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HORMONES AND THE DIRECT EFFECT OF PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) ON HIGHER PLANTS

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ABSTRACT

The direct effect of plant growth-promoting rhizobacteria (PGPR) on seed germination and seedling post-germination growth of canola (*Brassica campestris* cv. Tobin and *B. rapa* cv. *rosette*) and lettuce (*Lactuca sativa* cv. Grand Rapids) was examined using gnotobiotic growth pouches under fluorescent light or in darkness. It was demonstrated that: 1) *Rhizobium* spp. could promote growth of non-leguminous plants; 2) several spontaneous Rif^R mutants derived from *Pseudomonas putida* GR12-2 were highly promotive of plant growth; 3) the PGPR effect occurred in young seedlings during the early growth phase and was independent of light and nitrogen conditions; and 4) light promoted seedling root elongation of lettuce (but not canola), while inhibiting (de-etiolating) hypocotyl growth of both lettuce and canola.

Sixteen gibberellins (GAs) were identified from canola seedlings using gas chromatography-mass spectrometry (GC-MS) by full mass spectra and Kovats' Retention Indices (KRI), including two novel GAs (e.g. GA_{85} and GA_{89}) and two new GA-derivatives (e.g. 16α,17-dihydroxy GA_{34} and C/D ring rearranged GA_8), plus a putative new GA (12β-OH-GA_1) identified by GC-SIM and KRI.

Seed inoculation with GR12-2 reduced endogenous levels of abscisic acid (ABA) and growth-effective GAs in, and ethylene evolution from, canola roots. Levels of root indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) and malonyl ACC (MACC) were not significantly altered by GR12-2. Ethylene evolution rate from lettuce roots was reduced by GR12-2, but increased by the light.

The roles of plant hormones in the PGPR effect on root elongation are suggested to be: endogenous levels of ethylene, ABA, IAA and GAs in canola and lettuce seedling roots are (speculatively) supraoptimal for maximal root growth. The promotion of root elongation by PGPR may thus be due to a reduction of the levels of ABA, IAA, GAs and/or ethylene within the root. The mechanism (s) by which endogenous hormones in young seedling roots are altered by PGPR seed inoculation remains unknown.

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ABBREVIATIONS

ABA .....abscisic acid

ACC .....1-aminocyclopropane-1-carboxylic acid

amu .....atomic mass unit(s)

ASI.....after seed imbibition

BSTFA .....bis-(trimethylsilyl)trifluoroacetamide

bv .....biovar

C_{18}-PC .....C_{18} preparative column

CK(s) .....cytokinin(s)

cv .....cultivar

d .....day(s)

d.d. .....double glass-distilled water

dw .....dry weight

EI .....electron impact

EIMS.....EI mass spectrometry

EtOAc .....ethyl acetate

FID .....flame ionization detector

fw .....fresh weight

GA_n .....gibberellin A_n

GA(s) .....gibberellin(s)

GC .....gas chromatography

GC-MS .....GC-mass spectrometry

GC-SIM .....GC-MS-selected ion monitoring

h .....hour(s)

HOAc ..... acetic acid

HPLC .....high performance liquid chromatography

IAA .....indole-3-acetic acid

JA .....(-)jasmonic acid

KRI .....Kovats' Retention Index

M^+ .....molecular ion (m/z)

MACC .....1-malonylaminocyclopropane-1-carboxylic acid (malonyl ACC)

Me .....methyl ester derivative

MeOH .....methanol

MeTMSi .....methyl ester trimethylsilyl ether derivative

mol (M)..... molarity

m/z .....mass/charge

NMR .....nuclear magnetic resonance

OD .....optical density

PIA .....Bacto-P*seudomonas* isolation agar

PAF .....Bacto-P*seudomonas* agar F

PGPR .....plant growth-promoting rhizobacteria

PGR(s) .....plant growth regulator(s)

PRE .....promotion of root elongation (PGPR phenotype)

R/FR.....red radiation/far red radiation, e.g. I(655-665 nm)/I(725-735 nm)

Rif .....rifampicin

Rif^R .....Rif resistant

Rt .....retention time

SIM .....selected ion mornitoring

TSB .....tryptic soy broth

UV .....ultraviolet radiation

vs .....versus

CHAPTER 1

GENERAL INTRODUCTION

Generally speaking, higher plants are geographically immobile in the earth's biosphere and are responsive to local environmental factors, including soil microbes, especially those in the rhizosphere. Plant-associated microorganisms may thus play a significant part in the life process of the plant. The biotic relationships between plants and associated soil microbes are very complex. A soil microorganism can be either advantageous, neutral, or harmful, from the perspective of a plant (Peng and Paul, 1980; Kloepper and Schroth, 1978, 1981; Snellgrove *et al.*, 1982; Whipps and Lynch, 1983, 1985; Koch and Johnson 1984; Harris *et al.*, 1985; Okon and Hadar, 1987; Douds *et al.*, 1988; Jeffries, 1987; Kloepper *et al.*, 1980a, 1989; Chanway *et al.*, 1989; Fyson and Oaks, 1990; Chanway, 1990; Hebbar *et al.*, 1992).

Since the discovery that certain rhizosphere microorganisms can promote the early growth of higher plants and, often subsequently increase the yield, much effort has been expended in determining the mechanism(s). Even though the mechanism of this plant growth promotion by soil microorganisms is not yet clear, numerous microbial rhizosphere species have been shown to have beneficial effects on plant growth and development when applied to crop seeds or incorporated into the soil. The inoculation of economically important plants with beneficial microbes has become practical in agriculture, horticulture and forestry during the twentieth century (Cooper, 1959; Mishustin and Shilnikova, 1962; Mishustin, 1970; Kloepper and Schroth, 1978, 1981; Maronek, 1981; Torrey, 1982;

Menge, 1983; Torrey and Tjepkema, 1983; Okon, 1985; Lifshitz *et al.*, 1986; Jeffries, 1987; Okon and Hadar, 1987; Davidson, 1988; Chanway *et al.*, 1989; Kloepper *et al.*, 1980a, 1989; Chanway, 1990; de Freitas and Germida, 1992). It seems likely that many more species and strains of microbes beneficial to plants will become available for field use in the future.

1-1. Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) have been defined as a specific class of soil bacteria that live in the rhizosphere region (e.g., a narrow soil zone surrounding living roots), promote plant growth, and, thereby, increase the final yield (Kloepper *et al.*, 1978, 1980a, 1980b, 1988). The effects of PGPR on plants have been widely recognized, and yield increases of crops from PGPR inoculations can be up to 144% (Burr *et al.*, 1978; Suslow *et al.*, 1979; Suslow, 1980, 1982; Geels and Schippers, 1983; Schippers *et al.*, 1985; Bakker *et al.*, 1987; Okon and Hadar, 1987; Kloepper *et al.* 1989). The term, PGPR, was initially dedicated to strains of plant-growth promoting pseudomonads (Kloepper *et al.*, 1978, 1980a). However, the term is now widely used for many rhizosphere bacteria that are beneficial to the host plants. In this dissertation the term PGPR is used for all soil-borne, plant growth promotive bacteria. The biotic relationships between PGPR and higher plants can be symbiotic. For example, N_2-fixing nodule formation on roots of leguminous plants occurs with *Rhizobium*, *Bradyrhizobium*, and *Frankia* (Callaham *et al.*, 1978; Okon and Hadar, 1987; Boyd, 1988; Kloepper *et al.*,

1988; Sharma and Bajpai, 1989; Fyson and Oaks, 1990; Evans and Burris, 1992). There are also non-symbiotic relationships, including free-living bacteria and plant root-associated bacteria (Evans and Burris, 1992). Samples include *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas* and cyanobacteria (blue-green algae) strains (Cooper, 1959; Brown, 1974; Smith *et al.*, 1978; Schmidt, 1979; Stewart, 1980; Kapulnik *et al.*, 1983; Okon, 1985; Kloepper *et al.*, 1988; Redkina and Mishustin, 1989; Arsac *et al.*, 1990; Evans and Burris, 1992). Most PGPR can fix N_2 and some members may be classified into both symbiotic and non-symbiotic groups, for example, the wide-host-range cyanobacteria (Stewart, 1980; Evans and Burris, 1992).

Among PGPR, *Rhizobium* strains have been vigorously studied because of their global importance in N_2-fixation. Also, species of plant growth-promotive pseudomonads are showing increasing value in modern agriculture and forestry (Kloepper *et al.*, 1980a, 1988; Okon and Hadar, 1987). Although the effects, direct and/or indirect, of *Pseudomonas* and *Rhizobium* on plant growth are profound, we know very little about the mechanism(s) by which they work (Okon and Hadar, 1987; Kloepper *et al.*, 1988; Stacey *et al.*, 1992). A better understanding, and a more efficient use of plant-beneficial bacteria could provide the world with useful tools in resolving some global problems, such as food and energy shortages, and environmental damage. A study of the relationship of PGPR and important agricultural plants should thus be pursued from both fundamental and applied perspectives.

1-2. *Pseudomonas putida* GR12-2 and *Rhizobium* spp. PGPR

Pseudomonas, a genus of the Pseudomonadaceae, are aerobic, Gram-negative bacteria that possess considerable scientific and practical importance. Pseudomonads are among the most active participants in the process of mineralization of organic matter in nature, a role that can be inferred from their widespread occurrence in soil and water (Palleroni, 1975). The cells of *Pseudomonas* are typically straight rods. Strains of fluorescent pseudomonads, including *P. putida*, often have longer cells than other species in the genus. *Pseudomonas putida* can utilize a large number of organic compounds for growth in an otherwise purely mineral solution that contains only an ammonium salt as the nitrogen source (den Dooren de Jong, 1927). Growth and survival of *P. putida*, including its mutants and genetically engineered strains, in soil and rhizosphere have recently attracted the interest of researchers for their environmental and agricultural aspects (Compeau et al., 1988; Yeung et al., 1988; Tang, 1992).

Rhizobium, the best known genus of N_2-fixing bacteria within the Rhizobiaceae, are Gram-negative bacteria that selectively infect the roots of plants, causing the formation of root nodules in which symbiotic N_2-fixing and possible other interactions take place. The ability to form root nodules with *Rhizobium* is generally restricted to plants of the Leguminosae and plants in the genus *Parasponia* (family Ulmaceae) (Okon and Hadar, 1987; Becking, 1992). The symbiotic relationship between *Rhizobium* and legumes is one of the most widely studied mutually beneficial plant-microbial associations and has been utilized in agriculture and forestry for over a century. Inoculation with *Rhizobium* usually yields a beneficial effect upon legumes and non-legume companion plants and also on succeeding crops planted in the same soil (Sharma and Bajpai, 1989). This growth

promotive effect on non-legume species, such as maize, can even be achieved by just growing the plant in a low nutrient sandy loam inoculated with 4.1% (v/v) legume soils (Fyson and Oaks, 1990). However, Fyson and Oaks (1990) believed that fungi rather than bacteria in the alfalfa soil were involved in the shoot growth promotion of maize seedlings.

Actually, it is common knowledge in farming that rotation of maize and other non-legume crops with legumes can overcome yield declines of the primary crop. It has also been noted that the benefit of a previous legume crop is often more than can be accounted for residual nitrogen (Bolton *et al.*, 1976; Hesterman *et al.*, 1986).

