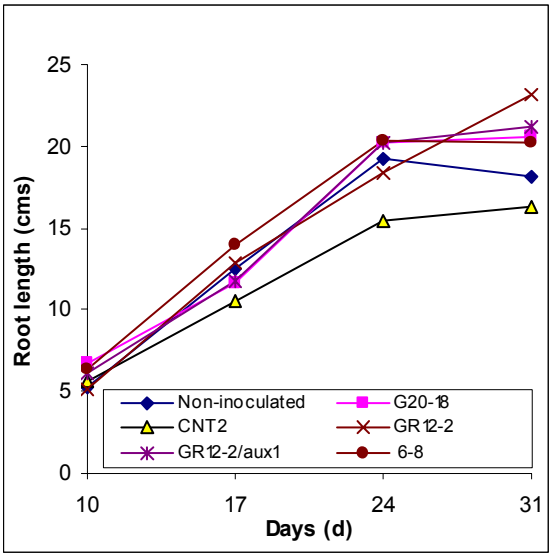
The effect of PGPR strains on root length was determined for 10-, 17-, 24- and 31-day-old lentil plants, but was significant only in trial one (Table 4.2 and Fig 4.7 A). There was no effect of the PGPR strains on root length of 10-day old lentil plants. In 17-day- old lentil plants strain 6-8 significantly enhanced root length compared to CNT2. Strain CNT2 significantly decreased root length of 24-day-old lentil plants compared to the control, G20-18, GR12-2/*aux1* and 6-8. The root length of 31-day-old lentil plants was significantly increased by strain GR12-2 compared to the control and CNT2. A significant interaction was observed with treatment and time of harvest in the first and third trial (Fig 4.7 A, C).

The root dry weight was significantly increased by PGPR strains in each of the three trials (Table 4.3, Fig 4.8). Strain G20-18 increased the root dry weight of 10-day-

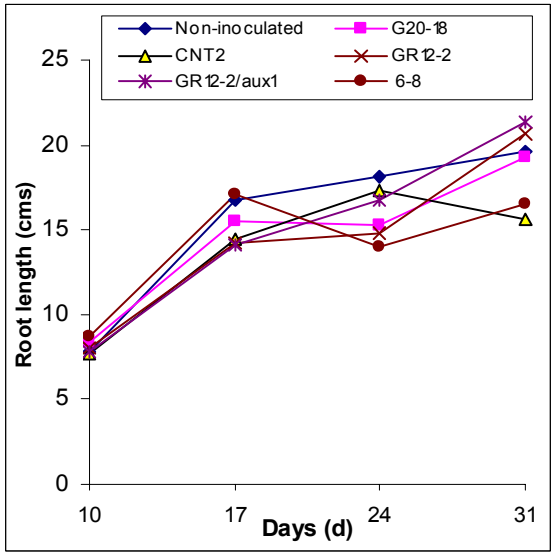
Table 4.2 Effect of PGPR strains on root length of lentil cv. Miletstone grown in pots in a growth chamber. Means obtained from an individual trial, with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

	Mean Root Length (cm)					
Trials	**Non-inoculated**	**G20-18**	**CNT2**	**GR12-2**	**GR12-2/*aux1***	**6-8**
Trial 1						
10-days	5.24 a*	6.75 a	5.58 a	5.11 a	6.11 a	6.39 a
17-days	12.54 ab	11.68 ab	10.57 b	12.9 ab	11.71 ab	14.01 a
24-days	19.26 a	20.16 a	15.39 b	18.36 ab	20.2 a	20.38 a
31-days	18.16 bc	20.62 ab	16.29 c	23.16 a	21.26 ab	20.2 ab
Trial 2	A.N.S					
Trial 3	A.N.S					

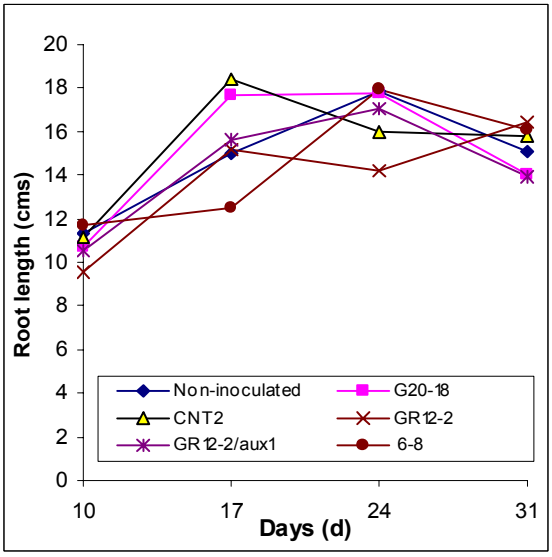
A.N.S – ANOVA not significant.
*- Means with the same letters for a single sample time within each trial indicate no significant difference between means (P=0.05).



A – Trial 1



B – Trial 2



C – Trial 3

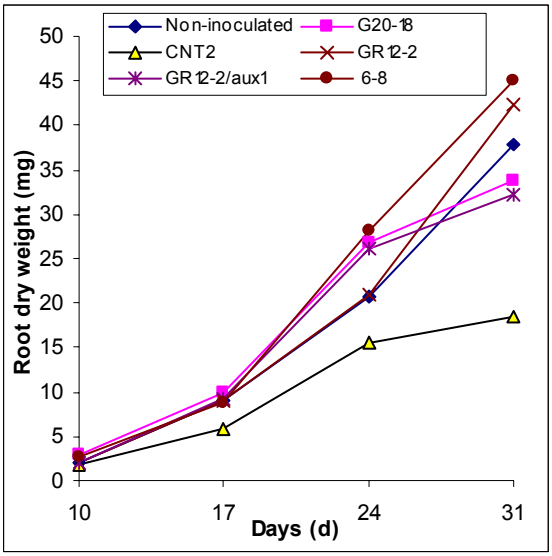
Figure 4.7 Effect of PGPR strains on root length of lentil grown under growth chamber conditions. A, B and C indicate the results obtained in each individual trial during 31 days of growth. Values are means of each harvest with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

Table 4.3 Effect of PGPR strains on root dry weight of lentil cv. Miletstone grown in pots in a growth chamber. Means obtained from an individual trial, with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

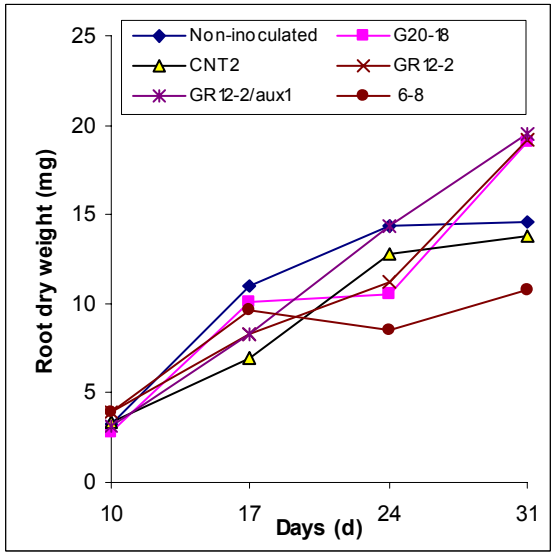
Mean Root Dry Weight (mg)

Trials	Non-inoculated	G20-18	CNT2	GR12-2	GR12-2/*aux1*	6-8
Trial 1						
10-days	2.06 b*	3.02 a	1.86 b	2.07 b	1.97 b	2.64 ab
17-days	8.99 a	9.93 a	8.94 b	8.97 a	9.18 a	8.81 a
24-days	20.68 b	20.93 b	15.44 c	18.36 ab	26.19 a	28.10 a
31-days	37.82 ab	33.86 b	18.40 c	42.35 ab	32.29 b	44.94 a
Trial 2						
10-days	3.22 ab	2.83 b	3.40 ab	3.95 a	3.09 ab	3.96 a
17-days	10.95 a	10.06 a	6.94 a	8.34 a	8.24 a	9.59 a
24-days	14.33 a	10.52 ab	12.83 ab	11.16 ab	14.37 ab	8.55 b
31-days	14.58 b	19.10 a	13.83 b	19.19 a	19.54 a	10.76 b
Trial 3						
10-days	5.75 a	4.98 ab	4.85 ab	4.43 ab	5.44 ab	4.10 b
17-days	8.72 abc	10.71 a	9.34 ab	7.96 bc	9.95 ab	6.57 c
24-days	13.48 a	16.11 a	12.56 ab	8.21 b	14.60 a	12.29 ab
31-days	11.59 a	11.82 a	12.46 a	13.17 a	7.85 b	10.66 ab

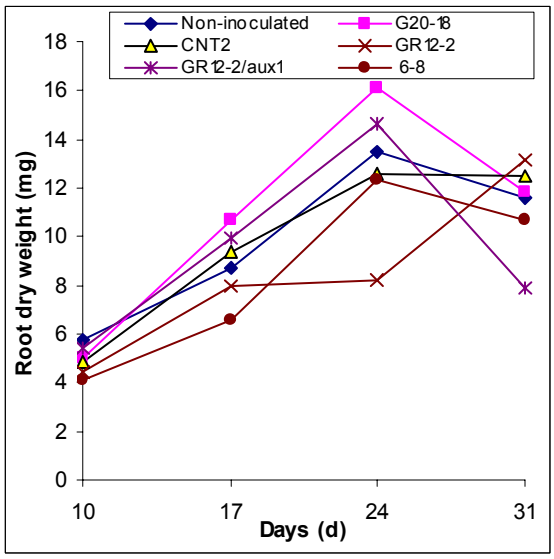
*- Means with the same letters for a single sample time within each trial indicate no significant difference between means (P=0.05)



A –Trial 1



B – Trial 2



C – Trial 3

Figure 4.8 Effect of PGPR strains on root dry weight of lentil grown under growth chamber conditions. A, B and C indicate the results obtained in each individual trial during 31 days of growth. Values are means of each harvest with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

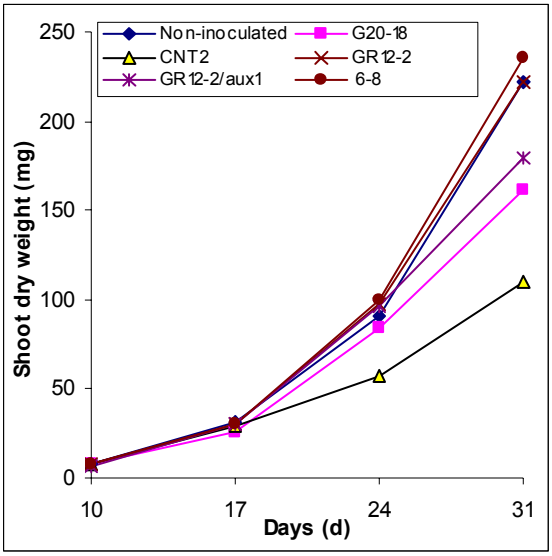
old lentil in the first trial compared to the control, CNT2, GR12-2 and GR12-2/*aux1*. Strain CNT2 exerted a significant negative effect compared to the control at 17, 24 and 31 days. Strains GR12-2/*aux1* and 6-8 significantly enhanced root dry weight of 24-day-old lentil plants when compared to the control, G20-18 and CNT2. Root dry weight of 31-day-old lentil plants was increased by strain 6-8 compared to strains G20-18, CNT2 and GR12-2/*aux1*. Wild-type strain G20-18 consistently increased root dry weight of lentil plants at all sample times compared to the mutant CNT2 (Table 4.3 and Fig 4.8 A). In the second trial, root dry weight of 10-day-old lentil plants was significantly increased by strains 6-8 and GR12-2 compared to G20-18 but not with that of the control (Table 4.3 and Fig 4.8 B). There was no effect of any of the PGPR strains at 17 days, but the root dry weight of 24-day-old lentil plants was significantly decreased by strain 6-8 compared to the control. Strains G20-18, GR12-2 and GR12-2/*aux1* increased root dry weight of 31-day-old lentil plants compared to the control, CNT2 and 6-8. There was no significant positive effect of PGPR strains on root dry weight of lentil during the third trial compared to the control (Table 4.3 and Fig 4.8 C). Strains 6-8, GR12-2 and GR12-2/*aux1* had a negative effect on 10-day-, 24-day- and 31-day-old lentil plants, respectively, compared to the control (Fig 4.8 C). Significant interaction was observed between treatment and time of harvest in all three trials (Table 4.3, Fig 4.8).

There was no effect of any PGPR strain on lentil shoot dry weight during the first two weeks of plant growth in the first trial (Table 4.4). Strain CNT2 had a negative effect on 24-day- and 31-day-old lentil plants when compared to that of the control and all other strains and in 31-day-old plants compared to that of the control, GR12-2, and 6-8 (Table 4.4 and Fig 4.9 A). In the second trial strains GR12-2 and 6-8 significantly increased shoot dry weight of 10-day-old-lentil plants compared to the control (Table

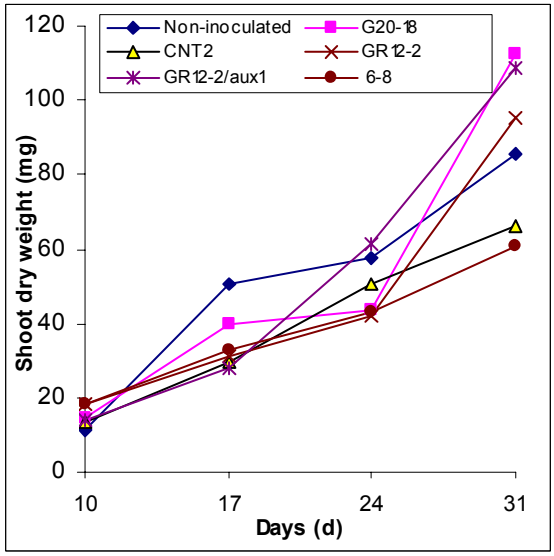
Table 4.4 Effect of PGPR strains on shoot dry weight of lentil cv. Milestone grown in pots in a growth chamber. Means obtained from an individual trial, with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

	Mean Shoot Dry Weight (mg)					
Trials	**Non-inoculated**	**G20-18**	**CNT2**	**GR12-2**	**GR12-2/*aux1***	**6-8**
Trial 1						
10-days	8.13 a*	7.67 a	8.00 a	7.33 a	6.25 a	8.02 a
17-days	30.90 a	25.25 a	28.80 a	30.04 a	30.26 a	30.06 a
24-days	91.35 a	84.49 a	57.62 b	97.16 a	95.86 a	99.26 a
31-days	221.43 ab	161.53 bc	109.97 c	221.94 ab	179.26 abc	234.96 a
Trial 2						
10-days	11.52 b	14.50 ab	13.54 ab	18.42 a	13.91 ab	18.21 a
17-days	50.64 a	39.81 ab	29.35 b	30.96 b	27.95 b	32.95 b
24-days	57.39 a	43.69 a	50.36 a	42.22 a	61.22 a	42.86 a
31-days	85.78 bc	112.28 a	65.95 cd	95.39 ab	108.64 ab	60.63 d
Trial 3	A.N.S					

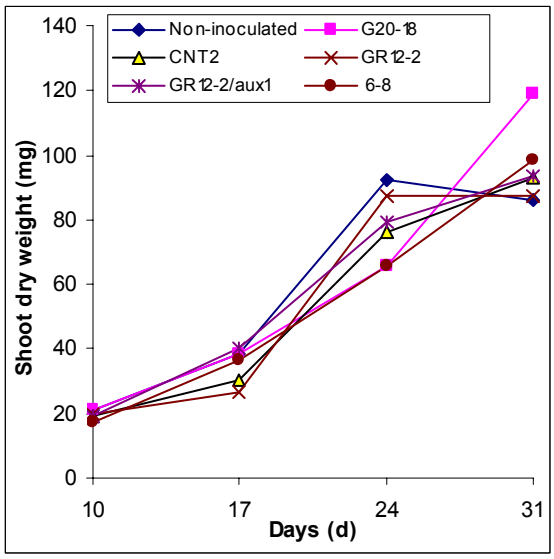
A.N.S- ANOVA not significant
*- Means with the same letters for a single sample time within each trial indicate no significant difference between means (P=0.05)



A – Trial 1



B –Trial 2



C –Trial 3

Figure 4.9 Effect of PGPR strains on shoot dry weight of lentil grown under growth chamber conditions. A, B and C indicate the results obtained in each individual trial during 31 days of growth. Values are means of each harvest with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

4.4). Shoot dry weight of 17-day-old lentil plants was significantly decreased by strains CNT2, GR12-2, GR12-2/*aux1* and 6-8 in comparison to the control. There was no effect of any of the PGPR strains during the third week of the study. The shoot dry weight of 31-day-old lentil plants was significantly increased by strain G20-18 compared to the control, CNT2 and 6-8 (Fig 4.9 B). The effects of treatment were not significant during the third trial (Table 4.4, Fig 4.9 C).

Strains G20-18, CNT2, GR12-2, GR12-2/*aux1* and 6-8 increased root surface area of 10-day-old lentil plants compared to the control in trial one (Table 4.5). Strain 6-8 increased the root surface area of 17-day-old lentil plants in comparison with the control, G20-18, CNT2 and GR12-2 (Table 4.5 and Fig 4.10 A). Root surface area of 17-day-old- and 24-day-old lentil plants was significantly decreased by CNT2 compared to the control and all other strains. Strains GR12-2 and 6-8 significantly increased the root surface area of 31-day-old lentil plants when compared to CNT2 but not with the control. There were no effects of treatment on root surface area during the second and third trial (Table 4.5).

