CHANGES IN ROOT HAIR MORPHOGENESIS AND NODULATION OF *Phaseolus vulgaris* L. IN THE PRESENCE OF *BACILLUS* AND *RHIZOBIUM*

by

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ABSTRACT

Twenty two *Bacillus* spp. isolates from the *Phaseolus vulgaris* rhizosphere were identified using Biolog™, GC-FAME and 23S rDNA analysis. Some *Bacillus* isolates produced copious amounts of indoleacetic acid *in vitro*, in culture medium supplemented with L-tryptophan. Spontaneous α-methyl tryptophan resistant mutants of *Bacillus megaterium* S49 exhibited altered IAA production and excreted tryptophan into the culture medium. Coinoculation of *Rhizobium etli* TAL 182 with some *Bacillus* spp. under gnotobiotic conditions promoted nodulation of *P. vulgaris.* In contrast, decreased nodulation was observed in response to low and high IAA-producing *Bacillus* mutants, suggesting that the nodulation response in bean was not dependent on the *in vitro* level of IAA production by the *Bacillus* coinoculant.

Coinoculation of *Bacillus* spp. and *Rhizobium* spp. resulted in a unique and significant synergistic enhancement of lateral root formation and root hair initiation which was independent of plant host. These observations provided direct evidence for a beneficial interaction on root morphology which may account for enhanced nodulation by creating additional infection sites for the *Rhizobium*.

The presence of live *Rhizobium* and *Bacillus* was necessary for the root hair proliferation response. Coinoculation of *Rhizobium* TAL 182 with cell extracts of *Bacillus* S49 failed to induce root hair proliferation or enhance nodulation of *P. vulgaris*. A biologically active *Rhizobium* TAL 182 was essential for the observed response, indicating that root hair proliferation and enhanced nodulation were direct consequences of the bacterial species interaction.

Nodulation and symbiotic specificity of *Rhizobium* TAL 182 were altered by the presence of *Bacillus* S49. Split-root experiments showed that coinoculation suppressed

host-controlled regulation of nodulation. *Bacillus* S49 facilitated heterologous nodulation of *Rhizobium* TAL 182 on *P. acutifolius*, indicating that a host plant response was also involved in the bacterial interaction.

Exogenous aminoethoxy vinyl glycine, a negative regulator of ethylene biosynthesis, inhibited root hair formation in *P. vulgaris*. These inhibitory effects were reversed by the presence of both *Bacillus* S49 and *Rhizobium* TAL 182. The interaction between bacterial species on root hair induction and nodulation patterns in *P. vulgaris* is discussed.

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
ACE	autoclaved cell preparations
ARA	acetylene reduction assay
AVG	aminoethoxy vinyl glycine
CCM	combined carbon medium
CFE	cell-free extract
CFU	colony forming units
Da	daltons
GC-FAME	gas chromatography-fatty acid methyl ester
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
IAA	indoleacetic acid
2iP	2-isopentyl adenine
MW	molecular weight
kD	kilo daltons
MHB	mycorrhization helper bacteria
NaOAc	sodium acetate
NFW	nodule fresh weight
NifTAL	nitrogen fixation in tropical agricultural legumes
NON	nodule number
NPR	nodulation promoting rhizobacteria
PCR	polymerase chain reaction
PGPR	plant-growth promoting rhizobacteria
SEM	scanning electron microscopy
TIBA	triiodo benzoic acid
t Z	*trans* zeatin
t ZR	*trans* zeatin riboside
WCE	Whole (French pressed) cell extract

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Foreword

Portions of this thesis have been submitted for publication as follows:

(i) Portions of Chapter 2 to *Canadian Journal of Microbiology* as "Influence of IAA producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions" by Srinivasan, M., Petersen D.J., and Holl, F.B.

This manuscript has been accepted for publication in *Canadian Journal* of *Microbiology*.

(ii) Portions of Chapter 3 to *Planta* titled " A unique rhizosphere bacteria synergism promoting root hair initiation" by Srinivasan, M., Petersen D.J., and Holl, F.B.

(iii) Portions of Chapter 5 to *Canadian Journal of Microbiology* titled " Nodulation of *Phaseolus vulgaris* by *Rhizobium etli* is enhanced by the presence of *Bacillus*" by Srinivasan, M., Petersen D.J., and Holl, F.B.

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(iv) Portions of Chapter 6 of this thesis have been accepted for publication in *Microbiological Research* as " Altered root hair morphogenesis in *Phaseolus vulgaris* in response to bacterial coinoculation and the presence of aminoethoxy vinyl glycine" by Srinivasan, M., Petersen D.J., and Holl, F.B.

The senior author initiated the investigations, carried out the majority of the laboratory work and wrote the manuscripts for journal submission. The second author was responsible for 23S rDNA sequencing and PCR analysis and helped in the analysis of the scanning electron microscopy experiments. All work was performed with the financial assistance and research supervision of Dr. Brian Holl.

(v) In addition, a portion of the material presented in Chapter 4 of this thesis has been accepted for publication in *FEMS Microbiological Letters* titled "*Bacillus polymyxa* stimulates increased *Rhizobium etli* populations and nodulation when co-resident in the rhizosphere of *Phaseolus vulgaris*" by Petersen, D.J., Srinivasan, M., and Chanway, C.P.

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Chapter 1

General Introduction

Basic research is what I am doing when I do not know what I am doing

Wernher von Braun (1912-1977)

The term rhizosphere refers to the unique soil environment which is under the influence of plant roots (Hiltner 1904, cited in Curl and Truelove 1986). Development of sustainable agricultural systems necessary to counteract the decline in quality of soils, demands that continuing advances in biological productivity be achieved in a sustainable manner (Schippers *et al.* 1995). Exploiting the biological potential of microbial species in the rhizosphere may offer an important target for sustained agricultural productivity. Bacteria are the most abundant of soil microorganisms by virtue of their ability to utilize a wide range of carbon and nitrogen sources and to grow rapidly (Glick 1995). While non-rhizosphere field soil may contain up to 100 million bacteria per gram dry weight of soil, root associated soil (rhizosphere) has been shown to contain over a billion bacteria expressed on a comparable basis (Curl and Truelove 1986). Since the majority of soil nutrients used by the plant pass through the rhizosphere, this region has significant potential for biological activity which may affect plant growth.

1.1. Mechanisms of plant-growth promotion by rhizobacteria

Plant-growth promoting rhizobacteria (PGPR) are free-living bacteria that are present on or near the roots of plants and are involved in a beneficial association with plants (Kloepper *et al.* 1988; 1989). PGPR have been isolated from plant parts and from rhizosphere soil, and selected for their ability to improve plant growth, either directly or indirectly (reviewed by Glick 1995). Bacteria belonging to the genera *Azospirillum*, *Bacillus*, *Pseudomonas*, *Streptomyces*, and *Serratia* have been identified as PGPR (Kloepper *et al.* 1989). Indirect effects of PGPR on plant growth include the suppression of deleterious rhizobacteria (DRB) or pathogenic microorganisms (De Freitas and Germida 1991), and the production of iron sequestering compounds (siderophores) (Bakker *et al.* 1993) or antibiotics (Keel *et al.* 1992; Thomashow and

Weller 1995). The direct promotion of plant growth by PGPR usually involves either providing the plant with a factor that is produced by the bacterium or by facilitating the uptake of nutrients/substances from the environment (Glick 1995). Direct PGPR effects on plant growth have been described in a variety of studies: (i) *Azospirillum* spp. enhanced plant growth by providing fixed atmospheric nitrogen (Volpin and Kapulnik 1994); (ii) *Azospirillum* spp. and *Pseudomonas* spp. enhanced mineral uptake of the plant (Murty and Ladha 1988; De Freitas and Germida 1992; Germida and De Freitas 1994); (iii) *Pseudomonas* spp. and *Bacillus* spp. solubilized minerals such as phosphorus so that they are readily available for plant growth (Kloepper *et al.* 1988); (iv) *Pseudomonas* spp. produced low molecular weight substances and enzymes leading to changes in plant growth and development (Lambert and Joos 1989; Glick *et al.* 1994b, 1995); (v) *Pseudomonas* spp. altered plant growth by changing endogenous phytohormonal levels (Glick *et al.* 1994b; Xie at al. 1996) and; (vi) phytohoromone production by *Azospirillum*, *Bacillus* and *Pseudomonas* enhanced various stages of plant growth (Tien *et al.* 1979; Kapulnik *et al.* 1985a; Holl *et al.* 1988; Chanway *et al.* 1991; Barbieri and Galli 1993; Xie *et al.* 1996). It is clear that PGPR are of great interest for their potential to enhance plant growth and to increase productivity in an ecologically sustainable manner.

1. 2. Are mixtures of compatible strains are more effective than single bacterial species in promoting plant growth and/or nodulation ?

The root systems of all higher plants are associated with a distinct community of beneficial and non-beneficial microorganisms capable of synthesizing phytohormones (Frankenberger and Arshad 1995). Most work with PGPR has been performed with single strains and under laboratory conditions. However, it is highly unlikely that a

single free-living bacterial strain is ever omnipotent in the rhizosphere and thus singularly responsible for enhanced plant growth (Lambert and Joos 1989). In this context, Garbaye (1994) reviewed recent work which indicates the ability of some bacterial species to enhance mycorrhizal root tip formation and subsequently plant (seedling) growth. These bacteria are referred to as mycorrhization helper bacteria (MHB). The ability of rhizobacteria to promote nodulation of legumes by *Rhizobium* was recognized by many workers and the term nodulation promoting rhizobacteria (NPR) was coined by Kloepper *et al.* (1988) to describe such microorganisms. Nodulation promoting rhizobacteria belonging to the genera *Azospirillum* (Schmidt *et al.* 1988; Yahalom *et al.* 1990), *Pseudomonas* (Grimes and Mount 1984; Bolton *et al.* 1990; De Freitas *et al.* 1993), *Streptomyces* (Li and Alexander 1990) and *Bacillus* (Halverson and Handelsman 1991) have been reported. The beneficial influence of NPR on nodulation of legumes by *Rhizobium* has been variously attributed to their ability to produce phytohormones (Schmidt *et al.* 1988), toxins (Knight and Langston-Unkefer 1988), or antibiotics (Li and Alexander 1990), as well as by other unidentified mechanisms (Halverson and Handelsman 1991). Such observations suggest that particular microorganisms have considerable potential to alter the activity of specific rhizosphere microflora such as *Rhizobium* (Schroth and Becker 1990).

Some of the most intimate beneficial interactions between plants and microbes, and between different microbes take place on the surface of the root (Lynch 1988). A variety of complex microbial interactions might be anticipated across the diversity of environments created in the plant rhizosphere. It is unlikely that any one rhizobacterium would be predominant and effective in all environments and hence mixtures of compatible strains might be more significant than a single bacterial species in promoting

plant growth.

1. 3. Role of phytohormones in the nodulation of legumes

1.3.1. Role of auxins

The role of phytohormones such as auxins and cytokinins in plant organogenesis is well established. Auxins may also be important for maintaining functional root nodules, as effective (nitrogen-fixing) nodules have a higher IAA content than ineffective (nitrogen non-fixing) nodules (Badenoch-Jones *et al.* 1983). The importance of auxin in nodule organogenesis was confirmed when external application of triiodo benzoic acid (TIBA), an auxin transport inhibitor, induced the formation of pseudonodules on a non-nodulating line of alfalfa (Hirsch *et al.* 1989). External application of IAA mimicked the effect of *Azospirillum* on nodulation in *Medicago sativa* (Schmidt *et al.* 1988), further implicating auxin in nodule organogenesis. Many *Rhizobium* spp. are also known to produce IAA *in vitro* (Ernstsen *et al.* 1987; Atzorn *et al.* 1988). While the above studies clearly suggest that bacterially derived IAA could be involved in nodule formation, there is conflicting evidence whether such involvement is essential for nodulation (Wang *et al.* 1982; Atzorn *et al* 1988).

1.3. 2. Role of cytokinins

Nodule initiation is a property often associated with plant hormones (Hirsch *et al.* 1989). Cytokinins are more effective than auxins in nodule initiation since they are primarily responsible for stimulating mitosis in plant tissues (Relic *et al.* 1993). Root nodules of both legumes and non-legumes generally contain elevated amounts of cytokinins which may be either of microbial origin or a response to microbial stimulation of host tissue synthesis (Puppo and Rigaud 1978). Cytokinin of rhizobial origin is involved in nodulation and early nodulin (nodule protein) induction in alfalfa (Long and

Cooper 1988). *Rhizobium* spp. are capable of synthesizing cytokinins *in vitro* (Morris 1986; Sturtevant and Taller 1989), while many rhizobacteria produce cytokinin-like compounds *in vitro* (Frankenberger and Arshad 1995) with mixed cultures showing higher production than single cultures (Cacciari *et al.* 1989). Nevertheless, unequivocal evidence for a direct role for microbially derived cytokinins in nodule formation or organogenesis in legumes has not yet been reported (Dehio and de Bruijn 1992).

1. 3. 3. Role of ethylene

Root hairs are tubular-shaped, tip-growing structures that arise from root epidermal cells (Ridge 1995). They serve as sites for rhizobial infection in legumes (Bauer 1981), in addition to enhancing water and nutrient uptake by roots (Clarkson 1985). Root hair formation and differentiation in *Arabidopsis* are dependent upon the ethylene/auxin ratios in the plant (Masucci and Schiefelbein 1994). Ethylene has also been shown to be a positive regulator of root hair formation in *Arabidopsis* (Tanimoto *et al.* 1995). Exogenous application of ethylene has been shown to suppress nodule formation (Lee and LaRue 1992), although this effect can be reversed by exogenous application of the ethylene biosynthesis inhibitor aminoethoxy vinyl glycine (AVG) (Zaat *et al.* 1989). Many rhizosphere microorganisms including *Rhizobium* spp. (Billington *et al.* 1979), *Pseudomonas* spp. and *Bacillus* spp. (Mansouri and Bunch 1989) are capable of synthesizing ethylene *in vitro*, in the presence of the precursor methionine (MET). Inoculation of canola with *Pseudomonas putida* strain GR12-2 resulted in root elongation, which was attributed to changes in endogenous ethylene levels in the plant (Glick *et al.* 1994a,b; Xie *et al.* 1996). Ethylene of microbial origin has been directly implicated in altered growth of etiolated pea seedlings (Arshad and Frankenberger 1988). The ability of microbially derived ethylene to influence nodulation on legumes

directly remains unproven. Nevertheless, ethylene-related changes in response to microbial infection leading to enhanced or suppressed nodulation have been observed in *Vicia sativa* (van Brussel *et al.* 1986; Zaat *et al.* 1989).

1. 4. Influence of rhizobacteria on the nodulation of legumes by *Rhizobium*

Genetic and biochemical mechanisms involved in *Rhizobium*-legume symbiosis have been well characterized. Studies on the influence of rhizobacteria on nodulation of legumes by rhizobia have primarily focused on the *Azospirillum-Rhizobium* interaction. In the legume-*Rhizobium* system, *Azospirillum* application promoted plant growth that was generally followed by increased nodule formation (Yahalom *et al.* 1990; 1991). However, many reports indicate that under gnotobiotic conditions, simultaneous application of *Azospirillum* and *Rhizobium* did not always result in a promotion of nodulation, and under some circumstances, even inhibited the ability of *Rhizobium* to nodulate its host (Plazinski and Rolfe 1985; Volpin and Kapulnik 1994). Enhanced nodulation responses under gnotobiotic conditions were however observed with the application of *Azospirillum* prior to, or following, inoculation with *Rhizobium* (Plazinski and Rolfe 1985; Volpin and Kapulnik 1994). In contrast, a positive influence of *Azospirillum* on nodulation of legumes by *Rhizobium* in field and greenhouse studies has been observed in response to the simultaneous application of *Azospirillum* and *Rhizobium*, indicative of the synergistic associations one might anticipate in natural ecosystems (Okon *et al.* 1988).

These results suggest that simultaneous cell-to-cell interaction(s) between the two bacterial species was not essential for the plant nodulation response, and that excreted bacterial products or soluble factors may be involved in the process. Therefore, it is reasonable to speculate that *Azospirillum* may exert its influence through

the host plant by causing morphological changes in root growth (for example, by inducing the plant to produce more root hairs), and not by a direct interaction with *Rhizobium*. Evidence implicating phytohormones produced by *Azospirillum* in enhanced nodulation of legumes by *Rhizobium* under gnotobiotic conditions has, to date, been circumstantial rather than conclusive.

1.4.2. Role of *Bacillus* spp. in promoting nodulation of legumes by *Rhizobium*

Bacillus spp. are widely distributed in temperate soils (Priest and Grigorova 1990) and have been implicated in plant growth promoting activity (Holl *et al.* 1988; Chanway *et al.* 1991; Shishido *et al.* 1996). *Bacillus cereus* and *Bacillus subtilis* have shown nodulation promotion of legumes with *Rhizobium* in both field experiments and under gnotobiotic conditions (Halverson and Handelsman 1991; Turner and Backman 1991). None of the above studies explain adequately the mechanism(s) of nodulation promotion by *Bacillus* spp. There is little evidence implicating a plant response that could be singularly attributed to the interaction between *Bacillus* and *Rhizobium* resulting in enhanced nodulation. Nodule formation is primarily controlled by the plant (Caetano-Anollés and Gresshoff 1991). Hence, any changes to plant root growth or morphogenesis in response to the presence of both rhizosphere bacteria may influence nodulation of the plant by *Rhizobium*. While substantial progress has been made in elucidating the mechanism(s) of the legume-*Rhizobium* interaction, information is scarce on rhizobacterial interactions and their potential to influence the legume-*Rhizobium* symbiosis.

1.5. Hypothesis and objectives of this study

Bacillus spp. are attractive as plant-growth promoting and nodulation promoting agents because they occur naturally in the rhizosphere, because they may be

endophytic in plant root tissues, and because the cells can be produced easily in large quantities and stored for long periods of time without loss of viability (Hwang *et al.* 1996). The general objective of this study was to evaluate the influence of *Bacillus* spp. in promoting nodulation of the legume, *Phaseolus vulgaris* (L.) by its homologous microsymbiont, *Rhizobium etli* . The study is predicated on the hypothesis that rhizosphere interactions between microorganisms may have significant potential to influence plant growth and plant-microbe interactions such as legume nodulation by *Rhizobium*.

Specific objectives include:

(i) to determine whether the ability of *Bacillus* spp. to promote nodulation of *Phaseolus vulgaris* by *Rhizobium etli* is dependent on phytohormone production (especially indoleacetic acid) *in vitro*,

(ii) to investigate the effect of the simultaneous presence of *Bacillus* spp. and *Rhizobium* on lateral root and root hair development in *P. vulgaris,*

(iii) to determine if viable, interacting *Bacillus* and *Rhizobium* are a prerequisite for invoking a plant response in terms of root hair morphogenesis and lateral root development,

(iv) to determine the effect of *Bacillus* spp. on nodulation pattern and nodulation specificity of *Rhizobium etli* and,

(v) to investigate whether the interaction between *Rhizobium etli* and *Bacillus* spp. is independent of ethylene-related changes within *P. vulgaris* with regard to root hair morphogenesis.

Chapter 2

Influence of IAA producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli*

Every method is imperfect

Charles-Jean-Henri-Nicollé (1932)

2.1. Introduction

The direct effects of PGPR have been most commonly attributed to the production of phytohormones, notably auxins (Brown 1972; Tien *et al.* 1979). The role of phytohormones in organogenesis in plants has been well established. Numerous rhizosphere-inhabiting, diazotrophic bacteria belonging to the genera *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus* produce copious amounts of phytohormones (Brown 1972; Tien *et al.* 1979; Holl *et al.* 1988). The capability of such bacteria to produce physiologically active amounts of growth hormones, particularly IAA, has been correlated to their growth-promoting ability (Barbieri *et al.* 1986; Loper and Schroth 1986).

Rhizobacteria of the genera *Azospirillum*, *Bacillus, Pseudomonas*, and *Streptomyces* also promote nodulation in many legume species (Iruthayathas *et al.* 1983; Grimes and Mount 1984; Li and Alexander 1990). The ability of *Azospirillum* to produce phytohormones has been hypothesized to play a major role in promoting nodulation in legumes (Schmidt *et al.* 1988; Yahalom *et al.* 1990). Auxins may also be important for maintaining functional root nodules, since effective nodules have a higher IAA content than ineffective nodules (Badenoch-Jones *et al.* 1983). Changes in the phytohormone balance in roots may also elicit nodule formation. External application of the auxin transport inhibitor triiodobenzoic acid (TIBA) induced the formation of pseudo-nodules in a non-nodulating line of alfalfa, indicating phytohormone involvement independent of Nod factor activity (Hirsch *et al.* 1989). Schmidt *et al.* (1988) demonstrated that application of IAA mimicked the effect of *Azospirillum* on nodulation in *Medicago sativa*. Many workers have demonstrated the accumulation of substantial

amounts of auxin in nodules compared to the surrounding root tissues in both legumes and non-legumes (Dullart 1970; Dangar and Basu 1987). Many *Rhizobium* spp. are known to produce IAA *in vitro* (Badenoch-Jones *et al.* 1983; Ernstsen *et al.* 1987; Atzorn *et al.* 1988). While some studies clearly implicate bacterially derived IAA in nodule formation (Kaneshiro and Kwolek 1985; Hunter 1987), there is conflicting evidence to suggest that such involvement may not be essential for nodulation (Wang *et al.* 1982; Atzorn *et al.* 1988).

Despite studies which suggest that IAA produced by bacteria can influence the biological response between plant and bacterial symbionts, the direct effect of phytohormone producing rhizobacteria on legume nodulation remains unproven. The objective of this study was to determine whether the nodulation promoting ability of *Bacillus* spp. is dependent on their ability to produce IAA *in vitro*.

2. 2. Materials and Methods

2. 2.1. Isolation and characterization of strains

Bacillus spp. isolates were obtained from the rhizosphere of *Phaseolus vulgaris* at seven and fourteen days after planting from a field in which beans had been cultivated for the previous ten years. Isolation of bacteria from the root surface and from the inside of roots was done according to the procedure described by O'Neill *et al.* (1992). Isolates of the genus *Bacillus* were obtained by enrichment under anaerobic conditions as described by Holl *et al.* (1988) using serial dilution of root-soil extracts. A total of 120 bacterial colonies were isolated and characterized in this study. Carbon source utilization was assayed using Biolog™ multiwell analysis. Classification was accomplished using the Biolog™ database supplemented by the inclusion of numerous

other *Bacilli* previously isolated (Massicotte and Chanway, 1994). Species identification was further refined using fatty-acid analysis (GC-FAME) at Auburn University according to Kloepper *et al.* (1992). Both Biolog™ and GC-FAME analysis were in agreement at the genus level. Where differences in species designation occurred, the GC-FAME classification was used. The comparative database for the GC-FAME analysis includes a substantial range of known rhizosphere bacteria. Phylogenetic analyses were conducted using 23S rDNA described by Petersen *et al.* (1995). *Rhizobium etli* TAL 182 was obtained from NifTAL, Paia, Hawaii.

2. 2 .2. Bacterial growth media

Bacillus spp. were routinely maintained on combined carbon medium agar slants (Rennie 1981). *Rhizobium etli* TAL 182 was maintained on yeast mannitol agar slants (Bohlool and Schmidt, 1970). Broth cultures were grown to early stationary phase on combined carbon medium for *Bacillus* spp. and in yeast mannitol broth for *Rhizobium etli* TAL 182. The cells were harvested by centrifugation, washed twice with sterile phosphate buffer (0.1M, pH 6.8-7.0) and resuspended in the same buffer to a desired cell density before inoculation.

2. 2. 3. Isolation of indoleacetic acid (IAA)

Screening of isolates for IAA production

IAA production by *Bacillus* spp. isolates was assessed by the rapid *in situ* assay described by Bric *et al.* (1991), using 2% 0.5M $FeCl_3$ in 35% perchloric acid as the reagent for color development. Bacterial colonies that formed distinct red halos were further analyzed for IAA production using thin layer chromatography (TLC), high performance liquid chromatography (HPLC), the avena coleoptile bioassay and gas

chromatography-mass spectrometry (GC-MS).

Extraction of indoleacetic acid

Indoleacetic acid (IAA) in *Bacillus* cultures grown in the medium described by Holl *et al.* (1988) was extracted using ethyl acetate described by Tien *et al.* (1979). *Rhizobium etli* TAL 182 was grown in a medium described by Ernstsen *et al.* (1987). Cultures were incubated on a rotary shaker in the dark at 30° C until early stationary phase in 500 ml flasks containing 200 ml of medium with or without supplemental L-tryptophan (100 µg/L). The cultures were harvested at early stationary phase (72 h) and centrifuged at 2000 x *g* for 30 minutes. The supernatant was supplemented with dithiothreitol (0.05g) to prevent oxidation of indole compounds and filtered (0.45 µm Millipore) to remove cell debris. The supernatant volume was reduced 75% by rotary evaporation at 37° C, adjusted to pH 2.7 with 1N HCL, and partitioned three times with an equal volume of ethyl acetate (99.9%; HPLC grade) as described previously (Tien *et al.* 1979). The ethyl acetate fractions were pooled and reduced to dryness by rotary evaporation at 37° C. The dry residue was dissolved in one ml of absolute methanol. The methanol extracts thus obtained were stored in the dark at -20°C until further use. Extraction of IAA was performed three times for each isolate using identical experimental conditions.

Thin layer chromatography

Detection of IAA using thin layer chromatography was accomplished by applying 10 µl of the methanol extracts to a 0.25 mm thick silica gel plate (Merck, Germany). The plates were developed with a freshly prepared solvent mixture of chloroform : methanol : water (84 :14:1). Detection of indole compounds on thin layer plates was

done according to Ehmann (1977). IAA was identified at R_f 0.53 as verified by an authentic IAA standard (Sigma Chemical Co, St. Louis, MO).

Bioassay

The avena coleoptile straight growth assay (Nitsch and Nitsch 1956) was used for the detection of biological activity resulting from auxin-like substances in the methanol extract. Aliquots (250 µl) of the methanol extracts were reduced to dryness under vacuum and the residues were dissolved in 0.1 M phosphate buffer, pH 6.4 containing 1% sucrose (w/v), and the bioassay was conducted as described by Holl *et al.* (1988). Specific fractions representing 10 R_f values were eluted from untreated portions of the thin layer chromatograms and tested for auxin-like activity by the same assay.

High performance liquid chromatography

Methanol extracts (20 µl) were injected onto a 250mm x 4.6mm (I.D.), C:18 Nucleosil column (Metachem) using a Gilson 714 liquid chromatograph equipped with a differential UV detector absorbing at 280nm. The indole compounds were eluted using an isocratic gradient of 2% acetic acid in acetonitrile as solvent B and 2% acetic acid in water (HPLC grade) as solvent A at a flow rate of 1ml/min. Each extract was analyzed at least three times by HPLC and the retention times for peaks were compared to those of an authentic standard for IAA. Indoleacetic acid peaks were also confirmed by co-chromatography with an authentic standard. Quantitation of IAA in the samples was done by comparison of peak areas. The IAA band corresponding to R_f 0.53 was removed from the TLC plate and eluted for three hours at 4° C in the dark in 2 ml of absolute methanol; the eluate was filtered and evaporated under vacuum and the

residue redissolved in 0.5 ml of absolute methanol. Aliquots (30 µl) of this extract was analyzed by HPLC as described above to confirm the presence of IAA.

Gas chromatography-mass spectrometry

The methanol extract from the TLC plate was analyzed by GC -MS using 2 µl of trimethylsilyl derivatized samples. Analysis was performed with a Fisons Trio 2000 mass spectrometer, using an ID DB-5 column at 50° C, programmed at 8 °/min. GC - MS analysis of an authentic IAA standard was done in the same manner.

2. 2. 4. Isolation of spontaneous α-methyl tryptophan resistant mutants

Isolation and characterization of α-methyl tryptophan resistant mutants was done as described by Smidt and Kosuge (1978). *B. megaterium* S49 at 10^6 cfu ml^{-1} was grown on Spizizien minimal-glucose agar (Spizizien 1958) with either a centrally located 6mm filter paper disk impregnated with 100 µl of 5mM filter-sterilized α-methyl tryptophan or a 6mm well in the agar filled with 100 µl of 5 mM α-methyl tryptophan. Single α-methyl tryptophan resistant colonies growing within zones of inhibition on these plates were streaked on to the same medium and resultant single colonies were streaked out three times for purification. The resistant mutants thus obtained were maintained in the same medium containing 2.5 mM α-methyl tryptophan. IAA production by resistant mutants was determined by TLC as described above, and by the colorimetric method of Gordon and Weber (1951), using 20 ml cultures grown in Spizizien minimal-glucose medium containing 2.5mM α-methyl tryptophan. For plant inoculation experiments the resistant mutant isolates were grown as described above to the required cell density.

Assay for tryptophan excretion

Strains were assayed for excretion of tryptophan as described by Hoch *et al.* (1971), using 0.05% acid hydrolyzed casein and/or 50 µg per ml of phenylalanine in the medium where appropriate. Samples were removed periodically (until early stationary phase) and centrifuged at 2000 *x* g for 10 minutes. The supernatants were examined fluorometrically using a Shimazdu RF 540 spectrofluorometer at an excitation wavelength of 280 nm and an emission wavelength of 350 nm and the amount of tryptophan excreted was determined from a standard curve obtained using L-tryptophan (Sigma Chemical Co, St. Louis, MO).

2. 2. 5. Plant material and growth

Seeds of *Phaseolus vulgaris* L. 'Contender' were cultivated in growth chambers using modified Leonard jar assemblies (Blauenfeldt *et al.* 1994), containing 1:1 (v/v) of sterile planting medium of industrial sand and Turface™. Seeds were surface sterilized by soaking in 95% ethanol for 30 seconds and then in 20% Chlorox (1.5% sodium hypochlorite) for 15 minutes and rinsed seven times with sterile distilled water. Surface sterilized seeds were germinated aseptically on moist filter paper in sterile petri plates. Two day old pregerminated seeds with uniform radicle length were inoculated with early stationary phase bacterial suspensions of *Rhizobium etli* TAL 182, *Bacillus* spp. and α-methyl tryptophan resistant mutants of *B. megaterium* S49 at a cell density of approximately 2 x 10^8 cfu/ml. Plants were grown under a controlled environment with a 16 h photoperiod and 22° C/18° C day night temperatures, and 350 µE m^2 s^{-1} illumination and were harvested at 24 to 26 days after planting. The experiment was laid out as a completely randomized design with seven replicates for each treatment.