The morphology, physiology and genetics of rhizobial bacteria have been investigated for many years in association with the process of biological N_2 fixation, and is now at quite an exciting stage (Stacey *et al.*, 1992; Long and Staskawicz, 1993). With the help of modern methods of biological analysis, especially the tools of molecular biology, information accumulation about PGPR and PGPR-plant interactions has been exponential over the past decade. Nevertheless, more questions arise as progress is being made in the area (Lifshitz *et al.*, 1987; Davidson, 1988; Kloepper *et al.*, 1989; de Bruijn *et al.*, 1992; Stacey *et al.*, 1992; Tang 1992; Young *et al.*, 1993).

1-3. Suggested hypotheses on the mechanism(s) of the promotive effects of PGPR on growth of higher plants

Effects of PGPR on plant growth and development are very complicated. The bacteria

may act directly or indirectly, or in both ways, to affect the life processes of the hosts. With regard to indirect effects, PGPR may benefit their host plants through depressing soil-borne pathogens, supplying readily available forms of nitrogen in soils (via N_2 fixation) and improving mineral nutrient uptake, especially of phosphorous and iron. On the other hand, for the direct effect of PGPR on their hosts, evidence points toward microbial-origin plant growth regulators (PGRs) which may subsequently influence biosynthetic and/or metabolic processing of endogenous hormones in the plants. Alternatively, PGPR may secrete biologically active compounds (known or unidentified PGRs) within or adjacent to the host. These bioactive compounds may then regulate expression of gene(s) finally responsible for growth and development of the plant.

Bacterial N_2 fixation

As available N in soils is usually not sufficient for optimal growth, plants often benefit from microbial N_2-fixers in the rhizosphere. Therefore, significant and consistent increases in growth and yield of *Rhizobium*-inoculated crops have been generally attributed to N_2 fixation by the symbiont, and the hypothesis that N_2 fixation was the sole reason why *Rhizobium*-inoculated plants had increased growth and yield was generally accepted for many years. Also, the promotive effect of plant growth by other N_2-fixing PGPR, such as *Azotobacter, Azospirillum* and *Pseudomonas*, is sometimes presumed to be via N_2 fixation (Okon and Hadar, 1987; Chanway *et al.*, 1989; Stacey *et al.*, 1992).

However, the widely accepted "biological N_2 fixation" mechanism has been challenged for many (Allison, 1947; Cooper, 1959; Mishustin and Naumova, 1962; Mishustin, 1970;

Brown, 1974; Elliott and Frederickson, 1987). There is an accumulating body of evidence which indicates that mechanism(s) other than N_2 fixation can also be involved in host-inoculant interactions where growth of the plant is enhanced (Brown, 1974; Van Berkum and Bohlool, 1980; Okon, 1985; Kloepper *et al.*, 1989). It has thus been reported that PGRs, either produced or modified by the microbial inoculant, may play an additional significant role in this beneficial effect of the "N_2-fixing" PGPR on growth and development of inoculated plants (Dullaart, 1967, 1970; Phillips and Torrey, 1970; Puppo and Riguard, 1978; Wang *et al.*, 1982; Badenoch-Jones *et al.*, 1982, 1983; Atzorn *et al.*, 1988; Yahalom *et al.*, 1990; Bhattacharyya and Basu, 1990).

Biological control of soil-borne phytopathogens

Evidence exists that PGPR can benefit higher plants through suppressing deleterious microorganisms in the rhizosphere, thereby reducing the chance that plants will be infected by those pathogens. Such a biological control of plant disease by PGPR could result from antibiosis and/or competition for nutrients against soil-borne pathogens (Kloepper *et al.*, 1980, 1989; Lifshitz *et al.*, 1986; Okon and Hadar, 1987). In fact, this was the mechanism first proposed as the basis of the PGPR effect on plant growth (Kloepper, 1979; Kloepper and Schroth, 1978, 1981; Weller and Cook, 1982; Suslow and Schroth, 1982; Bakker and Schippers, 1987). Applications of selected microorganisms to soil have proven to be a potential non-chemical method of plant disease control (Cook and Baker, 1983).

It has been reported that under low soil iron conditions some *Pseudomonas* PGPR strains may deprive other microorganisms of available Fe^{+++} by producing siderophores that can chelate the ferric ion in the rhizosphere, making it even less available to plant pathogenic or deleterious species, thus suppressing their growth (Kloepper *et al.*, 1980; Geels and Schippers, 1983; Wong and Baker, 1984; Neilands and Leong, 1986).

Alternatively, PGPR may control soil-borne phytopathogenic bacteria and fungi by producing a variety of antibiotics (Leissinger and Margraff, 1979). It has been long known that many microbes can effectively antagonize others (Papavizas and Lewis, 1981), e.g. *Trichoderma* spp. can control *Rhizotonia salani* and *Pythium*, two phytopathogens that induce damping-off disease of very young seedlings (Liu and Baker, 1980; Marshall, 1982; Papavizas *et al.*, 1982). A number of fluorescent *Pseudomonas* strains in the rhizosphere, including *P. putida*, have also been found to have inhibitory effects on the growth of pathogenic microbes (Leissinger and Margraff, 1979; Xu and Gross, 1986). It is thus suggested that pseudomonads and other rhizosphere species of PGPR may stimulate plant growth by production of antibiotic compounds and/or some simple molecules, such as HCN. Those compounds are toxic to, and thus preventive of deleterious soil microorganisms (Leissinger and Margraff, 1979; Howell and Stipanovic, 1979; Weller and Cook, 1983; Neilands and Leong, 1986; Kloepper *et al.*, 1989). Some PGPR strains have actually shown promise as biological control agents to reduce field crop damage caused by major plant pathogens, thereby increasing the yield (Suslow and Schroth, 1982; Weller and Cook, 1983; Bakker and Schippers, 1987; Schipper, 1988; Weller, 1988; Kloepper *et al.*, 1989).

Biological antagonists of phytopathogens, such as fluorescent *Pseudomonas* strains, may be capable of producing both siderophores and antibiotics. However, under certain soil iron conditions the bacterial antagonist may produce only siderophores, or only antibiotics (Leissinger and Margraff, 1979; Xu and Gross, 1986). Biosynthesis of microbial antibiotics, which are secondary metabolites, is usually stimulated in a low phosphorus and iron-rich medium. In contrast, in such a medium siderophore production is suppressed (Leissinger and Margraff, 1979; Xu and Gross, 1986). On the other hand, however, evidence inconsistent with this hypothesis is also considerable. First, most aerobic and facultative anaerobic bacterial species, and virtually all fungi, can produce siderophores (Neilands and Leong, 1986), but not all siderophore producers are plant growth promotive. Second, and most direct, it has been repeatedly shown that *P. putida* strain GR12-2, a siderophore-producing PGPR, can significantly enhance growth of canola seedlings under gnotobiotic (sterile) condition (Lifshitz *et al.*, 1987, 1988; Young *et al.*, 1993). This finding would appear to role out the siderophore mechanism as the sole reason for *Pseudomonas* promoted plant growth.

Improvement of soil mineral nutrient uptake

Improving mineral nutrient uptake of plants by rhizosphere microorganisms is mainly through mineralization of organic phosphorus compounds, probably through the action of phosphatase or by solubilization of inorganic phosphates through the secretion of acidic compounds (Brown, 1974). Either can increase phosphate availability to plant roots, and

thus benefit plants giving better growth and yield under low P soil conditions (Subba Rao, 1982, 1984; Kloepper *et al.*, 1989). In many countries considerable effort has been made to research the practical uses of the "soil bacterial fertilizers", especially phosphate-solubilizing bacteria. For example, "Phosphobacterin" was widely used in former USSR as a soil and seed inoculant, and it was claimed that an average increase in crop yield of approximately 10% occurred in 60% of the trials (Subba Rao, 1984). In India 27% of the trials showed significant increases in yields after soil inoculation (Subba Rao, 1984). The specific microorganism involved was *Bacillus megatherium* var. *phosphatium*. However, published evidence also shows that the mechanism by which the bacterium promotes plant growth may be something other than just an improvement of phosphorus uptake. It was reported that inoculation of *B. megatherium* var. *phosphaticum* could significantly promote the growth of a plant in an environment where the bacterium was already present in sufficient numbers in the soil to solubilize mineral phosphate. If so, then the significant promotion of plant growth after bacterial inoculation may not be an increase of soil P availability to the plant by the so-called "Phosphobacterin" (Smith *et al.*, 1961; Kucey, 1983). Nonetheless, fluorescent *Pseudomonas* PGPR strains, such as *P. putida*, do promote phosphorus uptake by inoculated plants, as shown by use of ^{32}P-labelled inorganic phosphate (Lifshitz *et al.*, 1987). In that study, correlations between root ^{32}P level and root elongation, and shoot ^{32}P level and shoot elongation were significant, suggesting that the increased growth in young canola seedlings by *P. putida* seed inoculation was likely due to improved P uptake (Lifshitz *et al.*, 1987). However, in the same report it was shown that whether or not external K_2HPO_4 was added, bacterial

inoculation always had the same promotive effect on growth of the canola seedlings. This PGPR effect occurred under gnotobiotic conditions with high or low external K_2HPO_4 (Lifshitz *et al.*, 1987). Hence, the bacterium may well have more direct effects on canola growth, in addition to any promotion of P uptake by the plant.

It is also known that some soil microorganisms, including fluorescent pseudomonads, can produce so-called "yellow-green water-soluble siderophores" under iron-deficient conditions. These siderophores can chelate the sparingly water-soluble ferric ions, thus increasing the ability of soil water to solubilize Fe^{+++} from mineral soils containing a low level of exchangeable iron (Powell *et al.*, 1980; Wallace, 1982; Jurkevitch *et al.*, 1988). Therefore, *Pseudomonas* PGPR may promote plant growth by providing plants with additional ferric ions in low iron soils (Lifshitz *et al.*, 1987; Kloepper *et al.* 1989).

1-4. The direct effect of PGPR on growth of higher plants

All of the above-listed mechanisms, e.g. N_2-fixation, biological control of phytopathogens and/or improvement of mineral nutrient availability in the rhizosphere, by which PGPR promote plant growth, and thus subsequentially increase yield, can be classified as indirect effects of the microbes on plants.

The interactions between PGPR and plants depend on very specific conditions. The relationship in a specific microbial-plant symbiosis is likely governed by both the stage of plant ontogeny and environmental factors, especially soil properties. Thus, at various plant life stages, under different environment conditions (especially soil type and quality)

the biotic-relationship may change (Bethlenfalvay *et al.*, 1983; Son and Smith, 1988; Velagaleti *et al.*, 1990; Herdina, 1991). A direct PGPR effect may overlap with a more effective indirect one, and *vice versa.* Thus, under certain circumstances a beneficial microbe might even become ineffective (Hetrick *et al.*, 1987; Anderson and Liberta, 1989) or even disadvantageous to the plant (Buwalda and Goh, 1982; Koide, 1985; Fitter and Nichols, 1988, and ref. therein). This appears to be because rhizosphere microbes rely on the continuous supply of energy and organic materials from the host plant. Thus, in wheat and barley up to 30-40% of photosynthate can be lost via the roots in a non-sterile system (Whipps and Lynch, 1983, 1985). This increased translocation of photosynthate, resulting from microbial infection, is associated with both increased root respiration and loss of organic matter into the soil (Snellgrove *et al.*, 1982). This is considered to be a large cost of energy to the host plant (Koide, 1985). Therefore, when organic assimilates are in short supply and/or when P, N, Fe^{+++}, etc., are abundant in the soil, a usually plant-growth beneficial microorganism may become parasitic to the plant. Hence, the final effects of rhizosphere microorganisms, including PGPR, on the host plant depend upon a wide variety of interacting factors.