4.4 Indole Production

Plant growth promoting rhizobacteria were assayed for their ability to produce indoles, such as ICA, IPA, IBA and IAA, in pure culture in the presence of various concentrations of the IAA precursor L-tryptophan (0 - 500 µg/ml). In the absence of L-tryptophan strain GR12-2 produced significant amounts of indole (1.19 µg/ml) compared to other PGPR strains (0.09-0.47 µg/ml) (Fig 4.11). As the concentration of L-tryptophan was increased in the medium significant differences in production of indole were identified among PGPR strains, ranging from 0.19 µg/ml to 9.80 µg/ml. In the

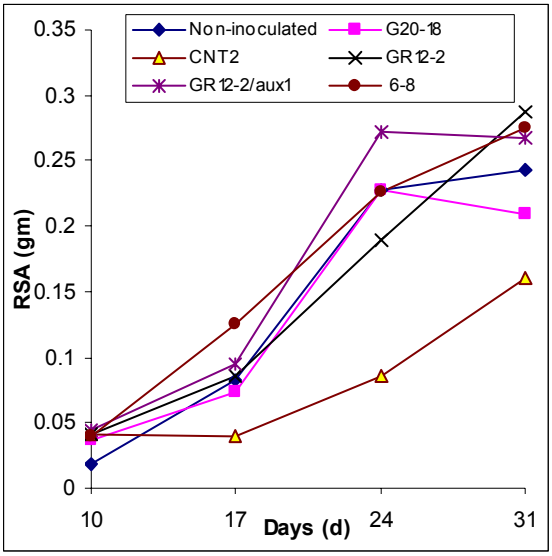
presence of 50 µg/ml of L-tryptophan strain GR12-2/*aux1* produced significantly higher concentrations of indole than other PGPR strains. When 100 µg/ml of L-tryptophan was added to the medium strains GR12-2/*aux1* and GR12-2 produced two and three times, respectively the concentrations of indole produced by G20-18, CNT2 and 6-8. The amount of indole produced by strains CNT2, GR12-2, GR12-2/*aux1* and 6-8 was 4 to 5 fold higher than that produced by strain G20-18 when L-tryptophan was present at 500 µg/ml.

Table 4.5 Effect of PGPR strains on root surface area of lentil cv. Milestone grown in pots in a growth chamber. Means obtained from an individual trial, with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

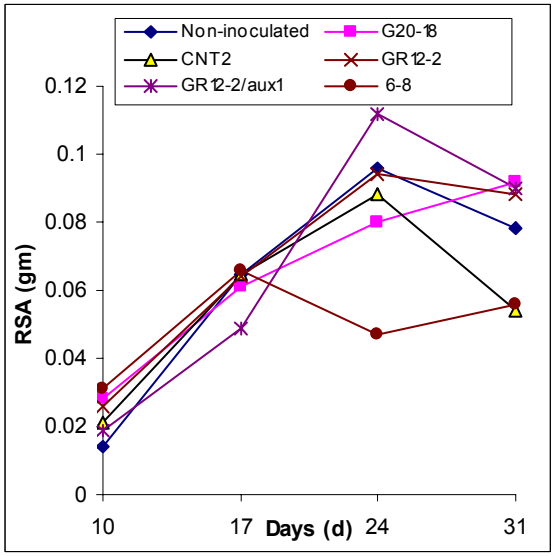
Root Surface Area (mg)

Trials	**Non-inoculated**	**G20-18**	**CNT2**	**GR12-2**	**GR12-2/*aux1***	**6-8**
Trial 1						
10-days	0.01 b*	0.03 a	0.04 a	0.04 a	0.04 a	0.04 a
17-days	0.08 b	0.07 b	0.03 c	0.08 b	0.09 ab	0.12 a
24-days	0.22 ab	0.22 ab	0.08 c	0.18 b	0.27 a	0.22 ab
31-days	0.24 ab	0.20 ab	0.16 b	0.28 a	0.26 ab	0.27 a
Trial 2	A.N.S					
Trial 3	A.N.S					

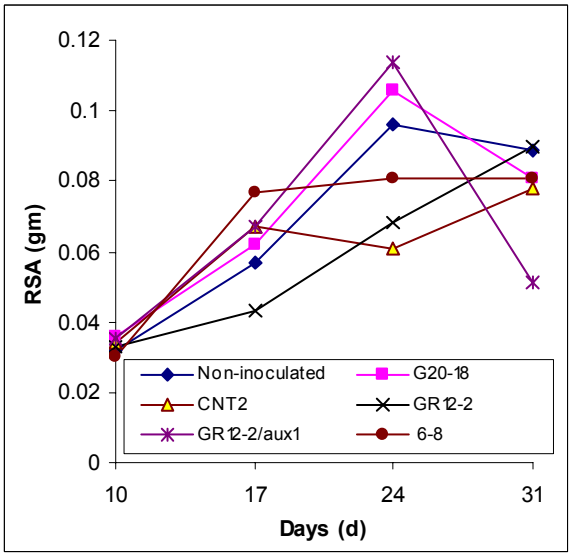
A.N.S-ANOVA not significant
*- Means with the same letters for a single sample time within each trial indicate no significant difference between means (P=0.05)



A – Trial 1



B – Trial 2



C – Trial 3

Figure 4.10 Effect of PGPR strains on root surface area (RSA) of lentil grown under growth chamber conditions. A, B and C indicate the results obtained in each individual trial during 31 days of growth. Values are means of each harvest with five replicates per treatment. Each replicate consisted of a single pot with two seedlings per pot.

The effect of increasing concentrations of precursor on indole production within PGPR strains was also analyzed. The amount of indole produced significantly increased as the concentration of the L-tryptophan was increased in the medium except for strain G20-18 (Fig 4.11). In the case of strain G20-18 there was no significant difference in indole production with 0, 50, 100 and 500 µg/ml of L-tryptophan, and the only significant increase in indole production was observed with L-tryptophan concentration of 200 µg/ml. The pattern of indole production was similar in strains GR12-2 and 6-8 with increasing concentrations of L-tryptophan.

4.4.1 Identification and quantification of indole-3-acetic acid (IAA)

Specific production of IAA by PGPR strains was identified using the ELISA technique. Three different samples for each culture from treatments amended with 0, 100 and 500 µg/ml of L-tryptophan were obtained from the previous assay, filter sterilized and methylated. There was no significant difference in the amount of IAA secreted by PGPR strains in the absence of L-tryptophan and with a concentration of 100 µg/ml (Fig 4.12). Strain GR12-2/*aux1* produced a significantly higher concentration of IAA compared to other PGPR strains when 500 µg/ml of L-tryptophan was added as the precursor.

Increasing concentrations of the precursor L-tryptophan had no significant effect on IAA production levels within strains G20-18, GR12-2 and 6-8 (Fig 4.12). Significant increases in IAA levels were observed in strains CNT2 and GR12-2/*aux1* amended with 500 µg/ml of L-tryptophan. The amount of IAA secreted by GR12-2/*aux1* was two fold higher when a concentration of 500 µg/ml of L-tryptophan was added in comparison with the amount of IAA secreted with 0 and 100 µg/ml of the precursor.

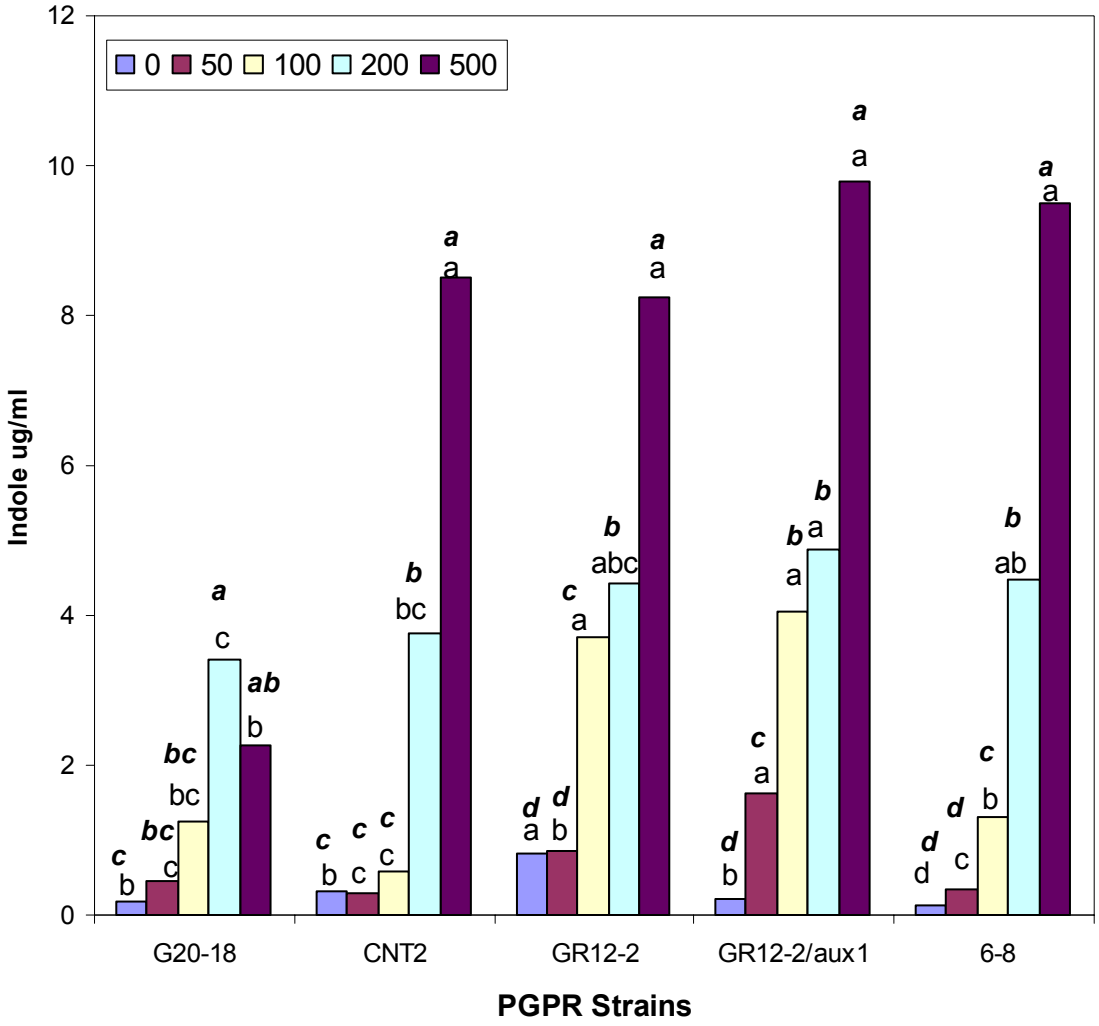


Figure 4.11 Production of indole (µg/ml) by PGPR strains 48 h after inoculation. L-tryptophan was added at varying concentrations. Bars are means of duplicates per treatment. Bars with the same letters within each concentration of L-tryptophan indicate no significant differences in indole production as determined by LSD test (P=0.05). Bars with the same bold italicized letters indicate no significant differences in indole production within each strain with varying concentrations of L-tryptophan (P=0.05).

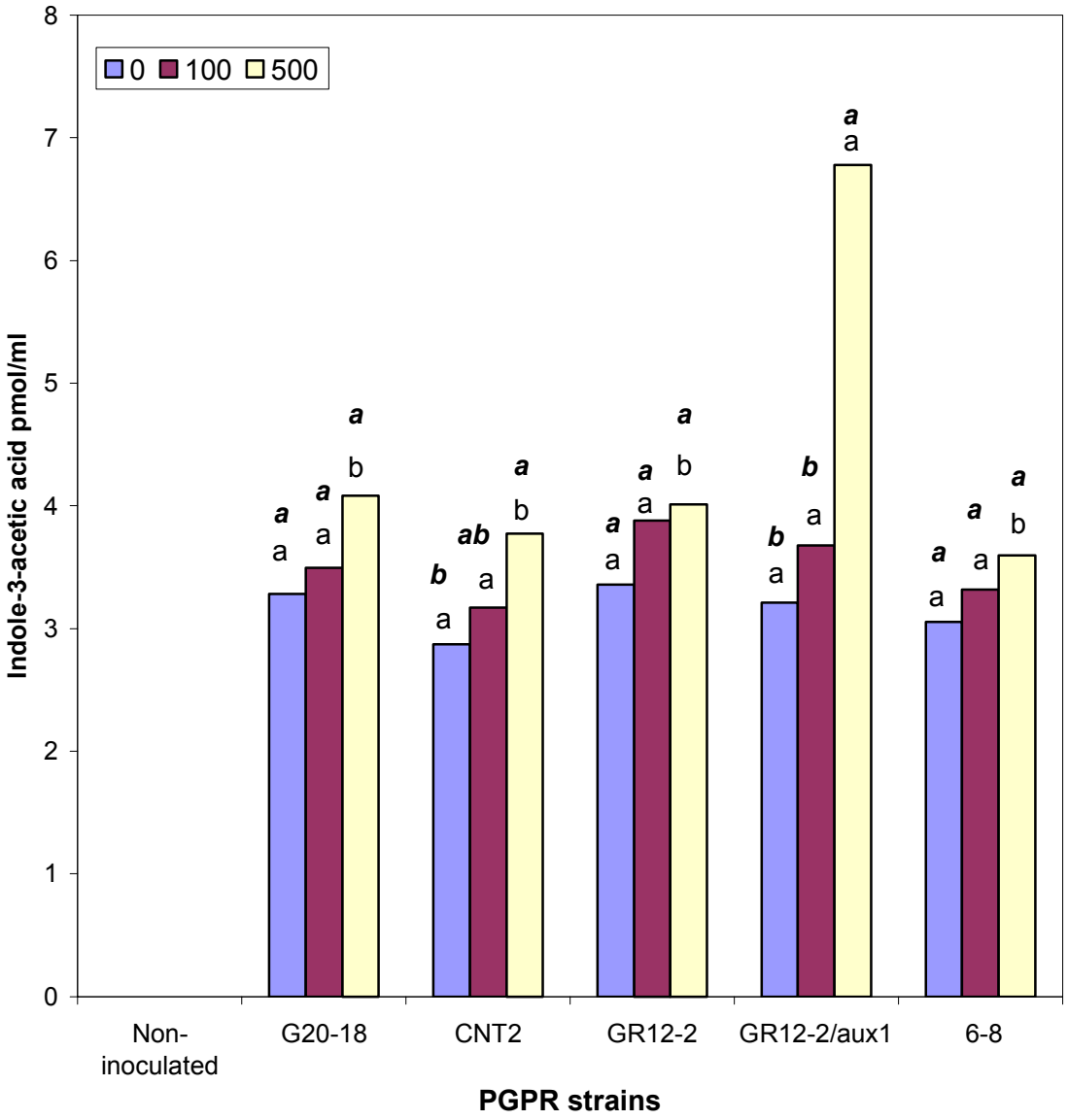


Figure 4.12 Production of IAA (pmol/ml) by PGPR strains 48 h after inoculation. L-tryptophan was added at varying concentrations (0, 100 and 500 µg/ml). Bars are means of duplicates per treatment. Bars with the same letters within each concentration of L-tryptophan indicate no significant differences in IAA production as determined by LSD test (P = 0.05). Bars with the same bold letters indicate no significant differences in IAA production within each strain with varying concentration of L-tryptophan (P = 0.05).

4.5 Identification and quantification of the cytokinin phytohormones, isopentenyl adenosine (IPA), dihydroxyzeatin riboside (DHZR) and zeatin riboside (ZR)

The production of the cytokinins, IPA, DHZR and ZR in pure cultures of strains G20-18, CNT2 and 6-8 was quantified using the ELISA technique. Strains G20-18 and CNT2 (Salamone 2000), previously characterized for production of the cytokinins, IPA, DHZR and ZR were used as positive controls. Production of IPA in PGPR strains G20-18, CNT2 and 6-8 was observed after 12 h of growth (Fig. 4.13). There was no significant difference in the concentrations of IPA secreted by all three strains between 12 and 96 h of growth. Strains G20-18 and 6-8 secreted significantly higher levels of IPA than CNT2 following 168 and 240 h of growth. At 336 h the amount of IPA produced by strain G20-18 was two fold higher (5.44 pmol/ml) than that by strain CNT2 (2.389 pmol/ml). Production of IPA increased in G20-18 with time, while in the case of CNT2 and 6-8 the synthesis of IPA did not increase after 96 h.

The production of ZR by PGPR strains was detectable after 72 h of growth in pure cultures as shown in Fig 4.14. Strains G20-18 and 6-8 produced significantly higher levels of ZR at 72 and 96 h than strain CNT2. However there was no significant difference in the amount of ZR produced by strains at 168 and 240 h. At 336 h strain 6-8 produced significantly higher amounts of ZR than CNT2. In all three strains the amount of ZR produced increased with time.