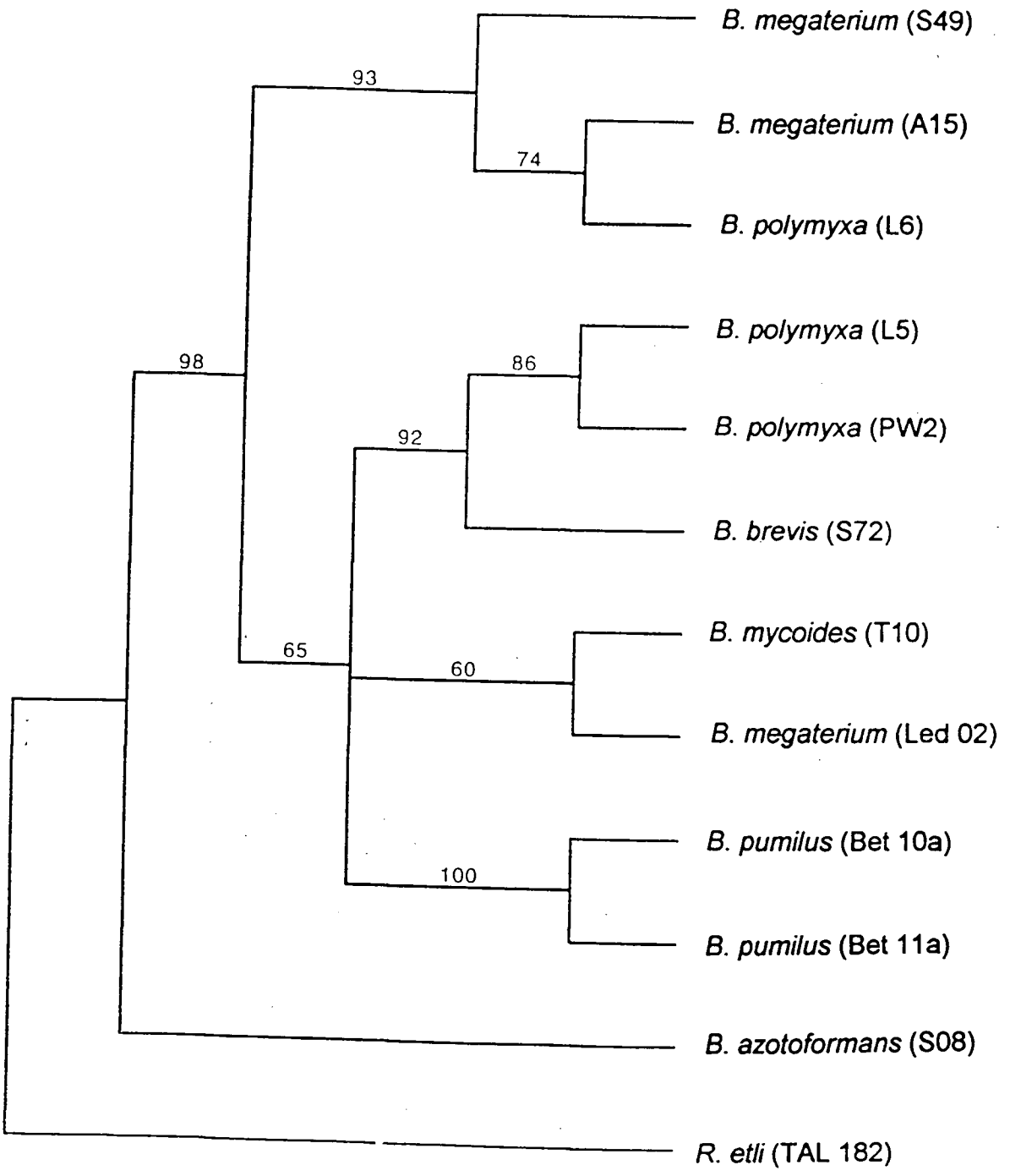
Acetylene reduction assay (ARA)

The ARA system as described by Dilworth (1966) was used to measure nitrogenase activity. After one hour incubation, the gas mixture was analyzed by flame - ionization gas chromatography using a Hewlett Packard 5830A gas chromatograph (Holl *et al.* 1988). Total soluble protein in nodules was estimated according to Bradford (1976). The difference -spectral method (Appleby and Bergersen 1980) was used to measure the leghemoglobin content in the root nodules. Statistical analysis was performed using standard analysis of variance (ANOVA) techniques (SAS Institute Inc., Cary, NC).

2.3. Results

Initial classification of strains isolated from the rhizosphere of *P. vulgaris* using Biolog™ and 23S rDNA analysis demonstrated the presence of several *Bacillus* spp. both in the rhizosphere (soil adhering to the root surface) and inside root tissues. Endophytic isolates were identified predominantly as *Bacillus megaterium* whereas those from outside of the roots included various *Bacillus* spp. - *B. megaterium*, *B. brevis*, *B. pumilus*, *B. licheniformis* and *B. azotoformans*. Direct sequencing of an approximately 850 bp band amplified from the 23S rDNA using PCR primers BAC11X and BAC19 (Petersen *et al.* 1995) yielded genotypic differences which were not distinguishable using metabolic fingerprinting or GC-FAME analyses. DNA sequence alignments were constructed and compared to other sequences included in the Ribosomal Database Project (Larsen *et al.* 1993) to yield the dendrogram shown in Figure 2.1. *Bacillus* spp. S89 and B17 were not included in the 23S rDNA analysis; sequence data for these isolates was unavailable at the time of original analysis, and

Figure 2.1. Phylogenetic dendogram showing the diversity of IAA producing *Bacillus* isolates. The dendogram from 23S rDNA nucleotide sequences was constructed by Phylogenetic Analysis Using Parsimony (PAUP 3.0). The numbers at the nodes are boot strap values for the nodes (based on 1000 bootstrap resamplings). *Rhizobium etli* TAL 182 was used to outgroup the tree.


B. megaterium (S49)
B. megaterium (A15)
B. polymyxa (L6)
B. polymyxa (L5)
B. polymyxa (PW2)
B. brevis (S72)
B. mycoides (T10)
B. megaterium (Led 02)
B. pumilus (Bet 10a)
B. pumilus (Bet 11a)
B. azotoformans (S08)
R. etli (TAL 182)
93
74
98
86
92
65
60
100

several other examples of *B. megaterium* were already included in the study. Bacterial identification using GC-FAME was consistent with the phylogenetic analysis.

The origin of different *Bacillus* isolates used in this study is shown in Table 2.1. The thirteen isolates in this table include nine obtained in the current study and an additional four isolates from other plant and ecosystem environments. The amounts of indoleacetic acid (IAA) produced by different *Bacillus* isolates in the presence and absence of supplemental L-tryptophan (100 µg/L) is shown in Table 2.2. Of the 22 *Bacillus* isolates obtained from the *P. vulgaris* rhizosphere, forty-five percent were IAA producers, with significant differences noted in the *in vitro* production by different bacterial strains. Addition of L-tryptophan to the medium resulted in production of significantly higher amounts of IAA in seven of the nine *Bacillus* isolates tested. Two strains (S72 and T10) which produced low amounts of IAA in unsupplemented culture medium showed no appreciable response in IAA production following addition of L-tryptophan. In contrast to the *Bacillus* spp., no IAA could be detected in extracts of TAL 182 (*Rhizobium etli)* in the unsupplemented culture medium, while the addition of exogenous tryptophan (100 µg/L) resulted in low detectable amounts of IAA (0.35 µg/ml). The detection limit of IAA using the HPLC method described was approximately 0.1-0.2 µg/ml. Quantification of IAA was carried out over several months and some variation in detection sensitivity might be anticipated due to HPLC column wear and ageing UV detector.

Thin layer chromatography of the methanol extracts revealed the presence of several indole compounds. IAA was detected at an R_f value of 0.53 which corresponded to the R_f of an authentic IAA standard.

Table 2.1. Identification and origin of *Bacillus* isolates used in this study

Isolate	GC-FAME ID	Isolate origin	Source
A15	*B. megaterium*	*P. vulgaris* root interior	This study
S49	*B. megaterium*	*P. vulgaris* root interior	"
Led 02	*B. megaterium*	*P. vulgaris* root interior	"
S89	*B. megaterium*	*P. vulgaris* root interior	"
B17	*B. megaterium*	*P. vulgaris* rhizosphere	"
S72	*B. brevis*	*P. vulgaris* rhizosphere	"
Bet 10a	*B. pumilus*	*P. vulgaris* rhizosphere	"
Bet 11a	*B. pumilus*	*P. vulgaris* rhizosphere	"
S08	-	*P. vulgaris* rhizosphere	"
L5	*B. polymyxa*	Mixture of *L. perenne* and *T. repens* root and soil	Chanway et al. (1991)
L6	*B. polymyxa*	Mixture of *L. perenne* and *T. repens* root and soil	Holl et al. (1989)
PW2	*B. polymyxa*	Lodgepole pine root interior	Shishido et al. (1995)
T10	*B. mycoides*	*Pseudotsuga menziesii* mycorrhizosphere	Massicotte and Chanway (1994)

- = not determined. This isolate was identified as *B. azotoformans* by the Biolog™ carbon utilization profile.

Table 2.2. Indoleacetic acid (IAA) production by *Bacillus* isolates and *Rhizobium etli*

	IAA production (µg/ml)**				
Isolate	(+trp)†	(SD)	(-trp)	(SD)	
TAL182(*Rhizobium etli*)‡	0.35	(0.05)	*nd*	*nd*	
S49(*B. megaterium*)	4.80	(0.33)	1.08	(.005)	*
A15(*B. megaterium*)	4.48	(0.86)	0.89	(.001)	*
B17(*B. megaterium*)	4.88	(0.28)	1.05	(.010)	*
S89(*B. megaterium*)	1.80	(0.25)	0.40	(0.07)	*
Led 02(*B. megaterium*)	2.70	(0.30)	0.70	(0.09)	*
S72(*B.brevis*)	0.40	(0.04)	0.30	(.010)	
Bet10a(*B. pumilus*)	3.86	(0.25)	0.45	(.010)	*
L6(*B. polymyxa*)	3.04	(0.37)	0.84	(.001)	*
L5(*B. polymyxa*)	2.59	(0.61)	0.41	(.008)	*
PW2(*B. polymyxa*)	2.39	(0.15)	1.03	(.016)	*
T10(*B. mycoides*)	0.58	(0.09)	0.54	(.010)	

** Mean of six replicates in two separate experiments using HPLC analysis as outlined in materials and methods. *nd* = not detected.

† Grown in a medium with supplemental L-tryptophan at 100 µg/L.

‡ Grown in medium as described by Ernstsen *et al.* (1987).

* IAA production was significantly higher ($p \leq 0.05$) in the presence of supplemental L-tryptophan as determined by t-test.

The presence of IAA in the methanol extracts of isolates S49 and L6 was further supported by the avena coleoptile straight growth assays, showing significant auxin-like activity compared to the control (Figure 2.2). TLC eluted fractions corresponding to R_f 0.4-0.6 (corresponding to the mobility of authentic IAA standard) of *B. megaterium* S49 and *B. polymyxa* L6 showed biologically responsive auxin-like activity, with a 155% increase in coleoptile growth. The authentic IAA standard on TLC eluted at R_f 0.4-0.6 also gave a significant increase in coleoptile growth compared to the control (0.1M phosphate buffer with 1% sucrose).

Methanol extracts and the TLC fractions corresponding to R_f 0.4-0.6 were analysed by HPLC and GC-mass spectrometry. In the HPLC analysis, peak activity corresponded to the elution time for the IAA standard (9.9 minutes) (Figure 2.3 A, B). The TLC band at R_f 0.4-0.6 also gave a prominent HPLC peak corresponding to the IAA standard (Figure 2.3C). Co-chromatography of the unknown samples from TLC with a TLC-derived authentic IAA standard produced a single enhanced peak in HPLC at the same retention time (9.9 min) as the standard alone (Figure 2.3D). Further confirmation that the TLC eluate (R_f0.4-0.6) contained IAA was obtained from the UV absorption spectrum which corresponded to that of an authentic IAA (data not shown). In addition to S49, IAA in the culture filtrate extracts derived from other *Bacillus* isolates was also identified by HPLC and TLC as described above.

The GC-mass spectral analysis of the TLC eluted band corresponding to R_f 0.4-0.6 for the *B. polymyxa* isolate L6, yielded two prominent ions (m/z 130 and m/z 189) in the spectrum of indole acetic acid and the mass spectrum of these two ions was very similar to that of indoleacetic acid (Figure 2.4). At the onset of this work, isolate L6 was

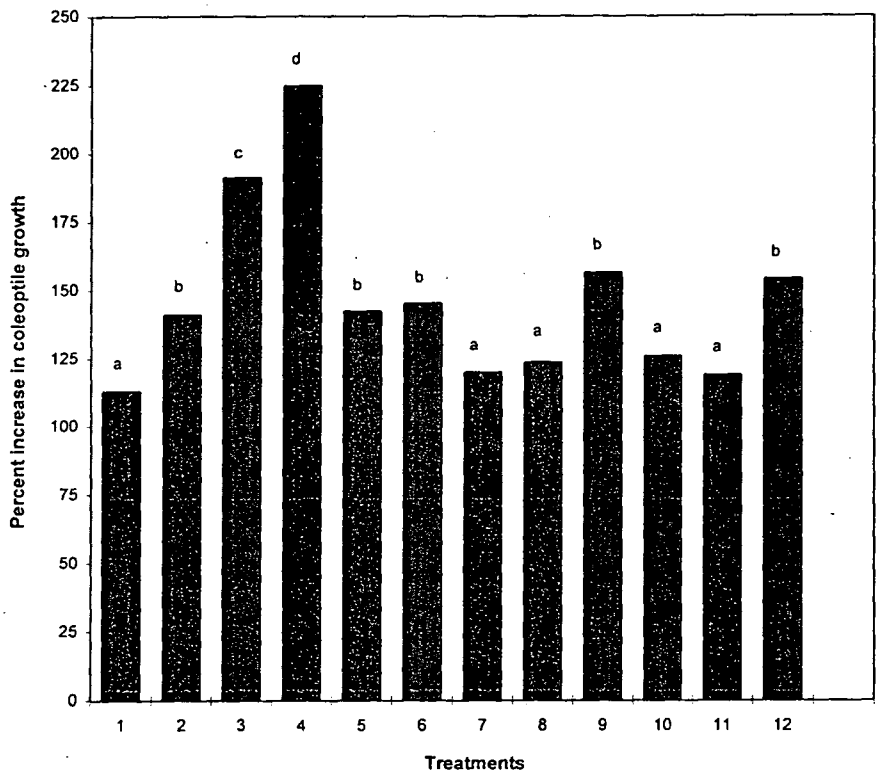


Figure 2.2. The avena coleoptile straight growth assay for auxin-like activity in *Bacillus* isolates extracts. Treatments were as follows: (1) Control, (2) IAA at 1μg / ml, (3) IAA at 5 μg / ml, (4) IAA at 10 μg / ml, (5) S49 cell free extract, (6) L6 cell free extract, (7) S49 R_f 0-0.2, (8) S49 R_f 0.2-0.4, (9) S49 R_f 0.4-0.6, (10) S49 R_f 0.6-0.8, (11) S49 R_f 0.8-1.0, (12) L6 R_f 0.4-0.6. Each value represents the mean of ten replicates. Means with the same letter are not significantly different at $p \leq 0.05$.

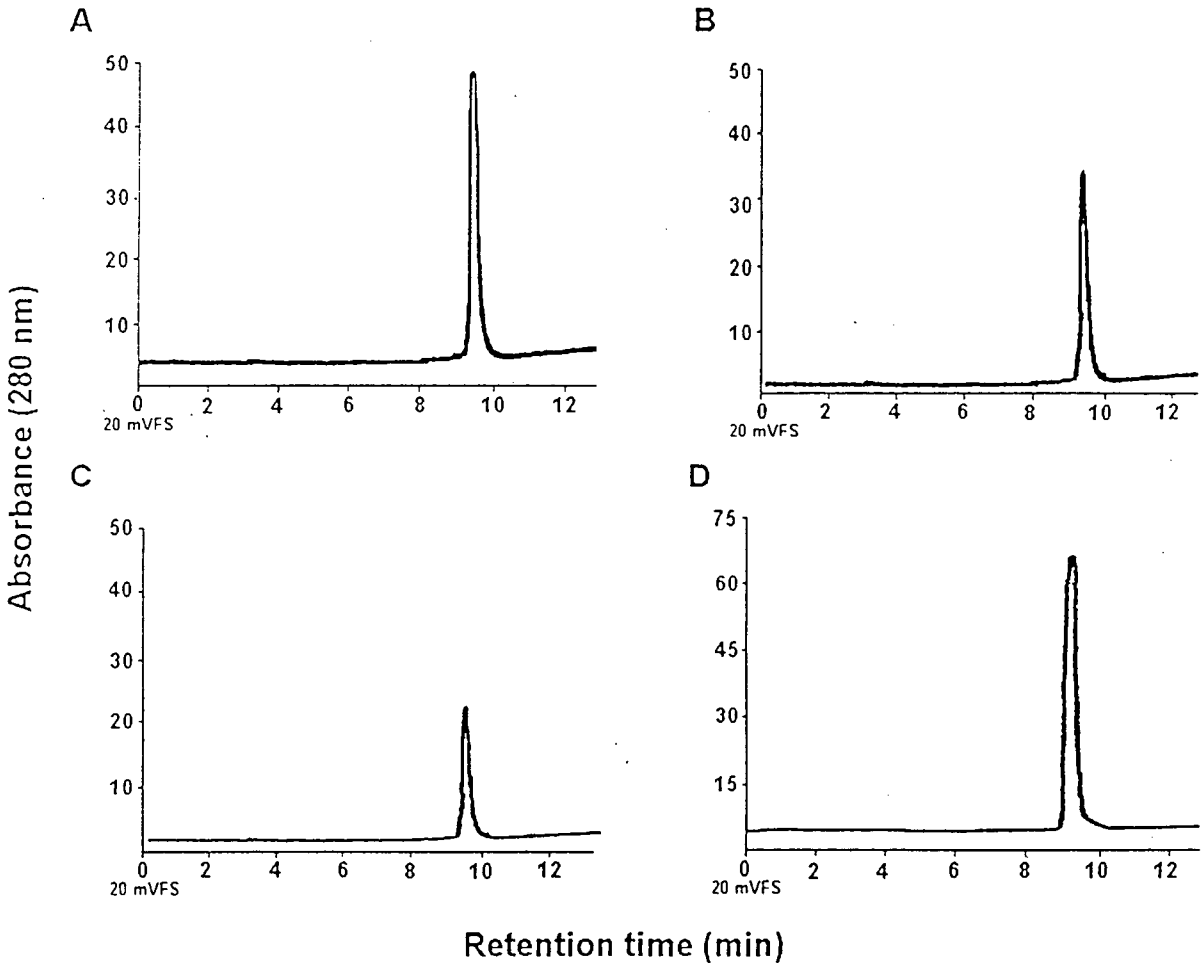
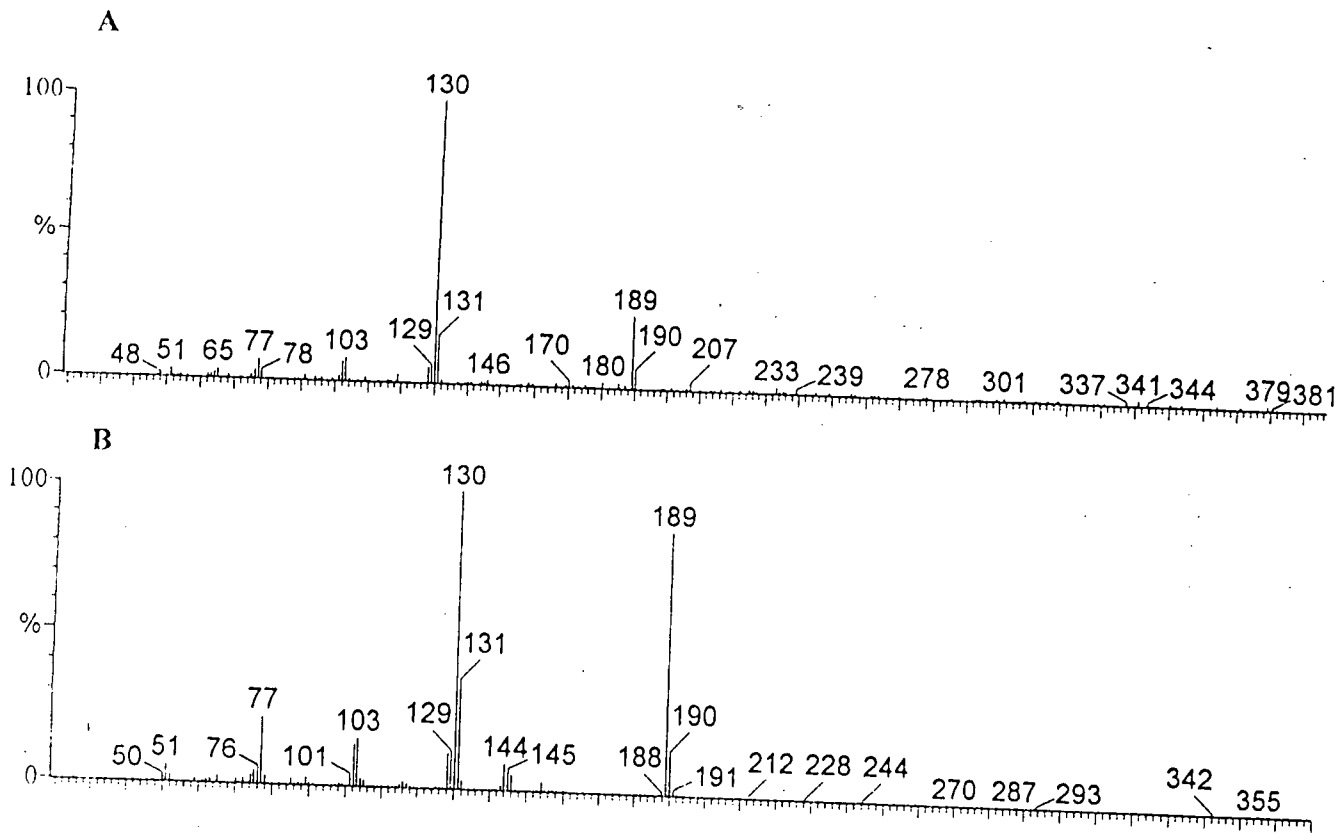


Figure 2.3. HPLC separation of (A) authentic IAA standard at 5 μg / ml, (B) IAA fraction in the methanol extract of *B. megaterium* S49, (C) TLC eluted fraction of R_f 0.4-0.6 of *B. megaterium* S49 and, (D) co-chromatography of the TLC eluted band of *B. megaterium* S49 (R_f 0.4-0.6) with authentic IAA eluted from TLC at R_f 0.4-0.6.

Figure 2.4. Mass spectrometric span of (A) 100 picograms of an authentic indoleacetic acid standard and (B) thin layer chromatograph eluted band corresponding to R_f 0.4-0.6 of *B. polymyxa* L6.


A
100
%
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130
131
189
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48
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78
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278
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B
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131
77
103
129
190
50
51
76
101
144
145
188
191
212
228
244
270
287
293
342
355

the most characterized growth promoting strain was selected for GC-MS analysis. The subsequent analogous biochemical analyses for IAA production by *B. megaterium* S49 were consistent with the earlier data derived from *B. polymyxa* L6. Cost constraints precluded repeating the GC-MS analysis on the *B. megaterium* S49 extracts.

The α-methyl tryptophan-resistant mutants of *B. megaterium* S49 were obtained using 5mM α-methyl tryptophan on Spizizien glucose-minimal agar plates (Spizizien 1958) as described previously (Smidt and Kosuge 1978). The growth rates of the α-methyl tryptophan resistant mutants derived from *B. megaterium* S49 are given in Table 2.3. Manifestation of α-methyl tryptophan inhibition is observed through an extension of the lag phase in the growth curve, while logarithmic growth remains unaltered (Smidt and Kosuge 1978). A similar growth response was observed for *Bacillus* S49-1D and S49-1W in this study. It is also evident from Table 2.3 that wild-type *B. megaterium* S49 was more susceptible to α-methyl tryptophan inhibition than the resistant mutants (S49-1D and S49-1W). Addition of 0.05% hydrolyzed casein and/or 50 μg/ml phenylalanine to the growing medium increased the growth rates of the resistant mutants. The α-methyl tryptophan resistant mutants of *Bacillus* S49 excreted tryptophan, which could be measured spectrofluorometrically, into the growing medium. Isolate S49-1D excreted higher amounts of tryptophan (2.40 μg/ml) at early stationary phase than isolate S49 - 1W (1.51 μg/ml) . The reverse was true for IAA production by these two resistant mutants (S49-1W > S49-1D) (Figure 2.5).

Having established the IAA-producing ability of our *Bacillus* isolates, we subsequently investigated the influence of coinoculation of these isolates with *Rhizobium etli* TAL 182 on nodulation of *P. vulgaris*. Initially nine IAA producing *Bacillus* isolates

Table 2.3. Growth of *Bacillus megaterium* S49 in the presence of 2.5mM α-methyl tryptophan

Isolate	Extension of lag phase (h)*		
	Minimal-glucose medium with 2.5mM α-MT		
	a	*b*	*c*
S49	18	18	-
S49 -1D	12	09	06
S49 -1W	18	12	12

* Difference between duration of lag phase in cultures simultaneously inoculated into Spizizien minimal-glucose medium and Spizizien minimal-glucose medium with 2.5mM α-methyl tryptophan.

a = Growth in Spizizen minimal-glucose medium with 2.5mM α-methyl tryptophan.

b = 2.5mM α-methyl tryptophan with 50 µg / ml of phenylalanine.

c = 2.5mM α-methyl tryptophan with 0.05% acid hydrolyzed casein.

- = not determined.

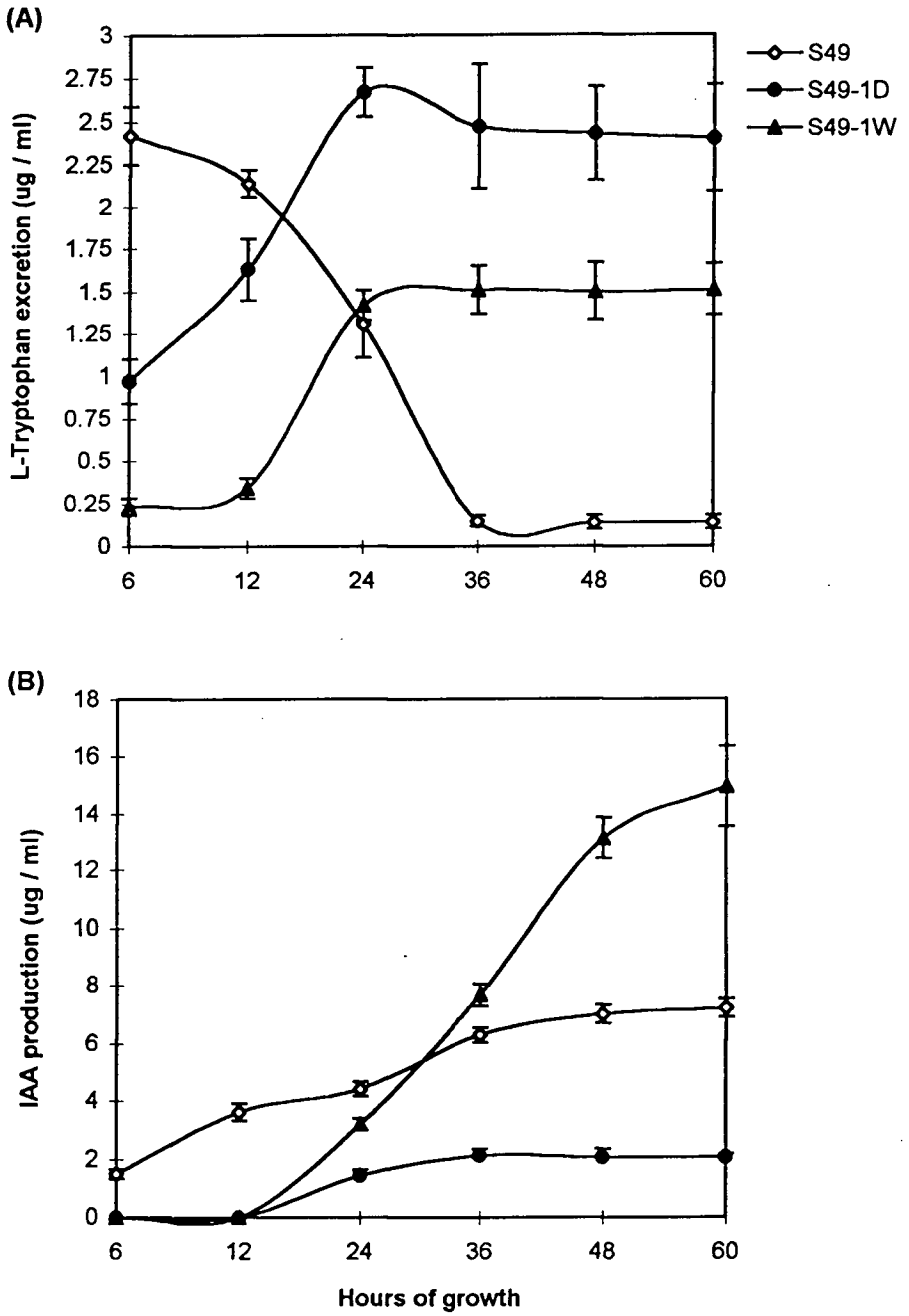


Figure 2.5. (A) Excretion of L-tryptophan and, (B) production of IAA by wild-type *B. megaterium* S49 and α-methyl tryptophan resistant mutants of S49 (S49-1D and S49-1W). Wild-type S49 was grown in Spizizien minimal medium with 100 μg /ml of L-tryptophan. The mutant isolates were grown in Spizizien minimal medium with 2.5mM α-methyl tryptophan , 0.05% acid hydrolyzed casein and 50 μg /ml of phenylalanine. Each value represents the mean of six individual samples in two separate experiments. Bars represent the standard error of the mean.

from the *P. vulgaris* rhizosphere and isolate L6 from a perennial pasture (Holl *et al.* 1988) were tested for their ability to promote nodulation in *P. vulgaris* in the greenhouse. Five Bacillus isolates promoted nodulation under these non-sterile conditions (Table 2.4-2.6). Three of these five isolates (S49, S72 and A15) also promoted nodulation under gnotobiotic conditions in growth chambers, suggesting a direct influence of these isolates on the nodulation of *P. vulgaris* by TAL 182 (Table 2.7-2.8). Some of the control plants that received one ml of sterile yeast mannitol medium as inoculum developed a few pseudonodules, devoid of any *Rhizobium*. The formation of spontaneous nodules or nodulation in the absence of *Rhizobium* has been reported (Caetano-Anollés *et al.* 1990; Blauenfeldt *et al.* 1994). The positive effects of coinoculation of such *Bacillus* isolates on nodulation in *P. vulgaris* 'Contender' were readily discernible. Nodule number (NON), nodule fresh weight (NFW), nitrogenase activity (ARA), leghemoglobin content, and total soluble proteins (TSP) were significantly increased by coinoculation compared to the TAL 182 single inoculation (Table 2.7). IAA-producing *Bacillus* isolated from the rhizosphere of other plant species were tested for their ability to promote nodulation of *P. vulgaris* by *Rhizobium* TAL 182. Amongst the *Bacillus* isolates tested, *B. polymyxa* L6 produced the greatest enhancement of nodule number while *B. megaterium* S49 produced the highest nodule fresh weights and nitrogenase activity, and *B. brevis* S72 resulted in the highest accumulation of total soluble proteins in the nodules (Table 2.7).