There is a fair body of evidence showing that inoculations with PGPR can promote plant growth directly, rather than by the indirect effects discussed earlier. The most notable is the inoculation of canola seeds with *Pseudomonas putida* strain GR12-2, where growth of young canola seedlings under gnotobiotic conditions is significantly promoted (Lifshitz *et al.*, 1987). This indicates a direct interaction occurs between the bacterium and the plant. A similar direct effect on plant growth has also been seen with some

isolates of *Arthrobacter, Bacillus, Enterobacter* and *Serratia* (Okon and Hadar, 1987 and references therein).

More than 100 species of soil bacteria have been shown to produce one or more classes of PGRs, as well as substances which are growth inhibitors (Kampert *et al.*, 1975a, 1975b; Barea *et al.*, 1976; Lieberman, 1979; De Francisco *et al.*, 1985; Lynch, 1985; Hucheson and Kosuge, 1985; Crozier *et al.*, 1987, 1988; Ernstsen *et al.*, 1987; Arshad and Frankenberger, 1988, 1989). Evidence showing the uptake of microbe-origin PGRs by the plant has also been reported (Libbert and Silhengst, 1970). It is a generally accepted concept that growth and development of a higher plant can be controlled by a wide variety of PGRs (see articles in Pharis and Reid, 1985; Davies, 1988). Thus, alterations in growth and development of a higher plant in response to soil microorganisms may be due to microbial-origin PGRs being taken up by the plant, and/or to the microbe's regulating the plant's biosynthesis of endogenous hormones through as yet unknown control mechanisms.

As a challenge to the common knowledge that *Rhizobium* inocula promote growth of leguminous plants primarily through N_2-fixation in root nodules, Bhattacharyya and Basu (1990) found that in two *Rhizobium*-inoculated legumes (one tree, one shrub) levels of endogenous IAA-like, GA-like and cytokinin-like compounds were higher in mature nodules than in young or old ones, and also higher in nodules than in roots. This implies that plant hormones produced during the microbe-plant interaction may be involved in the symbiotic precess. In a more direct experiment, it was shown that *Bradyrhizobium* strain 127E14, deficient in nitrate reductase activity, can significantly increase internode length

of cv. Henderson lima bean (*Phaseolus lunatus* L.) (Triplett *et al.*, 1981). Nodules formed on lima bean by strain 127E14 contained higher levels (500 ng/g f.w.) of GA-like substances, relative to nearby root tissue (<2.2 ng/g f.w.). Strain 127E14 also enhances stem and petiole length of cowpea (*Vigna unguiculata* L. Wap) (Dobert *et al.*, 1992a). Similar nodules were formed by another *Bradyrhizobium* strain (127E15), which while capable of fixing N_2, is less promotive for lima bean shoot elongation than 127E14 (Evensen and Blevins, 1981; Triplett *et al.*, 1981). Nodules infected with strain 127E14 contained up to 50 times the level of GA-like substances than nodules infected with strain 127E15 (Evensen and Blevins, 1981). It was later confirmed by GC-MS-SIM that the levels of GA_{19}, GA_{20} and GA_{44} (but not GA_1) are much greater in nodule tissue infected by strain 127E14 than for strain 127E15 nodules (Dobert *et al.*, 1992b). Also the effect of the *Bradyrhizobium* strains on elongation response of the inoculated plants can be mimicked by exogenous GA_3 application and suppressed by treating plants with inhibitors of GA biosynthesis (Evensen and Blevins, 1981; Dobert *et al.*, 1992a).

There is also other evidence that a plant's response to applied PGRs can mimic inoculations with beneficial soil microorganisms (Brown *et al.*, 1968; Azcòn *et al.*, 1978; Tien *et al.*, 1979; Hubbell *et al.*, 1979; Young *et al.*, 1993).

All five of the main classes of plant hormones may be involved in the interactions between plants and soil microflora, not only plant growth-beneficial microflora, but also phytopathogens. Supporting this hypothesis are papers by Puppo and Rigaurd (1978), Dullaart (1970), Wang *et al.*, (1982), Badenoch-Jones *et al.* (1982, 1983), Arshad and Frankenberger (1988), Yahalom *et al.* (1990), and Dobert *et al.* (1992a, 1992b).

1-5. Goals of the study

As noted above, plant growth and development responses to PGPR inoculation are unlikely to be due solely to indirect effects, e.g., N_2-fixation, biological control and/or enhanced mineral nutrient uptake. Thus, the mechanism(s) by which PGPR directly promote plant growth should be determined. This study proposed to examine the hormonal basis of the direct effect of PGPR on higher plants using *Pseudomonas putida* strain GR12-2 and *Rhizobium* spp. with canola cv. Tobin and lettuce cv. Grand Rapids as plant species.

Success in this study should improve our understanding of the mechanism(s) of the direct PGPR effect on the promotion of plant growth, and on subsequent increase in yields. Such an insight could be helpful in the development of theories and practical methods for the use of PGPR in agriculture, horticulture and forestry.

CHAPTER 2

GERMINATION AND EARLY GROWTH OF CANOLA AND LETTUCE, AND THE EFFECT OF LIGHT

2-1. Introduction

The Brassicaceae (mustard family) possesses a number of economically important species, among which is canola. Canola is a widely grown vegetable oil crop in many countries, including Canada (Agriculture Canada, 1988). The name "canola" is designated for oilseed rape varieties (*Brassica campestris* and *B. napus*), which have less than 2% erucic acid in the oil, less than 30 micromoles of glucosinilate per gram in the meal (Kneen, 1992) and contain high levels of mono-unsaturated fatty acids (Taylor *et al.*, 1990). Therefore, canola has attracted the interest of farmers all over the world in recent years, and now ranks second to wheat in Canada as a very economically important crop (More, 1992). Canola is also an attractive species to plant physiologists for studying growth and development processes which are regulated by endogenous and/or exogenously applied hormones, especially the gibberellins (GAs) (Suge and Takahashi, 1982; Rood *et al.*, 1987, 1989; Hedden *et al.*, 1989; Sheng *et al.*, 1992a, 1992b; Zanewich, 1993).

The physiology of lettuce (*Lactuca sativa*) seed dormancy and germination has been widely studied (see Khan, 1982; Mayer and Poljakoff-Mayber, 1989). However, post-

germination growth of very young lettuce seedlings is rarely investigated. Due to their economic and physiological significance, canola and lettuce were chosen in this study as the major plant species for examining the direct effect conferred by PGPR on germination and early growth of higher plants.

Germination of the seed is the resumption of growth of the embryo. Many laboratory workers take protrusion of the radicle, the seed embryonic root, through the testa (seed coat) as the culmination of germination. During germination the renewal of metabolic activity is ultimately towards growth. Different parts of the embryo start growing at different times after the onset of imbibition. In many species, radicle growth precedes epicotyl growth by many hours, hence much research has been carried out on the radicle of the germinating seeds. Although it is important to distinguish early growth of a seedling from the preceding germination process, it is not all that easy. The exact stage at which germination ends and growth begins is quite difficult to define. Figure 2-1 demonstrates the basic processes of seed germination and post-germination growth of canola and lettuce. It also demonstrates how I divided the two physiological stages, e.g. seed germination and seedling early growth, from each other. The terms "post-germination growth" and "early growth" are both used in this dissertation for the stage from the radicle protrusion to emergence of the first foliage leaf.

To examine the direct effects of PGPR on seed germination and seedling early growth, it is essential to understand all parts of the seed germination process, and of early seedling growth. This chapter thus examines the processes of canola and lettuce seed germination and seedling post-germination growth under microbe-free (gnotobiotic) conditions.

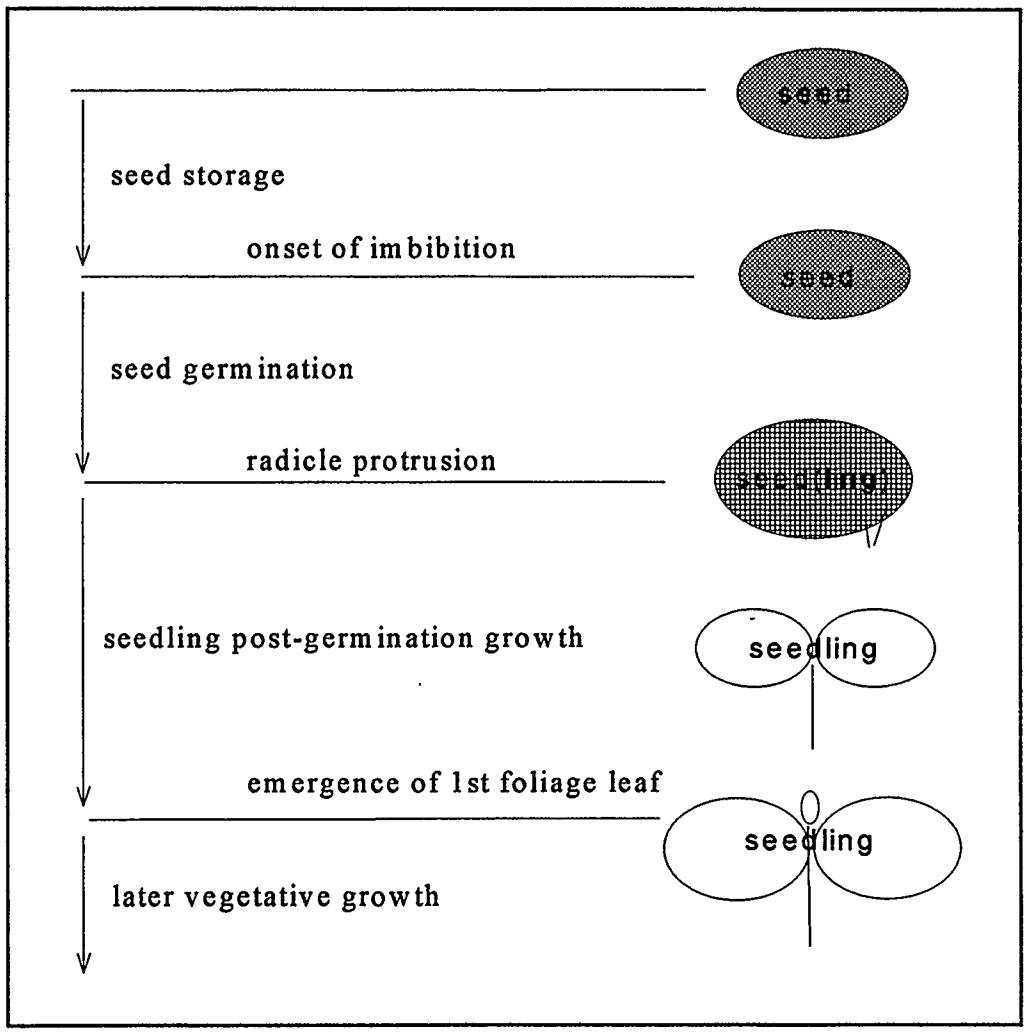


Figure 2-1. Scheme of seed germination and early seedling growth of canola and lettuce, showing the germination process starting from seed imbibition and ending with radicle protrusion. The post-germination growth stage starts from radicle protrusion and ends with emergence of the first foliage leaf.