Differences in the concentration of DHZR produced by strains G20-18, CNT2 and 6-8 were observed (Fig 4.15). Production of DHZR was initially observed after 12 h of growth for strain 6-8 but not until 96 h and 168 h for G20-18 and CNT2, respectively. The concentrations of DHZR secreted by strain 6-8 were always significantly higher

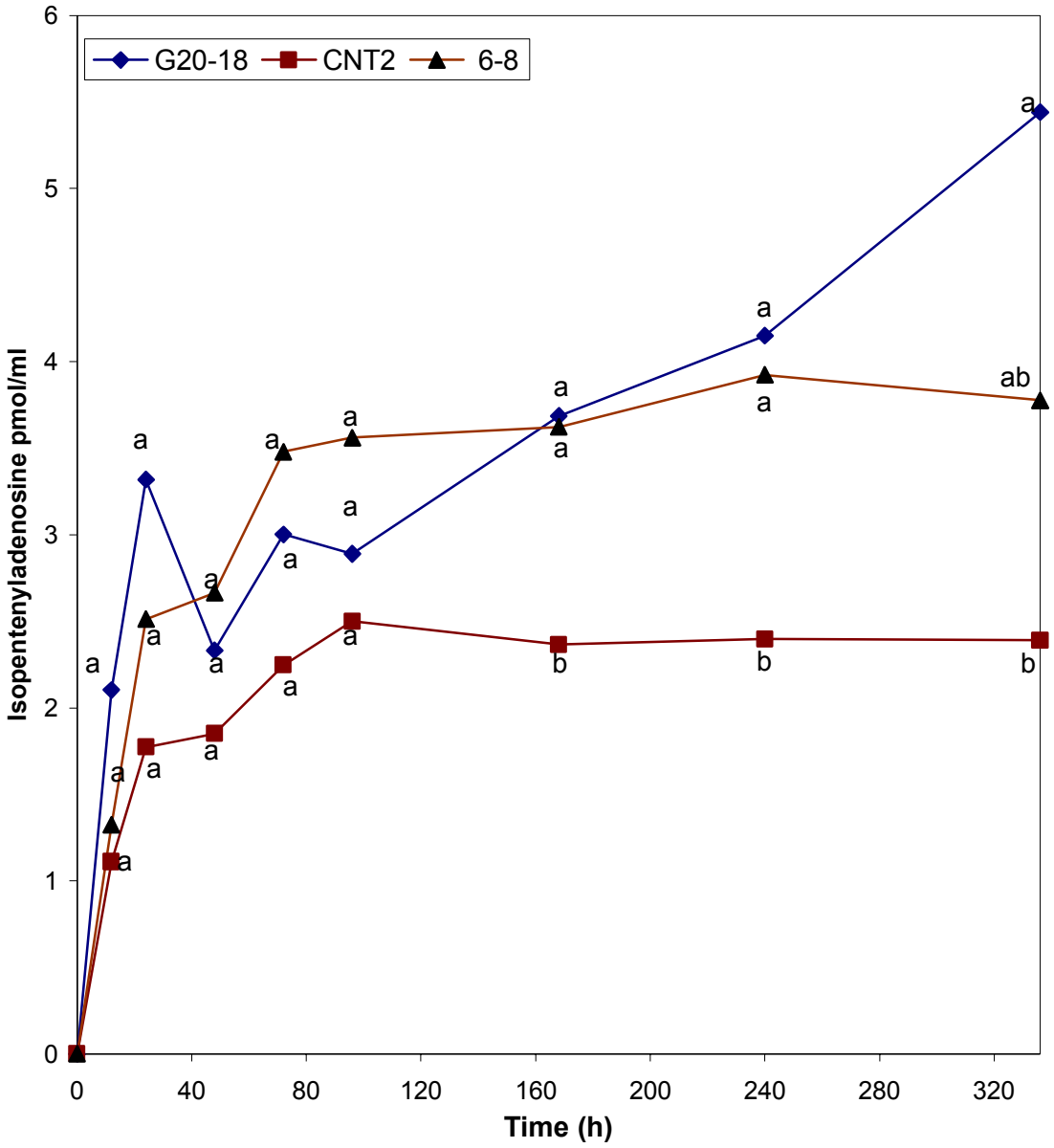


Figure 4.13 Production of the cytokinin, isopentenyladenosine (IPA) by PGPR strains G20-18, CNT2 and 6-8 at different time intervals in pure culture. Data are means of duplicates per treatment. PGPR strains with the same letters at each sampling time indicate no significant differences between means as determined by LSD test (P = 0.05).

than those by G20-18 and CNT2. Strain G20-18 produced significantly higher levels of DHZR than CNT2 after 240 h of growth.

4.6 Phytohormone production in the rhizosphere of canola cv Smart inoculated with PGPR strains in growth pouches

The presence of the phytohormones IAA, IPA, ZR and DHZR in the rhizosphere of canola cv. Smart grown in growth pouches and inoculated with PGPR strains was compared with that of a non-inoculated control. Of the three cytokinins IPA was present in the highest concentrations. The concentration of IPA in the rhizosphere of plants inoculated with strains G20-18 and 6-8 was 1.5 times higher than that of the control and CNT2 (Fig 4.16). The amount of ZR in the rhizosphere of canola roots treated with G20-18 and 6-8 was significantly higher than that of the control but not that of CNT2. There were no significant differences in the concentrations of DHZR in the rhizosphere of control and inoculated plants. The concentrations of IAA secreted in the rhizosphere of canola treated with GR12-2 and GR12-2/*aux1* were significantly higher than that of the control (Fig 4.17).

4.7 Production of siderophores, phosphate solubilization and ability to use ACC as sole nitrogen source

Strains GR12-2, GR12-2/*aux1* and 6-8 demonstrated the ability to solublize inorganic phosphate as shown by a zone of clearing around the colonies on agar plates supplemented with calcium phosphate as a source of inorganic phosphate (Table 4.6). All the PGPR strains produced siderophores and had the ability to grow in minimal medium with ACC as the sole nitrogen source.

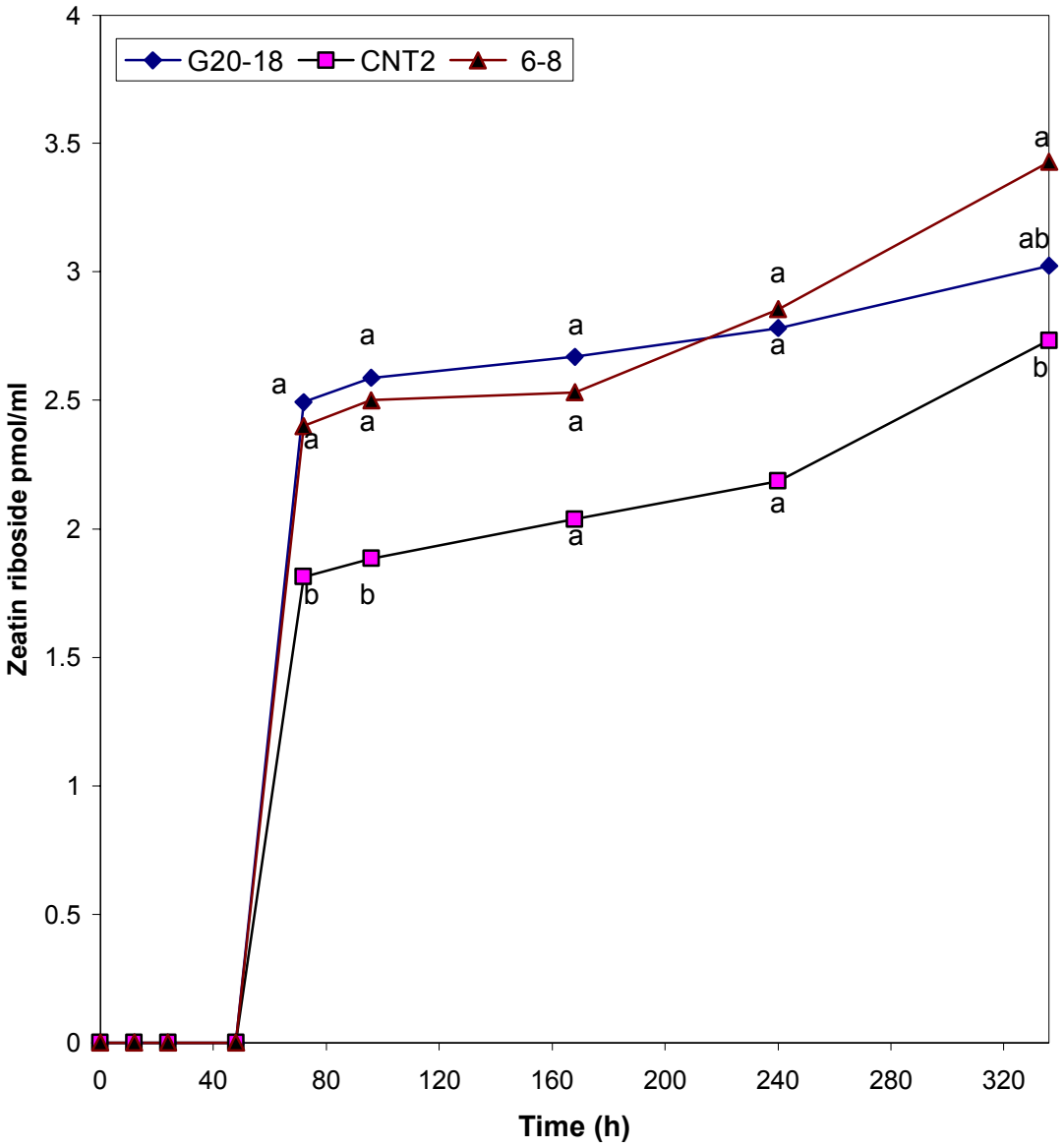


Figure 4.14 Production of the cytokinin, zeatin riboside (ZR) by PGPR strains G20-18, CNT2 and 6-8 at different time intervals in pure culture. Data are means of duplicates per treatment. PGPR strains with the same letters at each sampling time indicate no significant differences between means as determined by LSD test (P = 0.05).

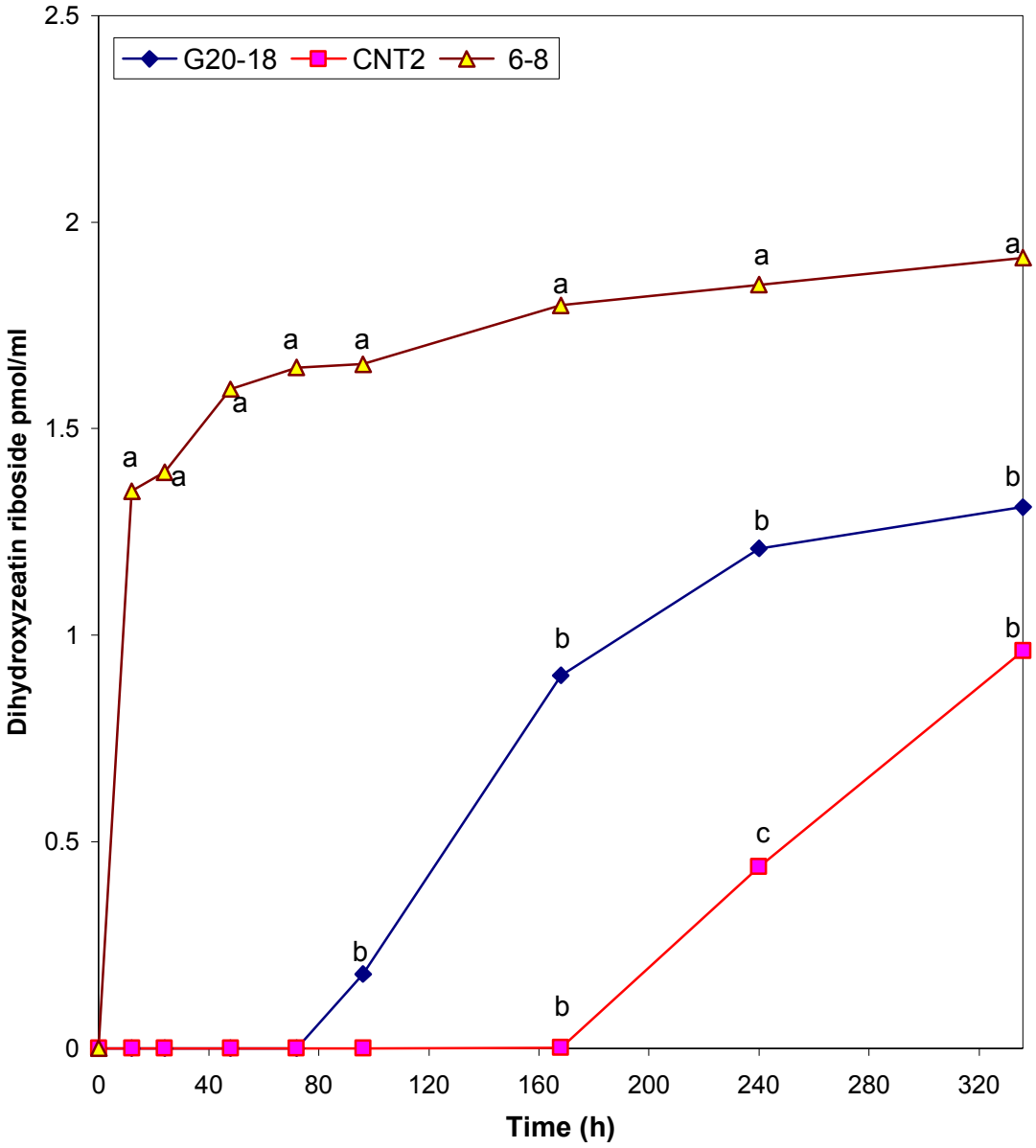


Figure 4.15 Production of the cytokinin, dihydroxyzeatin riboside (DHZR) by PGPR strains G20-18, CNT2 and 6-8 at different time intervals in pure culture. Data are means of duplicates per treatment. PGPR strains with the same letters at each sampling time indicate no significant differences between means as determined by LSD test (P = 0.05).

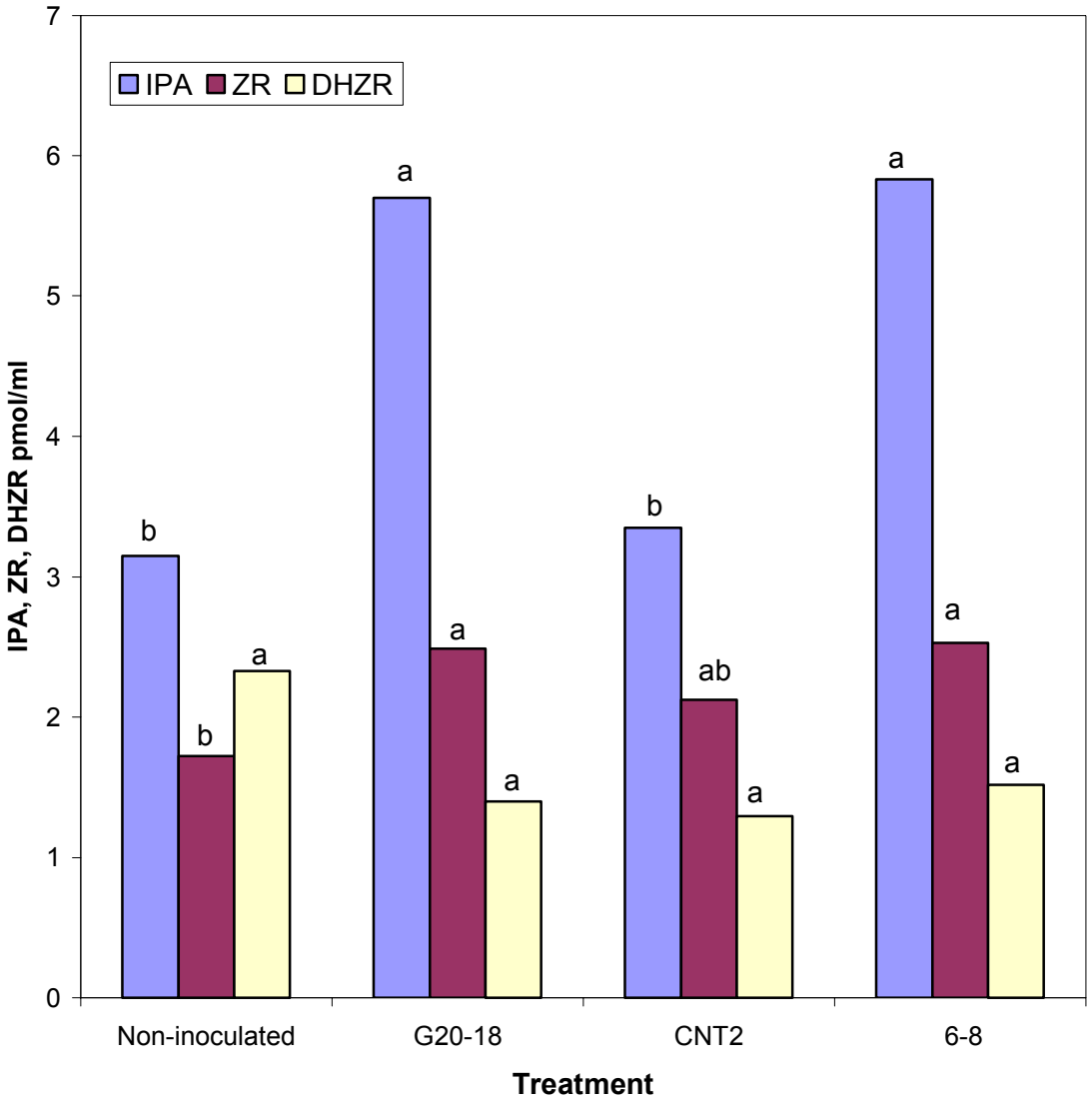


Figure 4.16 Concentrations of isopentenyladenosine (IPA), zeatin riboside (ZR) and dihydroxyzeatin riboside (DHZR) in the rhizosphere of canola cv. Smart inoculated with PGPR strains and grown in growth pouches for 7 days at 18ºC. Bars are means of three replicate GP within an experiment. Bars with the same letters indicate no significant differences between means as determined by LSD test (P=0.05).