Coinoculation of the α-methyl tryptophan resistant mutants of *B. megaterium* strain S49 (S49-1D and S49-1W) with *Rhizobium* TAL 182 inhibited nodulation in *P. vulgaris*. Significant reductions in nodule number (NON), nodule fresh weight (NFW),

Table 2.4. Influence of *Bacillus* isolates on nodule number (NON), nodule fresh weight (NFW) and nitrogenase activity (ARA) of *P. vulgaris* by *Rhizobium etli* (TAL 182) in the greenhouse[†]

Treatment	NON (± SE)	NFW (g/plant ± SE)	ARA (n moles C_2H_4 h^{-1} $plant^{-1}$ ± SE)
Control[•]	6.5 ± 2.46 *d*	0.05 ± 0.02 *c*	0.11 ± 0.05 *c*
Rhizobium TAL 182	43 ± 3.21 *c*	0.19 ± 0.02 *b*	4.92 ± 0.24 *b*
TAL 182 + L6 (*B. polymyxa*)	66 ± 7.16 *a*	0.28 ± 0.04 *a*	6.54 ± 0.53 *a*
TAL 182 + S49 (*B. megaterium*)	62 ± 6.54 *a*	0.26 ± 0.03 *a*	6.02 ± 0.36 *a*
TAL 182 + B17 (*B. megaterium*)	46 ± 4.95 *b,c*	0.17 ± 0.02 *b*	4.68 ± 0.27 *b*
TAL 182 + Led 02 (*B. megaterium*)	60 ± 5.51 *a,b*	0.24 ± 0.03 *a,b*	6.05 ± 0.35 *a*

[†] Surface-sterilized *P. vulgaris* seeds were grown in four-inch pots with 1:1 (v/v) of steam pasteurized soil and Turface® as growth medium. Plants were inoculated at three days after planting with one ml of 2 x 10^8 cfu / ml of bacterial suspensions. A booster inoculation was given five days after the first inoculation. Control plants received one ml of sterile one-tenth strength yeast mannitol broth as inoculum. Plants were harvested at 21 days after planting. The experiment was laid out as a randomized block design with seven replicates for each treatment.

[•] Nodules formed on control plants were smaller than those observed on plants that received *Rhizobium* inoculum. Generally, control plants were chlorotic in appearance.

Values represent the means of seven replicates. Means were separated using analysis of variance (ANOVA). Means followed by the same letter in a column are not significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test.

Table 2.5. Influence of *Bacillus* isolates on nodule number (NON), nodule fresh weight (NFW) and nitrogenase activity (ARA) of *P. vulgaris* by *Rhizobium etli* (TAL 182) in the greenhouse[†]

Treatment	NON (± SE)	NFW (g/plant ± SE)	ARA (n moles C_2H_4 h^{-1} $plant^{-1}$ ± SE)
Control	5.9 ± 1.45 *c*	0.03 ± 0.01 *c*	0.10 ± 0.05 *c*
Rhizobium TAL 182	44 ± 4.65 *b*	0.17 ± 0.02 *b*	4.66 ± 0.40 *b*
TAL 182 +A15 (*B. megaterium*)	68 ± 8.64 *a*	0.25 ± 0.03 *a*	6.68 ± 0.64 *a*
TAL 182 + T10 (*B. mycoides*)	37 ± 3.40 *b*	0.14 ± 0.02 *b*	3.95 ± 0.16 *b*
TAL 182 + Bet 10a (*B. pumilus*)	38 ± 3.80 *b*	0.14 ± 0.02 *b*	3.85 ± 0.23 *b*

[†] Plant growth conditions, bacterial inoculation, experimental conditions, and statistical analysis are outlined in Table 2.4. Means were separated using analysis of variance (ANOVA). Means followed by the same letter in a column do not differ significantly at $p \leq 0.05$, as determined by Duncan's multiple range test.

Table 2.6. Influence of *Bacillus* isolates on nodule number (NON), nodule fresh weight (NFW) and nitrogenase activity (ARA) of *P. vulgaris* by *Rhizobium etli* (TAL 182) in the greenhouse[†]

Treatment	NON (± SE)	NFW (g/plant ± SE)	ARA(n moles C_2H_4 h^{-1} $plant^{-1}$ ± SE)
Control	6.2 ± 2.39 *c*	0.03 ± 0.01 *c*	0.11 ± 0.04 *d*
Rhizobium TAL 182	47 ± 5.56 *b*	0.16 ± 0.02 *b*	4.80 ± 0.35 *b*
TAL 182 +S08 (*B. azotoformans*)	35 ± 3.80 *b*	0.13 ± 0.01 *b*	3.85 ± 0.30 *b,c*
TAL 182 + S72 (*B. brevis*)	71 ± 7.32 *a*	0.36 ± 0.06 *a*	7.54 ± 0.43 *a*
TAL 182 +S89 (*B. megaterium*)	37 ± 2.90 *b*	0.13 ± 0.01 *b*	3.70 ± 0.21 *c*

[†] Plant growth conditions, bacterial inoculation, experimental conditions, and statistical analysis are outlined in Table 2.4. Means were separated using analysis of variance (ANOVA). Means followed by the same letter in a column are not significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test.

Table 4. Effect of *Bacillus* isolates on nodulation of *Phaseolus vulgaris* cv. 'Contender' inoculated with TAL 182[1]

Treatment strain	# of nodules±SE	NFW[2]±SE (g)	ARA[3]±SE nmol C_2H_4/h/plant	Leghemoglobin µg/g FW nodules	TSP[4] µg/g FW nodules
Control[5]	3.5 ±1.8[d*]	0.02 ±0.01[d*]	-	-	-
TAL 182	74.0 ±5.7[c]	0.6 ±0.1[c]	7.5 ±0.7[c]	137.4 ±11.1[c]	867.6 ±82.4[c]
L6+TAL 182	138.4 ±6.0[a]	1.0 ±0.1[a]	14.3 ±0.6[a]	324.2 ±50.5[a,b]	1885.7 ±50.0[b,d]
L5+TAL 182	128.6 ±6.8[a,b]	0.9 ±0.1[a]	13.6 ±1.4[a]	302.2 ±46.3[a]	2190.5 ±97.0[a,b]
S49+TAL 182	132.4 ±4.6[a,b]	1.1 ±0.1[a,b]	16.1 ±1.2[a,b]	230.8 ±28.9[a]	1979.1 ±126.3[b,d]
S72+TAL 182	121.9 ±3.1[a,b]	0.9 ±0.1[a]	13.3 ±0.4[a]	210.8 ±20.0[a]	2452.1 ±156.4[a]
A15+TAL 182	101.1 ±6.2[b]	0.8 ±0.1[a]	13.2 ±1.2[a]	141.0 ±16.8[c]	1781.6 ±49.5[b]

[1] Plants were grown under gnotobiotic conditions in Leonard jars containing a sterilized industrial sand-Turface™ mixture as described in the text. Values represent means of seven replicates of each treatment taken at 24 DAP.

* Pseudonodules

Means were separated using analysis of variance and means followed by the same letter in a column do not differ significantly at $p \leq 0.05$ as determined by Duncan's multiple range test.

[2] NFW=Nodule fresh weight.

[3] ARA=Acetylene reduction assay expressed as n moles of ethylene per hour per plant.

[4] TSP=Total soluble proteins

[5] Sterile one tenth strength yeast extract mannitol broth used as an inoculant.

Table 2.8. Influence of *Bacillus* isolates on nodule number (NON), nodule fresh weight (NFW) and nitrogenase activity (ARA) of *P. vulgaris* by *Rhizobium etli* (TAL 182) under gnotobiotic conditions in the growth chamber[†]

Treatment	NON (± SE)	NFW (g/plant ± SE)	ARA (n moles C_2H_4 h^{-1} $plant^{-1}$ ± SE)
Control	1.30 ± 0.56 *c*	0.004 ± 0.002*c*	0.00 ± 0.00 *c*
Rhizobium TAL 182	89.80 ± 5.97*a*	0.470 ± 0.029*a*	9.80 ± 0.82 *a*
TAL 182 +Led 02 (*B. megaterium*)	78.10 ± 4.85 *b,a*	0.373 ± 0.035 *b*	7.23 ± 0.46 *b*
TAL 182 +T10 (*B. mycoides*)	67.00 ± 5.52 *b*	0.356 ± 0.030 *b*	6.48 ± 0.48*b*
TAL 182 +B17 (*B. megaterium*)	78.14 ± 4.62 *b,a*	0.392 ± 0.026 *b*	7.46 ± 0.53 *b*

[†] Plant growth conditions, bacterial inoculation, experimental conditions, and statistical analysis are described in Chapter two, section 2.2.5. Means followed by the same letter in a column are not significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test.

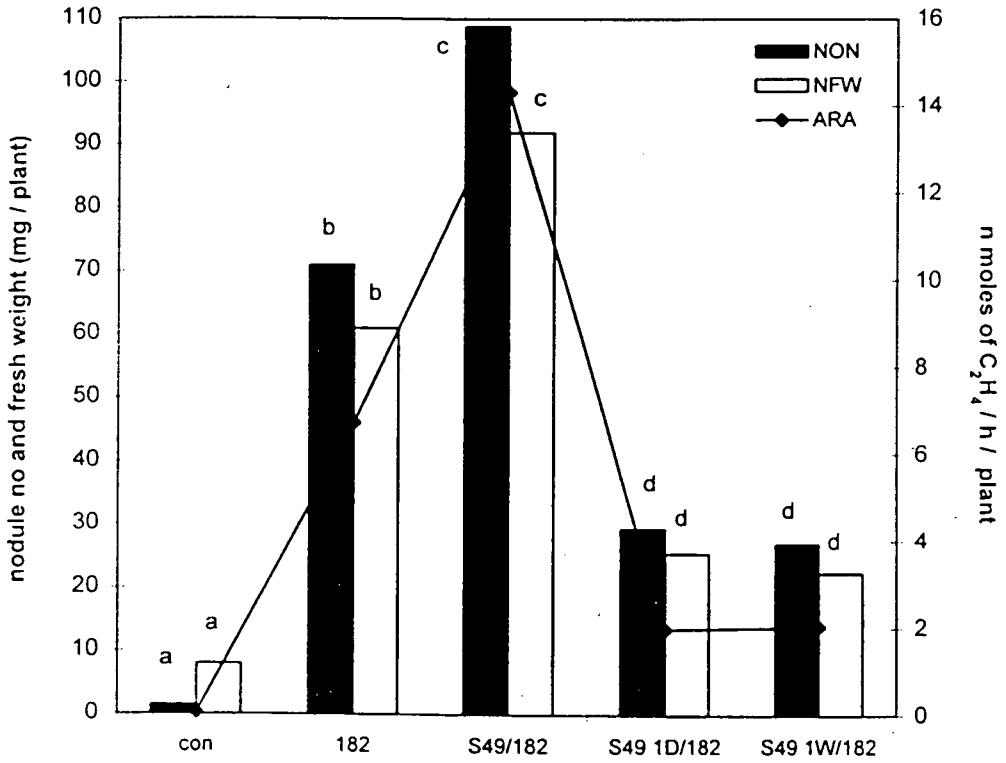


Figure 2.6. Effect of coinoculation of α-methyl tryptophan resistant mutants of *Bacillus megaterium* S49 with *Rhizobium etli* TAL 182 in nodule number (NON), nodule fresh weight (NFW), and nitrogenase activity (ARA) in *P. vulgaris* 'Contender'. Each value represents the mean of six replicates. Means were separated by Duncan's multiple range test using analysis of variance. Means followed by the same letter are not significantly different at $p \leq 0.05$. This experiment was repeated once with similar results.

and nitrogenase activity (ARA) also resulted from coinoculation (Fig 2.6). Root growth was also inhibited as a result of coinoculation of TAL 182 with the α-methyl tryptophan resistant mutants of *Bacillus* S49 (Table 2.9).

2. 4. Discussion

Of 120 isolates characterized from the rhizosphere of *P. vulgaris*, twenty two belonged to the genus *Bacillus* as identified by Biolog™ and GC-FAME analysis. The phylogenetic relationships of this multiplicity of *Bacillus* spp. associated with plant roots were demonstrated by 23S rDNA analysis. This diversity may reflect the dependence of rhizosphere microorganisms upon specific root exudates for their carbon sources. Mavingui *et al.* (1992) reported that *B. polymyxa* strains isolated from the rhizoplane of wheat (*Triticum aestivum* L.) were capable of metabolizing sorbitol whereas rhizosphere and non-rhizosphere isolates did not show such capability. Such diversity may also represent a closer specific association between the plant and *Bacillus* spp. which provides some benefit to the plant by producing phytohormones (Holl *et al.* 1988), suppressing deleterious rhizobacteria and/or by an unknown mechanism(s) (Chanway *et al.* 1991; Halverson and Handelsman 1991; Turner and Backman 1991).

Forty five percent of the 22 *Bacillus* isolates produced IAA as determined by the rapid *in situ* assay, although quantities of IAA produced by individual strains varied markedly. This variation was not phylogenetically-determined since 23S rDNA sequence analysis did not cluster the IAA producers. However, the complexity of the rhizosphere environment and the intricacies of plant host/bacterial interactions likely alter the metabolism of both host and symbiont. Hence, the quantity of phytohormone produced at the root surface may be expected to vary from those measured in this study; such

Table 2.9. Root fresh weight and root dry weight of *P. vulgaris* due to coinoculation of *Rhizobium etli* TAL 182 with *Bacillus megaterium* S49 or α-methyl tryptophan resistant mutants of S49, under gnotobiotic conditions in the growth chamber†

Treatment	Root fresh weight (g/plant ± SE)	Root dry weight•• (g/plant ± SE)
Control•	3.16 ± 0.12*c*	0.32 ± 0.04*c*
Rhizobium TAL 182	3.91 ± 0.11*b*	0.41 ± 0.05*b*
TAL 182 + S49	5.27 ± 0.19*a*	0.60 ± 0.07*a*
TAL 182 + S49-1D	1.34 ± 0.12*d*	0.15 ± 0.04*d*
TAL 182 + S49-1W	1.13 ± 0.09*d*	0.11 ± 0.03*d*

† Plant growth conditions, bacterial inoculation, experimental conditions, and statistical analysis are described in Chapter two, section 2.2.5. Means were separated using analysis of variance (ANOVA). Means followed by the same letter in a column are not significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test.
• Control plants received one ml of sterile phosphate buffer (pH 6.8) as inoculum.
•• Roots were dried at 70°C for 72 hours, to a constant dry weight.

differences may account for the different plant responses observed.

Previous investigations of the beneficial effects of coinoculation of *Rhizobium* spp. and *Azospirillum* spp. on nodulation attributed these effects to bacterial-derived phytohormones (Yahalom *et al.* 1990). Similarly, *Bacillus* isolates observed in this study that enhanced nodulation in *P. vulgaris* also produced significant amounts of IAA, *in vitro*. Further analysis of the morphogenic potential of indole compounds produced from *Bacillus* cultures showed a variable relationship between *in vitro* IAA production, plant growth stimulation and/or nodulation enhancement. For example, isolates such as *B. brevis* S72 produced only small amounts of IAA, but markedly enhanced nodulation over *Rhizobium* inoculation alone. Such observations suggest that the enhanced nodulation observed required the presence of both bacterial species and may not be solely dependent on *in vitro* IAA production by the *Bacillus* spp. This response supports the view that combinations of bacteria are more effective in promoting nodulation (Grimes and Mount 1984; Caetano-Anollés and Bauer 1988).

Support for the view that *Bacillus* isolates influenced nodulation on *P. vulgaris* by *R.etli* TAL 182 was provided by the observation that the α-methyl tryptophan mutants S49-1D and S49-1W inhibited nodulation by *Rhizobium* TAL 182, and that host plants showed poor root growth. These changes in root growth and nodulation may have occurred as a consequence of changes in the phytohormonal balance of the rhizosphere since IAA production by the α-methyl tryptophan mutants was appreciably different from that of the wild-type. Furthermore, other metabolic changes were likely associated with α-methyl tryptophan resistance; different carbon source utilization patterns (Biolog™), the presence of a prolonged lag phase, and excretion of tryptophan reflected such

changes. These differences may also have contributed to altered symbiotic efficiency of *Rhizobium* (TAL 182). Nevertheless, the ability of the α-methyl tryptophan mutants to affect nodulation of *P. vulgaris* adversely under gnotobiotic conditions leads to the speculation that *Bacillus* spp. may have a direct effect on either the *Rhizobium* or the plant. Similar results have been reported by Hunter (1987) with 5-methyl tryptophan resistant mutants of *Bradyrhizobium japonicum* on soybeans.

In addition to enhancement of nodulation, coinoculation of *Bacillus* isolates with *Rhizobium* TAL 182 increased nitrogenase activity and total soluble nodule protein content of the nodules. Higher nitrogenase activity may be attributed to the increase in leghemoglobin content in the root nodules. Interactions between plants and microorganisms are often associated with the induction in the plant of a complex set of biochemical responses and morphogenetic changes (Young *et al.* 1991; Kamoun *et al.* 1993). It is highly likely that genes encoding nodule proteins can be regulated by *Rhizobium* (Nap and Bisseling 1990). The increased leghemoglobin and total soluble protein content observed in this study may be due to unique physiological conditions imposed during nodule development by the presence of both bacterial species on the plant roots. The ability of *Rhizobium* to alter the regulation of an already functional gene(s) and impose physiological changes during nodule organogenesis has been reported previously (Bennett *et al.* 1989; Nap and Bisseling 1990).

This study implicates naturally occurring phytohormone-producing *Bacillus* spp. in promoting nodulation in *P. vulgaris* by *Rhizobium etli* TAL 182. Nodule organogenesis clearly involves phytohormones (Hirsch and Fang 1994), but it is not yet clear whether elevated phytohormonal levels in nodules are the result of a plant

response to bacterial infection or a direct function of bacterial activity (Dullart 1970). Realizing the importance of cytokinins in nodule organogenesis, the ability of the *Bacillus* isolates to produce cytokinins *in vitro* was evaluated. Some of the *Bacillus* isolates tested produced *trans* zeatin, isopentyl adenine (2iP) and *trans* zeatin riboside. Further detailed analysis of cytokinin production by *Bacillus* spp. was not carried out in this study for the following reasons: (i) inconsistent results obtained in the present study and, (ii) technical difficulties encountered in identifying and quantifying cytokinins. Preliminary results on the detection of cytokinins in the cell cultures of *Bacillus* isolates are given for information in Appendix I.

A possible explanation for increased nodulation in response to coinoculation in this study is the creation of additional infection sites for *Rhizobium*. In other studies, increased nodulation observed due to coinoculation of *Azospirillum* with *Rhizobium* has been attributed to the creation of more infection sites on the roots for *Rhizobium* by *Azospirillum* (Iruthayathas *et al.* 1983; Plazinski and Rolfe 1985). Further investigation of the physiological basis of the plant response to *Bacillus* inoculation should enhance the basic understanding of the plant-microbe interaction. The ability of some *Bacillus* species in this study to promote nodulation under gnotobiotic conditions irrespective of *in vitro* IAA production suggests a broader role for these microorganisms in directing the development of plant tissues in natural ecosystems. The primary objective of the work described in the next chapter was the evaluation of the response of root development in *Phaseolus vulgaris* to the presence of *Bacillus* and *Rhizobium*.

Chapter 3

Synergistic interaction between *Rhizobium etli* and *Bacillus megaterium* enhances root hair formation in *Phaseolus* spp. and in *Arabidopsis thaliana*

I have the result, but I do not yet know how to get it

Karl Friedrich Gauss (1777-1855)

3.1. Introduction

In the previous chapter, phytohormone-producing *Bacillus* spp. were shown to be capable of nodulation enhancement of *P. vulgaris* inoculated with *Rhizobium etli* TAL 182. Those observations are consistent with the involvement of phytohormones in the promotion of plant growth and the influence of bacteria on root growth (Tien *et al.* 1979). Both lateral root development (Barbieri *et al.* 1986) and root hair production (Barbieri and Galli 1993) have been shown to be enhanced by bacterially-derived indoleacetic acid (IAA) (Yahalom *et al.* 1991). Changes in root length and density of root hairs due to microbial activity have also been reported (Morgenstern and Okon 1987; De Freitas *et al.* 1993).

Root development and root hair morphogenesis are significant factors in the development of a healthy plant. In particular, root hairs play an important role in water and nutrient uptake (Clarkson, 1985) and, in both legumes (Bauer 1981) and non-legumes (Newcomb and Wood 1987), have been implicated in the symbiotic infection process. Abnormal root and root hair morphogenesis have been described in various studies of *Arabidopsis* auxin-resistant mutants (Mirza *et al.* 1984; Estelle and Somerville 1987). None of the reported root hair mutations affected the spacing or distribution of root hairs, however, or the performance of the plants under optimal water and nutrient conditions (Schiefelbein and Somerville 1990).

Deformation of legume root hairs in response to *Rhizobium* spp. inoculation has been correlated with the production of IAA by these microbes (Nutman 1956; Fahraeus and Ljunggren 1968). Phytohormone effects have also been implicated in the increased nodule numbers observed on legume hosts in response to combined inoculation with *Rhizobium* and *Azospirillum* (Sarig *et al.* 1986; Yahalom *et al.* 1990).

While phytohormones clearly participate in such developmental responses, other work suggests that IAA is not the sole, or primary, factor responsible (Wang *et al.* 1982; Plazinski and Rolfe 1985; Atzorn *et al.* 1988). Since auxins are most likely involved in both root development and nodulation, the main objective of this study was to determine the effect of IAA -producing and nodulation-promoting *B. megaterium* S49 and *Rhizobium etli* TAL 182, on lateral root and root hair development in the common bean (*Phaseolus vulgaris*), and on root hair development in *P. acutifolius* and *Arabidopsis thaliana.*

3. 2. Materials and methods

3. 2.1. Bacterial strains and growth media

The isolation and characterization of *Bacillus megaterium* S49 and α-methyl tryptophan resistant mutants of S49 (S49-1D) have been described previously (Chapter 2; sections 2.2.1; 2.2.4.). *B. megaterium* S49 was routinely maintained on combined carbon medium (Rennie, 1981) and *Rhizobium* TAL 182 was maintained on yeast mannitol agar slants (Bohlool and Schmidt, 1970). Growth conditions for α-methyl tryptophan mutants of S49 have been described previously (chapter 2; section 2.2.4.). Broth cultures were grown to early stationary phase in combined carbon medium for *Bacillus* spp. and in yeast mannitol broth for *Rhizobium* spp. The cells were harvested by centrifugation, washed twice with sterile 0.1M phosphate buffer (pH 6.8-7.0) and resuspended in the same buffer to the desired cell density before inoculation.

3. 2. 2. Plant material and growth

Seeds of *Phaseolus vulgaris* cv. 'Contender' were surface sterilized in 30% (v/v) hydrogen peroxide for 15 minutes. Sterile 2-day old pregerminated seedlings with

uniform radicle length (approximately 1 cm) were immersed for 15 minutes in 0.1M phosphate buffer (pH 6.8-7.0) either alone (control) or containing 10^6 cfu ml^{-1} of the appropriate bacterial inoculant. Seeds treated with IAA were incubated for 15 minutes in 5 or 30 µg ml^{-1} IAA in 0.1M phosphate buffer (pH 6.8-7.0). Seedlings were cultured axenically by transfer to a 20 x 150 mm test tube containing 10 ml of one-tenth strength plant growth medium (Broughton and Dilworth, 1971), in which the seed was supported above the medium by a 200 µl plastic pipette tip. Medium for treatments which included IAA were supplemented with appropriate concentrations of IAA (5 or 30 µg ml^{-1}). Tubes were incubated at 25° C with a 16h photoperiod at 200 µE m^2 s^{-1} illumination.

Seeds of *P. acutifolius* were obtained from NifTAL, Paia, Hawaii. The seeds were sterilized, pregerminated, treated and grown as described above. A random sample of lateral roots from approximately 10 plants/treatment was used for scanning electron microscopy. The base and tip (approximately 0.8 cm each) of the lateral roots were excised and discarded. The remaining central portion of the root was used for the scanning electron microscopy analysis.

Seeds of *Arabidopsis thaliana* derived from the Columbia wild type were obtained from the Arabidopsis Stock Centre (Columbus, OH). Seeds were surface sterilized and germinated on an agarose nutrient medium as described (Schiefelbein and Somerville, 1990). Roots of two day old seedlings were treated with 50 µl of buffer solution or bacterial suspension (10^6 cfu ml^{-1}) in a sterile plastic petri dish for 15 minutes. After inoculation, seedling roots were washed three times in sterile distilled water and transferred to petri plates containing the nutrient agar medium of Schiefelbein and Somerville (1990). Washing was necessary to prevent overgrowth of the culture medium by the bacteria. The plates were sealed with Parafilm® and incubated vertically

under continuous fluorescent illumination (approximately 100 $\mu E\ m^{2}s^{-1}$) at room temperature. At five days, the terminal 0.5-1.0 cm of radicle was excised and prepared for scanning electron microscopy as described below. Approximately 20 plants were scored for each treatment in each of two experiments.

3. 2. 3. Scanning electron microscopy (SEM)

The root segments were fixed overnight in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). Samples were washed three times (15 minutes each) with 0.05 M sodium cacodylate buffer, and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) for two hours. The samples were rinsed once with de-ionized water, dehydrated through a 30% to 100% series of ethanol rinses, and critical point dried from 100% ethanol in a Balzers CPD 020 system using liquid carbon dioxide as the exchange medium. After mounting on double-sided sticky aluminum stubs, specimens were coated with gold-palladium (approximately 25nm) in a Nanotech Semprep II Sputter coater, and viewed with a Cambridge 250T scanning electron microscope. All samples were viewed and the typical observed response was scored (e.g. no proliferation, slight proliferation, massive proliferation), and recorded photographically.

3. 2. 4. Estimation of lateral root numbers

For the estimation of lateral root numbers, seeds of *P. vulgaris* were sterilized, pregerminated, treated and grown as described above. Germinated seedlings were harvested at five days after seeding and lateral roots counted. Statistical comparisons were carried out by the SAS computer program (SAS Institute, Inc., Cary, NC) using Fisher's LSD.

3. 3. Results

3. 3. 1. Root hair production by *P. vulgaris* in response to various treatments

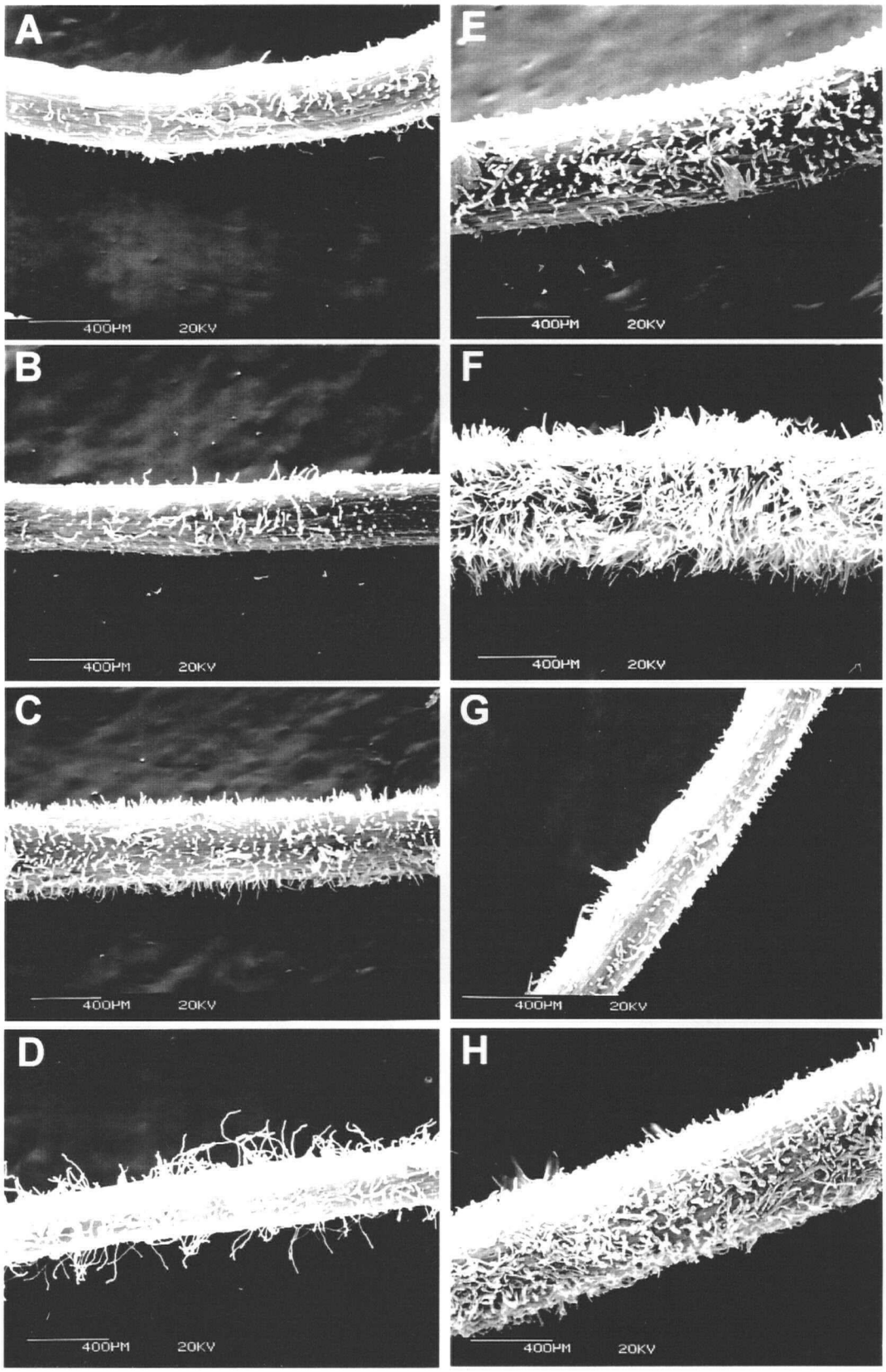
The root hair production responses in *P. vulgaris* to the various treatments are shown in Figure 3.1 (A-H). Single inoculation with either *Rhizobium* TAL 182 (Figure 3.1.C) or *Bacillus* S49 (Figure 3.1E) produced an increase in root hair production compared to control (Figure 3.1A). Dual inoculation of these two isolates resulted in greatly enhanced production of root hairs (Figure 3.1F). Application of IAA alone at 5 µg ml^{-1} (Figure 3.1B) did not show any evidence of increased numbers of root hairs. Application of IAA with *Rhizobium* TAL 182 resulted in the elongation of root hairs (Figure 3.1D), which may reflect its involvement in root hair morphogenesis.

Further evidence for the de-linking of IAA effects and bacterial interaction is found in the response to TAL 182 + S49-1D coinoculation (Figure 3.1.G). Although the *Bacillus* mutant S49-1D produces significantly lower IAA levels in culture (Chapter 2; section 2.3), it nevertheless does increase root hair production compared to the control (Figure 3.1.A). The results described were obtained from five separate experiments conducted over a period of several months. The presence or absence of prolific root hair initiation in response to the treatments was consistent across experiments. Of the 30 lateral roots examined from 15 plants receiving the dual inoculation, 10% showed proliferation equivalent to single inoculation by S49 alone, while 90% showed massive proliferation.