2-2. Materials and Methods

A gnotobiotic growth pouch assay system was adapted from Lifshitz *et al.* (1987) for use in this study. Growth pouches (Vaughan's Seed Company, Downers Grove, IL USA) were filled with deionized water (10 ml for each), wrapped in two layers of aluminium foil, and autoclaved at 121° C for 20 min before use.

Dry seeds of lettuce (*Lactuca sativa* cv. Grand Rapids) and canola (*Brassica campestris* cv. Tobin) were surface-sterilized in 1% sodium hypochlorite (1/6 dilution of Javex bleach) for 10 min, then thoroughly rinsed with autoclaved deionized water.

The surface-sterilized seeds were soaked in 0.1 M $MgSO_4$ solution for 60 min, then eight (canola) or nine (lettuce) seeds were placed (evenly-spaced) into each growth pouch. The seeded pouches were vertically positioned in a sterilized tray either under continuous cool-white fluorescent light (9.01 $\mu mol/cm^2/s$ of photosynthetically active rediation; R/FR ratio = 6.25) or in a dark room at 23° C. Thirty pouches were used for each species incubated in the light, and 90 pouches were used for each species incubated in the dark.

Germination was examined every 6 h, and growth of the seedlings was determined every 12 h until 180 h (7.5 days) after seed imbibition (ASI).

Canola *Brassica rapa* cv. *rosette* (Carolina Biological Supply Company, Burlington, NC, USA), was also assayed in the growth pouch system under the above conditions (e.g cool-white fluorescent light vs. dark) as was done for canola cv. Tobin. This *rosette* cultivar is a homozygous ros/ros mutant, deficient in growth-effective GAs, such as GA_1 and GA_3 (Rood *et al.*, 1989). Ten pouches were set up for each treatment, and the early

growth of *rosette* was examined 84 h ASI. It should be noted that the GA status in *rosette* seedlings at this time may still reflect cotyledon-stored GAs, and/or residual GA_3 applied to the maternal parent plant to induce flowering and normal seed development..

Length of roots and hypocotyls of young canola and lettuce seedlings were measured at each sampling time. Root and hypocotyl fresh weight (fw) of seedlings was determined at last sampling (180 h ASI). The roots and hypocotyls were divided by cutting at the root-shoot joint. Growth data were subjected to an independent t-Test (two tails), and were plotted as mean values with error bars at 95% confidence intervals using Sigma Plot Scientific Graphing Software (©1985-1993 Jandel Scientific Corporation, San Rafael, CA, USA). This data analysis method was used throughout unless otherwise stated.

2-3. Results

Germination and early root elongation of canola cv.Tobin

Figure 2-2 shows seed germination and early root elongation of canola cv. Tobin in continuous fluorescent light or in the dark. Tobin seeds did not start to break the testa until 12 h after seed imbibition (ASI). Germination (ca. 50%) occurred with radicle protrusion at about 18 h ASI. Neither germination speed nor frequency were significantly altered by the light, relative to the dark. Root elongation of young canola seedlings was slow in the first 6 h after germination (24 h ASI), and rapid from 24 h to 132 h ASI, then

again slowed slightly (Fig. 2-2). There was no significant difference in root elongation of canola seedlings grown in the light versus those grown in the dark, over the 7.5 day period of post-germination growth (Fig. 2-2).

Seed germination and early root elongation of lettuce

Unlike canola, the cool-white light had a profound effect on germination and early growth of lettuce cv. Grand Rapids (Fig. 2-3). Germination of lettuce seeds reached 50% about 12 h ASI in the light. However, it took 18 h in the dark. Lettuce seedling root elongation was also significantly altered (promoted) by light after 48 h ASI (Fig. 2-3). The pattern of lettuce root elongation was somewhat similar to canola during the post-germination growth. That is, rapid growth began about 6 h after radicle protrusion, lasting for about 100 h. The growth then slowed slightly towards the end of the observation period (Fig. 2-3).

Effect of light on hypocotyl elongation of canola and lettuce

Hypocotyl elongation of young canola and lettuce seedlings started about 12 h later than root elongation, and was significantly affected by use of the light (Figs. 2-4 & 2-5). Hypocotyl growth of both species was significantly inhibited by the cool-white fluorescent light, relative to dark, from about 60 h ASI. This effect became more pronounced with time, especially for canola. When measured at 84 h ASI, hypocotyl length of canola

seedlings grown in light was 6 mm shorter than for plants grown in the dark. At 168 h ASI, hypocotyls of light-grown seedlings were 16 mm shorter than those of dark-grown ones (Fig. 2-4). The trend is similar for lettuce, although the difference was not so pronounced as with canola (Fig. 2-5).

Growth responses of canola *rosette* seedlings to light conditions

The use of light did not significantly affect root elongation of young *Brassica rapa* cv. *rosette* seedlings grown in growth pouches (Fig. 2-6). Root length of *rosette* seedlings grown in the dark or in the light matched that (ca. 45 mm long) of the normal *B. campestris* cv. Tobin, when measured at 84 h ASI (Fig. 2-2). However, hypocotyl length of the 84-h-old *rosette* seedlings was highly significantly ($P \leq 0.001$) reduced under light, relative to dark (Fig. 2-6). The average hypocotyl length was 12 mm and 22 mm for light-grown and dark-grown *rosette* seedlings, respectively.

Effect of light on fresh weight of young canola and lettuce seedlings

The light vs dark regimens had a pronounced effect on fw of roots and hypocotyls of lettuce seedlings and hypocotyls (but not roots) of canola seedlings. The hypocotyl fw of dark-grown canola and lettuce was significantly greater than those grown in light. In contrast, average root fw of lettuce was significantly reduced in the dark (Table 2-1).

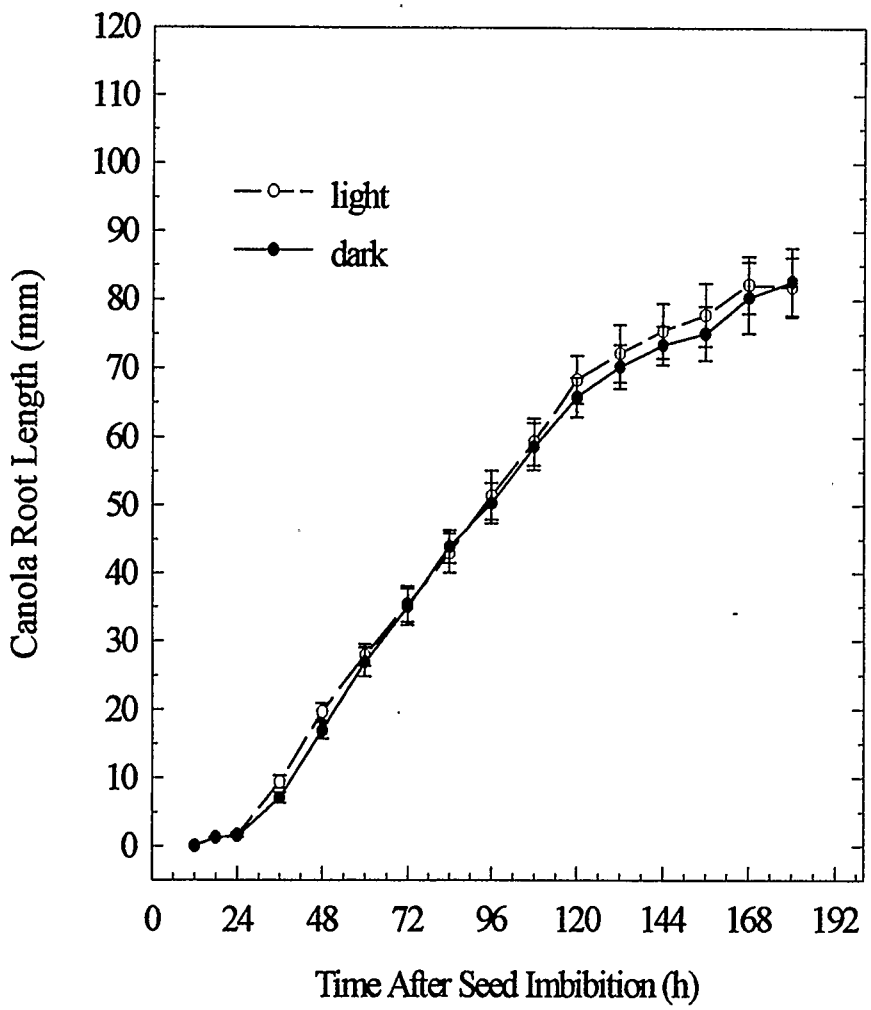


Figure 2-2. Germination and early root elongation of canola cv. Tobin seedlings grown in gnotobiotic growth pouches under fluorescent light vs. dark at 23° C. Values represent the mean of 40 measurements. Error bars represent 95% confidence intervals.

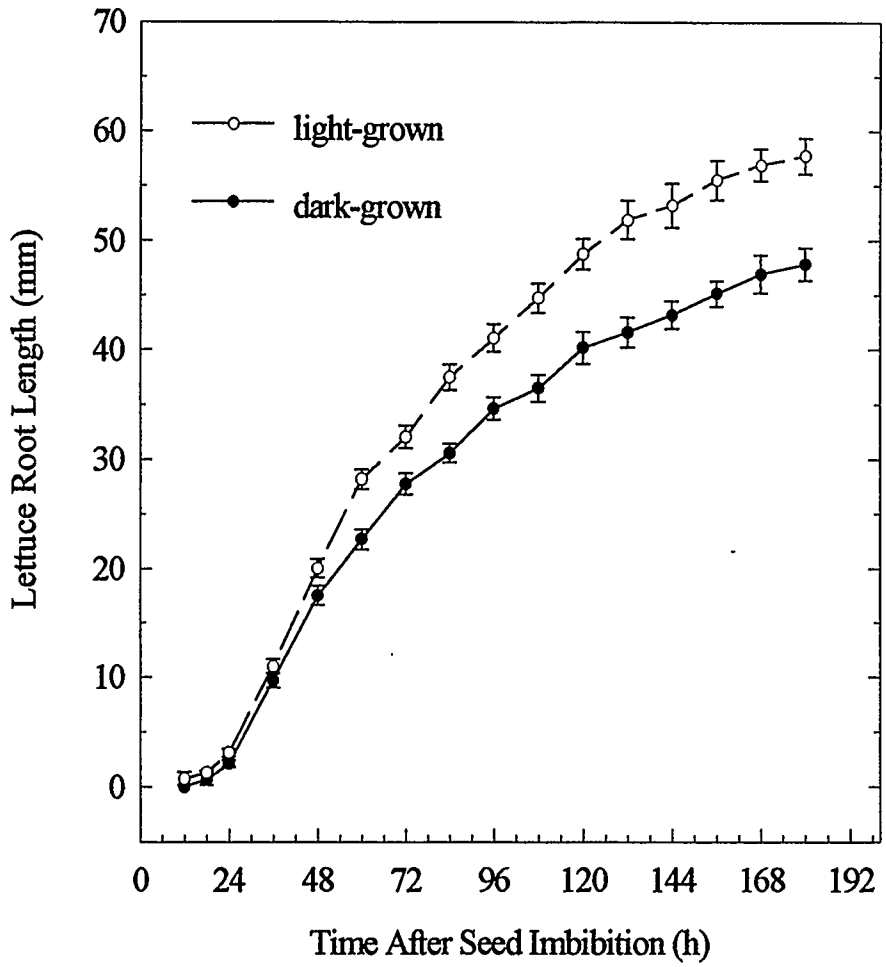


Figure 2-3. Germination and early root elongation of lettuce cv. Grand Rapids seedlings grown in gnotobiotic growth pouches under fluorescent light vs. dark at 23° C. Values represent the mean of 40 measurements. Error bars represent 95% confidence intervals.