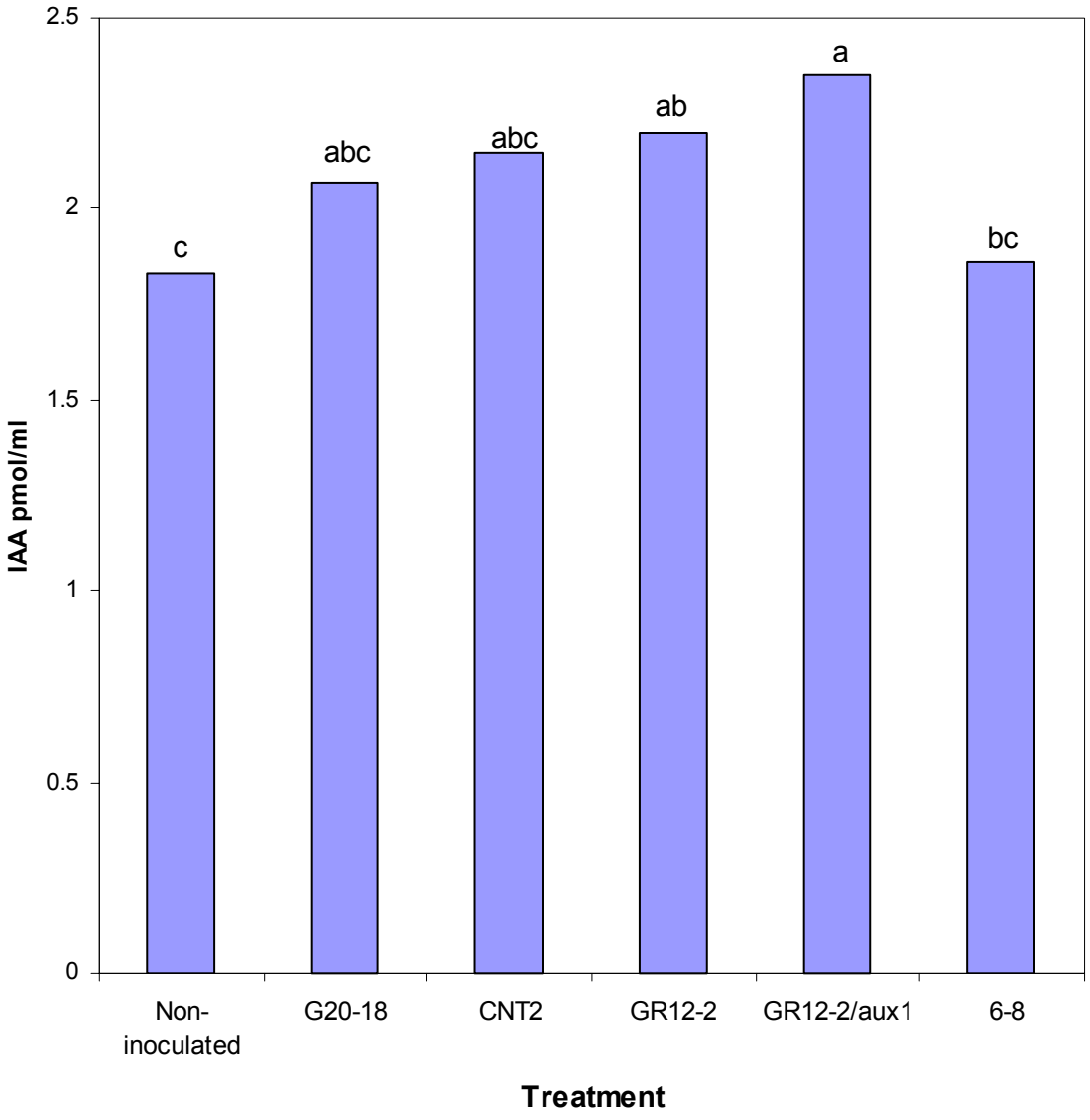


Figure 4.17 Concentrations of indole-3-acetic acid (IAA) in the rhizosphere of canola cv. Smart inoculated with PGPR strains and grown in growth pouches for 7 days at 18ºC. Bars are means of three replicate growth pouches within an experiment. Bars with the same letters indicate no significant differences between means as determined by LSD test (P=0.05).

Table 4.6 Production of siderophores, ability to solubilize inorganic phosphate and ability to use ACC as sole nitrogen source by strains G20-18, CNT2, GR12-2, GR12-2/*aux1* and 6-8.

Trait	PGPR strains				
	G20-18	CNT2	GR12-2	GR12-2/*aux1*	6-8
Solubilize inorganic phosphate	-	-	+	+	+
Siderophores	+	+	+	+	+
Utilize ACC	+	+	+	+	+

4.8 Root colonization studies

The pattern of root colonization by strain 6-8-GFP-Rif$^+$ on canola and lentil roots over 5, 7, and 9 days and 3, 6 and 10 days, respectively, was studied using the conventional dilution plating technique. The second approach utilized a novel technique, confocal laser scanning microscopy to obtain qualitative image analysis of colonization patterns by strain 6-8-GFP-Rif$^+$ on canola and lentil roots.

4.8.1 Recovery of strain 6-8-GFP-Rif$^+$ from roots of canola and lentil grown under gnotobiotic conditions

Strain 6-8-GFP-Rif$^+$ colonized both canola (Fig 4.18) and lentil roots (Fig 4.19) under gnotobiotic conditions in growth pouches. In canola roots the number of colony forming units was significantly higher on the root tip on day 5 when compared to the middle segments of the root. At 7 days significantly higher numbers of bacteria were found on the top and middle segments of canola root than on the root tip. Colonization by strain 6-8-GFP-Rif$^+$ of the top segment of the canola root was higher than that of the middle segment and the root tip 9 days after inoculation. The total numbers of colony forming

units of bacteria on all three segments of the root were 6.86 x 10^3, 1.59 x 10^4 and 9.73 x 10^3 CFU at 5, 7 and 9 days, respectively. This represents a 16, 39 and 23 fold increase in bacterial numbers on day 5, 7 and 9 when compared to the number of bacteria present on the seeds (4.07 x 10^2 CFU) at the time of inoculation.

For lentil significantly higher numbers of bacteria were found in the top portion of the root than in the middle and the root tip after 3 and 9 days of growth. Significantly higher numbers of bacteria were observed at day 9 in the top region when compared to the middle segment and root tip. The total number of bacteria colonizing the root tip was lower than that of other root segments at 3, 6 and 9 days. Bacterial populations on all three segments of the root were 1.57 x 10^3, 1.54 x 10^3 and 5.83 x 10^3 CFU after 3, 6 and 9 days of plant growth. This represents a 1.1, 1.1 and 4.4 fold increase in bacterial numbers on day 3, 6 and 9 when compared to the number of bacteria present on the seeds (1.31 x 10^3 CFU) at the time of inoculation.

4.8.2 Image analysis of colonization of canola and lentil roots by strain 6-8-GFP-Rif$^+$

Strain 6-8-GFP-Rif$^+$ was found in the depressions present on the canola seed surface following inoculation and before transferring to growth pouches (Fig 4.20). Five days after germination strain 6-8-GFP-Rif$^+$ was found on all segments of canola roots as single cells. The number of cells differed for each segment. The highest numbers were found in the top region of the root (Table 4.7). With time the number of bacteria colonizing each segment of the root decreased. Bacteria were found both on the external and internal surface of the root. Five days after germination bacteria colonized regions

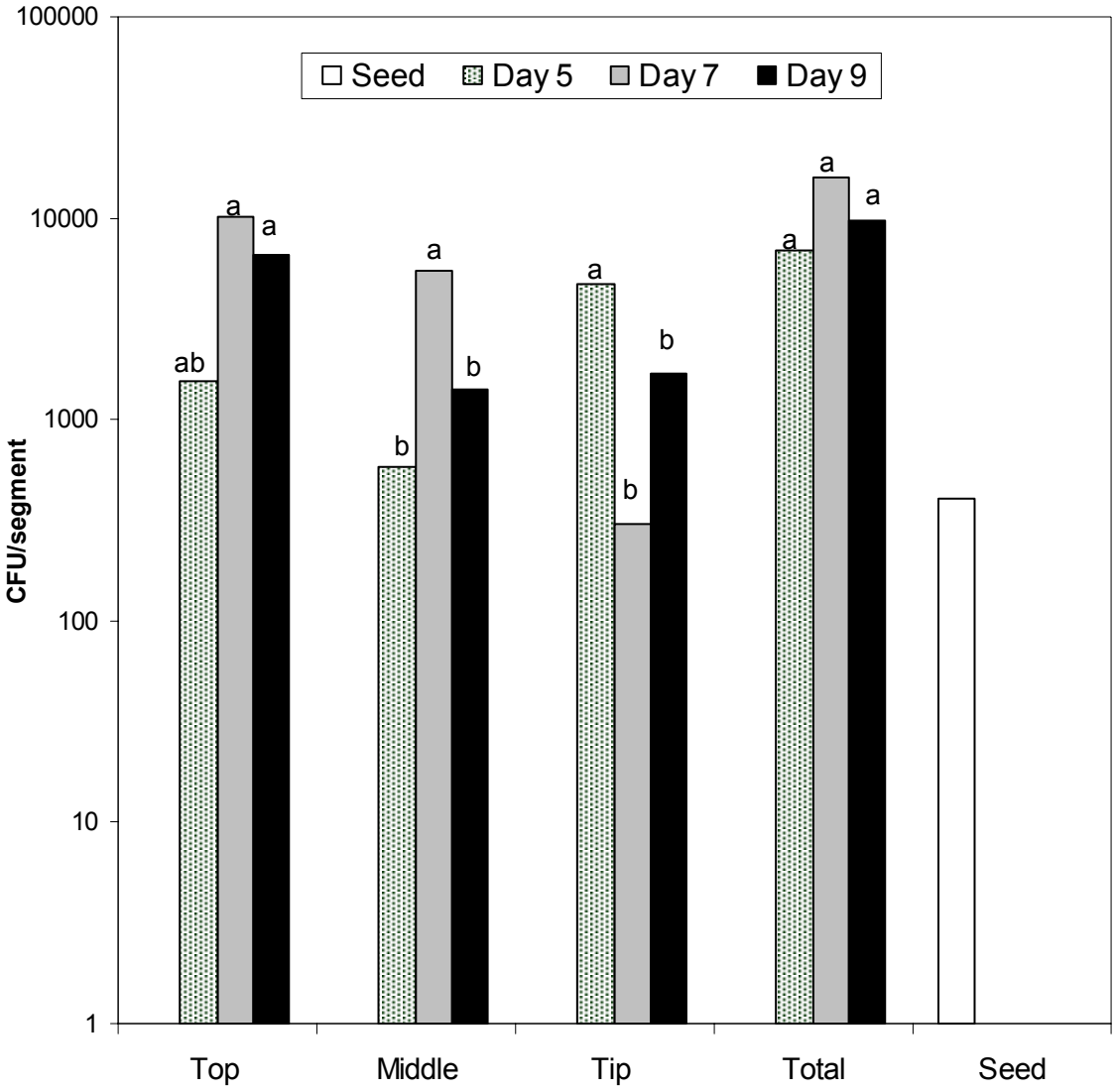


Figure 4.18 Colonization of the seed and root segments by strain 6-8-GFP-Rif^{+} 5, 7 and 9 days after inoculation of seeds of canola cv. Smart in gnotobiotic conditions at 18ºC. Bars are means of three replicate. Bars with the same letters between root segments indicate no significant differences between means as determined by LSD test (P=0.05).

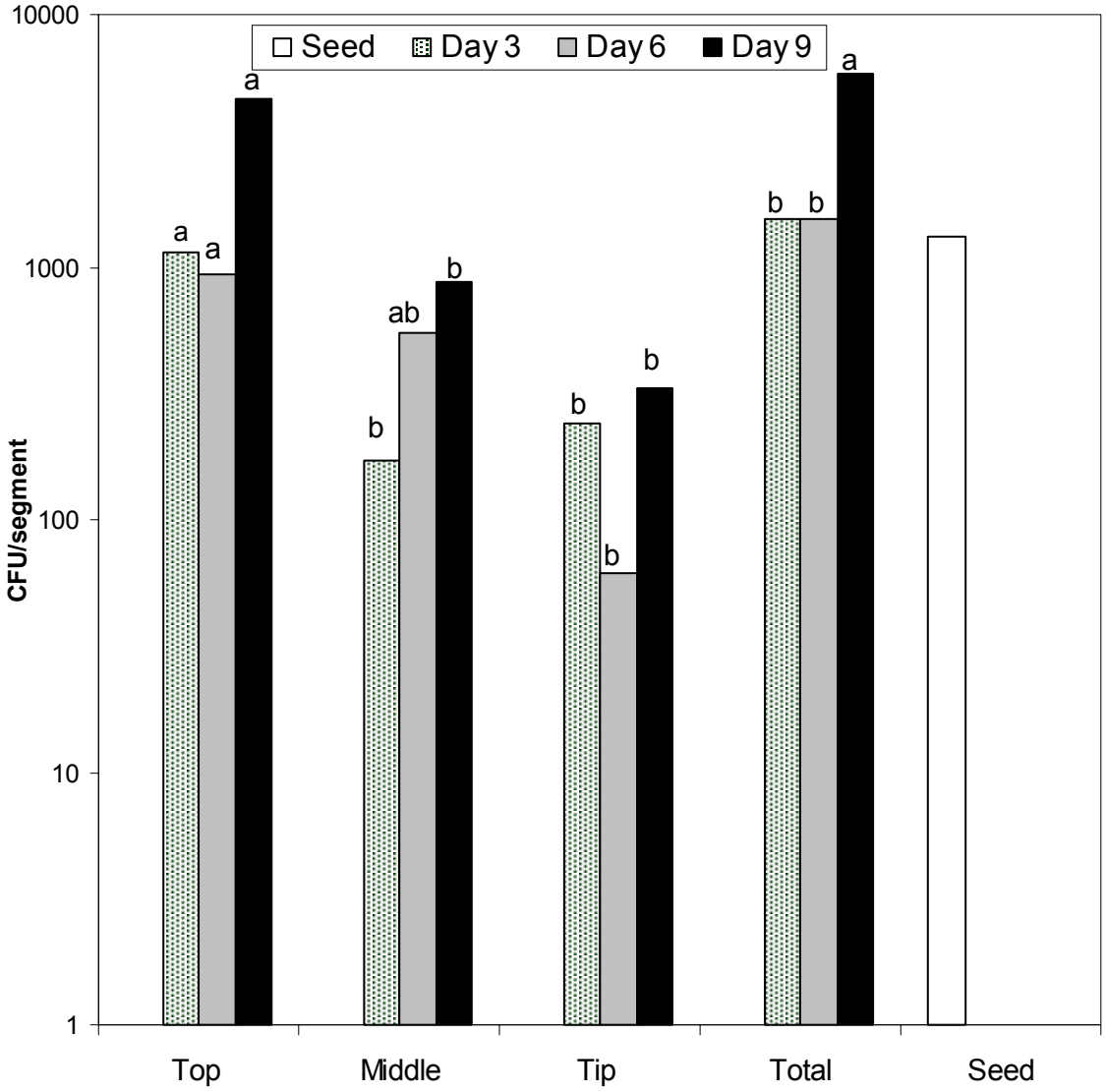


Figure 4.19 Colonization of the seed and root segments by strain 6-8-GFP-Rif^{+} 3, 6 and 9 days after inoculation of seeds of lentil cv. Milestone in gnotobiotic conditions at 18ºC. Bars are means of three replicate. Bars with the same letters between root segments indicate no significant differences between means as determined by LSD test (P=0.05).

around the epidermal cells, such as those found at the point of adjoining cells (Fig 4.21). Bacteria were not distributed evenly over the entire surface of the root. The colonization of the rhizoplane by strain 6-8-GFP-Rif^{+} decreased and by 7 days the numbers of bacteria on the middle and root tip were less than that on the top segment (Fig 4.22). Nine days after germination the total number of bacteria visualized in each segment of the root decreased (Fig 4.23).

Strain 6-8-GFP-Rif^{+} was present on the lentil seed surface following inoculation and before transferring to growth pouches (Fig 4.24). Three days after germination strain 6-8-GFP-Rif^{+} was found on all three segments of lentil root and the population of bacteria was significantly higher on the top segment than on the middle and root tip (Fig 4.25, Table 4.8). Six days after germination there was a decrease in the number of bacteria on each segment of the root (Fig 4.26). With time the number of bacteria colonizing each segment decreased and there were no detectable bacteria nine days after germination on the root tip, as observed on the control root tip (Fig 4.27). Bacteria were not evenly distributed over the entire root segment (Table 4.8). In contrast to the root tip, bacterial cells were observed on the top and middle segments of the lentil root (Fig 4.25). One of the difficulties in distinguishing fluorescent bacteria was the background fluorescence observed in plant and in root exudates (Fig 4.25, 4.26, and 4.27).