3. 3. 2. Compatible legume-*Rhizobium* combination is not required for root hair proliferation

The *P. acutifolius* used in these experiments is a line of bean which is normally nodulated by *Bradyrhizobium* spp. and is resistant to nodulation by *Rhizobium* TAL 182

Figure 3.1. Root hair production by *P. vulgaris* in response to bacterial coinoculation.
(A) 0.1M phosphate buffer (pH 7.0) (control)
(B) IAA (5 μg ml^{-1})
(C) *Rhizobium etli* (TAL 182)
(D) IAA (5 μg ml^{-1}) and *Rhizobium etli* (TAL 182)
(E) *Bacillus megaterium* (S49)
(F) *Rhizobium* (TAL 182) and *Bacillus* (S49)
(G) *Rhizobium* (TAL 182) and *Bacillus* (S49-1D)
(H) *Rhizobium* (TAL 182) and *Bacillus* (S49-1D) and IAA (5 μg ml^{-1})


A
400µM 20KV
B
400µM 20KV
C
400µM 20KV
D
400µM 20KV
E
400µM 20KV
F
400µM 20KV
G
400µM 20KV
H
400µM 20KV

(Somasegaran *et al.* 1991). The absence of any root hair stimulation comparable to dual inoculation by inoculation with *Rhizobium* TAL 182 alone (Figure 3.2B) is consistent with the known host nodulation specificity and the previous observations with *P. vulgaris.* Single inoculation with *Bacillus* S49 enhanced root hair production (Figure 3.2C) compared to the control (Figure 3.2A), while dual inoculation with *Rhizobium* TAL 182 and *Bacillus* S49 produced a more extensive proliferation of root hairs on the lateral root surface (Figure 3.2D).

3. 3. 3. *Arabidopsis* shows increased root hair production due to coinoculation

Since the root hair proliferation response did not require a compatible legume-*Rhizobium* combination, it was prudent to investigate whether a similar reaction could be observed following dual inoculation of an unrelated, non-legume genus. The root hair response of *Arabidopsis* to dual inoculation (Figure 3.3C) mimicked the extensive proliferation observed in the *Phaseolus* tests. Single inoculation of *Arabidopsis* yielded little evidence of changes in root hair morphogenesis (Figure 3.3B). The plant response was clearly not related to any natural association of *Arabidopsis* and the challenge bacteria. The results indicate that root hair proliferation required the participation of the bacterial partners, but was independent of the plant host.

3. 3. 4. Bacterial inoculation and root development

To determine the impact of bacterial inoculation and associated phytohormone production on root development, lateral roots were counted on five day old seedlings of *P. vulgaris* treated with various combinations of bacteria and IAA (Table 3.1). Lateral root production was clearly stimulated by exogenous IAA (5 μg ml^{-1}) and by inoculation with *Rhizobium* TAL 182 and/or *Bacillus* S49, whereas no effect was observed for treatments which included higher IAA levels (30 μg ml^{-1}) or the α-methyl tryptophan

Figure 3. 2. Root hair production by *P. acutifolius* in response to bacterial coinoculation.
(A) 0.1M phosphate buffer (pH 7.0) (control)
(B) *Rhizobium etli* (TAL 182)
(C) *Bacillus megaterium* (S49)
(D) *Rhizobium* (TAL 182) and *Bacillus* (S49)

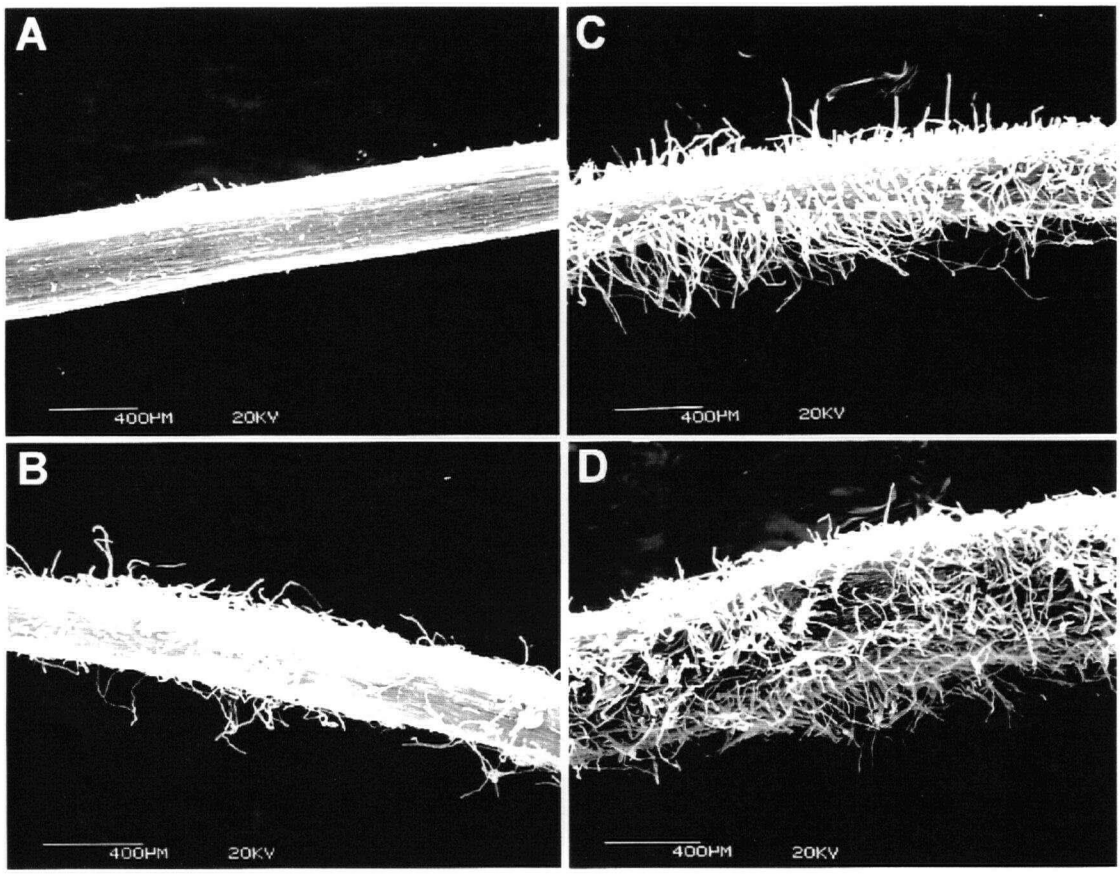

A
400µM 20KV
C
400µM 20KV
B
400µM 20KV
D
400µM 20KV

Figure 3. 3. Root hair production by *Arabidopsis thaliana* in response to bacterial coinoculation.
(A) 0.1M phosphate buffer (pH 7.0) (control)
(B) *Rhizobium etli* (TAL 182)
(C) *Rhizobium etli* (TAL 182) and *Bacillus megaterium* (S49)
Single inoculation of *Arabidopsis thaliana* with *B. megaterium* S49 gave similar results as treatment (B).

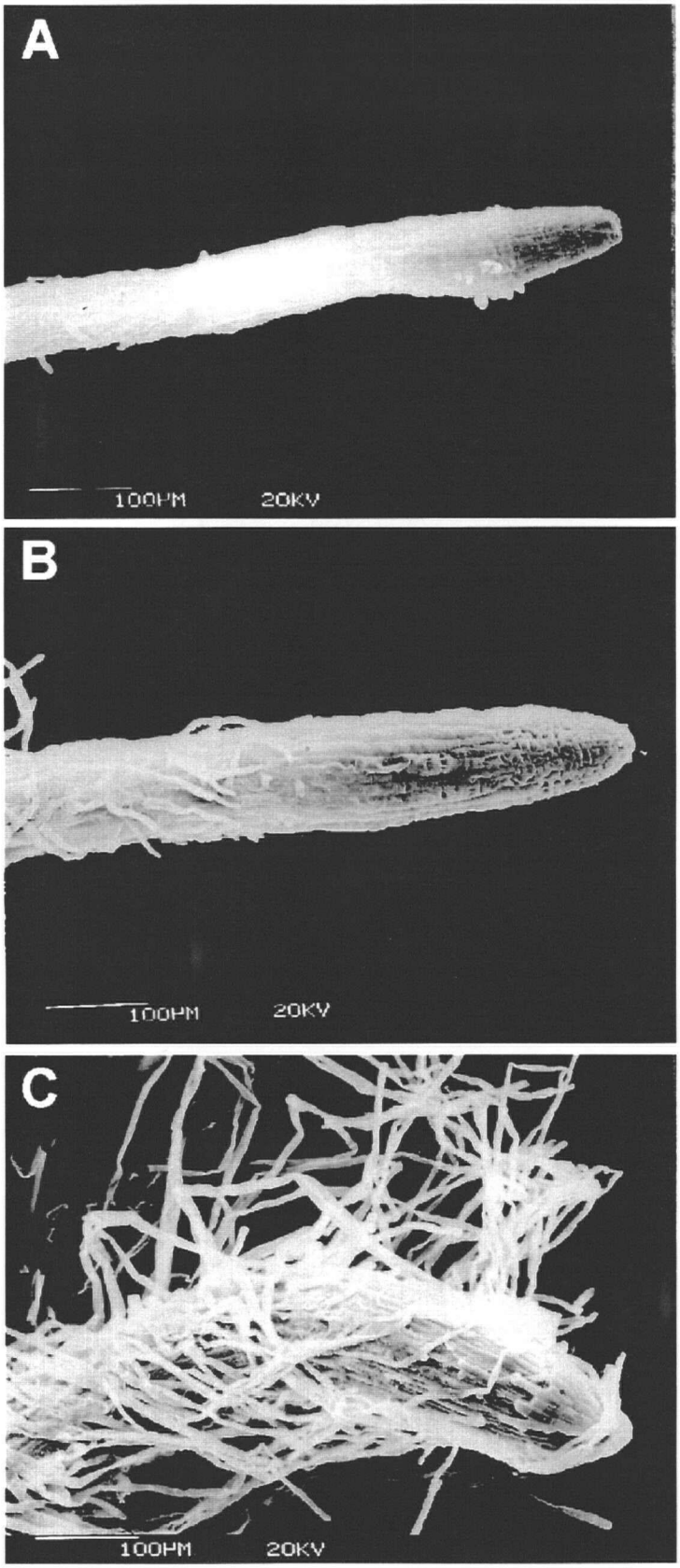

A
100PM 20KV
B
100PM 20KV
C
100PM 20KV

Table 3.1. Lateral root production on five day old seedlings of *Phaseolus vulgaris* treated with indoleacetic acid (IAA), or inoculated with *Bacillus megaterium* (S49 or S49-1D) and/or *Rhizobium etli* (TAL 182)

Treatment	(Number of lateral roots per sample[†])
Control	15.2 *a*
IAA (5 μg ml^{-1})	42.0 *d*
IAA (30 μg ml^{-1})	24.8 *a,b,c*
TAL 182	30.0 *a,b,c*
S49	40.5 *d*
S49-1D	23.6 *a,b,c*
TAL 182 + S49	30.2 *b,c,d*
TAL 182 + S49-1D	18.1 *a,b*
TAL 182 + S49 + IAA (5 μg ml^{-1})	32.4 *c,d*
TAL 182 + S49-1D + IAA (5 μg ml^{-1})	38.5 *c,d*

[†] Values shown are the means of ten replicates in two separate experiments. Means were separated using analysis of variance (ANOVA). Means followed by the same letter are not significantly different ($p \leq 0.05$; LSD = 11.9).

mutant S49-1D. Notably, the absence of lateral root enhancement by the combination of S49-1D was reversed by the addition of supplemental IAA (5 μg ml^{-1}).

3. 4. Discussion

Bacillus spp. are widely distributed in temperate soils. Phytohormone production by *Bacillus* has been shown to play a role in plant growth promotion (Holl *et al.* 1988). *Bacillus megaterium* S49 and *Rhizobium etli* TAL 182 were used to study the interaction of a plant growth-promoting rhizobacterium and the legume microsymbiont on lateral root and root hair development. *Bacillus* S49 produces IAA in culture and responds to the addition of supplemental tryptophan to the culture medium, whereas *Rhizobium* TAL 182 produces barely detectable amounts of IAA under similar growth conditions (Chapter 2; section 2.3). An α-methyl tryptophan resistant mutant of *Bacillus* S49 (S49-1D) has also been isolated; the mutant produces lower levels of IAA in culture and its growth cycle exhibits an extended lag phase compared to the parent strain (Chapter 2; section 2.3).

The importance of root hairs as infection sites for *Rhizobium* led to the investigation of the impact of dual inoculation on the production of root hairs in *Phaseolus*. Combined inoculations of *Rhizobium* and *Bacillus* gave a clear indication of a synergistic enhancement of root hair initiation. This enhancement was not mimicked by either supplemental IAA alone or by coinoculation with a low IAA producing *Bacillus* mutant. The inability of a low-IAA-producing *Bacillus* mutant to promote lateral root formation is consistent with the findings of Barbieri and Galli (1993), who reported similar effects on wheat root growth when inoculated with an analogous *Azospirillum* mutant, (SpM 7918). IAA supplementation of *Rhizobium* TAL 182 inoculated plants increased root hair elongation, but did not produce additional proliferation. Failure of

such treatments to produce a stimulatory response comparable to dual inoculation, as well as slightly increased root hair production using the *Bacillus* mutant S49-1D, indicates that the IAA effect on root hair elongation could be delineated from the bacterial interaction which produced prolific initiation of root hair development. In *Azospirillum*-plant interactions, changes in root morphology and root hair formation are attributed to the production of auxin by the bacteria. The effects of auxin are dependent on the concentration of *Azospirillum* on the plant roots (Harari *et al.* 1988). In clover, application of the synthetic auxin 2,4-D results in root hair elongation (Ridge 1995).

The stimulatory effect of dual inoculation was further demonstrated using a non-compatible legume host (*P. acutifolius*) for *Rhizobium etli* which also showed prolific root hair initiation in response to coinoculation with *Rhizobium* TAL 182 and *Bacillus* S49. These observations clearly indicate that the synergistic response is not linked to the host specificity of the *Rhizobium* isolate.

Given the positive response using a non-compatible host, the effect of inoculation on an unrelated plant genus which had no prior exposure to either of the bacterial isolates was examined. Not surprisingly, single species inoculation of *Arabidopsis thaliana* yielded little evidence of changes in root hair morphogenesis. In contrast plants receiving dual inoculation exhibited obvious proliferation of root hairs, confirming the view that the observed response was likely a consequence of interaction between the bacterial partners, rather than a host- directed event.

While considerable attention has been directed at the plant-microbe interaction, the observations in this study provide clear evidence of an bacterial "dialogue" which results in a significant change in plant root morphogenesis. Root hair proliferation in response to a single rhizosphere bacterial species has been reported previously (Umali-

Garcia *et al.* 1980; Kapulnik 1985a,b; De Freitas *et al.* 1993; Pacovsky, 1990). The present work describes the potential for interspecific bacterial interactions in the plant rhizosphere, and is the first report of a unique response in root hair morphogenesis induced by the interaction of two bacterial species. This interaction and its effect on root hair development are likely to have significant implications for the infection potential and nodulation of legumes, and for water and nutrient relations of plant species in natural ecosystems.

Chapter 4

Synergistic interaction between *Rhizobium* and *Bacillus* alters root morphogenesis and enhances nodulation in *Phaseolus vulgaris*

The *in vitro* axiom: "*Under the most rigorously controlled conditions of pressure, temperature, volume, humidity and other variables, the organism will do as it damn well pleases*".

Brian Ellis (1990)

4.1. Introduction

Root hairs are epidermal tubular outgrowths in the root system of higher plants (Ridge 1995; Schiefelbein and Somerville 1990). In legumes, they serve as attachment and infection sites for nitrogen-fixing *Rhizobium* (Bauer 1981, Wood and Newcomb 1989). Factors which adversely affect root hair formation in legumes also reduce nodulation by *Rhizobium* (Tu 1981). Many aspects of root hair development are under the genetic control of the plant (Cardus 1979; Mirza *et al.* 1984; Hochmuth *et al.* 1985), but non-specific factors that alter root hair growth include calcium ions (Weisenseel *et al.* 1975; Picton and Steer 1983) and water stress (Schnall and Quatrano 1992). Plant hormones such as auxins and ethylene (Cormak 1962; Mirza *et al.* 1984; Tretyn *et al.* 1991; Masucci and Schiefelbein 1994) also affect root hair growth. Many of the root hair morphogenetic changes induced by the non-specific and hormonal factors can be mimicked by nitrogen-fixing bacteria on compatible legume hosts (Callaham and Torrey 1981; Wood and Newcomb 1989).

Plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.* 1989) have also been reported to enhance nodulation of legumes by the *Rhizobium/Bradyrhizobium* microsymbiont (Grimes and Mount 1984; Halverson and Handelsman 1991). Indoleacetic acid (an auxin) has been associated with adventitious root initiation (Blakesley *et al.* 1991) and lateral root development (Wightman *et al.* 1980). Experimental evidence has implicated bacterially-mediated phytohormone production as a causal element in plant growth promotion (Tien *et al.* 1979; Holl *et al.* 1988) by altering the hormone balance within the affected plant (Glick 1995). Rhizosphere microbial production of indoleacetic acid (IAA) has also been implicated in lateral root

development (Barbieri *et al.* 1986; Dubeikovsky *et al.* 1993), root hair production (Barbieri and Galli 1993), root elongation (Kapulnik *et al.* 1985b), root hair branching and differentiation of root hairs (Patriquin *et al.* 1983; Jain and Patriquin 1984). However there is conflicting evidence regarding the primacy of bacterially derived IAA in causing these developmental changes (Atzorn *et al.* 1988; Harari *et al.* 1988).

Various reports implicate IAA in root hair deformation and curling (Farhaeus and Ljunggren 1968) and ascribe to it a critical role in nodule development (Hirsch *et al.* 1989). Although the influence of IAA produced by root-associated bacteria in promoting nodulation in legumes has been documented (Schmidt et al. 1988; Yahalom et al. 1990), other studies have indicated that IAA may not be involved in root hair deformation (Yao and Vincent 1976) and may not be essential for induction of nodule formation by *Rhizobium* (Wang *et al.* 1982). In chapter 2, it was shown that coinoculation of *Phaseolus vulgaris with Rhizobium etli* TAL 182 and *Bacillus megaterium* S49 induced massive proliferation of root hairs. This enhancement of root hair initiation was independent of the plant genotype or addition of exogenous IAA. Thus, a precise role for bacterial-derived IAA in root hair morphogenesis and nodule formation remains undefined.

The effect of dual inoculation using different *Bacillus* species, cell preparations and mutants of both *Rhizobium* and *Bacillus* on lateral root production and root hair proliferation in *P. vulgaris* has been examined. The results of this analysis clearly indicate a lateral root formation response to IAA, and that viable bacteria are likely required to induce root hair proliferation. Since root hairs are the infection sites for *Rhizobium*, the consequences of a unique interaction between *Bacillus* and *Rhizobium*

on nodulation of *P. vulgaris* by *Rhizobium etli* TAL 182 were also investigated.

4. 2. Materials and Methods

4. 2. 1. Bacterial strains and growth media

The origin, isolation and characterization of *Bacillus* S49 and *Rhizobium* TAL 182 have been described (Chapter 2). Two additional strains were used; *Bacillus megaterium* (Led 02) isolated from the *Phaseolus vulgaris* rhizosphere which shows high *in vitro* production of IAA (2.70 µg ml^{-1}) and *B. brevis* (S72) a low *in vitro* IAA producer (0.40µg ml^{-1}), as determined by HPLC analysis (Chapter 2). *Rhizobium etli* (MLC 35) a Tn5 mutant of *Rhizobium etli* TAL 182 which shows defective motility and nodulation (George and Robert 1991) was obtained from NifTAL, Paia, Hawaii. *Bacillus* spp. were routinely maintained on combined carbon medium (CCM) agar slants (Rennie 1981). *Rhizobium* TAL 182 was maintained on yeast mannitol agar slants (Bohlool and Schmidt, 1970). MLC 35 was maintained on TY plates (Beringer 1974) with nalidixic acid at 20 µg ml^{-1} and kanamycin at 50 µg ml^{-1} as described by George and Robert (1991). Broth cultures were grown to early stationary phase in combined carbon medium for *Bacillus* spp. and in yeast mannitol broth (YMB), with antibiotics, when appropriate, for *Rhizobium* spp. The cells were harvested by centrifugation, washed twice with sterile 0.1M phosphate buffer (pH 6.8) and resuspended in the same buffer to the appropriate cell density before inoculation.

4. 2. 2. Preparation of bacterial extracts

Rhizobium TAL 182 and *Bacillus* S49 were grown in YMB and CCM, respectively, to early stationary phase. Cell-free extracts (CFE) were prepared by harvesting 50 ml cultures by centrifugation at 10000 *x g* for 30 minutes in a Sorvall®

G13 rotor. The cell free supernatant was passed through a 0.22 µm syringe filter and stored at -20 °C prior to use. Whole cell extracts of both bacterial species were obtained by harvesting early stationary phase cultures as described above, and rupturing cells by passage through a French press (AMINCO, 5000 psi). The cell debris thus obtained is referred to as whole cell extracts (WCE). Autoclaved preparations (ACE) were obtained by autoclaving early stationary phase cultures for 15 minutes at 121°C and 15 psi. The absence of live bacteria in all the above preparations was tested by streaking a loopful of each preparation on to yeast mannitol agar plates for *Rhizobium* and nutrient agar (Difco) plates for *Bacillus* spp. Both the WCE and ACE samples were stored at -20 ° C prior to use.

4. 2. 3. Scanning electron microscopy (SEM)

Seeds of *P. vulgaris* 'Contender' were surface sterilized in 30% (v/v) hydrogen peroxide and rinsed seven times with sterile distilled water. Two-day old pregerminated seeds with uniform radicle length (approximately 1cm) were immersed for 15 minutes in 0.1M phosphate buffer (pH 6.8) either in the absence of bacteria (control) or containing 10^6 cfu ml^{-1} of the appropriate inoculant. For assessing the impact of whole cell extracts and/or cell-free preparations on root hair formation, pregerminated seeds were immersed for 15 minutes in the respective preparations, after dilution with sterile phosphate buffer to the desired cell equivalent. Media for treatments which included WCE, ACE or CFE were supplemented with one ml of the respective preparations. Seedlings were cultured axenically by transfer to a 20 x 150 mm test tube containing 10 ml of 1/10 strength nitrogen-free plant growth medium (Broughton and Dilworth 1971), in which the seed was supported above the medium by a 200 µl plastic pipette tip.

Tubes were incubated at 25° C with a 16h photoperiod at 200 μE m^2 s^{-1} illumination. Lateral roots in each treatment were counted prior to preparation for SEM. A random sample of lateral roots from at least 10 or more plants from each treatment was selected for SEM. In general, lateral roots of uniform length were chosen for SEM. The base and tip (approximately 0.8 cm each) of the lateral roots were excised and discarded. The remaining central portion of the root was fixed in 2.5% glutaraldehyde and prepared for SEM analysis as described in Chapter 3. An *in vitro* "cup assay" was developed to allow bacterial growth on the roots, while facilitating physical separation of the two bacterial species. A 30, 000 D MW cut off membrane was used to physically separate the two bacterial species, while allowing extracellular products to diffuse between the inoculants and the developing root system. This experiment helped to determine if the physical presence of both *Rhizobium* and *Bacillus* on the plant roots was necessary to induce root hair proliferation. Seedlings were grown as described above in 20 x 200 mm test tubes with 20 ml of 1/10 strength nitrogen-free plant growth medium. The seeds were supported above the medium by a 1000 μl sterile plastic pipette tip fused to a polypropylene cup containing a polysulfone filter of 30,000 D molecular weight cut off (Nalgene). Physical separation of bacterial strains was accomplished by adding 400 μl of either the *Rhizobium* or the *Bacillus* to the cup (10^6 cfu ml^{-1}) (Figure 4.1). Root samples were prepared for SEM analysis as described in Chapter 3.

4. 2. 4. Assessment of lateral root numbers in test tubes

To assess lateral root numbers, plant material and growth conditions were as described above. Seedlings were harvested at five days of growth in test tubes and the number of lateral roots counted. Statistical comparisons were made with the SAS

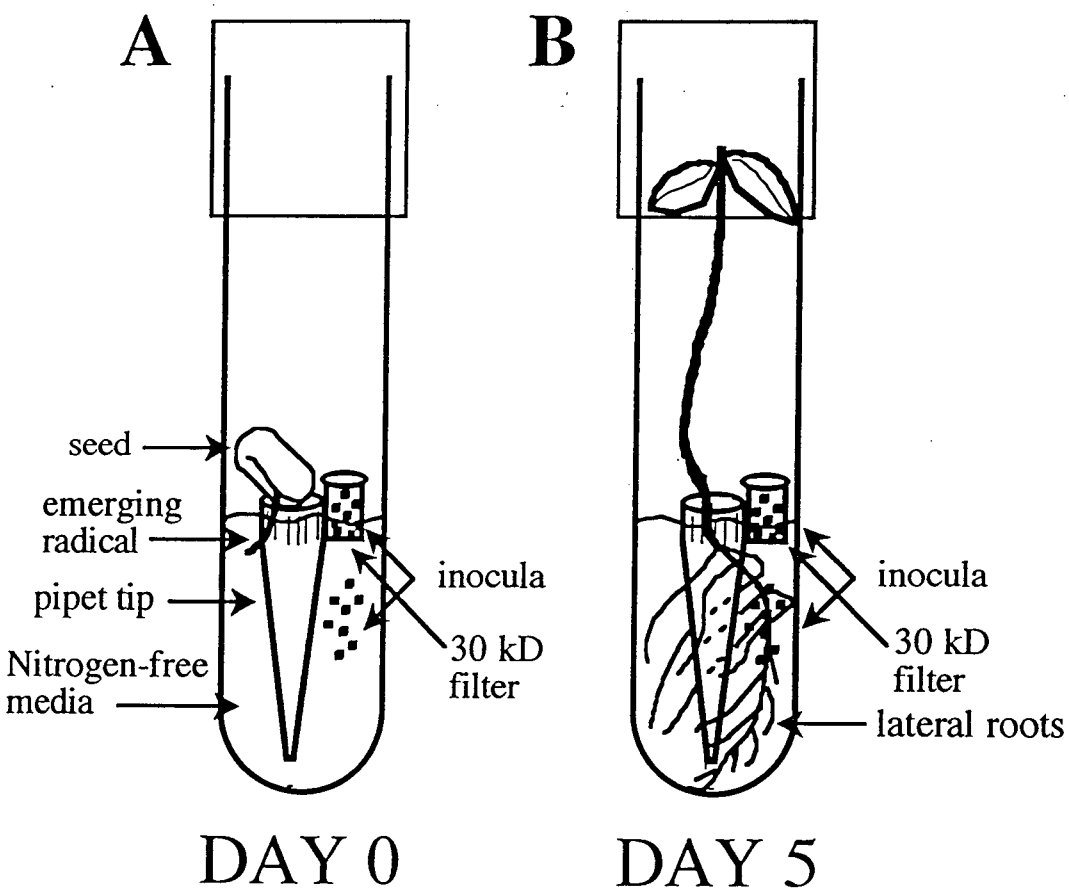


Figure. 4.1. Schematic diagram of the cup assay procedure used to facilitate physical separation of bacterial species on plant roots. (A) Initial set up at day one and (B) at five days of growth.

computer program (SAS Institute Inc., Cary, NC) using analysis of variance (ANOVA) and Duncan's multiple range test.

4. 2. 5. Assessment of lateral root numbers in Leonard jars

Seeds of *P. vulgaris* 'Contender' were surface sterilized, pregerminated and inoculated with bacterial suspensions as described above. Seeds were planted in modified Leonard jar assemblies (Blauenfeldt *et al.*1994) containing sterile industrial sand-Turface ® (1:1 v/v) of plant growth medium. Seedlings were grown under gnotobiotic conditions in the growth chamber with a 16h photoperiod at 350 $\mu E\ m^2\ s^{-1}$ illumination. Seedlings were harvested at four and seven days after planting and the lateral roots counted. Statistical comparisons were made as described above.

4. 2. 6. Early nodulation of *P. vulgaris* under gnotobiotic conditions in the growth chamber

This experiment was carried out to determine the effectiveness of nodules formed at early stages of plant growth in response to coinoculation of *Rhizobium* with *Bacillus* spp. Plant growth medium and experimental conditions in growth chambers were as described above. Plants were harvested at 8, 10, 12, and 14 days after inoculation and nodule numbers and fresh weights were determined. Acetylene reduction assays (Dilworth 1966), were determined by flame-ionization gas chromatography as described by Holl *et al.* (1988).

4. 2. 7. PCR determination of bacterial growth and physical presence

PCR was used to determine the efficacy of the "cup assay" experiments which allowed bacterial growth on plant roots while maintaining physical separation from either *Rhizobium etli* TAL 182 or *Bacillus* spp. in the cup. DNA of cells in one microlitre of

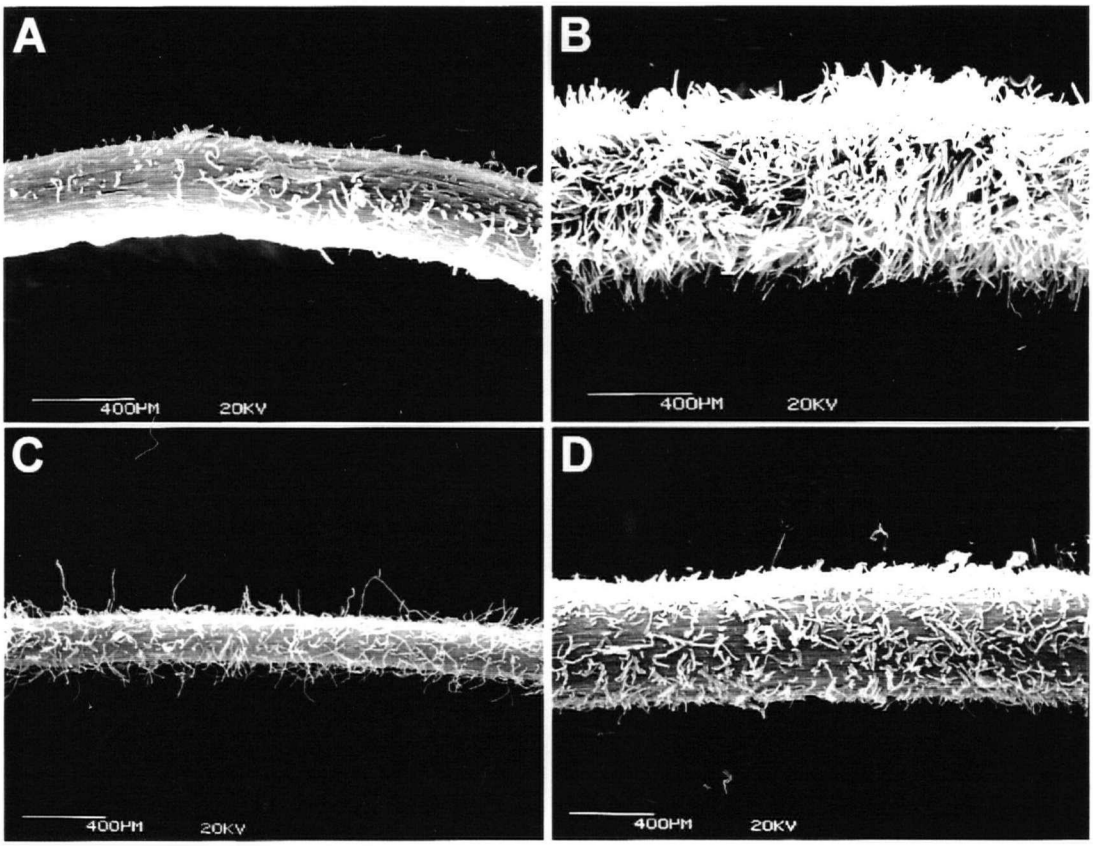
culture (plant growth medium vs cup) was amplified by multiplex PCR using 23S rDNA primers (BAC 11X, POL and TAL 182 primers), as described by Petersen *et al.* (1995). Amplification products of *Bacillus* and *Rhizobium* differ greatly in size (540 vs 650 bp respectively), and were separated by agarose gel electrophoresis, examined by staining with ethidium bromide and visualized under UV light.