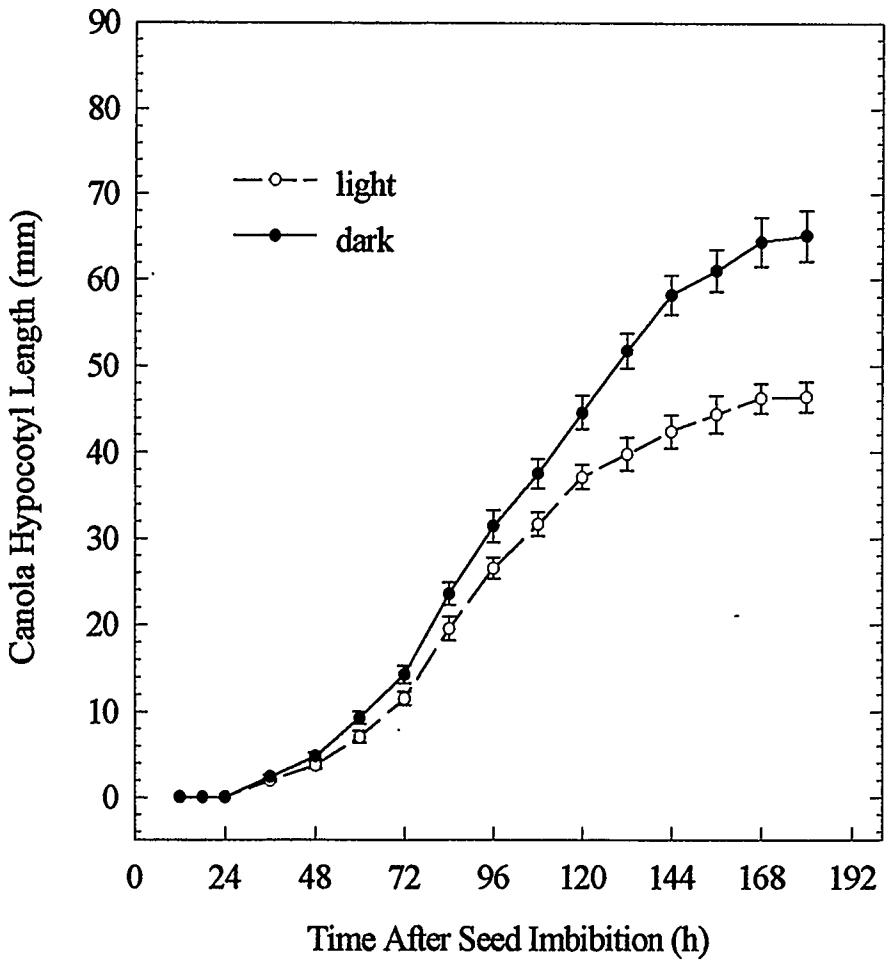


Figure 2-4. The effect of light on hypocotyl elongation of young canola cv. Tobin seedlings grown in gnotobiotic growth pouches at 23° C, under fluorescent light vs. dark. Values represent the mean of 40 measurements. Error bars represent 95% confidence intervals.

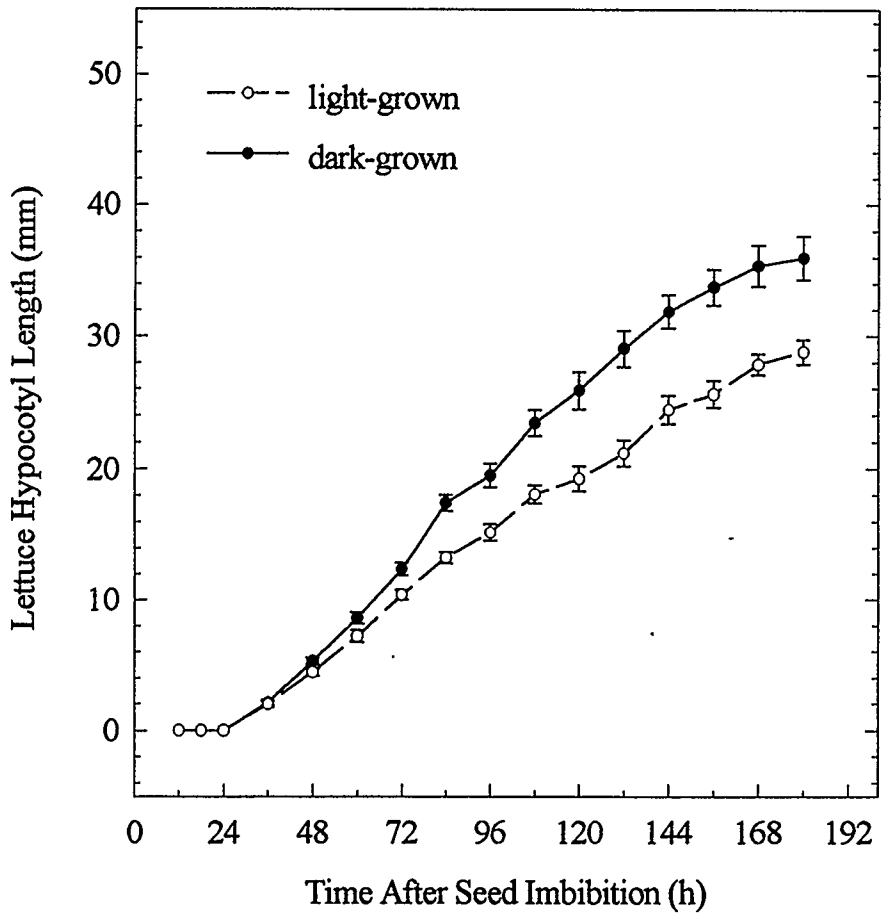


Figure 2-5. The effect of light on hypocotyl elongation of young lettuce cv. Grand Rapids seedlings grown in gnotobiotic growth pouches at 23° C, under fluorescent light vs. dark. Values represent the mean of 40 measurements. Error bars represent 95% confidence intervals.

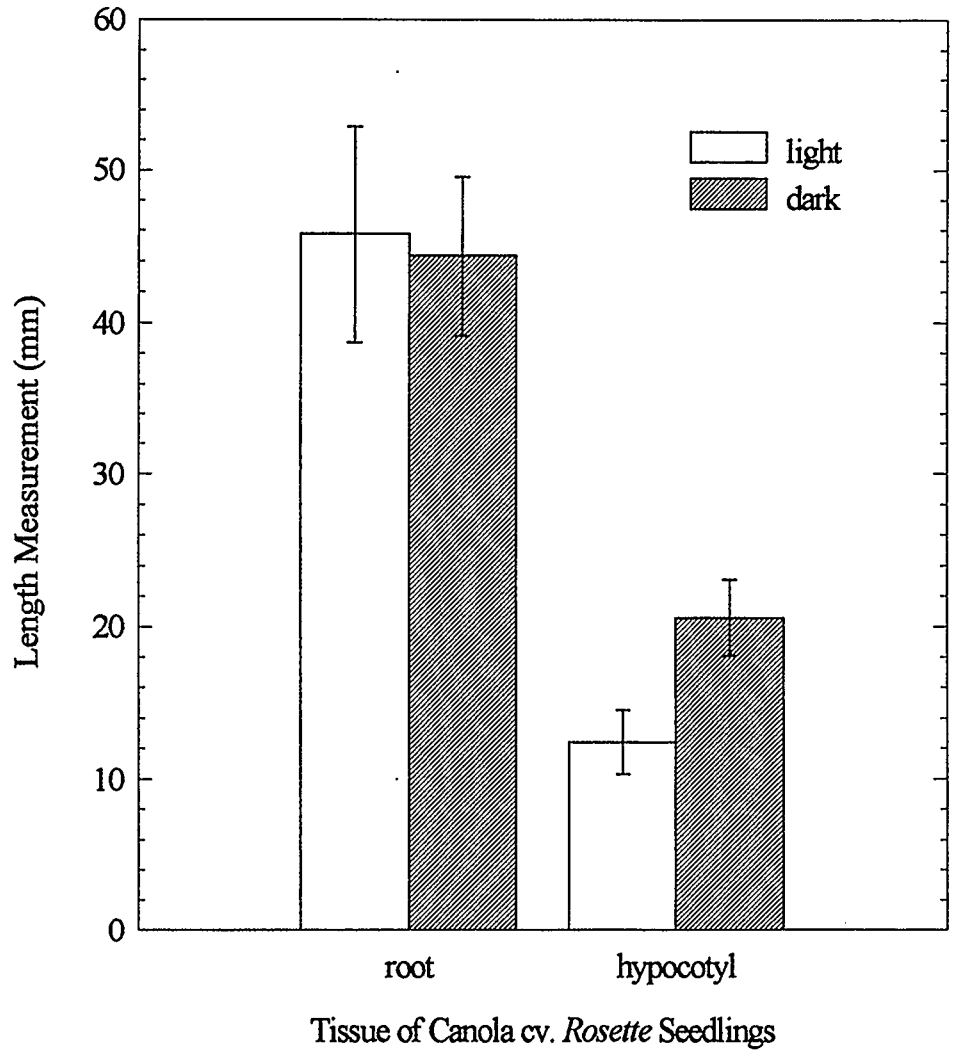


Figure 2-6. The effect of fluorescent light on root and hypocotyl elongation of 84-h-old canola cv. *rosette* seedlings grown in gnotobiotic growth pouches at room temperature (23° C). Values represent the mean of 20 measurements. Error bars represent 95% confidence intervals.