Table 4.7 Enumeration of total number of bacteria present in each segment of the canola root at different times following germination. Numbers were calculated based upon the images obtained from the confocal laser scanning microscope which had a field area of 219 x 146 μm.

Root segment	5 days	7 days	9 days
Top	> 200	90.2 ± 16.0 a	18.2 ± 4.3 a
Middle	53.9 ± 16.3*	19.5 ± 9.2 b	10.8 ± 2.7 ab
Tip	56.8 ± 16.9	20.7 ± 8.2 b	5.62 ± 3.3 b

*Means are of three replicates within an experiment. Each replicate had 5 images obtained from three segments of the canola root at each time. Means within a column followed by the same letter indicate no significant differences as determined by LSD test (P = 0.05).

Table 4.8 Enumeration of total number of bacteria present in each segment of the lentil root at different times following germination. Numbers were calculated based upon the image obtained from the confocal laser scanning microscope which had a field area of 219 x 146 μm.

Root segment	3 days	6 days	9 days
Top	15.2 ± 3.1 a*	13.3 ± 3.4 a	7.6 ± 2.4 a
Middle	7.4 ± 2.7 b	6.9 ± 1.8 ab	3.6 ± 1.1 ab
Tip	3.5 ± 1.5 b	1.1 ± 0.7 b	0 ± 0 b

*Means are of three replicates within an experiment. Each replicate had 5 images obtained from three segments of the lentil root at each time. Means within a column followed by the same letter indicate no significant differences as determined by LSD test (P = 0.05).

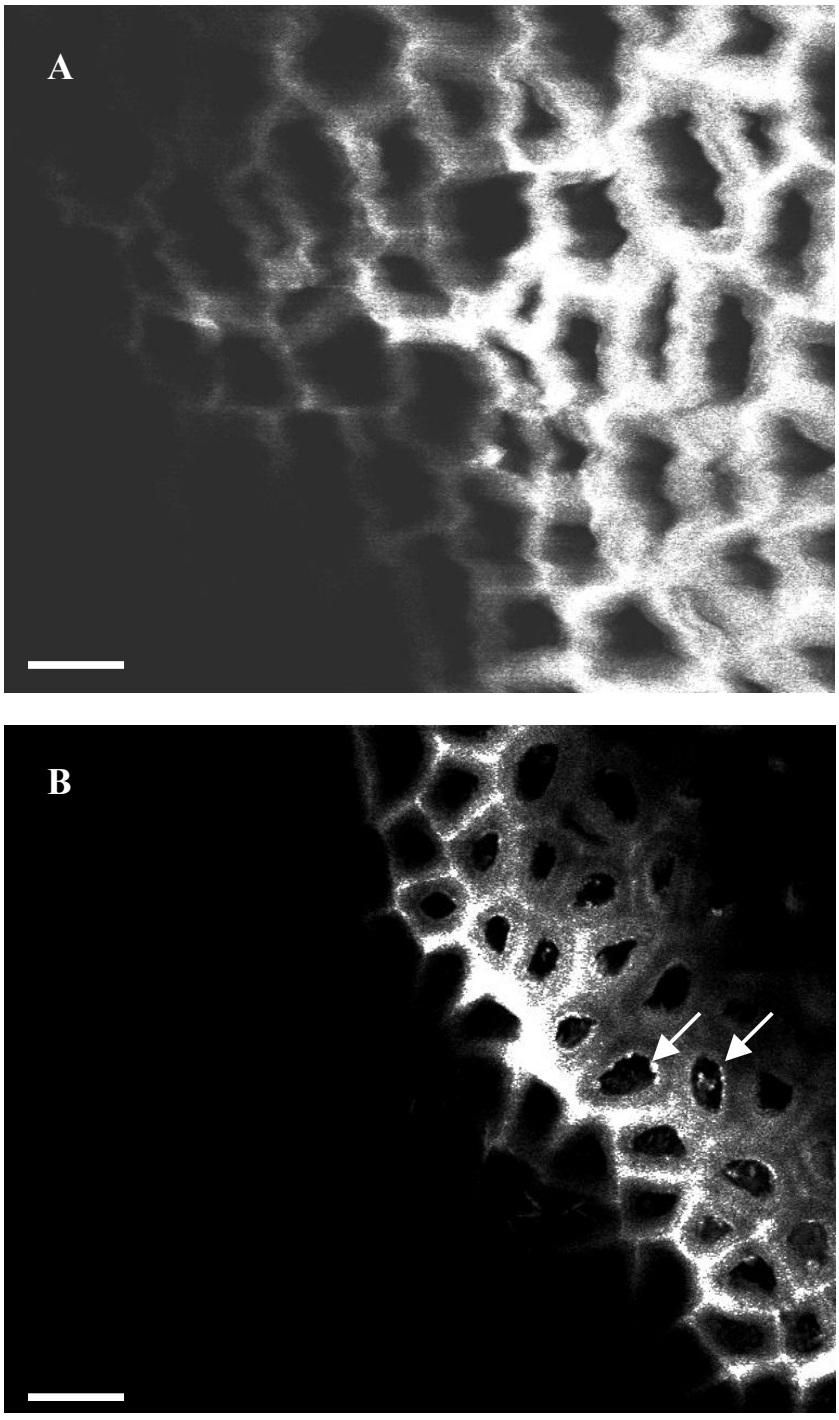


Figure 4.20 Confocal laser scanning micrographs of canola seeds at the time of inoculation (t = 0) with strain 6-8-GFP-Rif^{+}. (A) Control seeds treated with 0.1M $MgSO_4$; (B) Seeds inoculated with strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar in each image is equal to 60 µm.

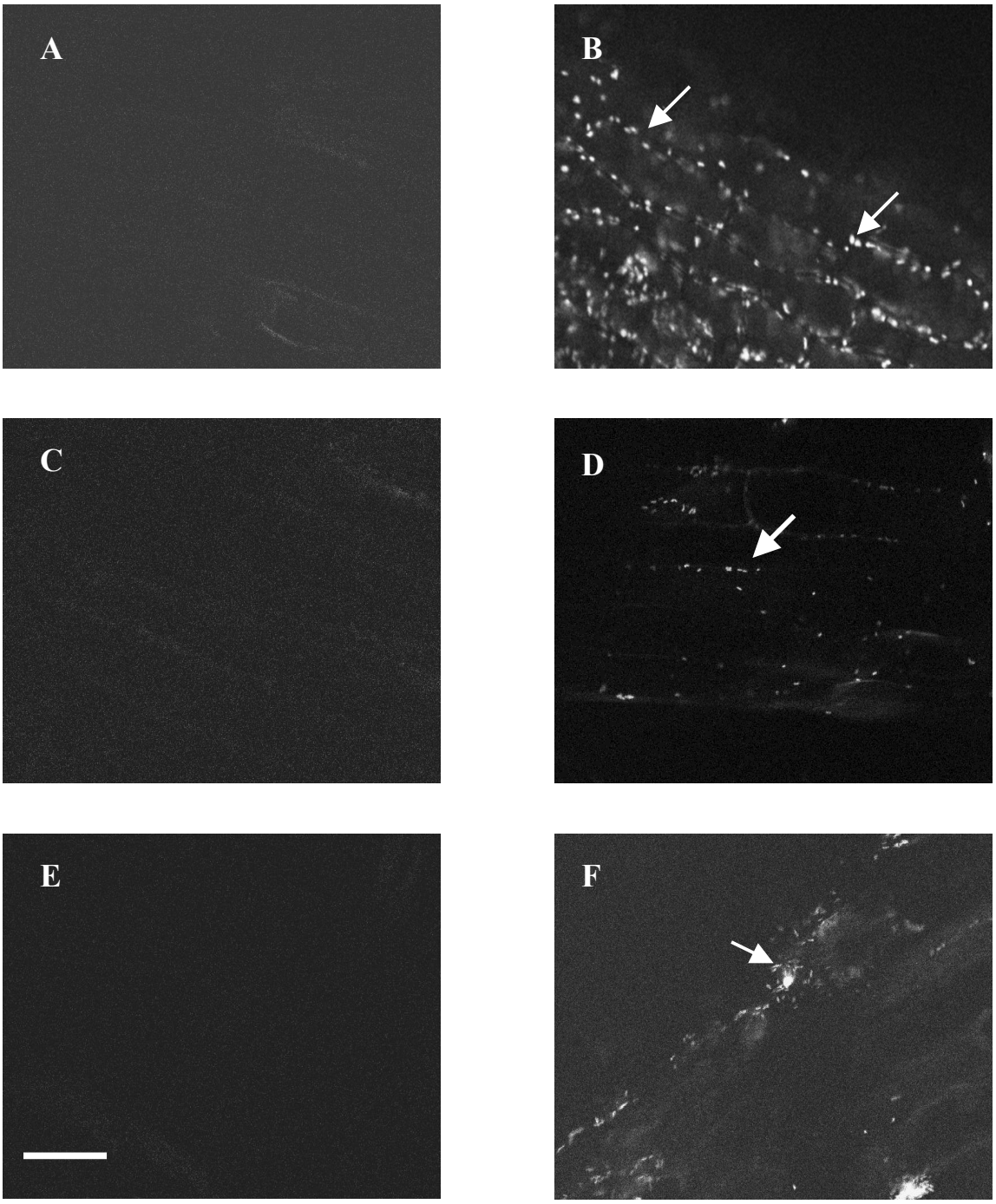


Figure 4.21 Confocal laser scanning micrographs of canola root segments 5 days after inoculation with strain 6-8-GFP-Rif^{+} following incubation in growth pouches. A, C, E: Top, middle and root tip of control roots treated with 0.1M $MgSO_4$; B, D, F: Top, middle and root tip of canola root colonized by strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar is equal to 60 μm.

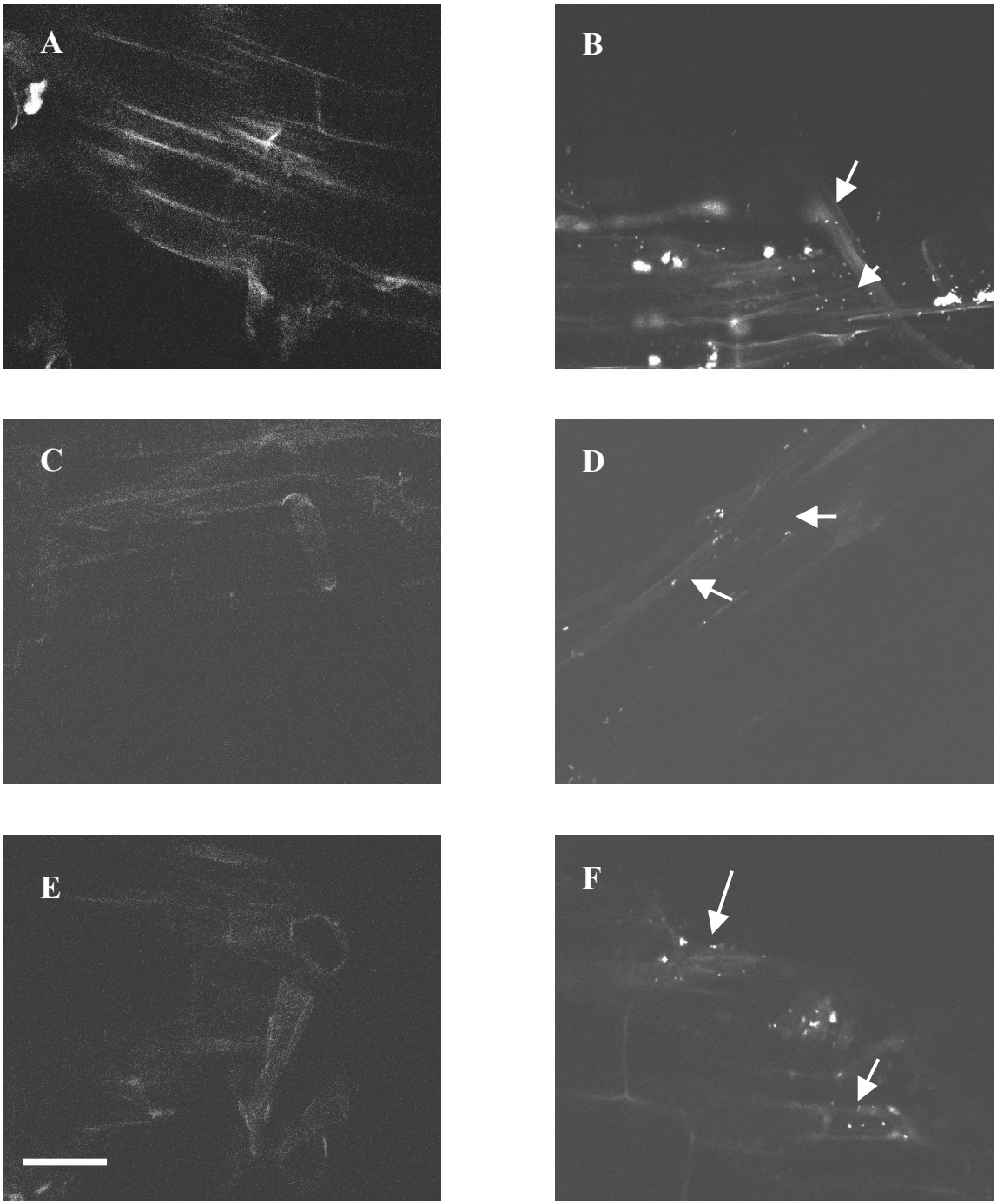


Figure 4.22 Confocal laser scanning micrographs of canola root segments 7 days after inoculation with strain 6-8-GFP-Rif^{+} following incubation in growth pouches. A, C, E: Top, middle and root tip of control roots treated with 0.1M $MgSO_4$; B, D, F: Top, middle and root tip of canola root colonized by strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar is equal to 60 μm.

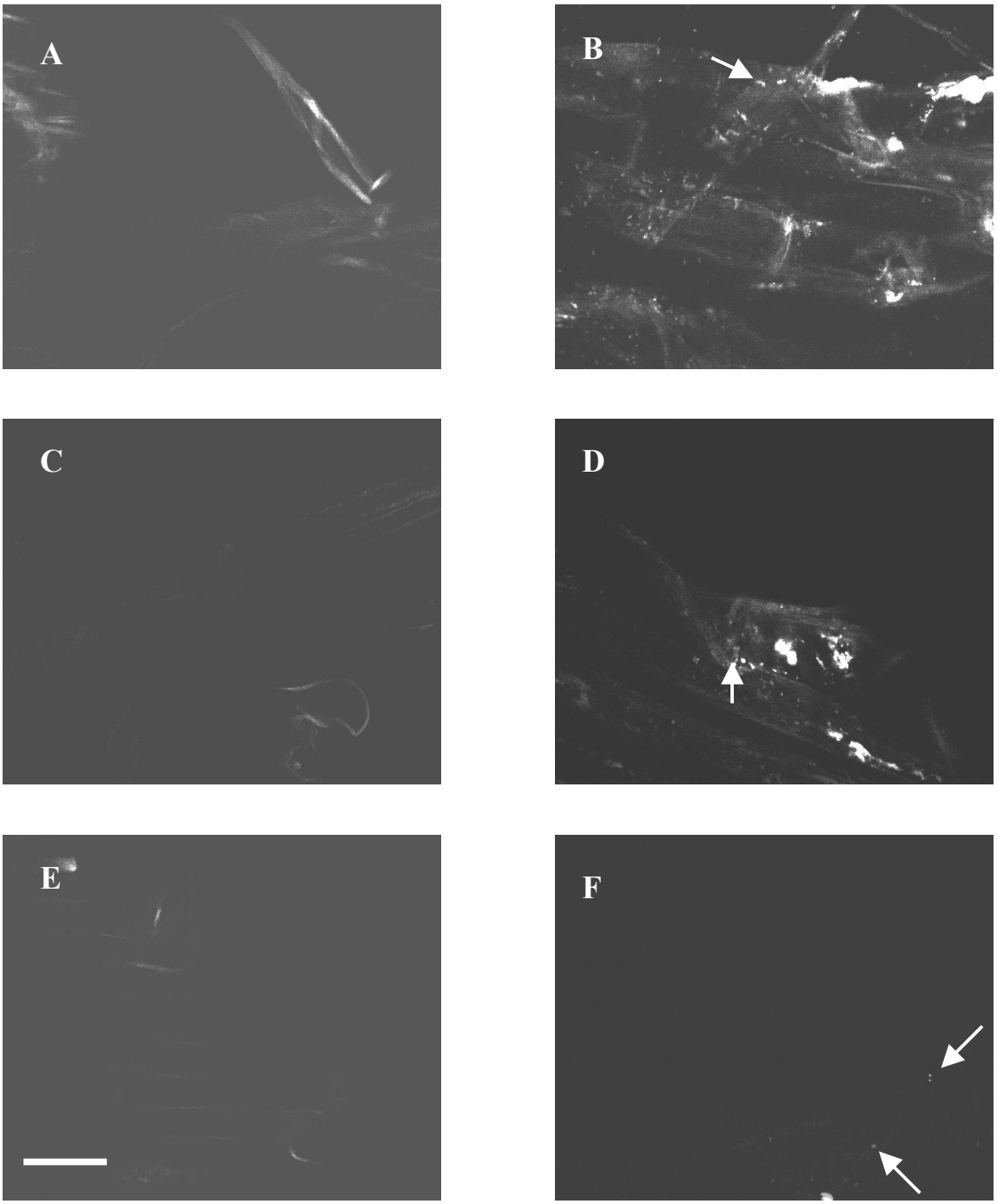


Figure 4.23 Confocal laser scanning micrographs of canola root segments 9 days after inoculation with strain 6-8-GFP-Rif^{+} following incubation in growth pouches. A, C, E: Top, middle and root tip of control roots treated with 0.1M $MgSO_4$; B, D, F: Top, middle and root tip of canola root colonized by strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar is equal to 60 μm.