4. 3. Results

4. 3. 1. Different *Bacillus* isolates induce different root hair morphogenetic responses when coinoculated with *Rhizobium etli* TAL 182

P. vulgaris plants grew well in liquid medium in test tubes, forming uniform normal root systems comparable to those plants grown on solid medium or in growth pouches. *B. megaterium* S49 induced root hair proliferation on *P.vulgaris* when co-inoculated with *R.etli* TAL 182, (Figure 4.2B) confirming the earlier observations reported in Chapter 3. The root hair induction responses due to inoculation with bacterial species were not influenced by lateral root size (diameter). Root hair proliferation responses for each treatment were judged by visual inspection by two independent observers and the figures shown represent the typical response for each treatment which consisted of 20 randomly chosen lateral roots from 10 different plants. Coinoculation of *Rhizobium* TAL 182 with either *Bacillus* spp. Led 02 or S72 did not enhance root hair formation on *P. vulgaris*, compared to coinoculation with *B. megaterium* S49 (Figure 4.2 B-D). This result also suggests that root hair proliferation due to coinoculation may not be solely dependent upon the ability of the *Bacillus* partner to produce IAA *in vitro*. Coinoculation of the higher IAA producing *B. megaterium* Led 02 with TAL 182 resulted in longer root hairs compared to the control and dual inoculation with the low IAA producer, *B. brevis* S72.

Figure. 4.2. Root hair production by *P. vulgaris* in response to dual inoculation of *Rhizobium etli* TAL 182 with different *Bacillus* spp.
A. Control (sterile 0.1M phosphate buffer [pH 6.8])
B. *Rhizobium* TAL 182 and *Bacillus* S49
C. *Rhizobium* TAL 182 with *Bacillus* Led 02
D. *Rhizobium* TAL 182 with *Bacillus* S72


A
400µM 20KV
B
400µM 20KV
C
400µM 20KV
D
400µM 20KV

Hence root hair proliferation in response to coinoculation can be discriminated from the effects of IAA produced by the *Bacillus* spp. These results are consistent with the previously observed effects on root hair morphogenesis using α-methyl tryptophan mutants of *Bacillus* S49 (Chapter 3).

4. 3. 2. Physical presence of both *Bacillus* and *Rhizobium* is necessary to induce root hair proliferation

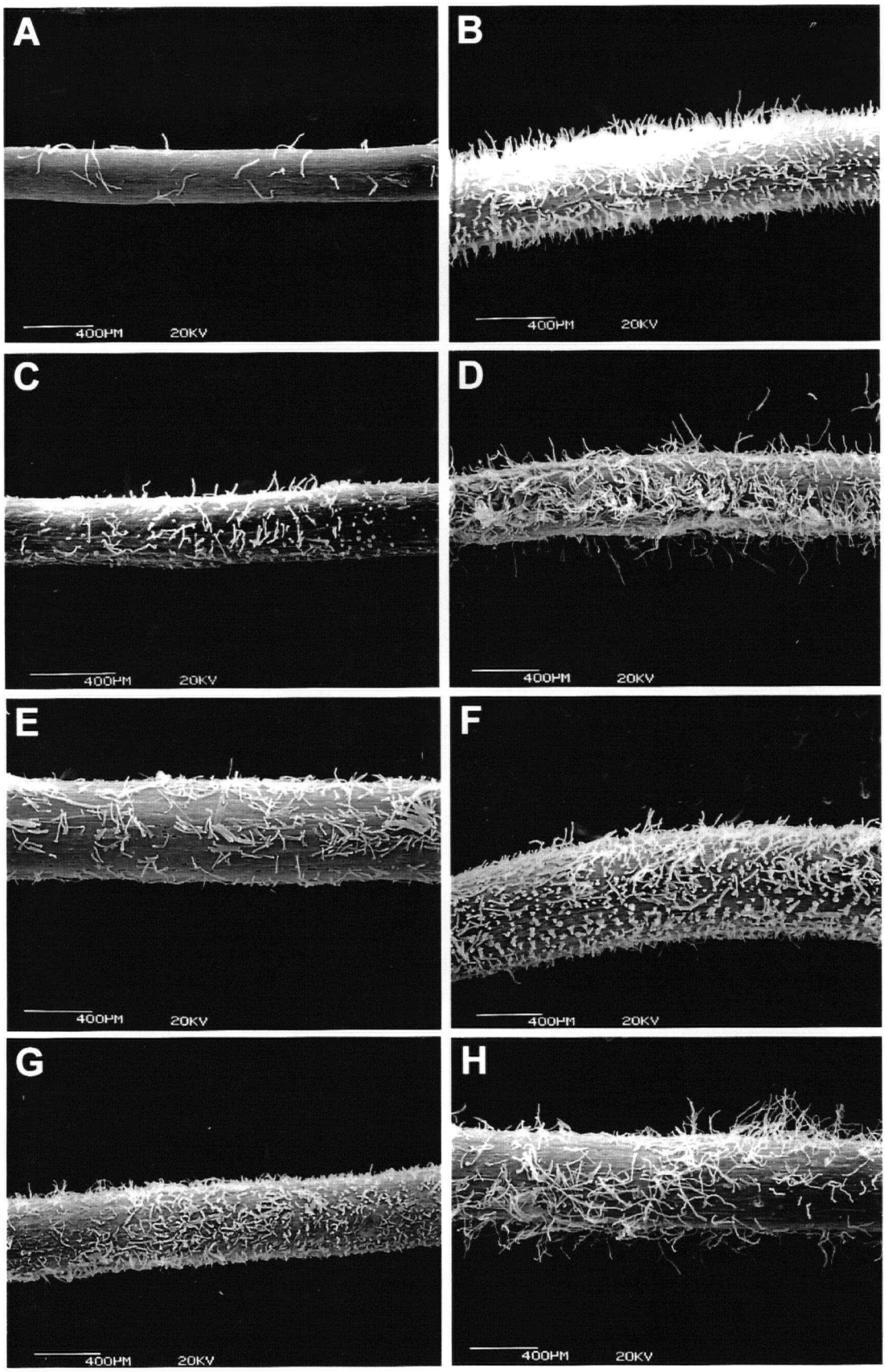
Separation of bacterial species using a 30,000 D MW cut off membrane prevented root hair proliferation, regardless of which bacteria was applied to the root system, indicating that both bacterial partners need to be physically present on the roots (Figure 4.3 H). The presence of either *Bacillus* or *Rhizobium* alone on the roots did not induce root hair proliferation (Figure 4.3 D-G), compared to dual inoculation of *Rhizobium* TAL 182 and *Bacillus* S49 (Figure 4.2 B).

4. 3. 3. Root hair proliferation is not induced in the absence of live bacterial species

Culture tests confirmed the absence of viable bacterial cells in WCE, ACE and CFE preparations. Inoculation of *P. vulgaris* with either filter-sterilized CFE or WCE of *Bacillus* S49 with live *Rhizobium* TAL 182 failed to elicit root hair proliferation (Figure 4.3 B-C) compared to dual inoculation of live bacterial species (Figure 4.2 B and 4.4 A). Inoculation of WCE of both *Bacillus* S49 and *Rhizobium* TAL 182 failed to show a root hair response on 85% of lateral roots examined (Figure 4.3 B) compared to dual inoculation with live bacterial species (Figure 4.2 B and 4.4 A) whereas 15% of lateral roots screened showed proliferation comparable to dual inoculation with viable cultures.

Figure. 4.3. Root hair production by *P. vulgaris* in response to inoculation with cell extracts of *Rhizobium etli* TAL 182 and *B. megaterium* S49

A. Control (sterile 0.1M phosphate buffer [pH 6.8])
B. *Rhizobium* TAL 182 WCE and *Bacillus* S49 WCE
C. *Rhizobium* TAL 182 CFE and *Bacillus* S49 CFE
D. *Rhizobium* TAL 182 WCE and *Bacillus* S49 (live cells)
E. *Rhizobium* TAL 182 (live cells) and *Bacillus* S49 WCE
F. *Rhizobium* TAL 182 CFE and *Bacillus* S49 (live cells)
G. *Rhizobium* TAL 182 (live cells) and *Bacillus* S49 CFE
H. *Rhizobium* TAL 182 (cup) and *Bacillus* S49 (live cells on plant roots)


A
400µM 20KV
B
400µM 20KV
C
400µM 20KV
D
400µM 20KV
E
400µM 20KV
F
400µM 20KV
G
400µM 20KV
H
400µM 20KV

Inoculation using ACE of S49 and TAL 182 gave similar results to the filter sterilized CFE (not shown). These results suggest that live (metabolically active) interacting bacterial partners may be necessary for inducing root hair proliferation in *P. vulgaris*.

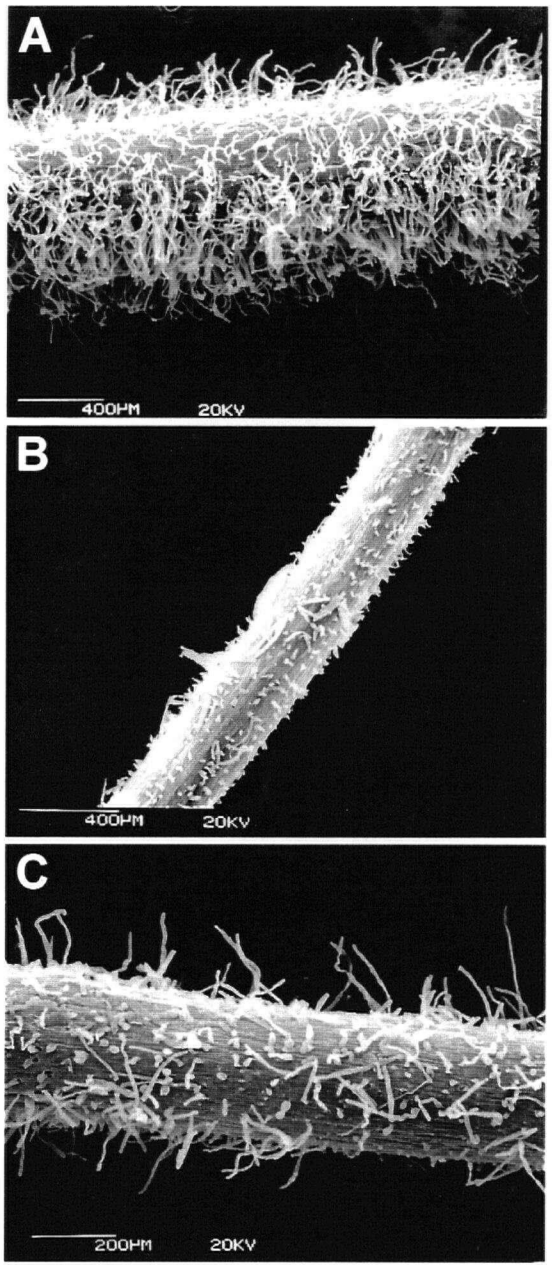
4. 3. 4. Functional *Bacillus* and *Rhizobium* are necessary to induce root hair proliferation

MLC 35 is a Tn5 mutant of *Rhizobium etli* capable of root hair deformation and nodule initiation, but which forms empty, non-fixing pseudonodules (George and Robert 1991). Coinoculation of *Bacillus* S49 with *Rhizobium* MLC 35 did not induce root hair proliferation (Figure 4.4 C), compared to dual inoculation of *Bacillus* S49 with wild-type *Rhizobium* TAL 182 (Figure 4.4 A). This observation is consistent with the non-proliferative response observed with coinoculation of an α-methyl tryptophan resistant mutant of *Bacillus* S49 (S49-1D) and *Rhizobium* TAL 182 (Figure 4.4 B). These results suggest that a fully functional (wild-type) *Bacillus* and *Rhizobium* may be necessary to induce root hair proliferation, even though the mechanism(s) of the interaction is unknown.

4. 3. 5. PCR verification of membrane separation of bacteria

The PCR was used to determine the efficacy of the cup assay to separate the bacteria from the root system, while simultaneously monitoring their viability during the course of the experiment. One microlitre samples from the root medium and the "cup" were analyzed for the presence of bacteria of either genera using multiplex PCR using species-specific primers. No contamination of the root medium or the cup contents was observed (Figure 4.5), confirming the ability of the 30 kD Nalgene membrane to function as a selective filter, allowing passage only of lower molecular weight diffusible

Figure 4.4. Root hair production by *P. vulgaris* in response to inoculation with Tn*5* mutant (MLC 35) of *Rhizobium etli* TAL 182 and *B. megaterium* S49
A. *Rhizobium* TAL 182 and *Bacillus* S49
B. *Rhizobium* TAL 182 and *Bacillus* S49-1D
C. *Rhizobium etli* MLC 35 and *Bacillus* S49


A
400µM 20KV
B
400µM 20KV
C
200µM 20KV

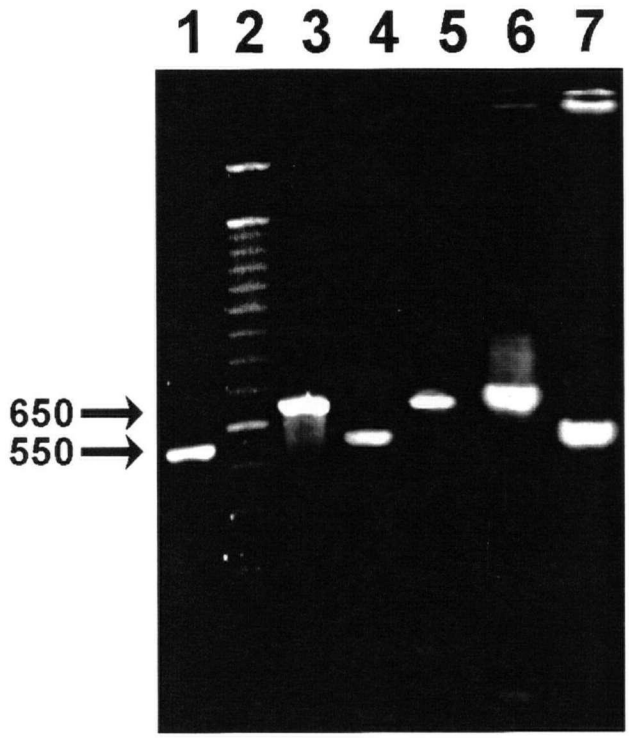


Figure. 4.5. Analysis of bacterial populations in cup assay experiments. Amplified fragments obtained by multiplex PCR using *Bacillus* S49 and *Rhizobium* TAL 182 specific primers, were separated on a 1.5% agarose gel. Amplified fragments obtained from pure cultures *Rhizobium* TAL 182 and *Bacillus* S49 were used as controls in lane 1 and 3, respectively. Samples removed from treatments with *Rhizobium* TAL 182 on roots and *Bacillus* S49 in the cup were analysed and run in lane 4 (root medium sample) and lane 5 (cup sample). Samples removed from treatments with *Bacillus* S49 on roots and *Rhizobium* TAL 182 in the cup were analysed and run in lane 6 (root medium sample) and lane 7 (cup sample). 100 bp ladder (BRL) was run in lane 2, and approximate sizes (in basepairs) of amplified fragments are indicated at left.

molecules.

4. 3. 6. Effect of coinoculation on lateral root growth

The effect of *Bacillus* and *Rhizobium* inoculation on lateral root formation in *P. vulgaris* grown under gnotobiotic conditions in the growth chamber is summarized in Table 4.1. At four days of growth, single inoculation of *B. megaterium* S49 resulted in a significantly higher lateral root production in *P. vulgaris* compared to uninoculated control and single inoculation with *Rhizobium etli* TAL 182. At seven days after planting, single inoculation with *B. megaterium* S49 resulted in a significantly higher lateral root formation compared to other treatments. When the bacteria were physically separated from one another, the presence of *Bacillus* S49 on the plant roots consistently induced significantly higher lateral root numbers (52.5 per plant), compared to the presence of *Rhizobium* TAL 182 (34.5 per plant) (Table 4.2). A similar effect on lateral root formation results was also observed when *B. polymyxa* L6 was present on the plant roots. Treatment combinations which included live *Bacillus* S49 cells on *P. vulgaris* roots consistently induced the formation of higher number of lateral roots compared to treatment combinations containing live *Rhizobium* TAL 182 cells (Table 4.3). The presence of live *Bacillus* S49 alone on the plant roots resulted in the formation of a significantly higher number of lateral roots compared to other treatments. Treatments which included live *Rhizobium* TAL 182 and cell preparations of *Bacillus* S49 resulted in a significantly lower number of lateral roots compared to treatments which had live *Bacillus* S49 with *Rhizobium* TAL 182 cell preparations.

4. 3. 7. Effect of coinoculation on nodulation and nitrogenase activity

To determine if early nodulation in coinoculated plants is accompanied by

Table 4.I. Effect of *Rhizobium* and *Bacillus* inoculation on lateral root formation[†] in *Phaseolus vulgaris* grown in Leonard jars under gnotobiotic conditions

Treatment	Lateral root numbers	
	Day 4	Day 7
Control[•]	14*a*	16*a*
Rhizobium etli (TAL 182)	15.4*a*	23*b*
B. megaterium (S49)	22.8*b*	29.6*c*
TAL 182 + S49 coinoculation	18*a,b*	23*b*

[•] Control plants were inoculated with one ml of sterile 0.1M phosphate buffer (pH 6.8).
[†] Lateral roots were counted at four and seven days after planting. Values represent the means of five replicates. Means were separated using analysis of variance (ANOVA). Means followed by the same letter in a column are not significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test. This experiment was repeated once with similar results.

Table 4.2. Influence of *Rhizobium* and *Bacillus* inoculations on lateral root formation[†] in *Phaseolus vulgaris* grown in test tubes under axenic conditions

Treatment	Lateral root numbers
Control[††]	19.25*a*
Rhizobium etli (TAL 182)	34.50*b*
S49* with *Rhizobium etli* TAL 182 on the roots	32.50*b*
Bacillus S49 with TAL 182* on the roots	52.50*c*
Bacillus L6 with TAL 182* on the roots	51.00*c*

[†] Lateral roots were counted at five days of growth. [††] Control plants received sterile 0.1M phosphate buffer (pH 6.8) as inoculum. Values represent the means of five replicates. Means were separated using analysis of variance (ANOVA). Means followed by the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's Multiple Range test. This experiment was repeated twice with similar results.

* Physically separated from the plant root by a membrane (MW cutoff 30,000 Da).

Table 4.3. Influence of cell-free extracts of *Rhizobium* and *Bacillus* on lateral root formation[†] in *Phaseolus vulgaris* grown in test tubes under axenic conditions

Treatment	Lateral root numbers
Rhizobium etli TAL 182	35.75*b,c*
Bacillus S49	48.38*a*
S49 + TAL 182 **ACE**	44.37*a,b*
S49 + TAL 182 **WCE**	45.87*a*
S49 + TAL 182 **CFE**	44.75*a,b*
TAL 182 + S49 **ACE**	31.86*c,d*
TAL 182 + S49 **WCE**	30.57*c,d*
TAL 182 + S49 **CFE**	26.28*d*

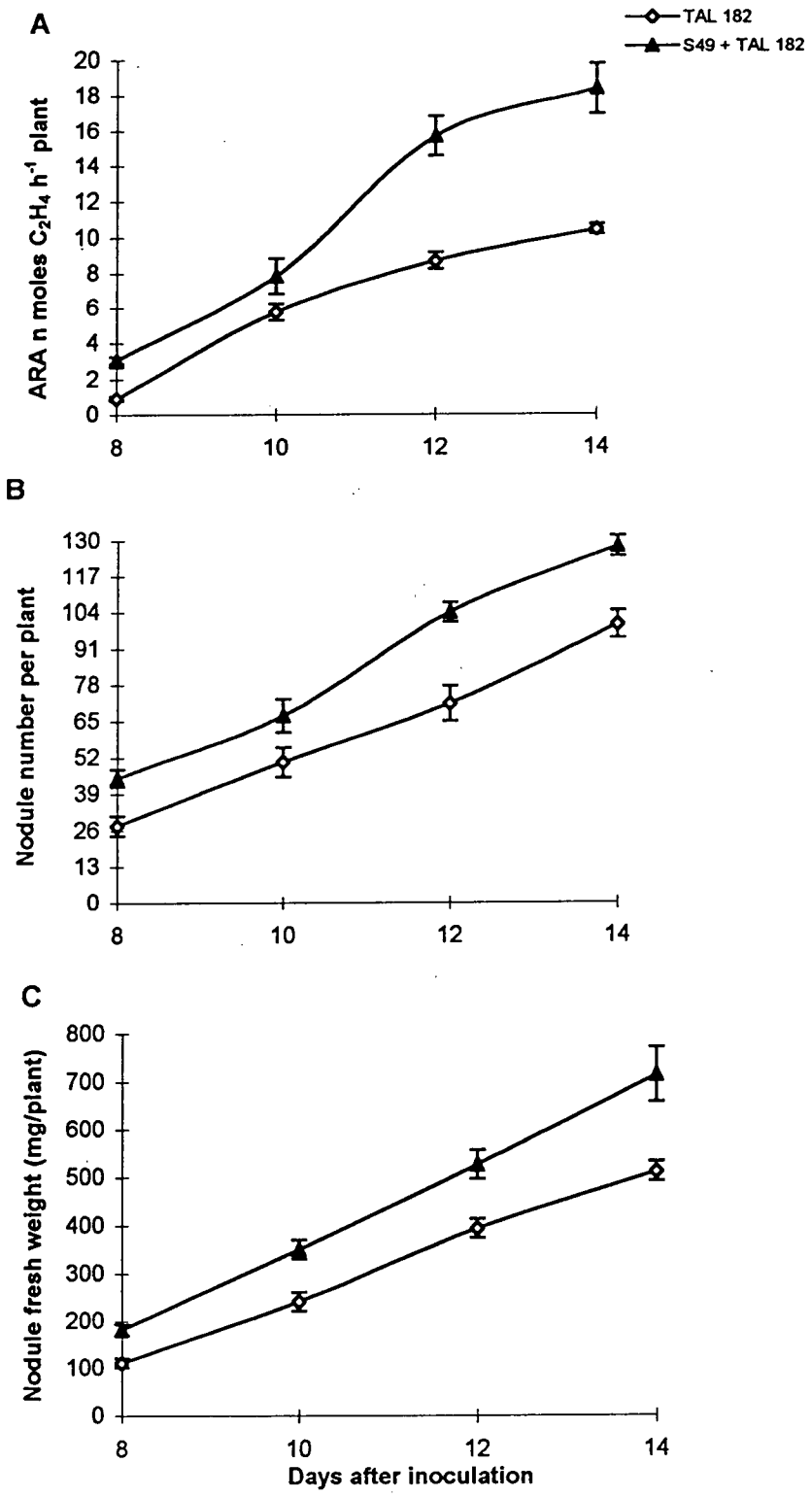
[†] Lateral roots were counted at five days of growth. Means were separated using analysis of variance (ANOVA). Means of eight replicates followed by the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. This experiment was repeated once with similar results.
Abbreviations used in the table:
ACE = autoclaved cell extracts; **WCE** = French pressed whole cell extracts; **CFE** = Filter sterile cell free supernatant.

increased nitrogen fixation activity, plants were grown in Leonard jars under gnotobiotic conditions and harvested at 8, 10, 12 and 14 days after inoculation. The plants were evaluated for nodule number, nodule fresh weight, and nitrogenase activity. Control plants that received sterile phosphate buffer as inoculum did not form any nodules and were chlorotic. Plants that received *Rhizobium* TAL 182 and *Bacillus* S49 coinoculation showed significantly higher values for all the above parameters (Figure 4.6 A-C).

4. 4. Discussion

Previous studies have implicated bacterially derived IAA in root hair morphogenesis (Tien *et al.* 1979; Morgenstern and Okon, 1987; Barbieri and Galli, 1993). However, the experiments in this study indicated root hair proliferation in *P. vulgaris* was most likely a function of the synergistic interaction between a *Rhizobium* and a *Bacillus* and may not be dependent upon *in vitro* IAA production by *Bacillus*. To ascertain the mechanism(s) involved, the influence of a high IAA producing *Bacillus megaterium* Led 02 and a low IAA producing *Bacillus brevis* S72 coinoculated with *Rhizobium* TAL 182 on root hair formation in *P. vulgaris* was examined. Elongation of root hairs in response to coinoculation of *Bacillus* Led 02, an IAA producer, with *Rhizobium* TAL 182 is consistent with earlier observations, using IAA alone or in combination with *B. megaterium* S49. Changes to the ultrastructure of tomato root hairs in response to application of auxin have been reported (Ayling and Pring 1995). The absence of root hair proliferation in response to IAA or to single inoculation by IAA-producing *Bacillus* suggests that the root hair proliferation response is a direct consequence of a *Rhizobium-Bacillus* interaction. Inoculation with IAA-producing *Azospirillum brasilense* induced changes in root hair density on pearl millet (Tien *et al.* 1979) and on wheat (Barbieri and Galli

Figure. 4.6. Effect of dual inoculation of *Rhizobium etli* TAL 182 and *Bacillus megaterium* S49 on **(A)** nitrogenase activity **(B)** nodule number and **(C)** nodule fresh weight of *P. vulgaris* grown under gnotobiotic conditions in the growth chamber. Control plants that received sterile 0.1M phosphate buffer (pH 6.8) did not form any nodules. Each value represents the means of six replicates. Means of values for each parameter tested were significantly different at $p \leq 0.05$,as determined by Duncan's multiple range test. Bars represent the standard error of the mean.


A
TAL 182
S49 + TAL 182
ARA n moles C_2H_4 h^{-1} plant
20
18
16
14
12
10
8
6
4
2
0
8
10
12
14
B
Nodule number per plant
130
117
104
91
78
65
52
39
26
13
0
8
10
12
14
C
Nodule fresh weight (mg/plant)
800
700
600
500
400
300
200
100
0
8
10
12
14
Days after inoculation

1993). However, the effect of microbially derived IAA on root hair morphogenesis still remains unresolved as other studies indicate that IAA production may not be the sole factor in causing changes to root morphology and root hair morphogenesis (Harari *et al.* 1988; Yahalom *et al.* 1991). The root hair proliferation observed in *P. vulgaris* is a consequence of viable interacting bacterial species and thus differs from *Azospirillum*-plant interaction(s) where only a single bacterium is involved in inducing changes to root hair morphogenesis.

The experiments in this study further confirm that the simultaneous physical presence of *Rhizobium* and *Bacillus* is necessary to induce root hair proliferation, since physical separation of either bacterial species precluded root hair proliferation in *P. vulgaris*. It is unlikely, therefore, that a low molecular weight substance is involved in the interaction, unless it is produced close to the site of action (root surface). Furthermore, viability of the bacterial inoculum was essential for the root hair proliferation response. These observations are consistent with the findings of Barbieri and Galli (1993) on the *Azospirillum*-wheat interaction. The most variable response was obtained when whole cell extracts of *Rhizobium* and *Bacillus* were applied to plant roots. Approximately 15% of such plants showed proliferation comparable to dual inoculation with live bacteria, thereby indicating that the mere presence of cell debris of the two bacterial species on plant roots may to a limited extent, trigger root hair proliferation. Such a phenomenon may be compared to the formation of shepherd's crooks in legumes, where *Rhizobium* needs to be physically present on the roots to cause such a response (Relic *et al.* 1993; van Rhijn and Vanderleyden 1995). Nevertheless, the results of this study are consistent with the view that soluble or extracelluar microbial

factors are not likely responsible for root hair proliferation in *P. vulgaris*.

Functional *Bacillus* and *Rhizobium* may also be prerequisites for root hair proliferation. Coinoculation of *B. megaterium* S49 with *Rhizobium etli* Tn*5* mutant MLC 35 failed to induce root hair proliferation in *P. vulgaris*. These data are consistent with the earlier observations that an α-methyl tryptophan resistant mutant of *B. megaterium* (S49-1D), also failed to affect root hair morphogenesis in dual inoculation experiments. However, changes to the IAA production potential of this mutant were also accompanied by other metabolic changes such as excretion of tryptophan into growing medium (Chapter 2), and hence this result should be viewed with caution. Nevertheless, it is interesting to note that changes to the growth pattern and/or metabolic phenotype of either *Rhizobium* or *Bacillus* resulted in a marked decrease in the synergistic induction of root hair proliferation response. This difference was also reflected in the decreased nodulation of *P. vulgaris* in response to dual inoculation of *Rhizobium* TAL 182 and *B. megaterium* mutant isolate, S49-1D (Chapter 2).

The physical presence of *B. megaterium* S49 on the roots resulted in the formation of more lateral roots than on control or co-inoculated plants. Such a response may be the consequence of *Bacillus*-derived IAA on plant root growth. At appropriate concentrations, IAA induces formation of lateral and adventitious roots (Scott 1972; Thimann 1972; Blakesley *et al.* 1991; King *et al.* 1995). Many authors have provided circumstantial evidence that bacterial production of plant hormones resulted in a stimulation of root branching or lateral root growth (Harari *et al.* 1988; Chanway *et al.* 1991). Lateral root development is known to result in significant disruption of existing root tissues and in the development of potential entry points for microorganisms

(Mahaffee *et al.* 1994). *Bacillus* S49 was originally isolated from the root interior of *P. vulgaris* 'Contender', and it is tempting to speculate that lateral root formation may help facilitate colonization of root internal tissues by this microorganism. Preliminary results of another study in this laboratory (Petersen *et al.* personal communication) have indicated a drastic decline in *Bacillus* S49 populations on the *P. vulgaris* root surface, five to seven days after inoculation, as determined by dilution-plating and PCR analyses. This decrease in rhizoplane population coincided with an increase in the population of *Bacillus* S49 in the root interior.

Further experimentation is required to relate internal root colonization of *Bacillus* S49 to lateral root development in *P. vulgaris*. Lateral root enhancement in response to dual inoculation may be attributed to the temporal and spatial changes in phytohormonal levels on the root surface in response to changing bacterial populations, which in turn may regulate aspects of plant root development. Such a response is similar to observations of lateral root formation and nodulation observed in *Medicago polymorpha* inoculated with an *Azospirillum-Rhizobium* combination (Yahalom *et al.* 1991).

Root hairs are infection sites for *Rhizobium* in legumes (Bauer 1981) and changes to root hair formation or density are likely to affect nodulation. Diaz *et al.* (1995) observed that *R. leguminosarum* bv. *trifolii* consistently induced formation of more nodules on *psl*-transformed white clover hairy roots than on wild-type clover; transformed plants also formed nodules much earlier than the wild type. In *P. vulgaris*, most nodules are formed on lateral roots (George *et al.* 1992) and the increased nodulation observed in this study due to coinoculation may be due to the availability of greater numbers of infection sites for the *Rhizobium* microsymbiont, as a result of

increased root hairs on lateral roots. In these experiments, coinoculated plants formed significantly higher numbers of effective nodules at early stages of plant growth. Increased nodulation in *P. vulgaris* due to dual inoculation suggests that normal regulation of nodule numbers has been at least partially overcome in the presence of *Bacillus* S49. Such a response is consistent with previously reported studies on nodulation of alfalfa by *R. meliloti* in the presence of *Pseudomonas* spp. (Knight and Langston-Unkefer 1988). These results are also consistent with the view that combinations of bacteria were more effective than single strain inoculation in promoting plant growth and/or nodulation (Grimes and Mount 1984; Park *et al.* 1988).