Table 2-1. The effect of light on fw of roots and hypocotyls of 180-h-old canola and lettuce seedlings germinated and grown in gnotobiotic growth pouches.

plant species	fw (mg) of five roots (mean ± s.e.)		fw (mg) of five hypocotyls (mean ± s.e.)	
	light	dark	light	dark
canola	38.05 ± 1.39	37.64 ± 1.65	122.75 ± 3.02	160.25 ± 4.69
95% interval	2.07	3.06	6.30	10.21
lettuce	18.37 ± 0.54	13.88 ±0.36	55.67 ± 1.26	61.04 ± 1.62
95% interval	1.11	0.76	3.17	3.43

2-4. Discussion

This study is one of only a few to assess the process of post-germination growth of very young seedlings under different light conditions (see Bewley and Black, 1983; Mayer and Poljakoff-Mayber, 1989; and references therein). The significant differences in hypocotyl growth of canola and lettuce, as well as in root growth of lettuce, caused by light vs dark are probably phytochrome mediated (see Kendrick and Kronenberg, 1986;

Amritphale *et al.*, 1992; Garcia and Martinez-Garcia, 1993; Kobayashi *et al.*, 1993; Weller *et al.*, 1993). It also likely involves PGRs, since light is known to be an effective regulator of biosynthesis of plant hormones, such as ABA (Tillberg, 1992), ethylene (Michalczuk and Rudnicki, 1993) and GAs in particular (Durley *et al.*, 1976; Garcia and Martinez-Garcia, 1993; Rood *et al.*, 1993; Toyomaso *et al.*, 1993; Weller *et al.*, 1993; Zeevaart, 1993). The fact that the fw of whole lettuce seedlings was not affected at all (Table 2-1) suggests that the effect of light on post-germination growth of lettuce is mainly on re-distribution of assimilate (from cotyledons) between the root and the hypocotyl. However, the mechanism of how light affects fw distribution between these tissues remains unknown.

Photomorphogenetic effects of light on plant stem elongation have been noted for many years (see Darwin, 1881; Kendrick and Kronenberg, 1986). In the very early growth stage, elongation of embryonic roots and hypocotyls of canola and lettuce is unlikely to be associated with photosynthesis. Light in low doses may thus act like a trigger to initiate photomorphogenetic events (Salisbury and Ross, 1985; Kendrick and Kronenberg, 1986). The light effect on seedling elongation occurs quite soon after emergence of embryonic root and shoot (Fig. 2-5). The inhibitory effect of light on hypocotyl elongation during post-germination growth of canola and lettuce is very possibly caused by modifications in GA biosynthesis (Rood, 1993; Weller *et al.*, 1993; García-Martínez and Martínez-García, 1993; Toyomaso *et al.*, 1993; Zeevaart, 1993).

A similar effect of light on canola shoot growth has been recently reported (Rood *et al.*, 1993; Potter and Rood, 1993). They showed that GA metabolism in *Brassica rapa*

was controlled by light, the taller dark-grown seedlings maintaining a higher proportion of free [^{3}H]-GA_{20} and [^{3}H]-GA_1 (noted after feeding [^{3}H]-GA_{20}) than the shorter light-grown ones. Conversely, levels of putative [^{3}H]-GA glucosyl conjugates were higher in light- than in dark-grown seedlings (Rood *et al.*, 1993). Endogenous GAs such as $GA_{1,3,8,19 \text{ and } 20}$, decreased under conditions with a higher light intensity, suggesting that low light intensity preferentially increases free GA content and this increase was probably responsible for the increased shoot elongation (Potter and Rood, 1993).

Root growth of Grand Rapids lettuce was effectively promoted by light (Fig. 2-3). However, the effects of light on lettuce root elongation during post-germination growth are not well documented. It is known that GA metabolism in lettuce roots can be regulated by light, and roots of dark-grown lettuce seedlings retained higher amounts of precursor [^{3}H]GA_4 and, yet still converted more [^{3}H]GA_4 to [^{3}H]GA_1 (e.g. 2x more) than roots of seedlings grown in the light (Durley *et al.*, 1976). On the other hand, Tanimoto and Watanabe (1986) suggested that plant roots need only very low levels of GAs for their normal growth. Thus GA content in lettuce roots may be high enough to be growth-inhibitory for root elongation, and this could be the situation for many plants. Thus, the promotion of lettuce root growth (Fig. 2-5) could also result from light-reduced free GA levels (Rood, 1993).

The promotive effect of dark on hypocotyl elongation of the dwarf mutant *rosette* has not been well studied. The very significant increase in shoot length growth under dark conditions may be due to a leaky expression of the ros/ros gene responsible for early

stages of GA metabolism. This, in turn, would increase the level of growth-effective GAs, such as GA_1, GA_{85} and/or GA_3 in the hypocotyls. Alternatively, there may be residual GA_3 in the cotyledons (from treatment of the maternal plant), and dark conditions may sensitize the effect of the hypocotyl to the residual GA_3, or influence its glucosylation.

Plant hypocotyl elongation can be also controlled (or co-controlled) by ethylene production in the tissue. Inhibition of shoot elongation of young lettuce and canola (cvs. Tobin and *rosette*) seedlings under the fluorescent light may thus be causally associated with an increased ethylene production induced by light (Craker *et al.*, 1971; Bucher and Pilet, 1981; Toppan and Esquerre-Tugaye, 1984; Tajiri, 1986; Rengel and Kordan, 1987; Wien *at al.*, 1989). However, ethylene production in plants can be either promoted or inhibited by light, depending on light quality and quantity, and on the type of plant tissue involved (Abeles *et al.*, 1992). As GA metabolism in the sunflower hypocotyl can be regulated by ethylene (Pearce *et al.*, 1991), and *vice versa* in the cowpea epicotyl (Garcia-Martinez *et al.*, 1984), the depressed hypocotyl elongation in light for young seedlings of cvs. Tobin, *rosette* and Grand Rapids could be the result of balance/interactions between more than one plant hormone (Stewart *et al.*, 1974; Garcia-Martinez *et al.*, 1984; Ross and Reid, 1986).

CHAPTER 3

DISTRIBUTION OF ENDOGENOUS HORMONES IN VERY YOUNG CANOLA SEEDLINGS

The literature and the results in Chapter 2 suggest that PGRs, especially GAs, are involved in the regulation of germination and early growth of canola, lettuce and possibly many other plant species.

A good understanding of the early growth processes of a young seedling requires a clear picture of the distribution of endogenous hormones during the post-germination rapid growth phase of the plant. For 'adult' canola plants the GA physiology of stem elongation, flowering induction and vernalization has been studied in reasonable depth (Rood *et al.*, 1987, 1989a, 1989b, 1993; Mandel *et al.*, 1991; Zanewich, 1993). However, hormonal aspects of germination and post-germination growth of canola has not yet been studied in any detail.

Therefore, the first step in investigating the direct effect of PGPR on growth of very young canola seedlings was to determine the presence and tissue distribution of GAs, IAA, ABA, JA, ACC, MACC, and ethylene production in the rapidly growing seedlings.

3-1. Identification of endogenous GAs from young seedlings of *Brassica campestris* cv. Tobin

Gibberellins, a hormone family with 90 defined structures at this moment, are defined

by their chemical structures rather than by their biological activities unlike some other plant hormones (e.g. auxins and cytokinins). The GAs are tetracyclic diterpenoid acids derived from four isoprene units. Gibberellins may be grouped into two types: the C_{20} GAs possessing a full complement of 20 carbon atoms, and C_{19} GAs in which carbon-20 has been lost and a γ-lactone bridge on ring A between carbons-10 and -19 has been formed (Crozier and Durley, 1983). A C_{19} GA skeleton in Figure 3-4 shows its carbon numbering and rings A, B, C and D. In canola shoots, over 10 of the known GAs (C_{20} and C_{19}, bioactive and non-bioactive) have been found, and roles in both vegetative growth and flowering have been suggested for certain of these (Suge and Takahashi, 1982; Hedden *et al.*, 1989; Rood *et al.*, 1987, 1989a, 1989b).

Each naturally-occurring GA has been given a trivial number (GA_n) upon definitive chemical characterization in accordance with a convention established by MacMillan and Takahashi (1968). This trivial nomenclature system is used in the present study. Since the introduction of GC-MS techniques, GAs can now be readily identified by comparison of their mass spectra and Kovats' retention indices (KRI, Kovats, 1958) with reference standards (MacMillan and Takahashi, 1968). The structure of a previously unknown compound can often be inferred from information provided by fragment ions present in the mass spectrum, and the authentic GA can then be synthesized. There have been several reports of mass spectrometric studies on GAs (Gilmour, 1983; MacMillan *et al.* 1992; Mander 1992; and references therein). Similarly, identification of other endogenous PGRs from canola seedlings can be also made by comparing their mass spectra and KRIs with those of the corresponding authentic compounds.

Materials and Methods

Plant material. Canola (*Brassica campestris* cv. Tobin) seeds (Pioneer Hybrids, Chatham, Ontario, Canada) were germinated and grown in a standard test sieve (W. S. Tyler Company of Canada Ltd., St. Catherines, Ontario, Canada) under cool-white dim fluorescent light at room temperature (23° C). Seedlings were harvested on day 7 and separated into roots, hypocotyls and cotyledons. The tissue was immediately frozen in liquid N_2 and then lyophilized as described previously (Sheng *et al.*, 1992a, 1992b).

Extraction and Purification. Ten g of lyophilized young cotyledons were ground in liquid N_2 and homogenized in pre-cooled (-25° C) 80% aqueous methanol (MeOH). Extraction of the tissue residue was repeated four times at room temperature. High specific activity standards of [^{3}H]GA_1, [^{3}H]GA_4 (Amersham), and [^{3}H]GA_9 (Yokota) were added to the MeOH extract (1500 Bq each). The 80% MeOH extract was purified by a preparative column of C_{18} material (C_{18}-PC; LiChroprep RP-18 ODS, EM Science, NJ USA). The C_{18}-PC eluate (after being taken to dryness) was then loaded onto a SiO_2 partition column with 5 g of SiO_2 that had been pre-saturated with 0.5 M formate. Free acid forms of hormones were eluted with 100 ml of 0.5 M formate-saturated hexane/ethyl acetate (5/95,v/v). The hexane/ethyl acetate fraction was dried *in vacuo*, and then subjected to high performance liquid chromatography (HPLC). The first HPLC used was a reversed phase C_{18} μBondapak column (30 cm x 3.9 mm i.d., Waters Associates Inc., USA) with a gradient elution program of 10% MeOH in 1% acetic acid (HOAc) for the first 10 min,

10 to 73% MeOH in 1% HOAc over 30 min, 73% MeOH in 1% HOAc for another 10 min, then 100% MeOH for 20 min, all at a flow rate of 2 ml per min. Sixty fractions, one min each from the C_{18} μBondapak HPLC, were taken to dryness in a vacuum desiccator and assayed for GAs by the modified dwarf rice (cv. Tan-ginbozu) micro-drop method (see later for details). Sequential fractions with GA-like activity were grouped and further purified by HPLC on a Nucleosil $N(CH_3)_2$ column (15 cm x 4.6 mm i.d., Alltech Associates Inc., USA) with isocratic elution by 0.1% HOAc in MeOH at 1 ml/min. The one-min fractions from the $N(CH_3)_2$ HPLC were again tested by the dwarf rice micro-drop assay. All fractions with GA-like activity were then subjected to GC-MS for identification of biologically active GAs. Fractions without GA-like activity on dwarf rice micro-drop assay were also scanned on GC-MS for biologically inactive GAs and their related catabolites.