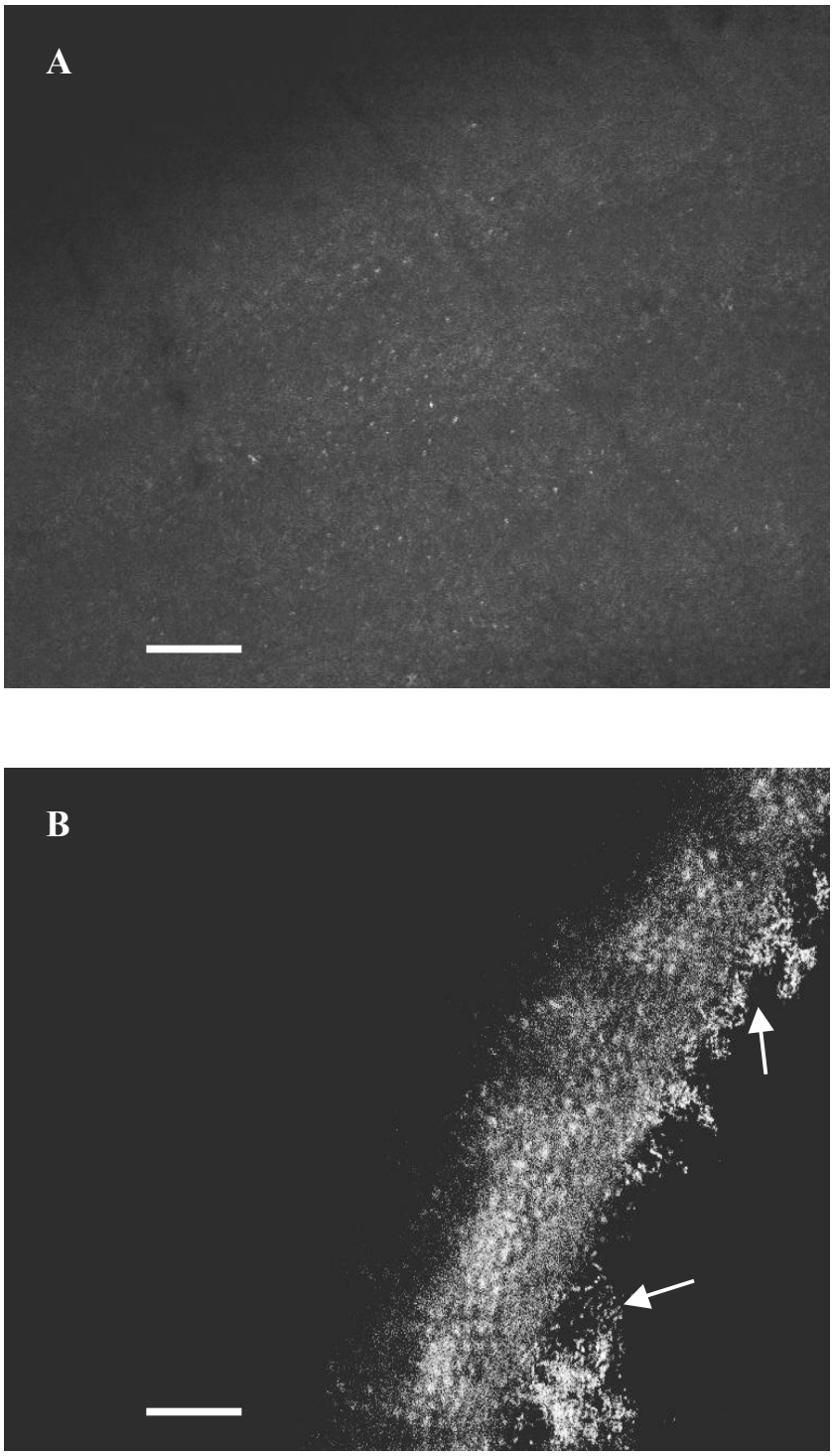


Figure 4.24 Confocal laser scanning micrographs of lentil seeds at the time of inoculation (t = 0) with strain 6-8-GFP-Rif^{+}. (A) Control seeds treated with 0.1M $MgSO_4$; (B) Seeds inoculated with strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar in each image is equal to 60 μm.

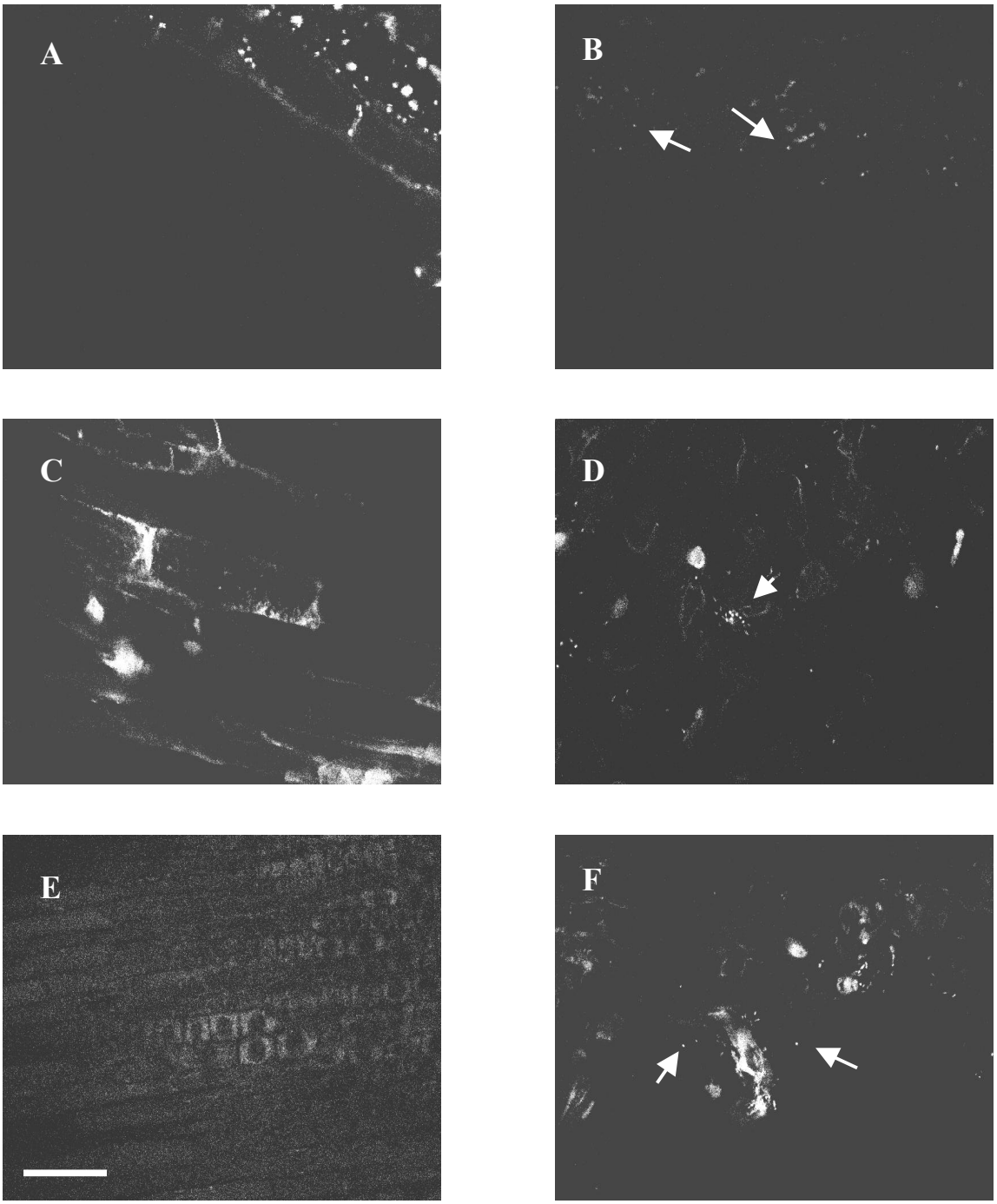


Figure 4.25 Confocal laser scanning micrographs of lentil root segments 3 days after inoculation with strain 6-8-GFP-Rif^{+} following incubation in growth pouches. A, C, E: Top, middle and root tip of control roots treated with 0.1M $MgSO_4$; B, D, F: Top, middle and root tip of lentil root colonized by strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar is equal to 60 μm.

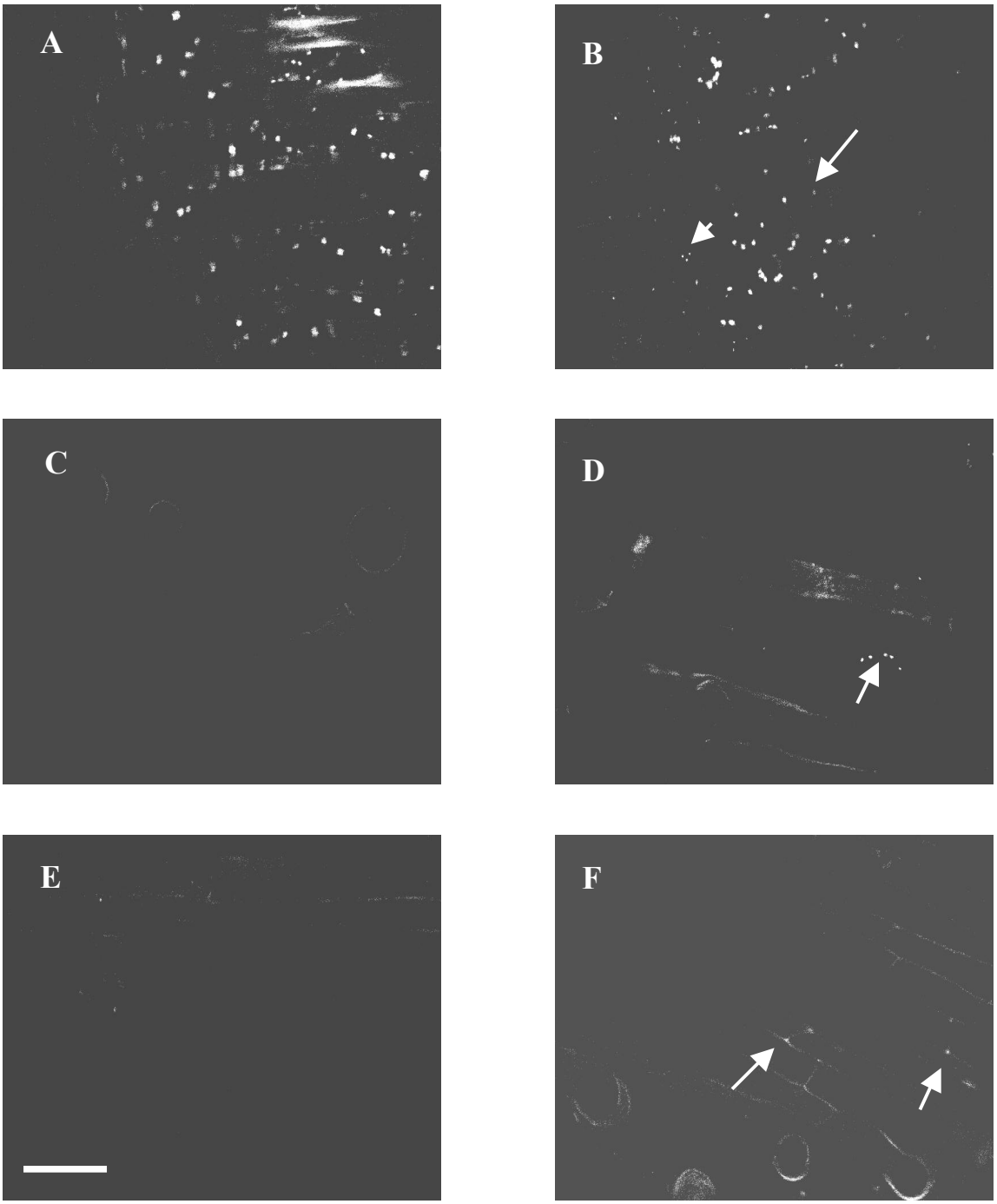


Figure 4.26 Confocal laser scanning micrographs of lentil root segments 6 days after inoculation with strain 6-8-GFP-Rif^{+} following incubation in growth pouches. A, C, E: Top, middle and root tip of control roots treated with 0.1M $MgSO_4$; B, D, F: Top, middle and root tip of lentil root colonized by strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar is equal to 60 μm.

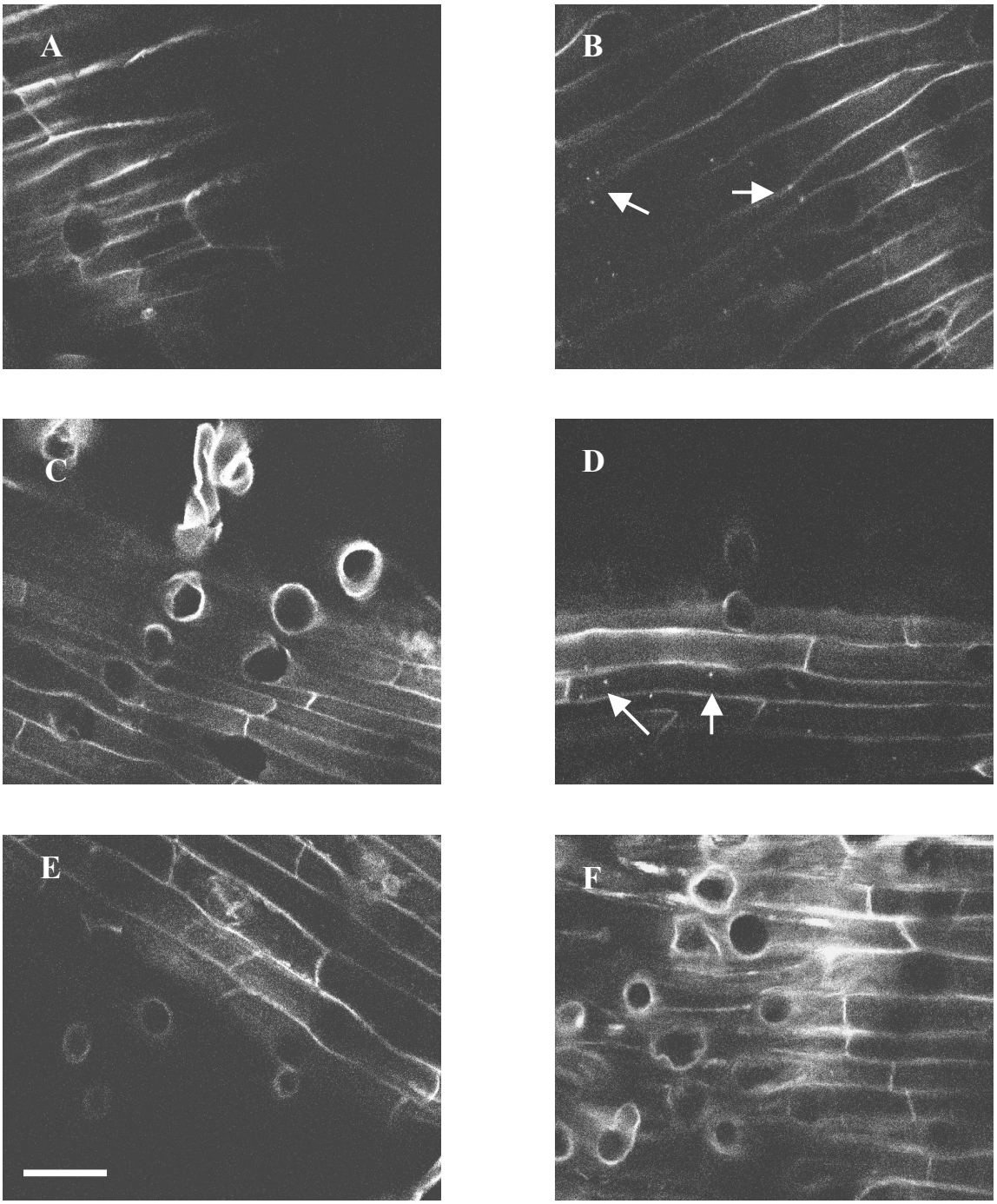


Figure 4.27 Confocal laser scanning micrographs of lentil root segments 9 days after inoculation with strain 6-8-GFP-Rif^{+} following incubation in growth pouches. A, C, E: Top, middle and root tip of control roots treated with 0.1M $MgSO_4$; B, D, F: Top, middle and root tip of lentil root colonized by strain 6-8-GFP-Rif^{+} (as indicated by arrows). The bar is equal to 60 μm.

5.0 DISCUSSION

The hypothesis that strain 6-8, when applied to canola cv. Smart and lentil cv. Milestone, enhances plant growth by direct mechanisms has been tested using various methodologies.