These data demonstrate a unique interaction between two rhizosphere bacteria in promoting lateral root development and root hair proliferation in *P. vulgaris*, which ultimately resulted in enhanced nodulation. A similar interaction between *Rhizobium* TAL 182 and nodulation promoting *Bacillus polymyxa* L6 in enhancing root hair induction and lateral root formation on *P. vulgaris* has also been observed. The results of coinoculation of *B. polymyxa* L6 and *Rhizobium* TAL 182 on lateral root formation and nodule formation in *P. vulgaris* are shown for information in Appendix II-III.

The results of this study also indicate that the root hair proliferation response may require a "dialogue" between two viable rhizosphere bacteria. Based on the currently available information, it is tempting to suggest a model which may account for the altered root hair morphogenesis in *P. vulgaris* in response to coinoculation. The presence of *Bacillus* spp. on the plant root might induce the plant to release compounds leading to the induction or derepression of *Rhizobium* genes that are involved in synthesis of a factor causing root hair proliferation. It is also possible that *a Rhizobium*-

derived factor may be modified by *Bacillus* or vice-versa, and that such a modified factor is involved in root hair proliferation. Evaluation of such models has been hindered by the lack of availability of suitable mutants of *Rhizobium etli* TAL 182 and/or *Bacillus megaterium* S49. In future, development of appropriate *Rhizobium* and *Bacillus* mutants may assist in the determination of the genetic and biochemical basis of this synergistic interaction.

Chapter 5

Nodulation of *Phaseolus vulgaris by Rhizobium etli* is enhanced by the presence of *Bacillus*

From beans to bacteria to the scavenger-hunter bond between larval crabs and jelly fish, symbiotic relationships show that peaceful existence is part of the very foundation of nature.

Robert Masello (1986)

5.1. Introduction

Root nodule formation is a complex developmental process involving sequential exchange of chemical signals between the bacterial microsymbiont and the host plant (Fisher and Long 1992; Fellay *et al.* 1995). Both plant and bacterial signals have been identified and shown to play a major role in the specificity of the *Rhizobium*-legume interaction (Relíc *et al.* 1994; Díaz *et al.* 1995; Fellay *et al.* 1995).

None of the cascade of steps that leads to nodulation is completely specific for a given host-microsymbiont combination (Fellay *et al.* 1995). Shantaram (1986) has observed ineffective (non-fixing) nodulation of soybean by a non-specific microsymbiont, *Rhizobium phaseoli.* Similar anomalous (heterologous) nodulation resulting in the formation of ineffective nodules has also been reported by other workers (Bal *et al.* 1982; Shantaram and Wong 1982). Autoregulation is one of several host factors controlling nodulation (Pierce and Bauer 1983). Jamming of nodulation (Jan), where pre-treatment of host roots with pure Nod-factor abolishes nodulation by subsequent, symbiotic bacteria (van Brussel *et al.* 1993), and competitive nodulation blocking (Cnb), where pre-treatment of plants with an incompatible *Rhizobium* blocks subsequent nodulation by compatible *Rhizobium* (Dowling *et al.* 1988) have been identified as regulatory phenomena in many legume hosts. Mellor and Collinge (1995) consider these processes to be extensions of autoregulation of nodulation. It is evident from the above studies that successful nodulation of the legume host is predicated on the genetic compatibility of the host plant and the rhizobial microsymbiont.

Specific microorganisms have considerable potential to alter the composition and activity of rhizosphere microflora such as *Rhizobium* (Schroth and Becker 1990). Nodulation-promoting bacteria (Kloepper *et al.* 1988) belonging to the *Azospirillum*

(Schmidt *et al.* 1988), *Pseudomonas* (Bolton *et al.* 1990), *Streptomyces* (Li and Alexander 1990) and *Bacillus* (Halverson and Handelsman 1991) genera have been reported. The beneficial effects of these bacteria have been variously attributed to their ability to produce various compounds including phytohormones (Schmidt *et al.* 1988), toxins (Knight and Langston-Unkefer 1988), or antibiotics (Li and Alexander 1990), to suppress deleterious rhizobacteria (Turner and Backman 1991) or through some other unidentified mechanism (Halverson and Handelsman 1991). Coinoculation of wild-type strains or *nod* gene mutants of *Rhizobium meliloti* along with an *Agrobacterium* cured of the Ti plasmid, enhanced nodulation of alfalfa by a wild type *Rhizobium meliloti* (Caetano-Anollés and Bauer 1988). None of these studies has adequately explained the mechanism of heterologous nodulation promotion or the influence of associated bacteria on the nodulation of legumes by rhizobia. While substantial progress has been made in elucidating the mechanisms of *Rhizobium*-legume interaction, there is little information available on the direct influence of rhizosphere bacteria on nodulation of legumes by *Rhizobium*.

It has been shown in previous chapters that coinoculation of *Rhizobium etli* TAL 182 with *Bacillus* spp. induced root hair proliferation in *Phaseolus vulgaris* and enhanced nodulation of *P. vulgaris* by *Rhizobium*. Since root hairs are the infection sites for *Rhizobium*, it was important to determine whether proliferation of root hairs in response to coinoculation would affect the nodulation capability of *Rhizobium etli*. The objective of this study was to demonstrate the specificity of the synergistic interaction between *Rhizobium* and *Bacillus* in enhancing root hair formation in *P. vulgaris* . The influence of *Bacillus* spp. on the nodulation capability of *Rhizobium etli* in overcoming host mediated autoregulation, and in inducing heterologous nodulation on *Phaseolus acutifolius* was

also examined in this study.

5. 2. Materials and Methods

5.2.1. Bacterial strains and media

Bacillus spp. were originally isolated from the rhizosphere of *Phaseolus vulgaris* as described previously (Chapter 2). *Rhizobium etli* TAL 182 and *Bradyrhizobium* spp. TAL 644 were obtained from NifTAL, Paia, Hawaii. Species identity was determined by analysis of carbon-source utilization (BIOLOG™). The distinct genera *Bradyrhizobium* and *Rhizobium* were further confirmed by sequencing a 320 bp region of the 23S rDNA (Petersen *et al.* 1995). *Rhizobium etli* TAL 182 and *Bradyrhizobium* spp.TAL 644 share only 68.7% nucleotide sequence identity within this region, and oligonucleotides were produced which distinguish each strain. *Bacillus* spp. were routinely maintained on combined carbon medium agar slants (Rennie 1981). Stock cultures of *Rhizobium etli* TAL 182 and *Bradyrhizobium* spp. TAL 644 were maintained on yeast mannitol agar slants (Bohlool and Schmidt 1970). Broth cultures were grown to early stationary phase in combined carbon medium for *Bacillus* spp. and in yeast mannitol broth for both *Rhizobium* TAL 182 and *Bradyrhizobium* TAL 644. The cells were harvested by centrifugation, washed twice with 0.1M sterile phosphate buffer (pH 6.8) and resuspended in the same buffer to a specified cell density before inoculation.

5.2.2. Preparation of bacterial extracts

Cell free extracts (CFE) of *Bacillus megaterium* S49 were prepared by harvesting 50-ml cultures by centrifugation at 10000 x *g* for 30 minutes using a Sorvall ® GSA-13 rotor. The supernatant was filtered (0.22 μm) to produce CFE. Autoclaved cell preparations (ACE) were obtained by autoclaving early stationary phase cultures for 15 minutes at 121°C and 15 psi. The absence of live bacteria in the above preparations

was confirmed by streaking a loopful of each preparation on nutrient agar (Difco) plates.

5.2.3. Plant material and growth

Seeds of *P. vulgaris* 'Contender' were obtained from Pacific Northwest Seed Co., Vernon, BC. Seeds of *P. acutifolius* were obtained from NifTAL, Paia, Hawaii. Seeds were surface sterilized by soaking in 30% (v/v) H_2O_2 for 15 minutes and rinsed seven times with sterile distilled water and were germinated aseptically on moist filter paper in sterile petri plates. Two-day old pre-germinated seeds with uniform radicle lengths were inoculated by incubation for 15 minutes in 20 ml early stationary phase bacterial suspensions of *Rhizobium etli* TAL182, *Bradyrhizobium* TAL 644 and *B. megaterium* S49 at a cell density of 2 x 10^8 cfu/ml. The experiment was established in a completely randomized design with six replicates per treatment and was repeated once with comparable results to those presented here.

5.2.4. Scanning electron microscopy (SEM)

Plant growth conditions and scanning electron microscopic analysis for studying root hair formation in *P. vulgaris* in response to dual inoculation of *Bacillus* S49 and *Bradyrhizobium* TAL 644 were as described previously (Chapters 2 and 3).

5.2.5. Delayed inoculation experiment

A time course analysis of inoculation effect was conducted using modified Leonard jar assemblies (Blauenfeldt *et al.* 1994) containing sterile planting medium of industrial sand and Turface™ (1:1 v/v). Cells of *Bacillus megaterium* S49 were inoculated on to two-day old pregerminated seedlings and plants grown in growth chambers with a 16 h photoperiod at 350 μE m^2 s^{-1} illumination and $22^0C/18^0C$ day night temperatures. *Rhizobium etli* TAL 182 inoculation was delayed by four, seven or ten days after *Bacillus* spp. inoculation. One ml of *Rhizobium etli* TAL 182 bacterial

suspension at 2 x 10^8 cfu/ml was applied directly to the lower stem of each plant at the point of emergence from soil. Coinoculated plants received simultaneous inoculation of both bacterial species (2 x 10^8 cfu/ml) at time zero. Control plants were inoculated with one ml of sterile 0.1M phosphate buffer (pH 6.8) at each inoculation period. One-tenth strength nitrogen-free sterile nutrient medium (Broughton and Dilworth 1971) was used to water the plants. Plants were harvested at 14 days after *Rhizobium* inoculation and the nodule number (NON), nodule fresh weight (NFW) and nitrogenase activity (acetylene reduction activity) were determined. The acetylene reduction assay described by Dilworth (1966) was used to measure nitrogenase activity. After one hour of incubation, the gas mixture was analyzed by flame-ionization gas chromatography using a Hewlett Packard 5830A gas chromatograph (Holl *et al.* 1988). Analysis of variance of the data was performed using the SAS computer program (SAS Inc., Cary, NJ).

5.2.6. Determination of speed of nodulation in growth pouches

To compare the speed of nodulation by *Rhizobium* TAL 182 in the presence and absence of *Bacillus* S49, seeds of *P. vulgaris* were surface sterilized and germinated as described above. Two-day old pre-germinated seeds were transferred to sterile plastic growth pouches (Mega International, MN) containing 15 ml of one tenth strength nitrogen-free sterile nutrient medium (Broughton and Dilworth 1971). The treatments were arranged in a completely randomized design with ten replicates. Two days after transfer, *Rhizobium etli* TAL 182, with and without *B. megaterium* S49, was inoculated individually on each of ten replicate plants at 10^8 cfu/ml. One ml of each bacterial suspension containing approximately 10^8 cfu was used as inoculum. Ten-ml of nitrogen-free nutrient solution was maintained in the growth pouches for the duration of the

experiment. The location of newly emerged nodules was marked on the growth pouch as soon as they were visible; new nodules were counted on alternate days up to 15 days after inoculation.

5.2.7. Nodulation of *P. vulgaris* using *Bacillus* spp. extracts

The growth pouches and experimental conditions were identical to those described above. For treatments involving autoclaved cell preparations (ACE) or cell-free extracts (CFE) extracts, the pre-germinated seeds were incubated in the respective preparations for 15 minutes after treatment with live *Rhizobium* TAL 182. Plants were grown in the growth chambers with a 16 h photoperiod at 350 $\mu E\ m^2\ s^{-1}$ illumination and $22^0C/18^0C$ day night temperatures.

5.2.8. Split-root experiment in growth pouches

Modified growth pouches for the split-root experiment were prepared as described by George *et al.* (1992). A ten-ml volume of sterile nitrogen-free nutrient medium was maintained on each side of the growth pouch. Seeds of *P. vulgaris* were surface sterilized and germinated as described above. The split-root systems were generated by cutting off approximately 75mm of the root tip of two day old radicles and transferring a single plant to each modified growth pouch. Five days after transfer, the primary side of the split-root was inoculated with a one ml suspension (10^8 cfu/ml) of *Rhizobium etli* TAL 182. The opposite (secondary) side received *Rhizobium* TAL 182 alone or in combination with *Bacillus* S49 (one ml suspension containing 10^8 cfu) after a given delay period. For each delay period, the positive control consisted of no inoculation on the primary side combined with a delayed inoculation on the secondary side. Plants that received sterile phosphate buffer at both inoculation events were used as controls and grown under the same conditions described above. Plants were

harvested 24 days after the primary inoculation and the nodules counted. Percentage suppression of nodulation was calculated based on the formula of George *et al.* (1992), which is given in Appendix IV for information. The data was analyzed using the analysis of variance in the SYSTAT PC computer package (SYSTAT Inc., Evanston) after arcsine transformation of percent values.

5.2.9. Nodulation studies on *P. acutifolius*

Bacterial inoculation of *P. acutifolius* using *B. megaterium* S49 in combination with either *Rhizobium etli* TAL 182 or *Bradyrhizobium* TAL 644 was performed on two-day old pre-germinated seeds as described above. Plant growth conditions were as described for the delayed inoculation experiment in Leonard jars. Nodules were harvested from 24 day old plants and stored at -70 ^{0}C .

5.2.10. Detection of *R. etli* TAL 182 in *P. acutifolius* nodules by fluorescent antibody staining

Fluorescein isothiocyanate conjugated (FITC) polyclonal antibodies specific to *Rhizobium etli* TAL 182 were obtained from NifTAL, Paia, Hawaii. Nodules were surface sterilized by sequential immersion in 95% ethanol for two minutes,1.2% sodium hypochlorite (three minutes) and five rinses with sterile deionized water. Nodules were halved with a sterile scalpel and incubated for three hours in microcentrifuge tubes containing 200 µl of sterile deionized water at 30° C, to release *Rhizobium*. The tubes were centrifuged briefly (2000 x *g* for 15 seconds) to remove nodule debris; 20 µl of the supernatant was applied to each well of a clean toxoplasmosis microscopic slide (Bellco Glass Inc., NJ) and dried on a slide warmer at 50° C. The samples were fixed by immersion in acetone for 10 minutes and air-dried. The fluorescent antibodies were diluted 1:100 in one ml of 10mM phosphate buffered saline (pH 7.2) containing 2%

blotto (10g skim milk powder, 0.2g NaN_3 in 100 ml de- ionized water), and 20 µl of antibody solution was used for each reaction. The slides were incubated in a moist chamber at 37^0 C for one hour, rinsed with water once, and dried in a slide warmer as described above. A small drop of mounting fluid was placed on each well and covered with a cover slip. The edges of the cover slip were anchored with nail polish and the samples were observed under epifluorescence using a Zeiss ™ axiophot microscope (100x objective; 10x occular) with a fluorescein filter-set. The excitation wavelength used was 400-450nm with an LP 520 barrier filter.

5.2.11. Polymerase chain reaction (PCR)

Nodules were surface sterilized as described above with the addition of a subsequent one minute rinse in 0.3M NaOAc, pH 4.2, followed by three rinses with sterile deionized water to remove extraneous DNA molecules. When the final water rinse was decanted from the sterilized nodules, a small volume of rinse water remained in the microcentrifuge tube with the nodules. A 50 µl aliquot of this liquid was removed for use as a negative control. Nodules were then crushed in 50 µl sterile deionized water to release bacteria. Whole-cell bacterial DNA amplification by multiplex PCR was conducted as described by Petersen *et al.* (1995) using the conserved forward primer BAC11X in combination with both *Rhizobium etli* TAL 182-specific and *B. megaterium* S49-specific reverse primers. The presence or absence of amplified product, as well as the fragment size (540 bp for *Rhizobium* TAL 182), was determined by electrophoretic analysis using 1.5% agarose gels stained with ethidium bromide and viewed under UV light.

5.3. Results

5.3.1. Root hair formation in *P. vulgaris* in response to dual inoculation with *Bacillus* S49 and *Bradyrhizobium* TAL 644

Scanning electron microscopy revealed that inoculation of *P. vulgaris* with *Bradyrhizobium* TAL 644 alone did not enhance root hair formation (Figure 5.1B), compared to the uninoculated control (Figure 5.1A). Coinoculation of *Bradyrhizobium* TAL 644 with *Bacillus* S49 did not result in enhanced root hair formation (Figure 5.1C), compared to *P. vulgaris* plants that received dual inoculation of *Rhizobium* TAL 182 and *Bacillus* S49 (Figure 5.1D). Nevertheless, the presence of *Bradyrhizobium* TAL 644 and *Bacillus* S49 resulted in the formation of more root hairs in *P. vulgaris* compared to the uninoculated control (Figure 5.1A) and plants that received a single inoculation of *Bradyrhizobium* TAL 644 (Figure 5.1B).

5.3.2. Nodulation suppression induced by *Rhizobium* TAL 182 is overcome by coinoculation with *Bacillus* S49

Inoculation of *Rhizobium* TAL 182 on the primary side of the split-root resulted in suppression of nodulation on the secondary side of the split-root after a given delay period (Table 5.1). However, when coinoculated with *Bacillus* S49, *Rhizobium* TAL 182 partially overcame this suppression; significant effects on nodulation were observed for both the four and seven day delay periods, compared to suppression with single inoculation of *Rhizobium* TAL 182 at time zero. These results suggest that coinoculation of *Rhizobium* TAL 182 and *Bacillus* S49 enhances the nodulation potential of TAL 182 by partially overcoming host-mediated suppression of nodulation. This effect was time-dependent, since a ten day delay in secondary inoculation produced no effect of coinoculation on nodulation suppression.

Figure 5.1. Root hair formation in *P. vulgaris* in response to bacterial inoculation.
(A) Uninoculated control (sterile 0.1M phosphate buffer, pH 6.8)
(B) *Bradyrhizobium* TAL 644
(C) *Bradyrhizobium* TAL 644 with *Bacillus* S49
(D) *Rhizobium* TAL 182 with *Bacillus* S49.

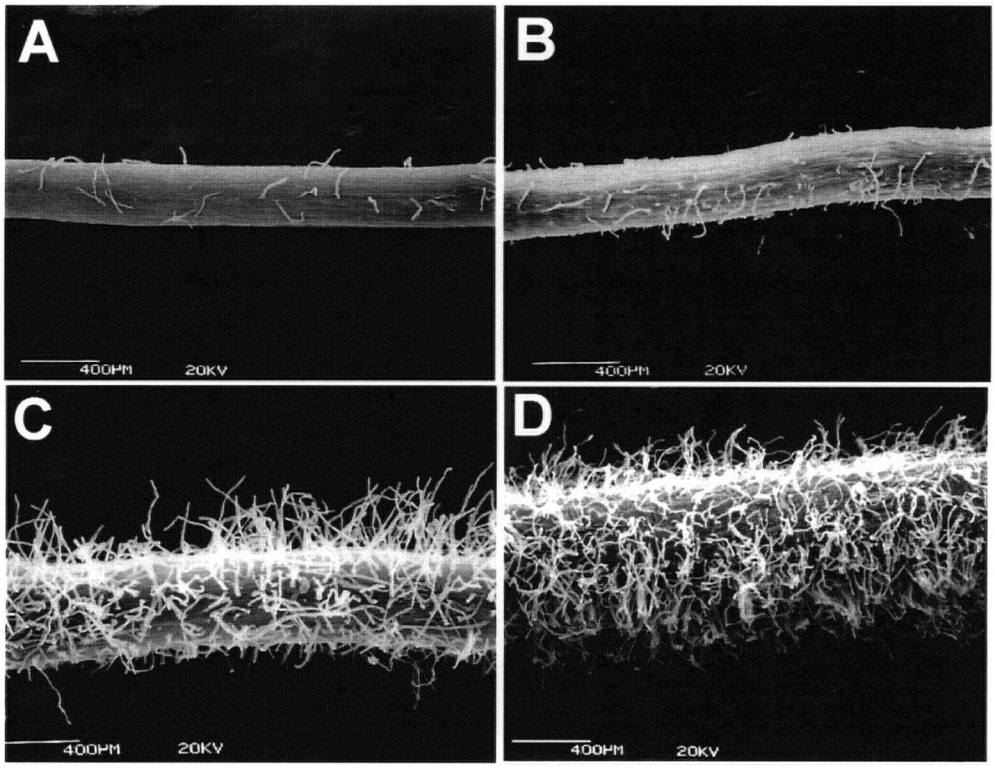

A
400PM 20KV
B
400PM 20KV
C
400PM 20KV
D
400PM 20KV

Table 5.1. Nodulation suppression induced by *Rhizobium etli* TAL 182 with and without *B. megaterium* S49 as co-inoculant on *P. vulgaris* 'Contender'

Treatment	Delay in secondary inoculation (days)	Percent suppression
TAL 182	1	7.79 *b*
	4	21.71 *c*
	7	51.36 *d*
	10	92.08 *e*
TAL 182 + S49	1	0.62 *a*
	4	7.90 *b*
	7	30.03 *c*
	10	90.59 *e*

Primary side of the split-root was inoculated with *Rhizobium* TAL 182 at day zero, while the secondary side was inoculated with *Rhizobium* TAL 182 or *Rhizobium* TAL 182 with *Bacillus* S49 following a given delay period. Values represent the means of five replicates. Means were separated using analysis of variance (ANOVA). Means followed by the same letter are not significantly different at $p \leq 0.05$, as determined by Fisher's LSD. Statistical analysis was carried out on arcsine transformed percentage values.

5.3.3. Rate of nodulation of *P. vulgaris* by *Rhizobium* TAL 182 is enhanced in the presence of *Bacillus* S49

The ability of a given *Rhizobium* strain to form nodules rapidly on a host plant can affect the outcome of competition (de Oliveira and Graham 1990). The ability of *B. megaterium* S49 to enhance the nodulation rate by *Rhizobium etli* TAL 182 was tested by observing the time course of nodule formation on *P. vulgaris*. Coinoculation of *Rhizobium* TAL 182 and *Bacillus* S49 resulted in early nodulation of *P. vulgaris*, compared to single inoculation with TAL 182 (Figure 5.2). These co-inoculated plants showed peak nodulation 11 days after inoculation, whereas singly inoculated plants showed peak nodulation 13 days after inoculation. In addition to earlier nodule formation, co-inoculated plants also formed more nodules during the first two weeks of plant growth (47 per plant) compared to plants that received a single inoculation of *Rhizobium* TAL 182 (34 per plant) (Figure 5.2). Coinoculation of *Bradyrhizobium* TAL 644 with *B. megaterium* S49 did not significantly enhance nodulation of *P. acutifolius* by TAL 644 (Table 5.2).

5.3.4. Effect of timing of inoculation on nodulation of *P.vulgaris* by *Rhizobium* TAL 182

The relative timing of inoculation of *Bacillus* S49 and *Rhizobium* TAL 182 had significant effects on the nodulation capability of TAL 182. When both bacterial species were simultaneously inoculated onto *P. vulgaris*, the nodulation of *P. vulgaris* by *Rhizobium* TAL 182 was significantly enhanced compared to single inoculation with *Rhizobium* TAL 182, as evidenced by higher values for nodule number (NON), nodule fresh weight (NFW) and nitrogenase activity (ARA) per plant (Figure 5.3). An initial

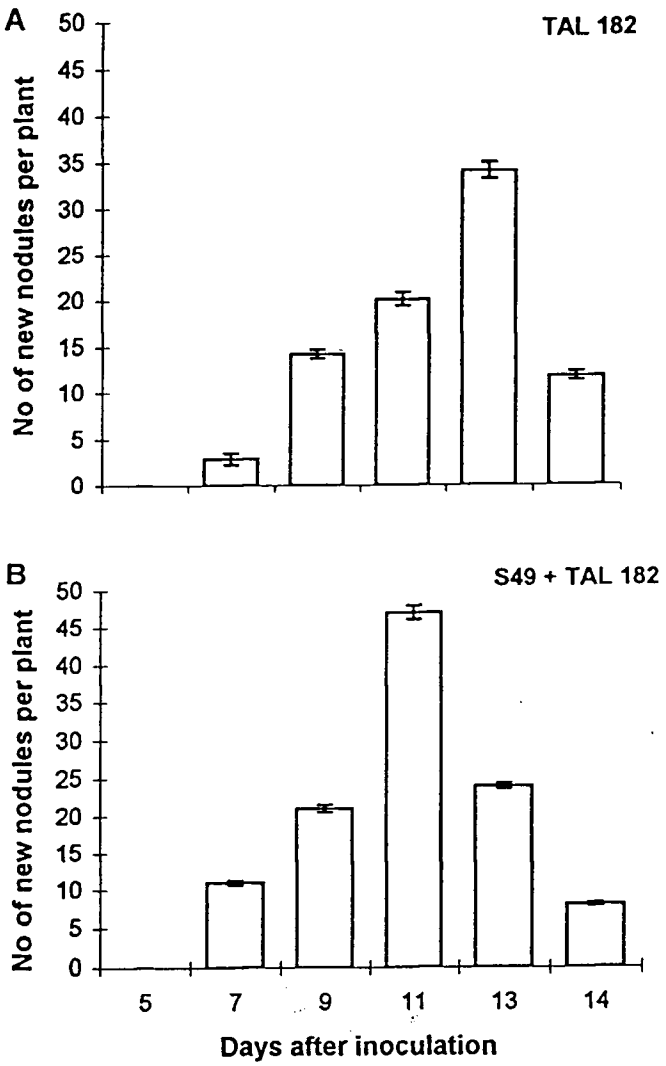


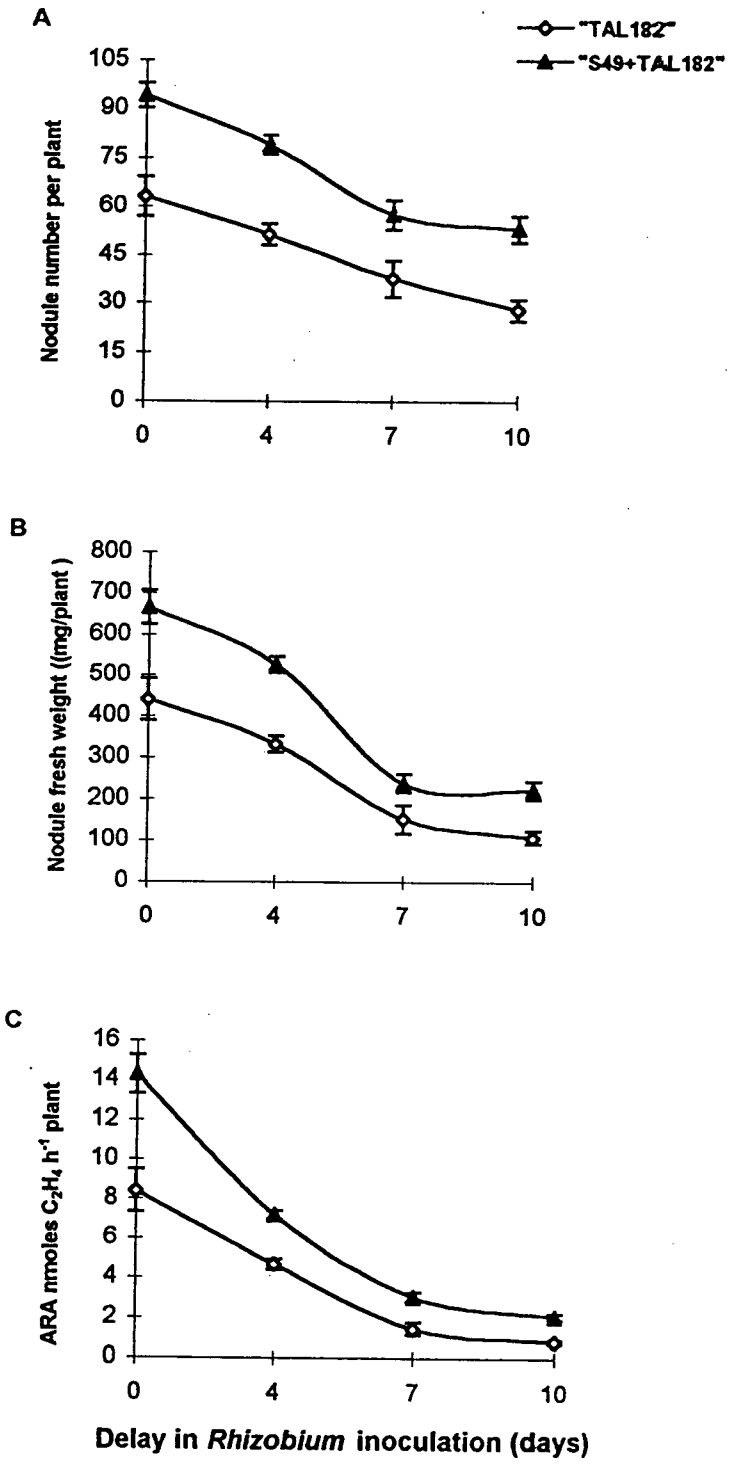
Figure 5. 2. Rate of new nodule formation of *R. etli* TAL 182 on *Phaseolus vulgaris* 'Contender' in growth pouches when inoculated (A) alone and, (B) with *B. megaterium* S49. Each value represent the means of nodule numbers from ten replicate plants.

Table 5.2. Effect of bacterial inoculation on nodule number (NON), nodule fresh weight (NFW) and nitrogenase activity (ARA) in *Phaseolus acutifolius* under gnotobiotic conditions in Leonard jars

Treatment	NON (±SE)	NFW (g/plant ±SE)	ARA (n moles C_2H_4 h^{-1} $plant^{-1}$±SE)
Control	0.00 *a*	0.000 *a*	0.000 *a*
Bradyrhizobium TAL 644	44.33 ± 4.55 *b*	0.34 ± 0.03 *b*	5.59 ± 0.29 *b*
Bradyrhizobium TAL 644 + *Bacillus* S49	41.00 ± 2.99 *b*	0.31 ± 0.02 *b*	5.50 ± 0.24 *b*
Bradyrhizobium TAL 644 + *Bacillus* L6	39.80 ± 3.24 *b*	0.31 ± 0.02 *b*	5.49 ± 0.23 *b*

Values represent the means of six replicates. Means were separated using ANOVA and means followed by the same letter in a column do not differ significantly at $p \leq 0.05$ as determined by Duncan's multiple range test.

Figure 5.3. Effect of timing of inoculation of *Rhizobium etli* TAL 182 and *B. megaterium* S49 on (A) nodule number, (B) nodule fresh weight and (c) nitrogenase activity of *P. vulgaris* 'Contender' grown under gnotobiotic conditions in the growth chamber. Control plants that received 0.1M sterile phosphate buffer did not form any nodules. Each value represent the means of six replicates. Means of values for each parameter tested were significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test. Bars represent the standard error of the mean.


A
"TAL182"
"S49+TAL182"
Nodule number per plant
105
90
75
60
45
30
15
0
0
4
7
10
B
Nodule fresh weight ((mg/plant)
800
700
600
500
400
300
200
100
0
0
4
7
10
C
ARA nmoles C_2H_4 h^{-1} plant
16
14
12
10
8
6
4
2
0
0
4
7
10
Delay in *Rhizobium* inoculation (days)

inoculation with *Bacillus* S49 followed by *Rhizobium* inoculation after a given delay period (4, 7,or 10 days) significantly reduced nodulation of *P. vulgaris* by TAL 182 relative to that observed in the absence of *Bacillus* S49. A similar delay in *Bacillus* spp. inoculation nullified the coinoculation nodulation enhancement of *P. vulgaris* by *Rhizobium* TAL 182 (data not shown). These results suggest that direct interaction between the two bacterial species may be necessary for enhanced nodulation of *P. vulgaris* by *Rhizobium* TAL 182.