Bioassay A modified dwarf rice (*Oryza sativa* L. cv. Tan-ginbozu) micro-drop assay (Murakami, 1968; Nishijima and Katsura, 1989) was utilized to monitor GA-like activities in the various HPLC fractions. The rice seeds were pretreated with 1 ppm active ingredient of Technical Grade uniconazole (Chevron Chemical Company, San Francisco, CA, USA) for 24 h at 23° C. This reduced the endogenous GA levels in the Tan-ginbozu rice seedlings, thereby making the assay more uniform and sensitive (Nishijima and Katsura, 1989). The uniconazole treated seeds were then placed under running tap water at 32° C for another 24 h to germinate. The germinating seedlings were planted on a 0.8% agar/water medium and grown in a growth chamber (32° C and near 100%

humidity) for 48 h. At this time, application of the HPLC fraction residues was made in either 0.5 or 1 µl of 95% ethanol (1/100 and 1/200 aliquots). The rice seedlings were then kept in the growth chamber for an additional 60 h, at which time the length of the second leaf sheath was measured (Sheng *et al.*, 1992a).

Gas Chromatography-Mass Spectrometry (GC-MS) Purified GA samples were methylated with ethereal diazomethane and trimethylsilylated with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS; Pierce, Rockford, Illinois, USA) as normal (Sheng *et al.*, 1992a). The methyl ester, TMSi ether (MeTMSi) derivative samples of each of authentic GAs and the putative endogenous canola GAs were analyzed by GC-MS. Introduction of each sample was made in one µl of *n*-hexane by cool on-column injection into a DB1-20HS fused silica capillary column (20 m x 0.18 mm i.d., 0.4 µm methyl silicone film) or a DB1-15N fused silica capillary column (15 m X 0.25 mm i.d., 0.25 µm methyl silicone film; J & W Scientific, Folsom, CA, USA) that was installed in a Hewlett Packard (HP) Model 5890 Series II GC system (Hewlett-Packard Co., Palo Alto, CA, USA). The temperature program was 60° C (0.1 min) to 200° C at 20° C/min, and then 4° C/min to 300° C. The helium flow rate was 40 cm/sec at 60° C, programmed for constant flow. The injector pressure was initially 20 psi (15 psi for DB1-15N column) at 60° C, and increased with the increase of temperature. The capillary column was led directly into a HP 5970B mass spectrum detector (MSD). Ionizing voltage was 70 eV. The mass range was set to 50-740 amu, and scanned once every sec. Data acquisition was controlled by a HP 59970 MS Chem Station computer

(Hewlett-Packard Co., Avondale, PA, USA). The KRI of each individual GA on GC-MS was determined according to a co-injected hydrocarbon mixture standard (MacMillan *et al.*, 1992; Sheng *et al.*, 1992a, 1992b).

Results

Identification of a new highly bioactive GA (GA_{85}) from canola

The dwarf rice micro-drop assay detected 4 major regions of GA-like activity over the 60 one-min fractions eluted from the C_{18} μBondapak HPLC (Fig. 3-1). The first region overlapped with the Rt of an external standard of $[^3H]GA_8$, and showed very high GA-like activity. The second bioactive region was located at and near the Rt of the internal $[^3H]GA_1$ standard. The third region occurred at the Rt of the external $[^3H]GA_{20}$ standard. The fourth region included the Rts from $[^3H]GA_4$ to $[^3H]GA_9$ (Fig. 3-1). As GA_8 is a deactivated form of GA_1, the high bioactivity found on the dwarf rice microdrop assay in μBondapak HPLC fractions near GA_8 region (Fig. 3-1) was postulated to be from another GA-like compound(s). Therefore, fractions of GA-like activity around GA_8 region were repurified on a second C_{18} μBondapak HPLC eluted isocratically with 10% MeOH in 1% HOAc. The bioactive GA-like compound(s) was separated from GA_8 on this HPLC using a combination of radioassay and bioassay monitoring (Fig. 3-2). The tentative new GA fraction was then further purified on $N(CH_3)_2$ HPLC prior to GC-MS (Sheng *et al.*, 1992a).

From GC-MS analysis, a novel trihydroxy GA was identified from the bioactive $N(CH_3)_2$ fraction (Rt, 28-30 min). The putative new canola GA (in MeTMSi form) possessed a molecular ion (M^+) of 594 (*m/z*) with the prominent peak at *m/z* = 491 (M^+ - 103). This fragmentation pattern is characteristic of C-12,13-dihydroxy GAs (Chu and Mander, 1988; Nakayama *et al.*, 1990) with one more hydroxy group located elsewhere on the gibbane skeleton. Based on its high bioactivity on dwarf rice micro-drop assay (Figs. 3-1 and 3-2), HPLC Rts and EIMS, the structure of the putative new GA was assumed to be C-12 hydroxylated GA_1. The final identification was based on a GC-MS comparison of the full mass spectrum (MeTMSi) and KRI of the putative new GA with those of authentic 12α-OH GA_1 and 12β-OH GA_1, both synthesized by Prof. L.N. Mander (Australian National University, Canberra, Australia). The new GA was confirmed to be 12α-hydroxy-GA_1, and has now been assigned the trivial name GA_{85} (Sheng *et al.*, 1992a). The full-scan mass spectrum of GA_{85} is shown in Figure 3-3, and a comparison of GC-SIM data (MeTMSi) between the novel GA and authentic standard 12α-hydroxy-GA_1 are shown in Table 3-1. The molecular structure of the new gibberellin (GA_{85}) is shown in Figure 3-4. The ^{1}H-NMR data of 12α-hydroxy GA_1 (GA_{85}) methyl ester in $CDCl_3$, at 300 MHz are given in Table 3-2. The NMR was performed with authentic 12α-hydroxy GA_1 by Prof. L.N. Mander's group (Research School of Chemistry, The Australian National University, Canberra, Australia).

Gibberellin A_{85} is equal in biological activity to GA_1 (Sheng *et al.*, 1992b), and is somewhat more biologically active than GA_3 on the dwarf rice micro-drop bioassay (see Fig. 3-5).

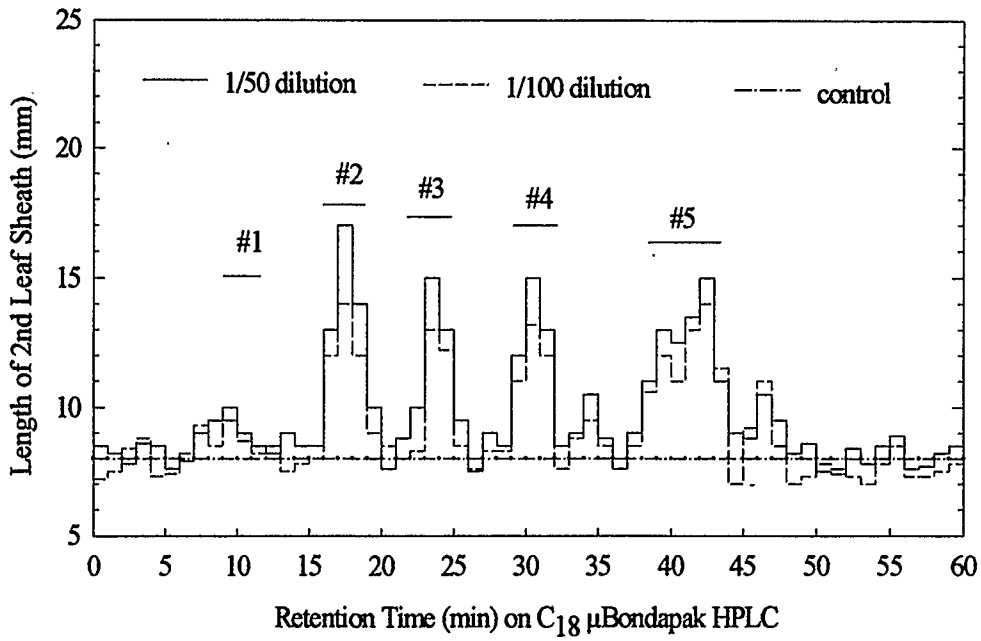


Figure 3-1. Dwarf rice (cv. Tan-ginbozu) micro-drop assay of GA-like activities in fractions of an extract from 7-d-old canola seedlings after C_{18} μBondapak HPLC.

The Rt regions of some authentic GAs (except for GA_{89}) are shown by horizontal bars ——— , based on radioassay, bioassay and/or GC-MS analysis. Symbols: #1, GA_{89}; #2, $GA_{8/85}$; #3, $GA_{1/3}$; #4, GA_{20}; #5, $GA_{4/7/9}$.

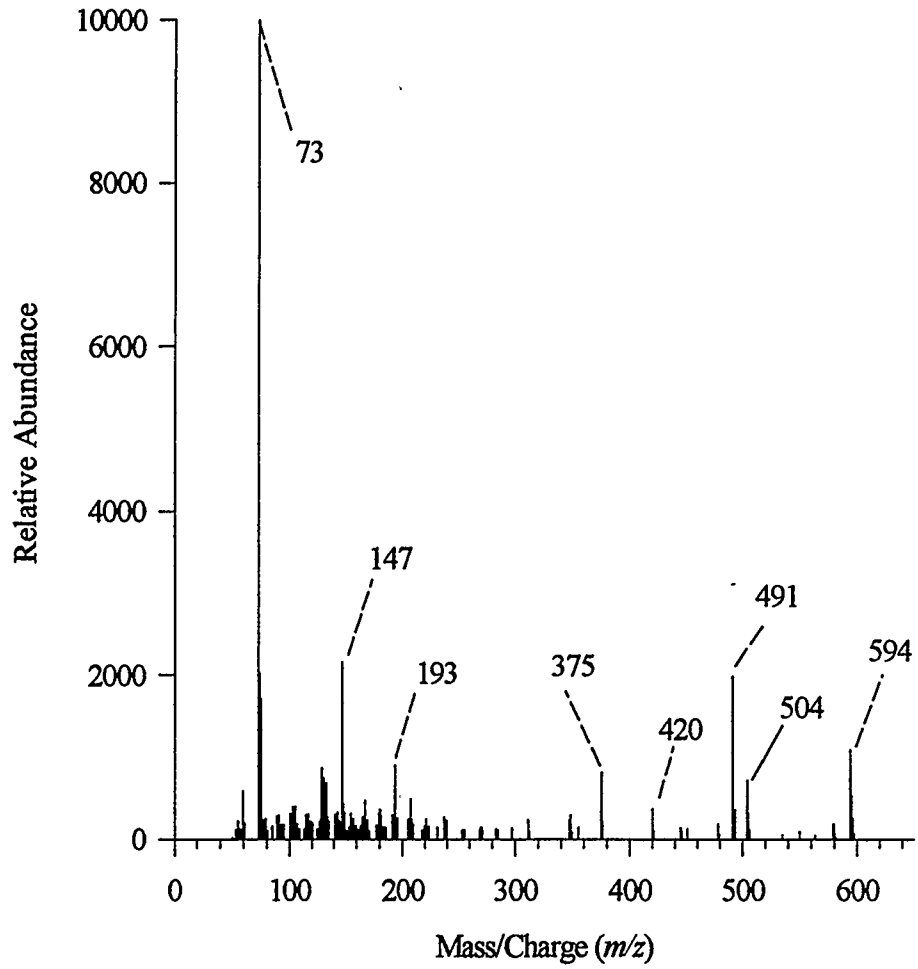


Figure 3-2. Separation of putative canola 12α-hydroxy-GA_1 (GA_{85}) from endogenous GA_8 in fraction No. 2 (Fig. 3-1) on second C_{18} μBondapak HPLC, eluting isocratically with 10% MeOH in 1% HOAc at 2 ml/min. The histogram represents dwarf rice microdrop bioassay activity. The Rt of [^{3}H]GA_8 was determined by radioassay, and the Rt of putative 12α-hydroxy-GA_1 was confirmed by GC-MS.