Initial studies were concentrated in studying the effect of strain 6-8 and other known PGPR strains on canola and lentil plants both in sterile growth pouches (gnotobiotic assay) and small pots (growth chamber studies). Variations in the experiments were observed when the experiments were repeated in both gnotobiotic and growth chamber studies. This required that statistical comparisons be made within each trial rather than combining the data together for analysis. Variability in the nature and magnitude of growth promotion due to bacterial inoculation is not uncommon and presents a significant barrier to the evaluation of bacterial inoculants (Schroth and Weinhold 1986).

The gnotobiotic studies indicated that inoculation of canola seeds with PGPR strains had some growth promoting effects on plants grown in sterile growth pouches in the absence of pathogens. Strain 6-8 increased the root length of canola compared to non-inoculated plants in trial one (fig 4.1). The results were similar to those observed by Lifshitz *et al*. (1987), Glick *et al*. (1997) and Salamone (2000). The effects of other known strains were compared to those of strain 6-8. Strains GR12-2, G20-18 and its

mutant CNT2 promoted root length of canola in trial one, but strain GR12-2/*aux1* had no effect (Fig 4.1). These results suggested that strain 6-8 was acting similarly to strains G20-18 and CNT2, previously identified as producers of the cytokinins (Salamone *et al*. 2001) and GR12-2, previously identified as an auxin producer (Xie *et al*. 1996). Further it was observed that all the PGPR strains, including strain 6-8 decreased lateral root formation in one of the three trials (Fig 4.2). Tien *et al*. (1979) showed that when the pure plant hormone cytokinin or PGPR strains with the ability to synthesize cytokinin were applied to plants, production of lateral roots was decreased but root length increased, which agrees with the present results. The ability of strain GR12-2 to decrease the number of lateral roots can also be accounted for by its synthesis of cytokinin (Salamone 2000). There was no difference between the wild-type (G20-18) and the mutants strain (CNT2) in promoting the growth of canola in gnotobiotic study. In an earlier study Xie *et al*. (1996) reported that wild-type strain GR12-2 promoted the growth of canola while the mutant (GR12-2/*aux1*) which synthesized four-fold higher levels of IAA did not. These results were similar to those obtained in one trial in the present study.

A second set of studies determined whether PGPR strains could promote the growth of canola in small pots under growth chamber conditions. There were variations in the results obtained (Fig 4.6). The differences among trials may be due to the absence of nitrogen in the nutrient solution, which may have caused a decrease in the normal growth of the plant in the later stages of the study. They also may have been due to the potting mixture, which may have initially affected the secretion of phytohormones by the microorganisms into the external environment; a similar result was observed by Chanway *et al*. (1989). Statistical analysis of data indicated that there was no significant

effect of any of the PGPR strains on root length, root dry weight and root surface area of canola over time but there was a significant effect on shoot dry weight. Variable effects of PGPR on shoot dry weight were observed during the first and second trial. Strains GR12-2 and GR12-2/*aux1* increased shoot dry weight of canola cv. Smart during the first trial, while strain G20-18 had a significant effect on 21-day-old canola plants during the second trial (Table 4.1).

Results of the canola gnotobiotic study were different from those observed for growth chamber studies, which may be due to differences in growth conditions in the two different systems. Strains 6-8, G20-18, CNT2 and GR12-2 increased the growth of canola plants in growth pouches,, while strains G20-18, GR12-2 and GR12-2/*aux1* increased shoot dry weight of canola in growth chamber studies in one of the three trials.

The results of the lentil gnotobiotic study were different from those observed in canola. Root length of lentil was increased by strains GR12-2 and GR12-2/*aux1* in trial three (Fig 4.3) when compared with other PGPR strains and the control. Strain GR12-2 and the mutant may have an effect on lentil root length due to their ability to secrete the phytohormone IAA (Xie *et al*. 1996). Lifshitz *et al*. (1986) have shown that strain GR12-2 promotes root elongation by increasing the nutrient uptake of phosphate when a concentration of 1mM of phosphate was added to the nutrient solution. This indicates that the strain has the ability to solubilize phosphate and provide it to the plants for better growth. Moreover root elongation does not necessarily imply plant growth as the correlation between root elongation and plant growth is not absolute (Kloepper and Schroth 1981). The decrease in root dry weight may occur due to the overproduction of IAA by the mutant GR12-2/*aux1*, which may interact with the enzyme ACC synthase in the plant and stimulate the synthesis of ACC the precursor of the hormone ethylene,

which inhibits root elongation (Xie *et al*. 1996). In most cases PGPR strains that secrete IAA increase the number of lateral roots (Barbieri and Galli 1993). In contrast, the results of the present study indicate that strain G20-18 increased lateral root formation in one trial and decreased it in another, while strains GR12-2 and CNT2 decreased lateral root formation in one trial . The effect on lateral roots can be attributed to the hormonal balance in secretion of IAA and cytokinins by strains GR12-2, G20-18, CNT2 (Salamone 2000) as it is critical to the growth and development of root tissues as indicated by Bent *et al*. (2001).

The effect of PGPR strains in promoting the growth of lentil under growth chamber conditions was also assessed and there were variations observed between trials (Fig 4.7, 4.8, 4.9, 4.10). Statistical analysis of data indicated significant effects of inoculation treatment on root length, root dry weight, shoot dry weight and root surface area of lentil over time. Strain GR12-2 increased root length during the fourth week of the study when compared to the control, CNT2 and GR12-2/*aux1*. In most of the trials CNT2, the mutant strain of G20-18, decreased root length when compared to other PGPR strains. Variable effects of PGPR on root dry weight were observed as strains G20-18, GR12-2, GR12-2/*aux1* and 6-8 had a significant effect in either one of the three trials. Strains G20-18, GR12-2 and 6-8 increased shoot dry weight while strain CNT2 significantly decreased shoot dry weight in one of the three trials; a similar response was observed by Chanway *et al*. (1989) with the PGPR strain G2-8. Root surface area was significantly increased by strains G20-18, GR12-2, GR12-2/*aux1* and 6-8 in trial one on week one, while the effect of strain 6-8 was significant only during the first two weeks of the study.

In general there was a good agreement between the results found in the growth pouch study and growth chamber studies for lentil. Both strains GR12-2 and GR12-2/*aux1* enhanced root development in both culture systems. Strain GR12-2 was more consistent in increasing root length, shoot dry weight and root surface area of lentil cv. Milestone in small pots. The effects of strains G20-18, GR12-2 and 6-8 on root dry weight and root surface area were variable (i.e. a significant effect was observed in one out of three trials). Strain CNT2 decreased growth of lentil. The results suggest that production of auxin (IAA) by strain GR12-2 and cytokinins by strains G20-18 and 6-8 may have enhanced the growth of lentil.

The effects of PGPR strains are considered to be highly specific with respect to plant and bacterial genotypic combination (Rennie and Larson 1979). Host variations in the interaction with beneficial plant-associated microbes are also considered to be an important factor (Smith and Goodman, 1999). The results of the present study support the hypotheses that strain 6-8 is able to promote the growth of canola cv. Smart in gnotobiotic conditions and that of lentil cv. Milestone in growth chamber studies. This may be attributed to host specific variations and different systems used for the study.

One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones (Kloepper *et al*. 1988; Glick 1995). In the present study strain 6-8 promoted the growth of canola cv. Smart in gnotobiotic conditions, and lentil cv. Milestone in growth chamber studies as did other known PGPR strains which produce auxins and cytokinins. I hypothesized that strain 6-8 may enhance the growth of canola and lentil plants by production of phytohormones.

All the PGPR strains were assayed for indole production, as auxin is one of the most frequently studied phytohormones (Frankenberger and Arshad 1995). The assessment of

indole production was performed in the presence of the precursor, L-tryptophan, as various authors have identified tryptophan addition to be necessary for indole-3-acetic acid production by microorganisms (Tien *et al*. 1979; Hartmann *et al*. 1983; Xie *et al*. 1996; Bent *et al*. 2001; Asghar *et al*. 2002). Strain GR12-2 produced indoles even in the absence of L-tryptophan, which is similar to the report of Fallik and Okon (1989) who showed that *Rhizobium* spp. synthesized IAA in the absence of L-tryptophan. The concentration of indole increased with increasing concentrations of L-tryptophan in the culture medium. Strain 6-8 was positive for indole production with varying concentrations of precursor added to the growth medium. In natural conditions L-tryptophan may be available in root exudates (Beniziri *et al*. 1998).

Xie *et al*. (1996) reported that the mutant strain GR12-2/*aux1*, which has the ability to secrete four fold higher levels of indole (IAA), inhibited root elongation of canola when compared to the parent strain. In the present study there was no significant difference observed between the wild-type GR12-2 and the mutant GR12-2/*aux1* in promoting the growth of canola in growth pouches. This suggests that indole (IAA) secreted by the strains had no significant effect on growth of canola cv. Smart.

There are several different kinds of indoles secreted by a particular microorganism into the environment, depending on the biosynthetic pathways and genotype (Patten and Glick 1996). Earlier studies by Gordon and Weber (1951) indicated that the Salkowski reagent was more sensitive to IAA, but at the same time interfered with other indole compounds such as indole-3-aldehyde, indole-3-carboxylic acid, indole-3-propionic acid, indole-3-butyric acid. The non-specificty of the Salkowski reagent in identifying indole compounds such as indole lactate, indole acetaldehyde and indole acetamide has also been mentioned by Hartmann *et al*. (1983). Due to the non-

specificity of Salkowski reagent strains G20-18, CNT2, GR12-2, GR12-2/*aux1* and 6-8 were screened specifically for IAA synthesis using the ELISA technique (Salamone, 2000; Bent *et al*. 2001). There were no significant differences in the production of IAA among the PGPR strains except for the mutant strain, GR12-2/*aux1* which produced higher concentrations of IAA (6.78 pmole/ml) in the presence of L-tryptophan (500 µg/ml). Also IAA production was very low in these strains (2.87 – 6.78 pmole/ml) when compared to the standards (78-2500 pmoles/ml). A modified standard curve with a minimum value of 0.75 pmol/0.1 ml as indicated by Bent *et al*. (2001) would have been useful for better interpretation of the data. The concentrations of IAA secreted in pure culture by PGPR strains (2.87 – 3.36 pmol/ml) was different from that found in the rhizosphere of canola inoculated with PGPR strains (1.83 – 2.35 pmol/ml) suggesting that IAA production was significantly less *in situ* in the canola rhizosphere, resulting in little effect of the strains. In addition there were no significant differences among PGPR strains in the IAA concentrations in the supernatants obtained from the canola rhizosphere, but strains GR12-2 and GR12-2/*aux1* produced significantly more IAA than the non-inoculated plants.

It should be emphasized that phytohormones such as auxin do not act alone, but may interact with other known phytohormones (Barendse and Peeters 1995). Strains G20-18, CNT2 and 6-8 were assayed for production of IPA, ZR and DHZR in pure cultures over time. The results showed that strain 6-8 produced IPA and the concentration of IPA produced was similar to that of the wild-type strain, G20-18 at stationary phase of growth. Strain 6-8 produced both ZR and DHZR in pure culture and

the amounts of ZR and DHZR produced were significantly higher than that of mutant CNT2, which has been shown to produce lower concentrations of these phytohormones (Salamone *et al*. 2001). The ability of strain 6-8 to synthesize the phytohormone cytokinins such as IPA, ZR and DHZR similar to the wild-type strain G20-18 in pure cultures is consistent with the hypothesis that the effects of strain 6-8 on canola growth were due to cytokinin production rather than IAA synthesis. However, a mutant of strain 6-8 lacking cytokinin synthetic ability would permit more definitive testing of this hypothesis.

Cytokinin concentrations in the canola rhizosphere in the presence of strains G20-18, CNT2 and 6-8 were increased; the concentration of IPA was always higher than that of ZR and DHZR. Inoculation with strains 6-8 and G20-18 increased production of IPA and ZR when compared to the non-inoculated canola plant. There were no differences in the concentrations of DHZR by the PGPR strains and the non-inoculated control, which is in contrast with the results obtained by Salamone (2000).

The results of the present study are consistent with the hypothesis that strain 6-8 promotes the growth of canola in growth pouches by production of cytokinins, a direct mechanism of growth promotion. The concentrations of IAA secreted in pure culture and present in the supernatants obtained from canola growth pouches were very low when compared to the standard concentrations. Thus the data suggest that the positive effect of strain 6-8 on canola plants was not associated with IAA production, since all the strains produced low concentrations of IAA both in bacterial cultures and in the rhizosphere of canola and 6-8 did not differ from the control in the latter. However, the possibility that strain 6-8 may possess other direct mechanisms of plant growth promotion can not be ruled out.

Apart from production of phytohormones, various other direct mechanisms of action have been associated with PGPR for enhancing plant growth. It is always difficult to ascertain that a PGPR promotes plant growth by using only a single mode of action as, for example, strain GR12-2 produces IAA (Xie *et al*. 1996), solubilizes phosphate (Lifshitz *et al*., 1986) and also produces ACC deaminase which helps in lowering ethylene concentration (Glick *et al*., 1994a). In the present study strain 6-8 was positive for PGPR traits such as siderophore production, ability to use ACC as sole nitrogen source and ability to solubilize inorganic phosphate. The growth promotion of canola by strain 6-8 in growth pouches may be attributed to any one of these mechanisms or a combination. For instance, the ability to use ACC as sole nitrogen source can be linked to the model proposed by Glick *et al*. (1998) where the bacteria utilize the plant-exuded ACC and decrease the concentration of ethylene, thereby breaking down ACC to ammonia. This helps in promoting plant growth by increasing root elongation, which may be a possible mechanism of action. Strain 6-8 may also have increased the nutrient uptake of canola by solubilizing inorganic phosphate present in nutrient solution and making it available for uptake by the plant, as suggested by Lifshitz *et al*. (1986) for other phosphate-solubilizing PGPR. The third alternative is by production of siderophores, which are low molecular weight iron-binding molecules that are synthesized under low-iron conditions (Neilands 1981b). A direct mechanism of action of bacterial siderophores is that they may be available to the plant, as a source of iron, which directly helps in the growth of the plant. In the present study strain, 6-8 may help in the uptake of iron from the nutrient solution or from the potting mixture through the production of siderophores, which would enhance the growth of canola. Cattelan *et al*. (1999) observed a similar result, where strains screened for more than one PGPR trait

were found to promote the growth of soybean. Development of mutants of 6-8 lacking the ability to produce siderophores, to use ACC as sole nitrogen source or to solubilize phosphate may help in determining, which if any of these mechanisms is important in increasing growth of canola in laboratory conditions.

One of the generally accepted concepts is that beneficial PGPR are effective only when they successfully colonize and persist in the plant rhizosphere (Elliot and Lynch, 1984; Lugtenberg *et al*. 2001). The pattern of root colonization on canola and lentil roots was studied using two different approaches. The first approach was the use of traditional dilution plating technique, which allows quantitative analysis of bacterial populations adhering to the surface of the root. The second approach was a novel method where the colonization by strain 6-8 of canola and lentil roots was monitored using a molecular marker and CLSM for qualitative image analysis. The results of the dilution plating technique showed that the population of strain 6-8 was 3.6 fold higher on canola roots per plant than on lentil roots in growth pouches. An increase in the total number of bacteria also indicated that strain 6-8 was able to grow within the plant rhizosphere making use of the root exudates as a possible source of carbon.

The results for CLSM image analysis were similar to those obtained from dilution plating as they showed higher populations of bacteria on canola roots than on lentil roots. The bacterial populations were higher in the top region of the root than in the middle and the root tip. This may be due to seed application, which limits the population initially to the upper region of the tap root (Roberts *et al*. 1999). In both plant systems the number of bacteria in the roots decreased with time. The decrease in number may be related to changes in exudation pattern during the life cycle of the plant, leading to lower numbers of pseudomonads in the later phase of plant growth (Miller *et al*. 1989).

Results from both the studies showed that strain 6-8 colonized roots of canola in higher number than those of lentil. This may be an important factor in the ability of strain 6-8 to promote growth of canola. The production of phytohormones may influence the growth and branching intensity of the root (Höflich *et al*. 1992) and the phytohormones secreted by strain 6-8 may be responsible for the observed growth promotion of canola in gnotobiotic studies when compared to that of lentil. The number of bacteria colonizing the root also has a significant effect in increasing or decreasing the beneficial effect of PGPR (Chiarini *et al*. 1998; Frey-Klett *et al*. 1999). The use of CSLM has been an advantage in following colonization patterns on different segments of the roots without any additional need for staining and sectioning. The difference in the bacterial population obtained from the two different methods may be due to the total area scanned for the entire study. For image analysis a small portion of the root is being scanned while in the plating technique the entire segment was assayed. Moreover dilution plating gives the results of bacteria present only on the external surface while CLSM gives a picture of bacteria colonizing the external and internal root tissues.