5.3.5. Effect of non-viable cell preparations of S49 on the nodulation of *P. vulgaris* by *Rhizobium* TAL 182

Autoclaved cell preparations (ACE) and cell-free preparations (CFE) of *Bacillus* S49 were tested for viability before they were mixed with *Rhizobium* TAL 182 for dual inoculation. Neither inhibition nor stimulation of nodulation of *P. vulgaris* by *Rhizobium* TAL 182 was observed in the absence of live *Bacillus* S49 cells, compared to single inoculation with *Rhizobium* TAL 182 (Table 5.3).

5.3.6. Heterologous nodulation of *P. acutifolius* by TAL 182 in the presence of *Bacillus* S49

The nodulation response of *P. acutifolius* to inoculation with *Rhizobium* TAL 182 alone, or in combination with *Bacillus* S49 was examined and is presented in Table 5.4. Inoculation of *P. acutifolius* with *Rhizobium* TAL 182 alone resulted in the formation of a few very small white swellings (pseudonodules) on the roots. Such structures showed no evidence of *Rhizobium* TAL 182 internally as revealed by fluorescent antibodies specific to *Rhizobium* TAL 182 (Figure 5.4 D). The specificity of the fluorescent antibody was confirmed by using a pure culture of *Rhizobium* TAL 182 (10^{-6}

Table 5.3. Nodulation of *P. vulgaris* by *Rhizobium etli* TAL 182 in the presence of cell preparations of *B. megaterium* S49

Treatment	No of nodules/plant (means ± SE)	Nodule fresh weight (g/plant ± SE)
Control	2.66 ± 1.14 *a*	0.001 ± 0.0007*a*
TAL 182	46.66 ± 3.79 *b*	0.28 ± 0.04 *b*
TAL 182 + S49	69.50 ± 3.37 *c*	0.40 ± 0.02 *c*
TAL 182 +S49 (CFE)	46.30 ± 3.14 *b*	0.31± 0.02 *b*
TAL 182 + S49 (ACE)	46.50 ± 2.90 *b*	0.27 ± 0.03*b*

Abbreviations: CFE= Cell free supernatant; ACE = Autoclaved cell preparation
Values represent the means of six replicates. Means followed by the same letter in a column are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test.

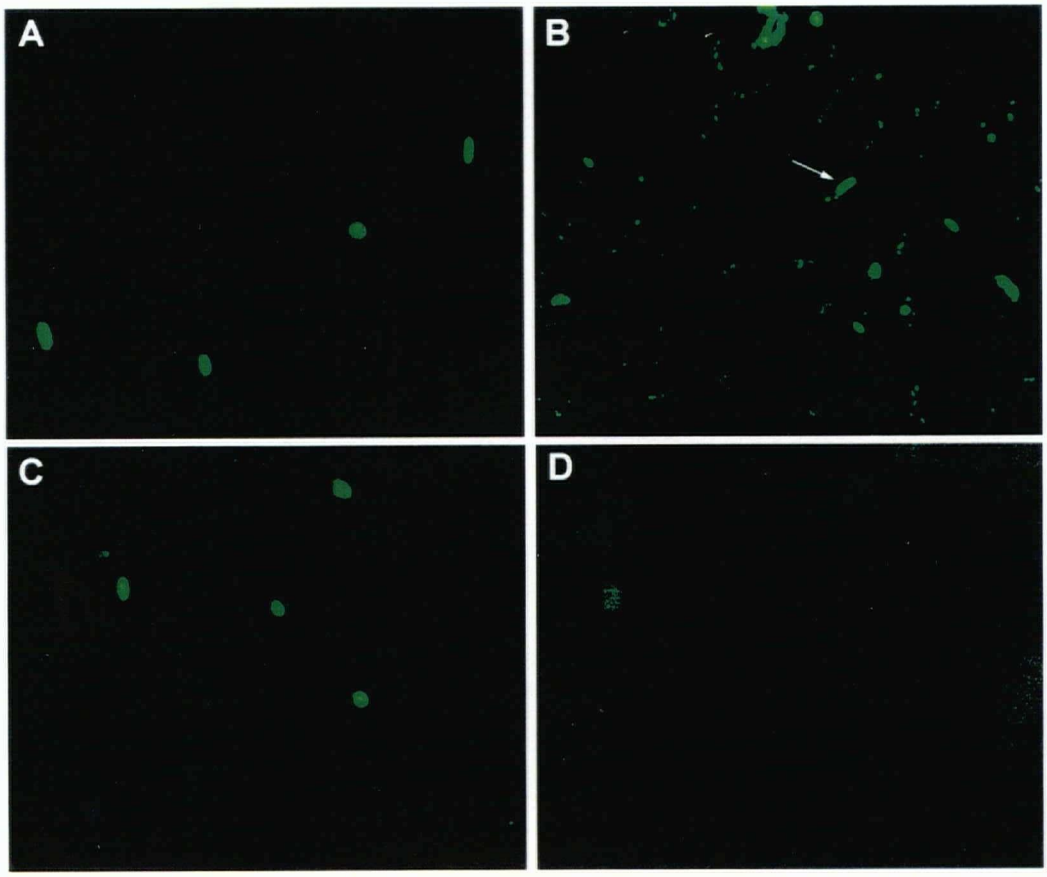
dilution) as a positive control. Fluorescent antibody specific to *Rhizobium* TAL 182 did not cross react with pure cultures of *Bradyrhizobium* TAL 644 or with the same microsymbiont within the nodules of *P. acutifolius*. *Rhizobium* TAL 182 could not be detected inside such pseudonodules even when an undiluted nodule eluate was used for antibody detection. However, coinoculation of *Rhizobium* TAL 182 and *Bacillus* S49 resulted in the formation of a few nodules that were slightly pink and contained *Rhizobium* TAL 182 as detected by fluorescent antibodies in an undiluted nodule eluate (Figure 5.4 C). Dilutions greater than 10^{-2} of the nodule eluate did not show the presence of *Rhizobium* TAL 182. Direct smearing of the nodules onto microscopic slides and subsequent detection with fluorescent antibody was precluded by the

Table 5.4. Nodulation of *P. acutifolius* 24 days after inoculation with *R. etli* TAL 182 alone or with *B. megaterium* S49

Treatment	No of plants	No of nodules analyzed *	No of nodules bearing TAL 182	Percent nodules bearing TAL 182
Control	10	0	0	0
TAL 182	14	11†	0	0
TAL 182 + S49	14	23†	4¥	17.4

* Total number of nodules observed on fourteen plants in two separate experiments.
† Small white nodule-like outgrowths (pseudonodules)
¥ Nodules pink in colour, slightly bigger compared to the rest

Figure 5.4. Detection of heterologous nodulation of *P. acutifolius* by *Rhizobium etli* TAL 182 in the presence of *Bacillus megaterium* S49 by using fluorescent antibodies specific to *Rhizobium* TAL 182. (A) pure culture of *Rhizobium* TAL 182, (B) *Rhizobium* TAL 182 in the nodule of its homologous host, *P. vulgaris*, (C) *Rhizobium* TAL 182 in the nodule of *P. acutifolius* and, (D) the nodule smear from singly inoculated plants. Arrow indicates the presence of *Rhizobium* TAL 182.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B
C
D

presence of heavy background fluorescence. The ineffectiveness of nodules formed by *Rhizobium* TAL 182 on *P. acutifolius* was apparent by the chlorotic appearance of the plants and stunted growth. Acetylene reduction assays of plants showed no evidence of nitrogenase activity. Only three of the 15 (20%) coinoculated plants formed such nodules and the frequency of heterologous nodulation was low (17.4%) (Table 5.4). Thepresence of *Rhizobium* TAL 182 inside the ineffective nodules of coinoculated plants was further confirmed by PCR amplification of surface sterilized nodules using *Rhizobium* TAL 182 specific primers, which showed an approximately 540 bp band corresponding to TAL 182 (Figure 5.5). PCR analysis of the pseudonodules of *P. acutifolius* plants that received single inoculation of *Rhizobium* TAL 182 did not show the presence of this microsymbiont inside these nodules (data not shown). Simultaneous PCR analysis with a second primer specific for *B. megaterium* showed no evidence of S49 within these nodules.

5.4. Discussion

This study has shown that the presence of viable *B. megaterium* S49 could significantly influence the *Rhizobium*-legume symbiotic interaction. The presence of both bacterial species is apparently necessary to enhance nodulation of *P. vulgaris* by *Rhizobium etli* TAL 182, as delayed inoculation of *Rhizobium* or *Bacillus* or coinoculation of *Rhizobium* TAL 182 with non-viable cell preparations of *B*acillus S49 failed to show enhancement of nodulation comparable to dual inoculation. It has been shown in previous chapters that coinoculation of *Bacillus* S49 with *Rhizobium* TAL 182 enhances root hair formation in *P. vulgaris* and on *P. acutifolius*. Since root hairs are infection sites for rhizobia (Bauer, 1981), it is possible that the nodulation enhancement observed for *P. vulgaris* in response to coinoculation may be attributed to increased numbers of

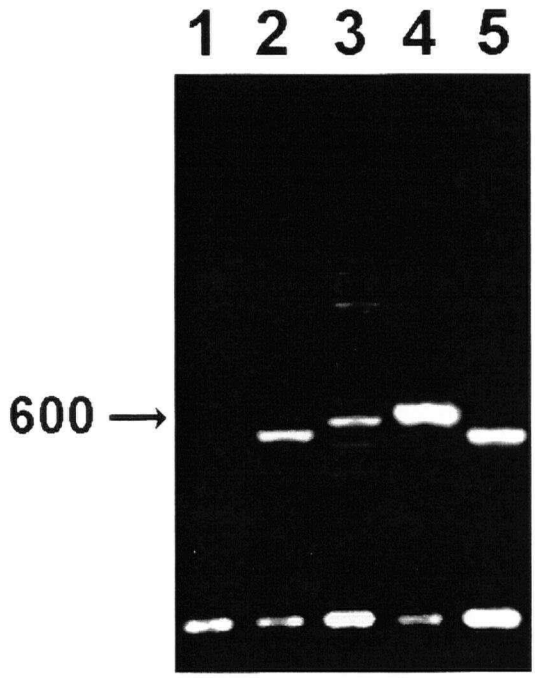

Figure 5.5. Presence of *R. etli* TAL 182 inside the surface-sterilized nodules of *P. acutifolius* as determined by multiplex PCR. Amplification products from PCR using *Rhizobium* TAL 182-specific and *Bacillus* S49-specific primers as described in text (section 5.2.11.) were separated on 1.5% agarose gels. Lane (1) Surface-sterilized nodule exterior, Lane (2) surface-sterilized nodule interior, of dual inoculated plants. *B. megaterium* S49 pure culture in Lane (4) and *R. etli* TAL 182 pure culture in Lane (5) were used as controls. Bands corresponding to *R. etli* TAL 182 (540 bp) were clearly distinguished from 650 bp *B. megaterium* S49 fragment as shown by the bright 600 bp fragment of the 100 bp ladder (Lane 3), and indicated by an arrow at left. The lower band denotes unincorporated primers and nucleotides.

infection sites available for *Rhizobium* TAL 182. Under the gnotobiotic conditions used in these experiments, enhanced nodulation may reflect an increased susceptibility of *P. vulgaris* root hairs to infection by *Rhizobium* TAL 182, triggered by the presence of *B. megaterium* S49. Coinoculation of *Rhizobium etli* TAL 182 with *Bacillus polymyxa* L6, a demonstrated plant growth promoter (Holl *et al.* 1988), produced similar nodulation responses on *P. vulgaris* (Appendix V). The presence of a few pseudonodules on control plants may be attributed to spontaneous nodulation by *P. vulgaris* in the absence of *Rhizobium*; such a phenomenon has been previously observed in clovers (Blauenfeldt *et al.* 1994).

It has been shown by others that coinoculation of symbiotically deficient mutants of *R. meliloti* and heterologous members of the Rhizobiaceae contributed to enhanced nodulation by wild-type *R. meliloti* on alfalfa (Caetano-Anollés and Bauer 1988). Similar enhancement of nodulation of legumes due to coinoculation of bacterial mixtures has been reported previously (Rolfe and Gresshoff 1980; Grimes and Mount 1984). The failure of cell-free preparations of *B. megaterium* S49 to stimulate nodulation of *P. vulgaris* by *Rhizobium etli* TAL 182 suggests that simple triggering of nodulation responses may not be solely due to extracellular substances produced by the *Bacillus*. These data reinforce the hypothesis that viable bacteria are required to participate in nodulation enhancement, as well as the possible involvement of a stimulatory substance(s) produced by *Bacillus* which may be subject to regulation by *Rhizobium* or vice-versa. Such an hypothesis has been proposed by Caetano-Anollés and Bauer (1988) to account for the beneficial association of *Agrobacterium* and *R. meliloti* in enhancing alfalfa nodulation. *Bacillus* S49 may also prime the plant to facilitate late rhizobial infection, similar to the results observed for *Azospirillum-Rhizobium* interactions

on clovers (Plazinski and Rolfe 1985). The inability of *Rhizobium* TAL 182 to form effective nodules after delayed inoculation may be a consequence of decreased susceptibility to infection of the root hairs of *P. vulgaris* . Susceptibility of host root cells to infection by *Rhizobium* is both transient and developmentally regulated (Bhuvaneswari *et al.* 1981).

Autoregulation by the plant host ultimately restricts nodulation (Pierce and Bauer 1983). Although *Rhizobium* TAL 182 shows nodulation suppression in split-root experiments (George *et al.* 1992), coinoculation with *Bacillus* S49 was able to partially offset that host-mediated suppression. It is tempting to speculate that coinoculation may induce the production of nod off compounds (compounds that suppress the expression of common *nod*-genes in *Rhizobium*) in the plant (Peters and Long 1988; Grandmaison and Ibrahim 1994; Mellor and Collinge 1995), to enable late infections on the secondary side of the split-root. Such mobilization of nod off compounds in split-root systems has been hypothesized by Caetano-Anollés and Gresshoff (1990). In *P. vulgaris*, delayed suppression of nodulation may be attributed to the requirement for proliferation of bacteria inside the nodules; *Rhizobium* infection *per se* does not trigger the suppression response observed in other legumes (George and Robert 1991). Partial reversal of suppression in this study may also reflect higher numbers of infection sites (root hairs) facilitated by the presence of *Bacillus* S49.

Coinoculation of *Rhizobium* TAL 182 and *Bacillus* S49 also induced heterologous nodulation of TAL 182 on *P. acutifolius*. Although *P. vulgaris* and *P. acutifolius* belong to the same genus, they are in different cross-inoculation groups (Allen and Allen 1981; Somesegaran *et al.* 1991). It was not possible to detect or re-isolate *Rhizobium* TAL 182 from the few pseudonodules observed on singly inoculated plants using PCR and

dilution-plating techniques. Heterologous nodulation of *P. acutifolius* by *Rhizobium* TAL 182 coinoculated with *Bacillus* S49 implicates the *Bacillus* more directly in the nodulation process. The enhancement of root hair production in *P. vulgaris* in response to coinoculation may have influenced both the infection process and the potential for heterologous nodulation. Similar heterologous nodulation on hairy roots of clover plants by a non-specific symbiont, *R. leguminosarum* bv. *viciae*, has been reported (Díaz *et al.* 1995). It is interesting to note that coinoculation of *Bacillus* S49 (or *B. polymyxa* L6) with *Bradyrhizobium* TAL 644 neither induced root hair proliferation on *P. vulgaris* nor enhanced nodulation by *Bradyrhizobium* TAL 644 on its host, *P. acutifolius*. These results suggest that the *Rhizobium* genotype might be more decisive than *Bacillus* in expressing the synergistic effect. Thus, reduced root hair proliferation in *P. vulgaris* in response to dual inoculation with *Bradyrhizobium* TAL 644 and *Bacillus* S49 reflects a degree of specificity in the synergistic interaction of *Rhizobium* and *Bacillus* on root hair formation and nodulation enhancement. Specific interaction between *Rhizobium* genotypes and *Azospirillum* in enhancing nodulation of legumes has been reported (Iruthayathas *et al.* 1983). Since the legume host can control and optimize events that lead to the formation of nodules (Caetano-Anollés and Gresshoff 1991), the lack of enhanced root hair formation and nodulation in *P. acutifolius* in response to coinoculation may be attributed to the host plant regulation of such events. Further work using well-characterized *Bacillus* and *Rhizobium* mutants may help elucidate the mechanism(s) of such an interaction and its consequences on plant nodulation and productivity.

Chapter 6

Altered root hair formation in *Phaseolus vulgaris* in the presence of *Rhizobium*, *Bacillus* and the ethylene biosynthesis inhibitor aminoethoxy vinyl glycine

If I have seen a little further it is by standing on the shoulder of giants.

Isaac Newton (1642-1727)

6.1. Introduction

Ethylene has been implicated in a number of processes that affect plant growth and development (reviewed by Kieber and Ecker 1993). External application of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) directly enhances ethylene production in plant tissues (Adams and Yang 1979; Lürssen *et al.* 1979; Tanimoto *et al.* 1995). Both ethylene and auxin are involved in root hair formation in *Arabidopsis thaliana* (Masucci and Schiefelbein 1994) and induction of root hair formation and inhibition of longitudinal root growth have been attributed to the effects of ethylene on plant roots (Goodlass and Smith 1979; Feldman 1984; Zaat *et al.* 1989). Exogenously applied ethylene promoted root hair formation in pea, faba bean and lupine (Abeles et al. 1992). External application of aminoethoxy vinyl glycine (AVG), which is an inhibitor of ethylene biosynthesis in plants, resulted in complete loss of root hair formation in *Arabidopsis* (Tanimoto *et al.* 1995), suggesting that ethylene may be a positive regulator of root hair formation (Dolan *et al.* 1994; Tanimoto *et al.* 1995).

Among the diverse group of soil microorganisms capable of producing ethylene (Primrose 1979), members of both *Rhizobium* and *Bacillus* genera are capable of producing ethylene *in vitro*, when grown in the presence of the precursor, methionine (Billington *et al.* 1979; Mansouri and Bunch 1989). Ethylene of microbial origin is capable of influencing plant growth either directly (Arshad and Frankenberger 1988) or indirectly (Glick *et al.* 1995). Root elongation in canola in response to *Pseudomonas putida* inoculation was postulated to involve deamination of plant-derived ACC, leading to lower concentrations of ethylene in plant tissues (Glick *et al.* 1994a, b). Nod-factors produced by *Rhizobium* may also induce localized production of ethylene in root tissues leading to the aberrant thick and short root (Tsr) phenotype observed in *Vicia sativa*

subspp. *nigra* (van Spronsen *et* al. 1995). Such observations suggest that microbial activity in the vicinity of plant roots may elicit ethylene-mediated changes to root morphology.

It has been shown in previous chapters of this thesis that co-inoculation of *Rhizobium etli* TAL 182 and *Bacillus megaterium* S49 induced proliferation of root hairs in *P. vulgaris* and that the plant response was dependent upon two viable interacting bacterial species. To investigate the possible role of endogenous ethylene in the proliferation response, root hair formation in *P. vulgaris* was examined in the presence of *Bacillus*, *Rhizobium*, ACC (an ethylene precursor) and AVG (an ethylene biosynthesis inhibitor). The main objective of this study was to determine the effect of AVG on the *Bacillus-Rhizobium* induced root hair proliferation in *P. vulgaris*.

6. 2. Materials and Methods

6. 2. 1. Bacterial strains and growth media

The isolation and characterization of *Bacillus megaterium* S49 has been described previously (Chapter 2). The media and growth conditions of both *Bacillus megaterium* S49 and *Rhizobium etli* TAL 182 have been described in chapter two. Cells were harvested by centrifugation, washed twice with sterile 0.1M phosphate buffer (pH 6.8) and resuspended in the same buffer to a specified cell density before inoculation.

6. 2. 2. Plant material and growth conditions

Seeds of *P. vulgaris* 'Contender' were surface sterilized in 30% hydrogen peroxide for 15 minutes, followed by seven rinses with sterile distilled water. The seeds were germinated aseptically on moist filter paper in petri plates. Stock solutions of aminoethoxy vinyl glycine (AVG) (2.5 mM), and 1-aminocyclopropane-1-carboxylic acid (ACC)(25 mM) (Sigma, St. Louis, MO), were prepared in distilled water, filter-sterilized

and stored at -20° C. Sterile two-day old pregerminated bean seedlings with uniform radicle length (approximately 1cm) were immersed for 15 minutes in either sterile 0.1M phosphate buffer (pH 6.8) (control) or buffer containing 10^6 cfu ml^{-1} of bacterial inoculant. Seeds treated with AVG or ACC were incubated for 15 minutes in sterile phosphate buffer (pH 6.8) containing AVG (50 µM or 100 µM) or 100 µM ACC. Seedlings were cultured axenically in a 20 x 150 mm test tube containing 10 ml one-tenth strength nitrogen-free plant growth medium (Broughton and Dilworth 1971), in which the seed was supported above the medium by a 200 µl plastic pipette tip. Medium for treatments which included AVG or ACC were supplemented with 50 µM and/or 100 µM AVG or 100 µM ACC. Tubes were incubated at 25° C with a 16h photoperiod at 200 µE m^2 s^{-1} illumination. A random sample of 20 lateral roots from approximately ten plants per treatment was used for scanning electron microscopy (SEM). The base and tip (approximately 0.8 cm each) of the selected lateral roots were excised and discarded. The remaining central portion of the root was used for SEM analysis.

6. 2. 3. Morphological characterization of root hair formation using scanning electron microscopy

Root segments from each treatment were fixed overnight in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer (pH 7.2). Samples were washed three times (15 minutes each) with 0.05M sodium cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.2) for two hours. The samples were rinsed once in de-ionized water, dehydrated through a 30% to 100% series of ethanol rinses, and critical point dried from 100% ethanol in a Balzers CPD 020 system using liquid carbon dioxide as the exchange medium. After mounting on double-sided sticky aluminum tabs, specimens were coated with gold-palladium (approximately 25

nm) in a Nano-tech II sputter coater, and viewed with a Cambridge 250T SEM. All samples were viewed by two independent skilled observers and typical observed response scored (e.g. no proliferation, slight proliferation, massive proliferation) and recorded photographically.

6. 3. Results

6. 3. 1. ACC induces root hair formation in *P. vulgaris*

Enhancement of root hair formation in response to dual inoculation with *Rhizobium* and *Bacillus* are shown in Figure 6.1 A, B as previously reported in Chapter three. The concentrations of AVG and ACC used in this study were comparable to those reported by Tanimoto *et al.* (1995) in *Arabidopsis thaliana*. No aberrant root phenotypes were observed in response to treatment with ACC, AVG and/or either bacterial species. Root hair formation was enhanced by the addition of 100 μM ACC (Figure 6.1C), and single inoculation with either *Rhizobium* TAL 182 (Figure 6.1D) or *Bacillus* S49 (Figure 6.1E). The addition of 100 μM ACC to dual inoculated plants showed no obvious synergistic enhancement of root hair formation (compare Figure 6.1F and 6.1B).

6. 3. 2. AVG inhibits root hair formation in *P. vulgaris*

The presence of 50 μM AVG in the growth medium completely inhibited root hair formation in uninoculated control plants (Figure 6.2 A), and severely suppressed root hair formation in plants inoculated with either *Rhizobium* TAL 182 (Figure 6.2 B) or *Bacillus* S49 (Figure 6.2 C). The suppression of root hair formation by 50 μM AVG was largely reversed in dual inoculated plants (Figure 6.2 D). However, higher concentrations of AVG (100 μM) inhibited root hair formation even in dual inoculated

Figure 6.1. Root hair formation in *P. vulgaris* in response to inoculation with *Rhizobium* , *Bacillus* and treatment with 1-aminocyclopropane-1-carboxylic acid (ACC).
(A) Uninoculated control (0.1M sterile phosphate buffer, pH 6.8)
(B) *Rhizobium etli* TAL 182 and *Bacillus megaterium* S49 dual inoculated control
(C) Uninoculated control with 100 μM ACC
(D) *Rhizobium* TAL 182 and 100 μM ACC
(E) *Bacillus* S49 and 100 μM ACC
(F) *Rhizobium* TAL 182 and *Bacillus* S49 supplemented with 100 μM ACC

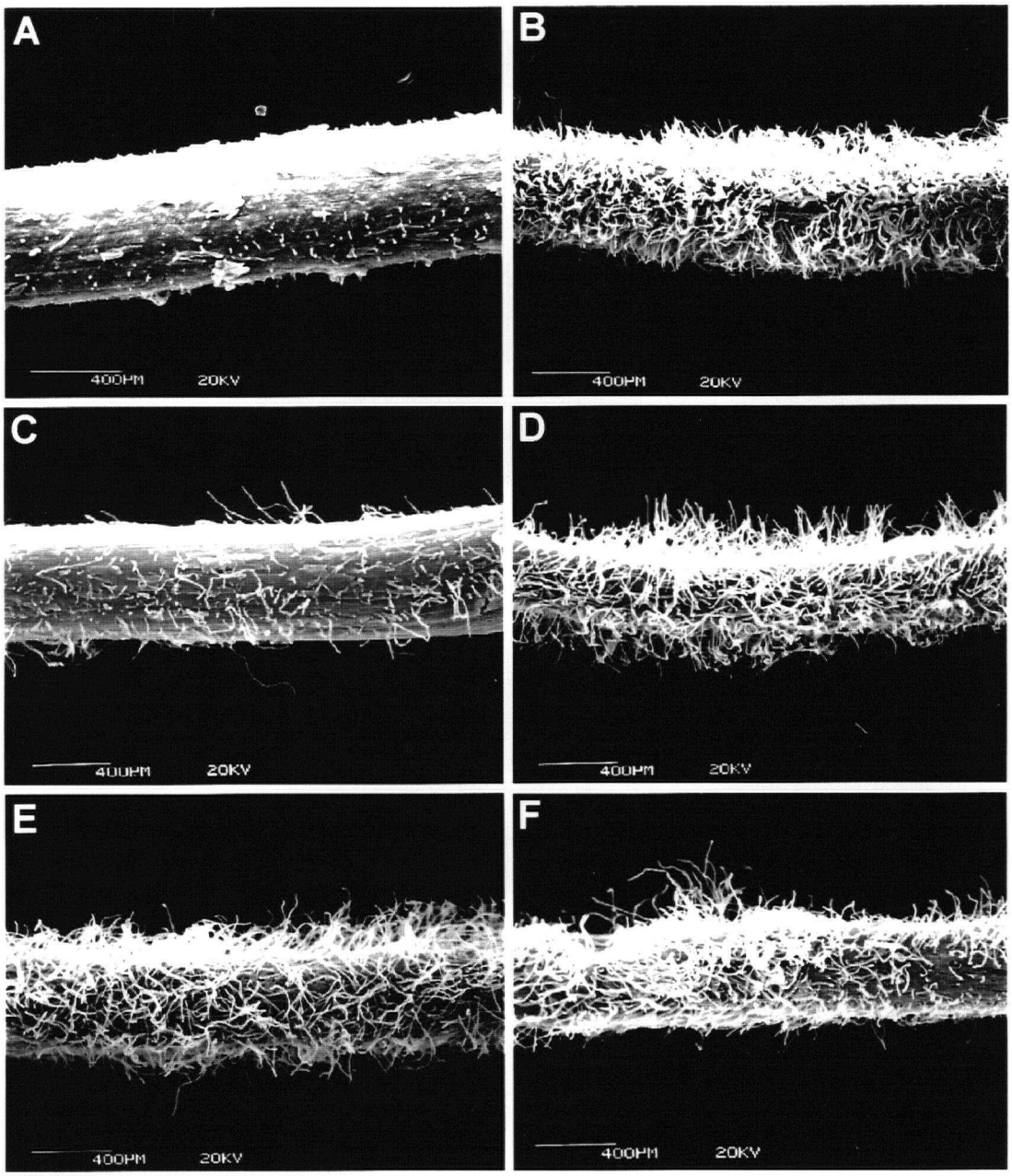
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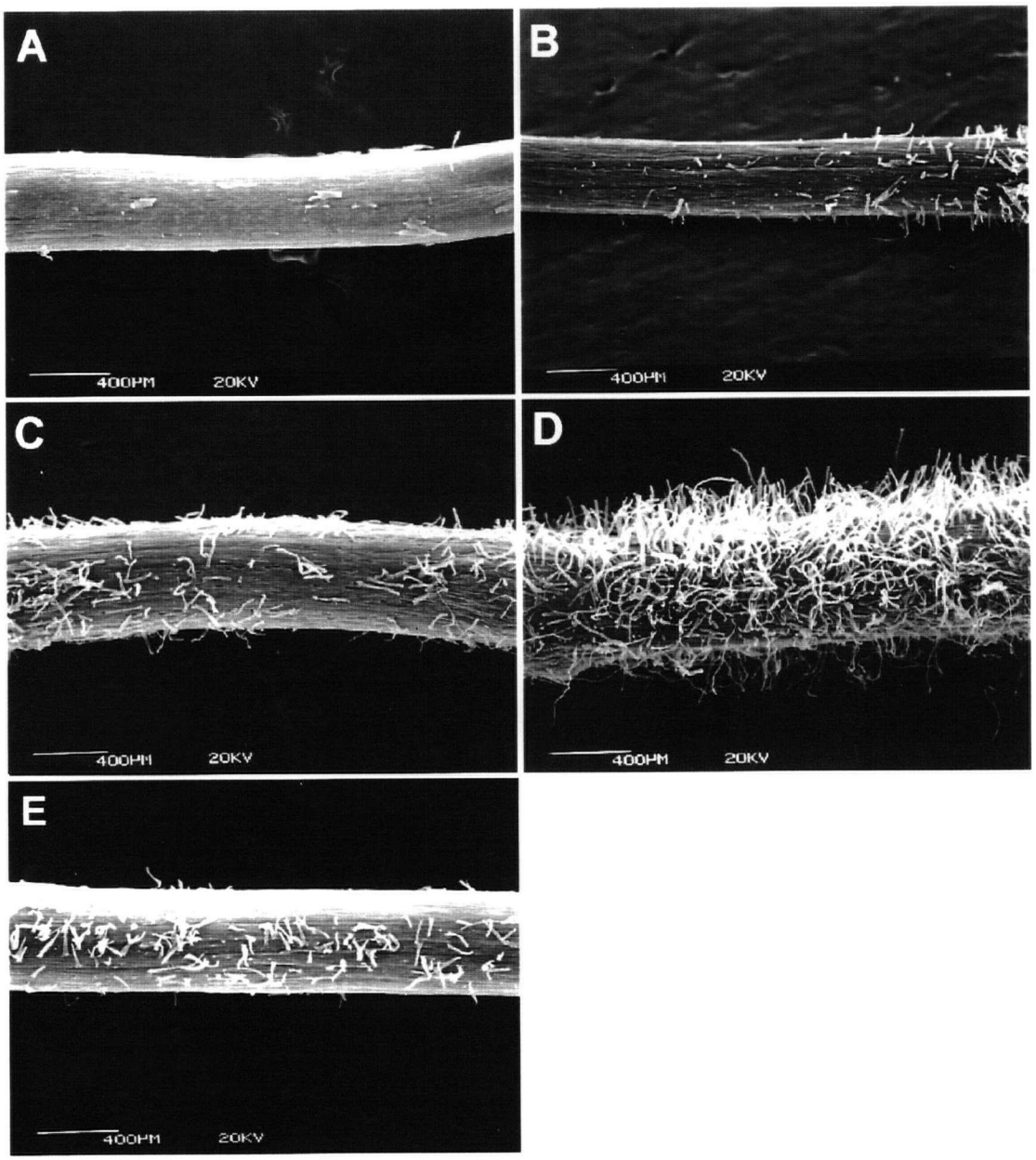
Figure 6. 2. Root hair formation in *P. vulgaris* in response to inoculation with *Rhizobium*, *Bacillus* and treatment with aminoethoxy vinyl glycine (AVG)
(A) Uninoculated control with 50 µM AVG
(B) *Rhizobium etli* TAL 182 and 50 µM AVG
(C) *Bacillus megaterium* S49 and 50 µM AVG
(D) *Rhizobium* TAL 182 and *Bacillus* S49 supplemented with 50 µM AVG
(E) *Rhizobium* TAL 182 and *Bacillus* S49 supplemented with 100 µM AVG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plants (Figure 6.2 E); root hair morphogenesis in these plants was similar to that observed for *B. megaterium* S49 single inoculated plants supplemented with 50μM AVG (Figure 6.2 C).