6.0 CONCLUSIONS

The results of the present study are as follows:

1. Strain 6-8 enhanced the growth of canola cv. Smart in growth pouches by increasing the root length in one of three trials and in lentil cv. Milestone in pots in growth chamber by increasing root dry weight (one trial), shoot dry weight (one trial) and root surface area (two trials).
2. Strain G20-18 increased the root length of canola cv. Smart in one of three trials and the number of lateral roots in lentil cv. Milestone in one of three trials in gnotobiotic assay. The root dry weight, shoot dry weight and root surface area of lentil cv. Milestone was significantly increased in one of the trials in pots in growth chamber studies.
3. Strain GR12-2 promoted root length in canola cv. Smart in one of three trials grown under gnotobiotic conditions. Strains GR12-2 and GR12-2/*aux1* promoted growth of lentil cv. Milestone in gnotobiotic conditions by increasing root length in one of three trials.
4. Strains GR12-2 and GR12-2/*aux1* increased shoot dry weight of canola cv. Smart grown in pots in growth chamber in two and one trials of three, respectively. The root length of lentil cv. Milestone was increased by strain GR12-2 in one trial of three. Root dry weight of lentil cv. Milestone grown in pots in growth chamber was significantly increased by strains GR12-2 in one trial of three and by GR12-2/*aux1* in two trials of three. The root surface area of

lentil cv. Milestone grown in pots in growth chamber was significantly increased by strains GR12-2 and GR12-2/*aux1* in one trial of three.

5. All the PGPR strains produced indole compounds when the precursor L-tryptophan was added to the culture medium. The concentration of indole increased with increasing concentrations of L-tryptophan.
6. The concentrations of IAA secreted by PGPR strains were extremely low in pure culture and in the rhizosphere of canola grown in growth pouches.
7. Strain 6-8 produced the cytokinins, IPA, ZR and DHZR in pure culture.
8. Strain 6-8 produced significantly higher concentrations of IPA and ZR in the canola rhizosphere than the non-inoculated control.
9. Strain 6-8 produced siderophores, used ACC as a sole nitrogen source and solubilized inorganic phosphate.
10. Strain 6-8 colonized roots of canola more efficiently than those of lentil, which correlates with the results obtained from the gnotobiotic assay of canola and lentil plants.

The above results support the hypothesis that strain 6-8 utilizes either one of the growth promoting mechanisms or a combination of actions to increase the growth of canola in sterile growth pouches and lentil in growth chamber studies. Moreover the ability to colonize canola roots in growth pouches efficiently may contribute to the ability of strain 6-8 to promote the growth of canola in a gnotobiotic assay better than that of lentil.

Further research in developing mutants of strain 6-8 with decreased cytokinin production, siderophore production, phosphate solubilization or ACC activity would

help in elucidating the major direct mechanism of action of strain 6-8 in promoting the growth of canola and lentil under laboratory and field conditions. This would help in developing a potential inoculant for use in agriculture in the future.

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8.0 APPENDIX

8.1 Statistical Analyses

Table 8.1.1 Summary of ANOVA for the root length, root dry weight and number of lateral roots for canola cv. Smart grown under gnotobiotic conditions. Data were analyzed using one way randomized block design where blocks indicate trials.

Source	df	Mean Square	F ratio	Probability
Root length				
Blocks	2	78.04	64.87	0.00***
Treatment	5	3.71	3.09	0.01*
Root dry weight				
Blocks	2	3.10	8.22	0.00***
Treatment	5	0.49	1.30	0.26 ns
Lateral roots				
Blocks	2	3448.86	31.90	0.00***
Treatment	5	555.44	5.13	0.00***

* Significant at p=0.05
*** Significant at p=0.001
ns Not significant

Table 8.1.2 Summary of ANOVA for the root length, root dry weight and number of lateral roots for canola cv. Smart grown under gnotobiotic conditions. Data obtained from three trials were analyzed individually.

Source	df	Mean Square	F ratio	Probability
Root length				
Trial 1	5	5.70	3.59	0.00**
Trial 2	5	0.86	0.86	0.51 ns
Trial 3	5	2.07	3.06	0.02*
Root dry weight				
Trial 1	5	0.17	0.60	0.70 ns
Trial 2	5	0.93	1.68	0.17 ns
Trial 3	5	0.21	0.76	0.58 ns
Lateral roots				
Trial 1	5	94.46	1.20	0.33 ns
Trial 2	5	527.54	4.53	0.00**
Trial 3	5	523.15	6.89	0.00***

* Significant at p=0.05
**Signficant at p=0.01
*** Significant at p=0.05
ns Not significant

Table 8.1.3 Summary of ANOVA for the root length, root dry weight and number of lateral roots for lentil cv. Milestone grown under gnotobiotic conditions. Data were analyzed using one way randomized block design where blocks indicate trials.

Source	df	Mean Square	F ratio	Probability
Root length				
Blocks	2	147.36	29.64	0.00***
Treatment	5	15.28	3.07	0.01*
Root dry weight				
Blocks	2	118.59	4.92	0.00***
Treatment	5	91.96	3.82	0.00***
Lateral roots				
Blocks	2	2696.03	31.90	0.00***
Treatment	5	384.20	1.66	0.14 ns

* Significant at p=0.05
*** Significant at p=0.001
ns Not significant

Table 8.1.4 Summary of ANOVA for the root length, root dry weight and number of lateral roots for lentil cv. Milestone grown under gnotobiotic conditions. Data obtained from three trials were analyzed individually.

Source	df	Mean Square	F ratio	Probability
Root length				
Trial 1	5	12.66	2.13	0.08 ns
Trial 2	5	4.31	0.76	0.58 ns
Trial 3	5	10.00	3.23	0.01*
Root dry weight				
Trial 1	5	83.15	5.26	0.00**
Trial 2	5	66.93	2.42	0.05 ns
Trial 3	5	30.97	1.35	0.26 ns
Lateral roots				
Trial 1	5	416.66	2.78	0.03*
Trial 2	5	564.11	3.50	0.01*
Trial 3	5	1033.86	4.68	0.00**

* Significant at p=0.05
** Significant at p=0.01
ns Not significant

Table 8.1.5 Summary of ANOVA for root length, root dry weight, shoot dry weight and root surface area of canola cv. Smart from the growth chamber studies analyzed using two way randomized block design where blocks indicate trials.

Source	Root length		Root dry weight		Shoot dry weight		Root surface area	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**
Blocks	25.89	.00 ***	.46	.63 ns	9.37	.00 ***	21.05	.00 ***
Treatment	1.78	.11 ns	.65	.65 ns	1.59	.16 ns	2.05	.07 ns
Time	366.21	.00 ***	421.12	.00 ***	618.70	.00 ***	228.58	.00 ***
Treatment x Time	.54	.91 ns	1.26	.25 ns	.88	.58 ns	.077	.65 ns
CV	25.46%		42.18%		40.91%		40.60%	

*** Significant at p=0.001
ns Not significant
CV Coefficient of variation

Table 8.1.6 Summary of ANOVA for root length, root dry weight, shoot dry weight and root surface area of canola cv. Smart from the growth chamber studies analyzed using two way randomized design for each individual trial.

Source	Root length		Root dry weight		Shoot dry weight		Root surface area	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**
Main effects								
Trial 1								
Treatment	1.69	.14 ns	1.65	.15 ns	3.81	.00 **	1.84	.11 ns
Time	327.44	.00 ***	473.02	.00 ***	402.01	.00 ***	77.58	.00 ***
Treatment x Time	1.20	.28 ns	2.92	.00 **	2.93	.00***	3.66	.00 ***
Trial 2								
Treatment	1.49	.19 ns	1.25	.29 ns	4.41	.00 **	0.79	.55 ns
Time	250.41	.00***	218.04	.00 ***	456.52	.00 ***	127.96	.00 ***
Treatment x Time	0.59	.87 ns	1.63	.11 ns	2.95	.00 ***	0.47	.90 ns
Trial 3								
Treatment	1.57	.17 ns	0.46	.80 ns	0.14	.98 ns	1.89	.10 ns
Time	101.92	.00 ***	186.83	.00 ***	291.08	.00 ***	113.95	.00 ***
Treatment x Time	9.89	.61 ns	0.19	.99 ns	1.05	.40 ns	0.54	.85 ns
CV								
Trial 1	18.36 %		30.08 %		31.70 %		34.24 %	
Trial 2	18.59 %		31.11%		28.25 %		34.51 %	
Trial 3	23.12 %		33.31%		33.84 %		37.39 %	

** Significant at p=0.01
*** Significant at p=0.001
ns Not significant.
CV Coefficient of variation.

Table 8.1.7 Summary of ANOVA for root length, root dry weight, shoot dry weight and root surface area of lentil cv. Milestone from the growth chamber studies analyzed using two way randomized block design where blocks indicate trials.

Source	Root length		Root dry weight		Shoot dry weight		Root surface area	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**
Blocks	.52	.59 ns	55.39	.00 ***	30.04	.00 ***	106.34	.00 ***
Treatment	1.10	.35 ns	2.30	.04 *	2.39	.03 *	3.79	.00 **
Time	181.22	.00 ***	131.07	.00 ***	214.21	.00 ***	91.79	.00 ***
Treatment x Time	1.37	.15 ns	1.28	.21 ns	1.09	.35 ns	1.76	.03 *
CV	21.55 %		51.41 %		51.84 %		53.26 %	

* Significant at p=0.05
**Signficant at p=0.01
*** Significant at p=0.001
ns Not significant.
CV Coefficient of variation.

Table 8.1.8 Summary of ANOVA for root length, root dry weight, shoot dry weight and root surface area of lentil cv. Milestone from the growth chamber studies analyzed using two way randomized design for each individual trial.

Source	Root length		Root dry weight		Shoot dry weight		Root surface area	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**
Main effects								
Trial 1								
Treatment	6.29	.00 ***	11.98	.00 ***	6.06	.00 **	8.98	.00 ***
Time	271.72	.00 ***	297.46	.00 ***	239.05	.00 ***	124.62	.00 ***
Treatment x Time	1.86	.03 *	4.82	.00 ***	3.10	.00***	2.34	.00 ***
Trial 2								
Treatment	1.09	.36 ns	3.05	.01 *	5.57	.00 **	2.20	.59 ns
Time	79.56	.00***	97.27	.00 ***	177.36	.00 ***	51.32	.00 ***
Treatment x Time	1.65	.07 ns	2.90	.00 ***	4.76	.00 ***	2.45	.00 **
Trial 3								
Treatment	1.16	.33 ns	3.12	.01 *	0.45	.80 ns	1.13	.34 ns
Time	41.83	.00 ***	61.71	.00 ***	126.49	.00 ***	38.42	.00 ***
Treatment x Time	2.35	.00 **	2.76	.00 ***	1.44	.14 ns	2.58	.00 **
CV								
Trial 1	15.29 %		27.04 %		36.61 %		33.62 %	
Trial 2	19.33 %		29.36 %		26.86 %		34.21 %	
Trial 3	15.24 %		25.40 %		30.70 %		32.19 %	

* Significant at p=0.05
**Signficant at p=0.01
*** Significant at p=0.001
ns- Not significant.
CV Coefficient of variation.

8.2 Media Used

8.2.1 N-free Hoagland's Nutrient Solution (Hoagland and Boyer, 1936)

Stock solutions were prepared as follows :

Macronutrients	**Stock solutions**
KH2PO4 (1M)	136.09 gl^{-1}
K_2SO_4 (0.5M)	87.135 gl^{-1}
$MgSO_4.7H_2O$ (1M)	246.48 gl^{-1}
Micronutrients	
Boric acid	1.00 g l^{-1}
Manganous chloride	1.00 g l^{-1}
Zinc sulfate	0.58 g l^{-1}
Cupric sulfate	0.13 g l^{-1}
Sodium molybdate	0.10 g l^{-1}

Iron stock solution: 20 g l^{-1} (Obtained from PBI, Saskatoon, SK, Canada)

The final medium contained:

KH_2PO_4: 2 ml l^{-1} of stock

K_2SO_4: 4 ml l^{-1} of stock

$CaSO_4$: 1 g l^{-1} of stock

$MgSO_4.7H_2O$: 1 ml l^{-1} of stock

Microstock: 1 ml l^{-1} of stock

IRON: 1 ml l^{-1} of stock

The pH was adjusted to 7.0 using 0.5 M KOH and sterilized for 20 minutes at 121°C for 15 minutes.

8.2.2 Salkowski's Reagent (Gordon and Weber – 1951)

150 mL concentrated Sulphuric acid

250 mL distilled water

7.5 mL (0.5M) $FeCl_3.6H_2O$

8.2.3 DF salts minimal medium utilized for indole production (Dworkin and Foster-1958)

KH_2PO_4	4.0 g
Na_2HPO_4	6.0 g
MgSO4.7H2O	0.2 g
$FeSO_4.7H_2O$	0.001 (Stock solution of 100 mg/10 ml)
Glucose	2.0 g
Gluconic acid (Ksalt)	2.0 g
Citric acid (Tri-Na salt)	2.0 g
$(NH_4)_2SO_4$	2.0 g

Dissolved in 1000 ml of distilled H_2O

Micro-nutrients (Stock solution: 0.1ml l^{-1} was added to above DF salts minimal medium)

H_3BO_3	10 mg
$MnSO_4$	11.2 mg
$ZnSO_4$	124.6 mg
$CuSO_4$	78.2 mg
MoO_3	78.2 mg

Dissolved in 1000 ml of distilled H_2O

8.2.4 Minimal medium + Glucose (Salamone 2000)

Component 1:

KH_2PO_4 1.36 g

K_2HPO_4 1.74 g

in 408 ml of deionized water.

Component 2:

$MgSO_4$ 0.5 g

NH_4Cl 1.0g

in 572 ml of deionized water

The two components were autoclaved separately to avoid formation of a phosphate precipitate and mixed after autoclaving. Forty milliliters of glucose 25 g dissolved in 100 ml of distilled water and filter sterilized was added to the minimal medium.

8.2.5 Rhizosphere Medium (RSM)

In one liter

Ca(NO3)2.4H2O 0.75 g

$MgSO_4.7H_2O$ 0.246 g

ACES 18.22 g

NaOH 2.0 g

Deionized Water 853.0 ml

The medium was autoclaved at 121ºC for 15-20 minutes and the following stock solutions were added

KH_2PO_4 (1M pH7) 1.0 ml

$ZnSO_4$ 7H2O ($7.0X10^{-4}M$) 1.0 ml

Mn SO_4 .4H2O($9.0X10^{-4}M$) 1.0 ml

Biotin(1 mgl^{-1}) 1.0 ml

Thiamine HCl (20 mgl^{-1}) 1.0 ml

Case amino acids (10%) 100.0 ml

Sucrose (30%) 33.3 ml

KH_2PO_4, Case amino acids and sucrose were autoclaved before adding to the medium $ZnSO_4$ 7H2O, Mn SO_4 .4H2O, Thiamine HCl and Biotin were filter sterilized with 0.2µm filter before being added to the medium.

8.2.6 CAS (Chrome Azurol S) Solution – (Schwyn and Neilands, 1987)

A. Chrome Azurol S	12.2 mg
Deionized water	10.0 ml
B. HCl (concentrated)	84.0 µl
Deionized water	100.0 ml
$FeCl_3$ 6H2O	27.0 mg
C. HDTMA	21.9 mg
Deionized water (warm)	25.0 ml

Seven and half millilitres of (A) were mixed with 1.5 mL of (B), then added slowly to (C) while stirring and then placed in a 100-ml volumetric flask and autoclaved.

D. Anhydrous Piperazine

12 M Hydrocholoric acid 6.25 ml,

HCl was added to the anhydrous piperrazine (4.307 g disolved in 40 ml of water) to a pH 5.6, Solution (D) was rinsed into the volumetric flask and made to a volume of 100 ml with sterile distilled water. This coloured solution was stored at 5°C and covered to keep the solution from breaking down.

8.2.7 YEDP (Phosphate solubilizing medium)

Yeast extract	5 g l^{-1}
Dextrose	10 g l^{-1}
Calcium phosphate	2 g l^{-1}
Agar	12 g l^{-1}

8.2.8 Modified DF salts minimal medium utilized for ACC assay (Dworkin and Foster- 1958)

KH_2PO_4	4.0 g
Na_2HPO_4	6.0 g
MgSO4.7H2O	0.2 g
$FeSO_4.7H_2O$	0.001 g (Stock solution of 100 mg/10 ml)
Glucose	2.0 g
Gluconic acid (Ksalt)	2.0 g
Citric acid (Tri-Na salt)	2.0 g

All of the above were dissolved in 1000 ml of distilled H_2O

Micro-nutrients (Stock solution: 0.1ml/l was added to above DF salts minimal medium)

H_3BO_3	10 mg
$MnSO_4$	11.2 mg
$ZnSO_4$	124.6 mg
$CuSO_4$	78.2 mg
MoO_3	78.2 mg

Dissolved in 1000 ml of distilled H_2O

ACC, $(NH_4)_2SO_4$ and 0.1M $MgSO_4$ (stock solution)

ACC	30.33 mg in 10 ml of distilled H_2O
$(NH_4)_2SO_4$	13.21 g l^{-1}
$MgSO_4$	24.64 g l^{-1}

8.2.9 Luria Bertani medium (Luria and Burrous, 1955)

Peptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
Sodium chloride	4 g l^{-1}
Agar	15 g l^{-1}

8.2.10 Minimal citrate agar – selective media (Modified from Simmons citrate agar)

Magnesium sulfate	0.2 g l^{-1}
Ammonium dihydrogen phosphate	1 g l^{-1}
Sodium citrate	2 g l^{-1}
Sodium chloride	5 g l^{-1}
Agar	15 g l^{-1}
Kanamycin	50 µg/ml
Gentamycin	30 µg/ml
Rifampicin	100 µg/ml