Plants treated with both AVG and ACC showed variable root hair formation responses; hence the results of this treatment are not presented here. Approximately 60% of treated plants showed little root hair formation, similar to uninoculated control with 50 μM AVG (Figure 6.2 A), while the remaining plants showed root hair formation similar to the uninoculated control with 100 μM ACC (Figure 6.1 C).

6. 4. Discussion

It was shown in Chapter 3 that a synergistic interaction between *Rhizobium etli* TAL 182 and *Bacillus megaterium* S49 enhanced root hair formation in *P. vulgaris* and this enhancement was independent of the plant host. This study further demonstrates that enhancement of root hair formation likely overcomes the inhibitory effects of ethylene biosynthesis inhibitor aminoethoxy vinyl glycine (AVG), in the presence of the two interacting bacterial species. The inhibitory effects of AVG on root hair formation were clearly evident in uninoculated control plants and in those which received a single inoculation of either *Rhizobium* TAL 182 or *Bacillus* S49. The reversal of AVG inhibition by dual inoculation of *Rhizobium* TAL 182 and *Bacillus* S49 indicated the ability of *P. vulgaris* roots in the presence of the two bacteria, to overcome the inhibitory effects of AVG at 50 μM concentration. This observation was surprising since AVG is considered to strongly inhibit ethylene biosynthesis and hence root hair formation (Tanimoto *et al.* 1995). This study is consistent with the data presented in (Chapter 3 and Chapter 4) on root hair proliferation in *P. vulgaris* in response to coinoculation with the two bacteria.

At higher concentrations of AVG (100 μM), root hair formation in *P. vulgaris* was

inhibited even in the presence of both bacterial species, which is consistent with the hypothesis that AVG is a negative regulator of root hair formation (Dolan *et al.* 1994; Tanimoto *et al.* 1995). Based on the root hair formation response in the presence of AVG, the effect of the addition of the ethylene precursor ACC to the plant growth medium on proliferation of root hairs in *P. vulgaris* was investigated. The presence of ACC in the growing medium of plants that received a single inoculation of either *Rhizobium* TAL 182 or *Bacillus* S49 resulted in a significantly enhanced root hair formation compared to the uninoculated bacterial control. This enhanced root hair formation may be due to increased ethylene biosynthesis in plant tissues, in the presence of exogenous ACC. Thus, the results of this study implicate ethylene as a positive regulator of root hair formation in *P. vulgaris* and are consistent with previously reported studies (Abeles *et al.* 1992; Dolan *et al.* 1994; Tanimoto *et al.* 1995). However, the presence of ACC in the growth medium of plants that received dual inoculation of *Rhizobium* TAL 182 and *Bacillus* S49 showed no obvious enhancement of root hair formation, compared to singly inoculated plants supplemented with ACC.

Ethylene production in plants can also be induced by a variety of biological and physical stimuli (Adams and Yang 1981; Yang and Hoffmann 1984). The thick and short root (Tsr) phenotype in *Vicia sativa* is attributed to excess ethylene production, induced by *Rhizobium* nod factors (van Spronsen *et al.* 1995). It is likely that the presence of both *Rhizobium* and *Bacillus* on plant roots may induce an increase in ethylene biosynthesis in the root epidermal cells, leading to root hair differentiation. Ethylene and auxin are involved in root hair formation in *Arabidopsis* (Masucci and Schiefelbein 1994). *B. megaterium* S49 produces IAA *in vitro* (Chapter 2) and it is tempting to speculate that nod factors produced by *Rhizobium* TAL 182 may act

synergistically with IAA produced by *Bacillus* S49 to cause local changes in ethylene concentrations in root epidermal cells. The root hair proliferation observed in this study may also reflect systemic cell changes in response to the interacting bacteria, similar to the mechanism described for microtubule reorientation in cortical cells in *Rhizobium*-vetch interaction (Zaat *et al.* 1989; van Spronsen *et al.* 1995). Nevertheless, a role for ethylene in causing root hair proliferation in the presence of AVG/ACC and interacting *Rhizobium* and *Bacillus* cannot be ruled out, since it is not clear to what extent AVG could reduce or ACC induce endogenous ethylene production in *P. vulgaris*. The variable root hair formation observed on plants treated with both AVG and ACC may be due to differential uptake of these compounds by the plant roots.

Ethylene of microbial origin could directly affect plant growth (Arshad and Frankenberger 1988). Under the experimental conditions of this study, both *Rhizobium* TAL 182 and *Bacillus* S49 did not produce detectable amounts of ethylene *in vitro*, even in the presence of its precursor L-methionine in the growing medium. Hence it is unlikely that ethylene production by these microorganisms could be a factor in the root hair proliferation responses observed. Nevertheless, the possibility of ethylene production by *Rhizobium* and/or *Bacillus* at the site of action (root surface) cannot be ruled out.

The root hair proliferation observed in *P. vulgaris* in response to dual inoculation with *Rhizobium* and *Bacillus* may be analogous to the formation of shepherd's crooks as described for the *Rhizobium*-legume interaction (Relic *et al.* 1993; van Rhijn and Vanderleyden 1995); both responses appear to require the physical contact between the plant and bacteria. Dual bacterial inoculation apparently disrupts the type of epidermal cell patterning described for root hair cells and non-root hair cells (Dolan *et al.* 1994).

The ACC/AVG response in combination with inoculation, suggest that the bacterial-plant interaction is mediated, at least in part, through ethylene. The root hair response reflects the unique potential for interaction between plants and their respective rhizosphere microbial populations in influencing the growth and physiology of the host. This is the first study to show unequivocal direct influence of interacting bacterial species on root hair formation in a leguminous plant, which has subsequently been shown to significantly influence nodulation of *P. vulgaris* by *Rhizobium etli* TAL 182.

Chapter 7

Summary

I do not mind if you think slowly. But I do object when you publish more quickly than you think.

Wolfgang Pauli (1900-1958)

The legume-*Rhizobium* symbiosis is considered to be the most efficient system for biological nitrogen fixation. Roots of *Phaseolus vulgaris* (common bean) are nodulated by *Rhizobium etli* and *Rhizobium tropici*. In contrast to other legumes, the common bean is considered a poor nitrogen fixer, which may be a function of several genetic and environmental factors. Nodules formed in *P. vulgaris* are of the determinate type and plants have a bushy type habitat with a short growth cycle. Hence nitrogen fixation may not contribute as significantly to the nitrogen nutrition of the plant as in other nitrogen fixing legumes.

The free-living, nitrogen-fixing bacteria of the genus *Bacillus* live in close association with plants, and may promote plant growth under appropriate conditions. The importance of *Bacillus* as plant-growth promoting rhizobacteria has received much attention in the last decade mainly from experiments under greenhouse and growth chamber conditions, and in some field inoculation experiments. Positive effects of *Bacillus* inoculation are mainly attributed to improved root development (i.e., increased root dry weight, root branching and root surface area), promotion of seedling emergence and general plant growth. Available evidence indicates that production of plant-growth promoting substances by *Bacillus* is at least partly responsible for these observed effects. The ability of *Bacillus* to enhance plant growth by suppression of deleterious rhizobacteria (DRB) and plant pathogenic fungi has been the focus of many researchers in the past few years. In this context, it is worth mentioning that suppression of deleterious microflora by *Bacillus* was achieved only in the presence of associated bacteria on plant roots. Thus, the growth promoting ability of *Bacillus* by suppressing deleterious rhizosphere microflora may be dependent upon its interaction with other root-associated microflora.

The effect of coinoculation of legumes with *Rhizobium* and *Bacillus* has not been extensively studied, unlike the *Azospirillum-Rhizobium* associations. Nevertheless, positive effects due to combined inoculation of *Rhizobium* and *Bacillus* in enhancing nodulation of legumes, mainly on soybeans, has been observed. Increases in dry-matter production and nitrogen content, increased number of nodules and, a general improvement in root development were attributed to the effects of coinoculation. However, none of the studies explain adequately the mechanism(s) of nodulation promotion due to coinoculation. A specific plant response to the presence of *Bacillus* alone or in combination with *Rhizobium*, has not yet been implicated, to explain enhanced nodulation. As in *Azospirillum-Rhizobium* associations, increases in root growth and enhanced nodulation in coinoculation experiments have been attributed to the effect of plant growth substances produced by *Bacillus*. Such evidence has to date, been circumstantial, rather than conclusive.

In the present study, attempts were made to evaluate the effects of combined inoculation of *Rhizobium* etli TAL 182 and *Bacillus* on nodulation of *P. vulgaris* under controlled conditions, and to investigate possible mechanism(s) involved in this interaction. The results of the study are summarized as follows:

(i) Some of the *Bacillus* isolates produced IAA *in vitro*, both in the presence and absence of L-tryptophan in the growing medium. The amount of IAA produced in a culture medium supplemented with 100 μg ml^{-1} of L-tryptophan was significantly higher than the amounts produced in unsupplemented medium. *Bacillus* isolates that enhanced nodulation of P. vulgaris by *Rhizobium* TAL 182 also produce IAA *in vitro*. A variable relationship was observed between *in vitro* IAA production by *Bacillus* isolates and their ability to promote nodulation on *P. vulgaris* by *Rhizobium* TAL

182. Enhanced nodulation responses likely require the presence of both bacterial species and may not be solely dependent on *in vitro* IAA production by *Bacillus* isolates. Spontaneous α-methyl tryptophan resistant mutants of *B. megaterium* strain S49 showed altered IAA production compared to the wild-type and excreted tryptophan into the growing medium. Both low and high IAA producing mutants decreased nodulation of *P. vulgaris* by *Rhizobium* TAL 182, suggesting that *Bacillus* isolates may have a direct effect on either the *Rhizobium* or the plant. Some of the *Bacillus* isolates also produced cytokinin-like compounds *in vitro*. A detailed study of cytokinin production by *Bacillus* isolates was precluded by inconsistency and lack of reproducibility in the HPLC analysis and hence was not pursued further.

(ii) Combined inoculation of *Rhizobium* TAL 182 and *B. megaterium* S49 resulted in a synergistic enhancement of root hair initiation. This enhancement was not mimicked by either supplemental IAA or by coinoculation of *Rhizobium* TAL 182 with a low IAA producing *B. megaterium* mutant, S49-1D. The stimulatory effect of dual inoculation was a primary consequence of an interaction between *Rhizobium* TAL 182 and *Bacillus* S49, rather than a host directed event. The lack of host determination was illustrated by root hair proliferation induced on *P. acutifolius* and *Arabidopsis thaliana* in response to dual inoculation of *Rhizobium* TAL 182 and *Bacillus* S49.

(iii) Simultaneous physical presence of viable *Rhizobium* and *Bacillus* was necessary to induce root hair proliferation. Physical separation of either bacterial species precluded root hair proliferation in *P. vulgaris*, which suggests that a low molecular weight diffusible substance is not involved in the interaction leading to root hair proliferation. *P. vulgaris* did not show altered root hair formation when inoculated with

non-viable cell preparations of both *Rhizobium* and *Bacillus*. The root hair proliferation responses observed in response to dual inoculation of *Rhizobium* with *Bacillus* isolates were not dependent on the ability of the *Bacillus* isolates to produce IAA *in vitro*. Fully functional *Rhizobium* and *Bacillus* may also be prerequisites for the synergistic interaction leading to root hair proliferation. Coinoculation of *Bradyrhizobium* spp. TAL 644 with *Bacillus* S49 did not enhance root hair formation in *P. vulgaris*, suggesting a degree of specificity for the microbial partners in the interaction.

(iv) Presence of viable *Bacillus* significantly influenced *Rhizobium* TAL 182- *P. vulgaris* symbiosis by inducing formation of effective nodules at early stages of plant growth. Coinoculation of *Bacillus* S49 with *Rhizobium* TAL 182 partly offset the host-mediated suppression of nodulation, indicative of a plant response to the interacting bacterial species. Enhancement of nodulation was not observed if *Rhizobium* inoculation was delayed, even when *Bacillus* was present on the roots. Coinoculation of *Rhizobium* TAL 182 with non-viable cell preparations of *B. megaterium* S49 also failed to promote nodulation of *P. vulgaris* by *Rhizobium* TAL 182. Heterologous nodulation of *P. acutifolius* by *Rhizobium* TAL 182 in the presence of *Bacillus* S49 implicates *Bacillus* directly in the nodulation potential of *Rhizobium*. Nodulation promotion appears to be *Rhizobium* specific, as coinoculation of *Bacillus* with *Bradyrhizobium* TAL 644 failed to enhance nodulation of *P. acutifolius* by its homologous microsymbiont, *Bradyrhizobium* TAL 644.

(v) The presence of aminoethoxy vinyl glycine (AVG) in the growth medium resulted in a complete loss of root hair formation in *P. vulgaris*. Plants that received a single inoculation of either *Rhizobium* TAL 182 or *Bacillus* S49 in the presence of AVG

also did not show enhancement of root hair formation. The inhibitory effect of AVG on root hair formation was nullified by the dual presence of both *Rhizobium* TAL 182 and *Bacillus* S49 on *P. vulgaris* roots. The ACC/AVG response in combination with bacterial inoculation, suggest that the bacterial-plant interaction is mediated, at least in part, through ethylene and dual inoculation apparently disrupts the epidermal cell patterning described for root hair cells and non-root hair cells.

The results of this study show that *Bacillus* enhances nodulation of *P. vulgaris* by *Rhizobium* TAL 182. This enhancement could be explained, at least in part, by the synergistic enhancement of root hair formation leading to increased infection sites for *Rhizobium*. This study provides clear evidence of a synergistic bacterial interaction which results in a significant change in plant root morphogenesis. The results reflect the potential for interspecific bacterial interactions in the plant rhizosphere, and this is the first study to demonstrate a plant response in root hair morphogenesis induced by the interaction of two bacterial species. This interaction, and its effect on root hair morphogenesis, has also been shown to have significant implications for the infection potential and nodulation of *P. vulgaris* by *Rhizobium* TAL 182 under gnotobiotic conditions.

7.1. Proposal of a working hypothesis for future research

Much progress has been achieved in recent years in understanding the nature of nodulation, the regulation of its physiological processes and in identifying the chemical nature of *Rhizobium* signals that trigger nodulation. Little is understood of the mechanism(s) by which associated rhizobacteria promote nodulation of legumes by *Rhizobium*. Based on the results of this study, it is tempting to present an hypothetical model on which future research may be carried out to determine the basis of enhanced nodulation in response to coinoculation of *Rhizobium* and *Bacillus*. The proposals are based on the model presented in the diagram. The letters for each heading refers to the relevant portion of the schematic diagram. This model is purely intended to stimulate experimentation and, by any standards, is not comprehensive.

A. Legume plant-*Rhizobium* interaction

This interaction has been well characterized and hence will not be considered further here.

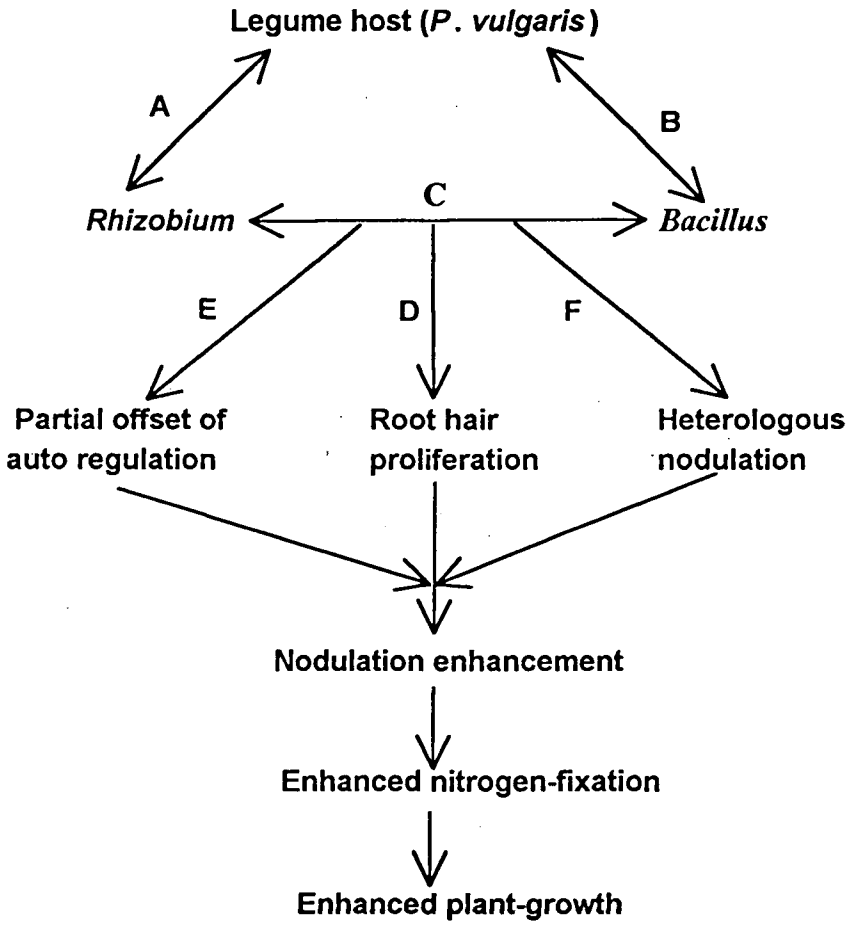
B. Legume plant-*Bacillus* interaction

The following may be appropriate for future studies:

(I) To determine whether seed or root exudates of *P. vulgaris* contain a compound(s) that might be used exclusively by *Bacillus* for spermosphere and/or rhizosphere colonization. The endophytic habitat of some *Bacillus* spp. may also reflect their ability to use a particular substrate as a carbon source.

(ii) To determine whether a plant-derived compound might be modified by *Bacillus* to enhance *Rhizobium* populations in the rhizosphere, or alter symbiotic activity. The influence of such a modified compound(s) on root growth might also be relevant.

(iii) To determine whether *Bacillus* enhances or alters the secretion of *nod* gene



Simplified scheme outlining events leading to nodulation enhancement and alteration of nodulation potential of *Rhizobium* in the presence of *Bacillus*.

inducing compounds from seeds/roots of *P. vulgaris*, that lead to altered *Rhizobium* activity.

C. *Rhizobium-Bacillus* interaction

The following investigations may provide evidence for the mechanism(s) involved in the synergistic interaction.

(i) To determine whether *Bacillus* directly alters or enhances the expression of *nod* genes in *Rhizobium*.

(ii) To determine whether a factor or compound produced by *Bacillus* is transferred to or is communicated to the plant by *Rhizobium*.

(iii) To determine whether a *Rhizobium* gene product or signal is modified by *Bacillus* or vice-versa as a trigger to root hair proliferation.

(iv) To examine the interaction between the nod factor produced by *Rhizobium* and IAA produced by the *Bacillus* on root hair proliferation. This interaction can further be characterized by use of *Rhizobium* mutants deficient in Nod factor production and *Bacillus* mutants deficient in IAA production.

D. *Rhizobium-Bacillus* interaction and root hair proliferation

(i) To determine whether the presence of interacting *Rhizobium* and *Bacillus* induces altered gene expression in root epidermal cells involved in root hair differentiation. Quantitative and qualitative variation of known root hair development (*RHD*) gene products may contribute to elucidating the mechanism(s) involved in root hair proliferation in response to coinoculation.

E. Alteration of regulation of nodulation due to interacting *Rhizobium* and *Bacillus*

(i) "Nod off" compounds are primarily the products of plant secondary metabolism (Mellor and Collinge 1995). Analysis of the quantitative and qualitative variations of

"nod off" compounds in root tissues in response to dual inoculation may contribute to our understanding of the mechanism(s) influencing autoregulation in root nodulation.

F. Heterologous nodulation of *Rhizobium* in the presence of *Bacillus*

(i) To examine whether there is a variation of oligosaccharides (nod factor binding ligands) in root hairs induced by the presence of *Bacillus*. For example, oligosaccharide metabolism is involved in the production of specific nod factor binding ligands. Variation in carbohydrate profiles in root hairs in response to the presence of *Bacillus* may influence legume-microsymbiont recognition. Interference with the process (by the presence of *Bacillus*) may lead to heterologous nodulation.

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Appendix

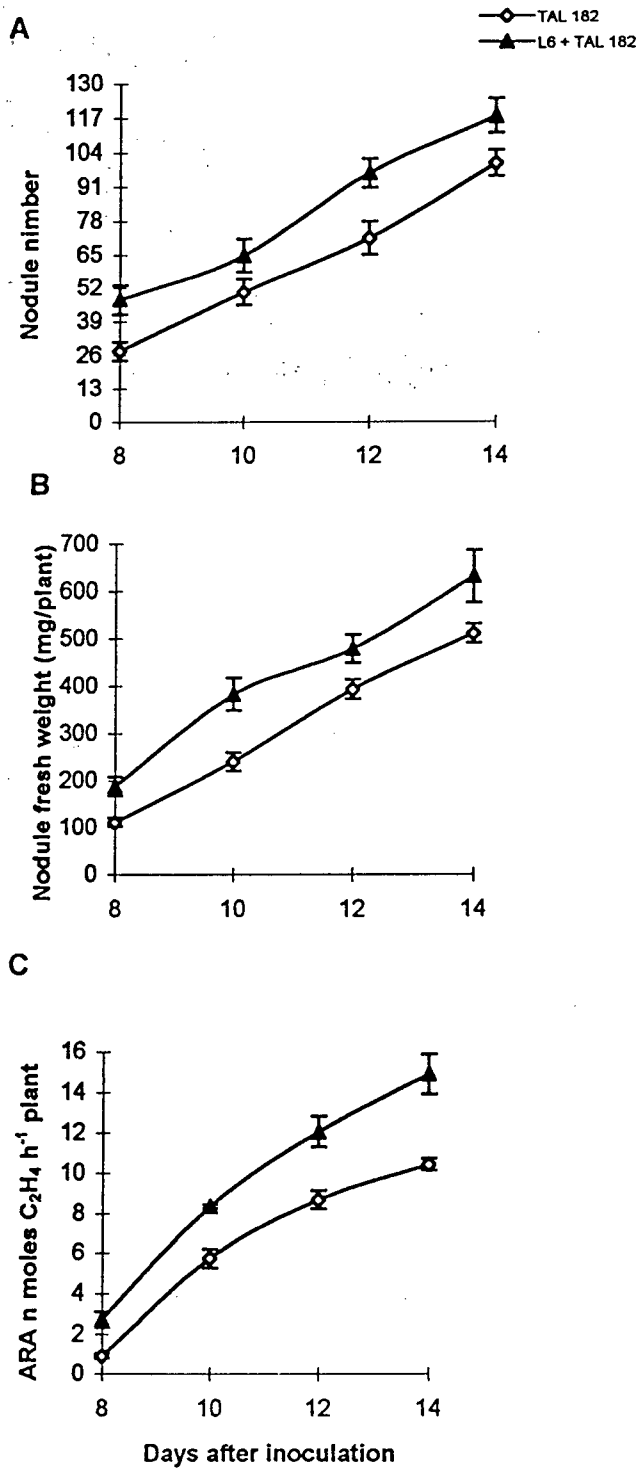
Appendix I. Preliminary results on the detection of cytokinins in the cell free supernatant of some *Bacillus* isolates

Strain	Cytokinin production		
	t-Zeatin[a]	t-Zeatin riboside[b]	2iP[a]
B. megaterium S49	+	+	+
B. megaterium A15	+	-	+
B. megaterium B17	-	nd	+
B. polymyxa L6	+	+	+
B. polymyxa PW2	+	+	+
B. brevis S72	+	+	+
B. mycoides T10	-	-	+
B. pumilus (Bet 10a)	+	nd	+

[a] As detected by TLC, Sephadex LH-20 chromatography, HPLC and radish cotyledon expansion bioassay.
[b] As detected by Phytodetek® immunoassay.
nd= not determined
+/- signs indicate the presence or absence of the compound.

Trace enrichment of cytokinin-like compounds in the cell free supernatant of *Bacillus* isolates was accomplished using Amberlite XAD-2 chromatography, as described by (Kim *et al.* 1985). Separation of cytokinin-like-compounds after trace enrichment was accomplished using Sephadex LH-20 chromatography (Sturtevant and Taller 1989). Fractions obtained after separation by Sephadex chromatography were analysed by HPLC, to identify *trans* zeatin and isopentyl adenine (2iP), as desribed by either Horgan and Kramers (1979) or Andersen and Kemp (1979). Sephadex LH-20 fractions were also used for radish cotyledon expansion bioassay (Letham 1971), to ascertain for cytokinin-like activity. Identification of *trans* zeatin riboside was accomplished using the Phytodetek ® immunoassay after reverse-phase chromatography of cell free supernatant using C18 Sep-pak column (Sturtevant and Taller 1989).

Appendix II. Effect of dual inoculation of *Rhizobium etli* (TAL 182) and *Bacillus polymyxa* (L6) on (A) nodule number, (B) nodule fresh weight, and (c) nitrogenase activity of *P. vulgaris* 'Contender' grown under gnotobiotic conditions in the growth chamber. Control plants that received sterile 0.1M phosphate buffer did not form any nodules. Each value represents the means of six replicates. Means of values for each parameter tested were significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test. Bars represent the standard error of the mean.


A
TAL 182
L6 + TAL 182
Nodule nimber
130
117
104
91
78
65
52
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13
0
8
10
12
14
B
Nodule fresh weight (mg/plant)
700
600
500
400
300
200
100
0
8
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12
14
C
ARA n moles C_2H_4 h^{-1} plant
16
14
12
10
8
6
4
2
0
8
10
12
14
Days after inoculation

Appendix III. Mean number of lateral roots on *Phaseolus vulgaris*[†] three and five days after the germinants were inoculated with *Rhizobium etli* TAL 182 and *Bacillus polymyxa* L6

Treatment	Number of lateral roots	
	Day 3	Day 5
Uninoculated control*	4.75	18.25
Rhizobium TAL 182	2.50	25.0
Rhizobium TAL 182 and *Bacillus* L6	7.75**	27.5

[†] Plants were grown in modified Leonard jar assemblies as described in Chapter 2.
* Sterile 0.1M phosphate buffer (pH 6.8)
* * Significantly different from *Rhizobium* treatment, $p < 0.001$ (t-test).

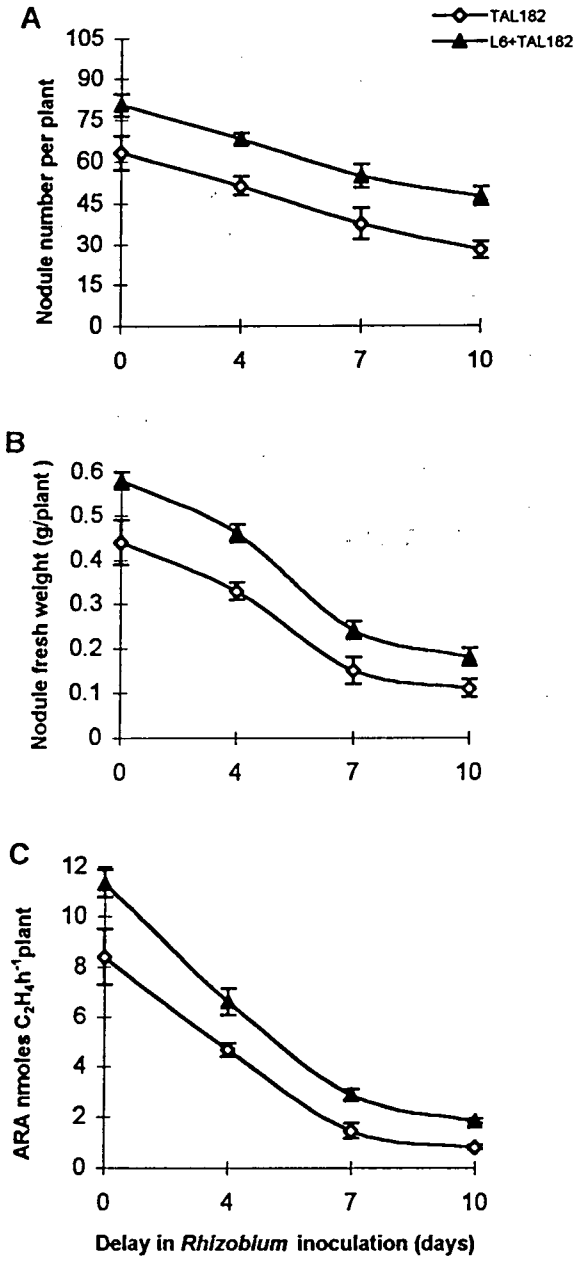
Appendix IV. Calculation of percent suppression in split-root experiments (adapted from George *et al.* 1992).

One side of the split-root system (primary side or E side) was inoculated at time zero, and the second side (D side) with increasing delay. At time zero, the uninoculated control consisted of early inoculation on both sides of the split-root with sterile 0.1M phosphate buffer (pH 6.8). For each delay period, the control consisted of a delayed inoculation only on the secondary side of the split-root (O/D control). In this experiment *R. etli* TAL 182 was the primary inoculant (E) and *R. etli* TAL 182 alone or in combination with *B. megaterium* S49 was the secondary inoculant (D). Inhibition of nodulation on the secondary side of the split-root was expressed as percent suppression, using the formula:

$$\% \text{ suppression} = 100 - \% \text{ nodulation}$$

The percent nodulation was derived by expressing the extent of secondary nodulation (D side of E/D treatments) as percentage of the nodulating potential of that side of the split-root at the time of the delayed inoculation (D side of O/D control) at each delay period. By quantifying suppression this way, the analysis was standardized with the comparison of nodules at the same developmental stage.

Appendix V. Effect of timing of inoculation of *R. etli* (TAL 182) and *B. polymyxa* (L6) on (A) nodule number, (B) nodule fresh weight, and (c) nitrogenase activity of *P. vulgaris* 'Contender' grown under gnotobiotic conditions in the growth chamber. Control plants that received sterile 0.1M phosphate buffer did not form any nodules. Each value represents the means of six replicates. Means of values for each parameter tested were significantly different at $p \leq 0.05$, as determined by Duncan's multiple range test. Bars represent the standard error of the mean.


A
TAL182
L6+TAL182
Nodule number per plant
105
90
75
60
45
30
15
0
0
4
7
10
B
Nodule fresh weight (g/plant)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
7
10
C
ARA nmoles $C_2H_4h^{-1}$plant
12
10
8
6
4
2
0
0
4
7
10
Delay in *Rhizobium* inoculation (days)