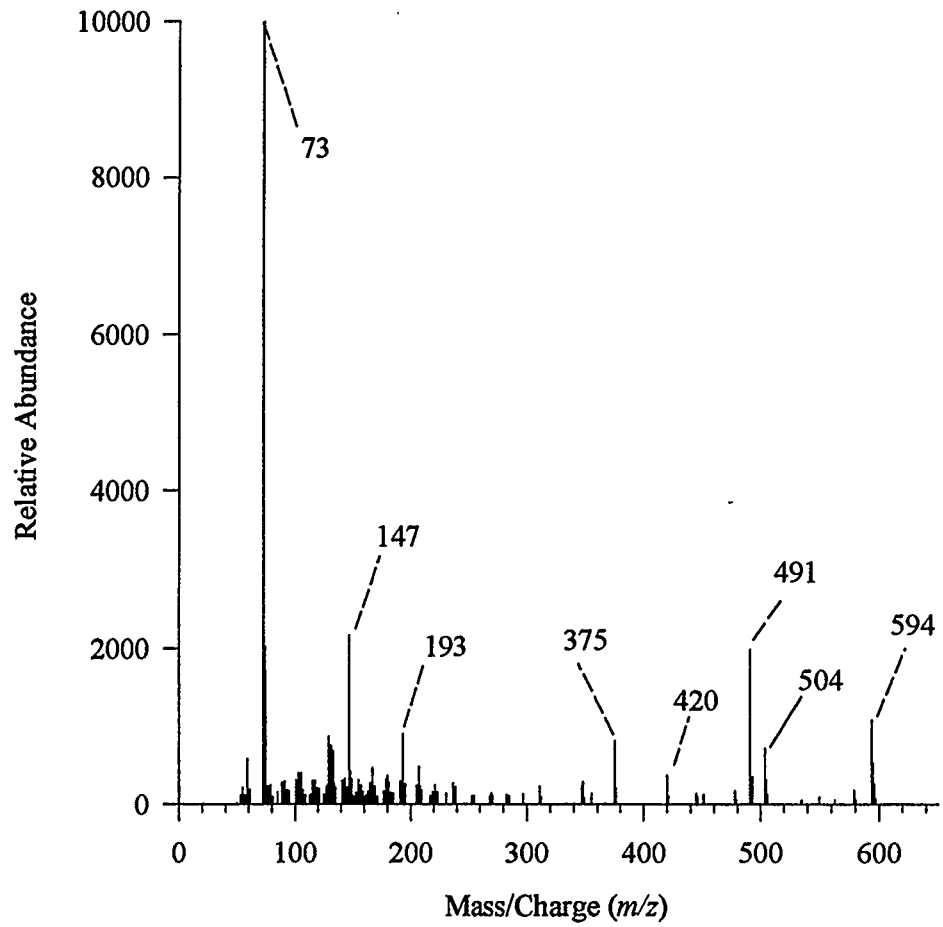
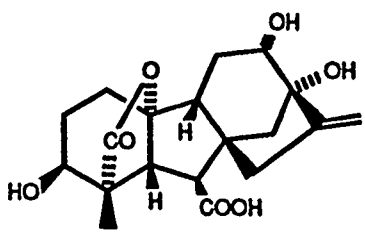
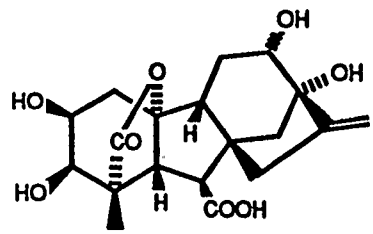


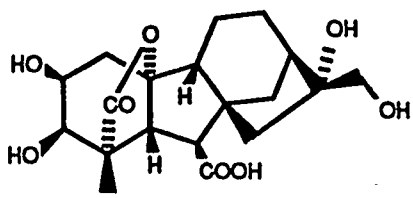
Figure 3-3. Mass spectrum of the highly bioactive novel canola gibberellin (GA_{85}) (as MeTMSi). The spectrum (ion range from *m/z* 50 to *m/z* 600) was determined by GC-MS (EIMS at 70 eV).



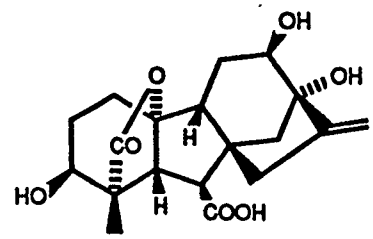
GA_{85}
(12α–OH GA_1)



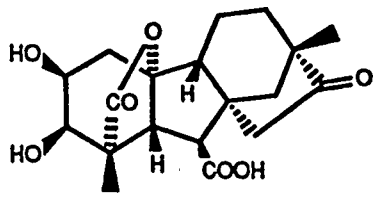
GA_{89}
(2β-OH GA_{85})



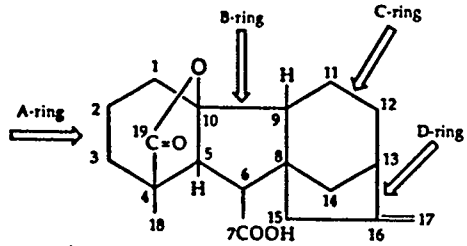
16α,17-dihydroxy GA_{34}



12β–OH GA_1



C/D rearranged GA_8



A skeleton of C_{19} GA

Figure 3-4. Molecular structures of novel GAs and GA-analogues identified from very young canola cv. Tobin seedlings by their full mass spectra and KRIs. For 12β-OH GA_1 KRI and GC-MS-SIM were utilized.

A C_{19} GA chemical skeleton showing carbon atom numbering and rings A, B, C and D is presented here for reference.

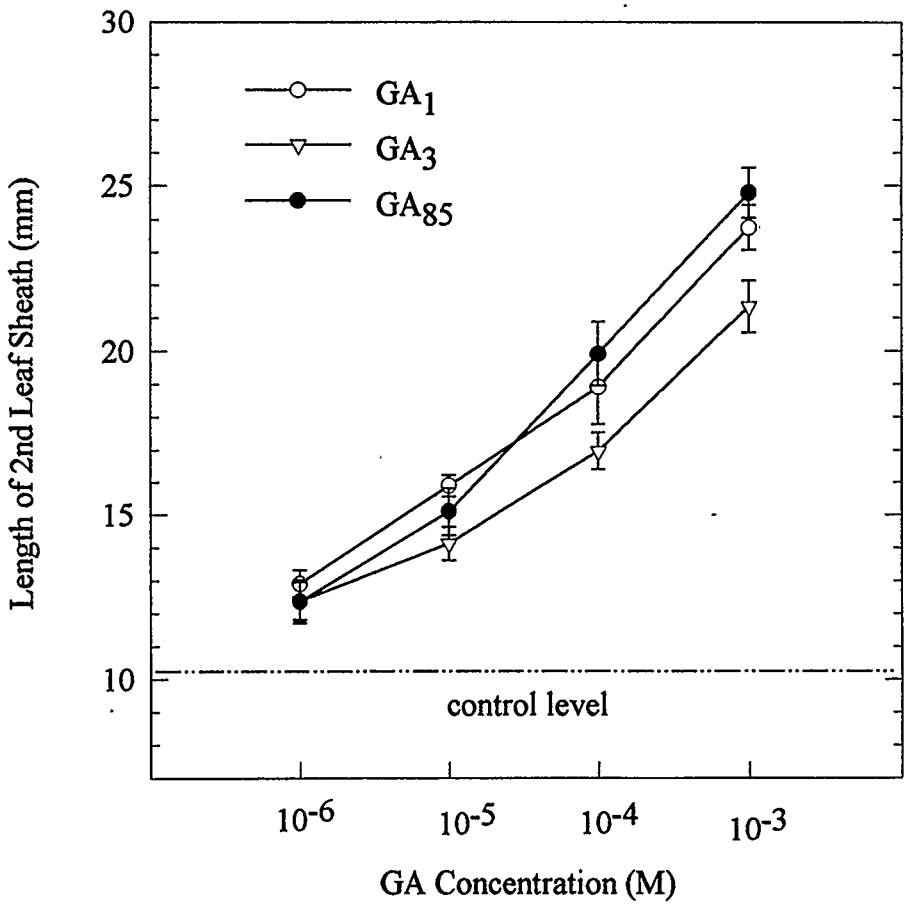


Figure 3-5. Biological activity of GA_{85} (authentic) on dwarf rice (cv. Tan-ginbozu) micro-drop assay relative to GA_1 and GA_3. Rice seeds were pretreated with 1 ppm uniconazole. One µl of each of the GA concentrations was applied to each rice seedling. Values represent the mean of 18 measurements. Error bars represent 95% confidence intervals.

Table 3-1. Comparison of Rts, KRIs and relative abundance of the principal *m/z* ions (normalized to the abundance of the molecular ions) of new canola GAs and GA metabolites (MeTMSi) with those of the corresponding authentic GAs (MeTMSi) on GC-MS-SIM analysis.

GA	Source	KRI	*m/z* Ion /Relative Abundance				
12α-OH-GA_1 (GA_{85})	Puta.	2821	594/100	504/80	491/214	420/41	375/97
	Auth.	2821	594/100	504/80	491/210	420/40	375/95
2β-OH-GA_{85} (GA_{89})	Puta.	2910	682/100	667/11	592/52	579/158	420/70
	Auth.	2910	682/100	667/12	592/52	579/150	420/63
16α,17-diOH GA_{34}	Puta.	3034	684/100	669/33	581/1745	535/36	491/34
	Auth.	3034	684/100	669/32	581/1682	535/34	491/30
C/D re-arranged GA_8	Puta.	2808	522/100	507/2	490/4	447/6	432/3
	Auth.	2808	522/100	507/2	490/3	447/6	432/3

Table 3-1 continued

12β-OH-GA_1	Puta.	2814	594/100	504/67	491/211	420/43	311/43
	Auth.	2814	594/100	504/62	491/210	420/41	311/38

Note: Puta. = putative new GA from canola seedlings; Auth. = authentic GA.

Table 3-2. ^{1}H-NMR data of 12α-hydroxy GA_1 (GA_{85}) methyl ester at 300 MH_Z in $CDCl_3$ provided by Prof. L.N. Mander.

C position	$^{\delta}$H(ppm)	(multiplicity, *J*:H_Z)
1	1.16	(*s*)
2	1.15	(d,d; 11.6,1.5)
3	2.02	(d,11.6)
4	2.2-1.55	(m)
5	2.43	(d,d; 12.9,6.4)
6	2.75	(d,10.8)
7	3.08	(d,10.8)
8	3.64	(d,d; 7.6,6.4)
9	3.78	(s,-OMe)
10	3.84	(d,d; 2.5,2.2)
11	4.18	(d,d; 3.1,2.5)
12	5.4	(d,2.5)
13	5.43	(d,3.1)

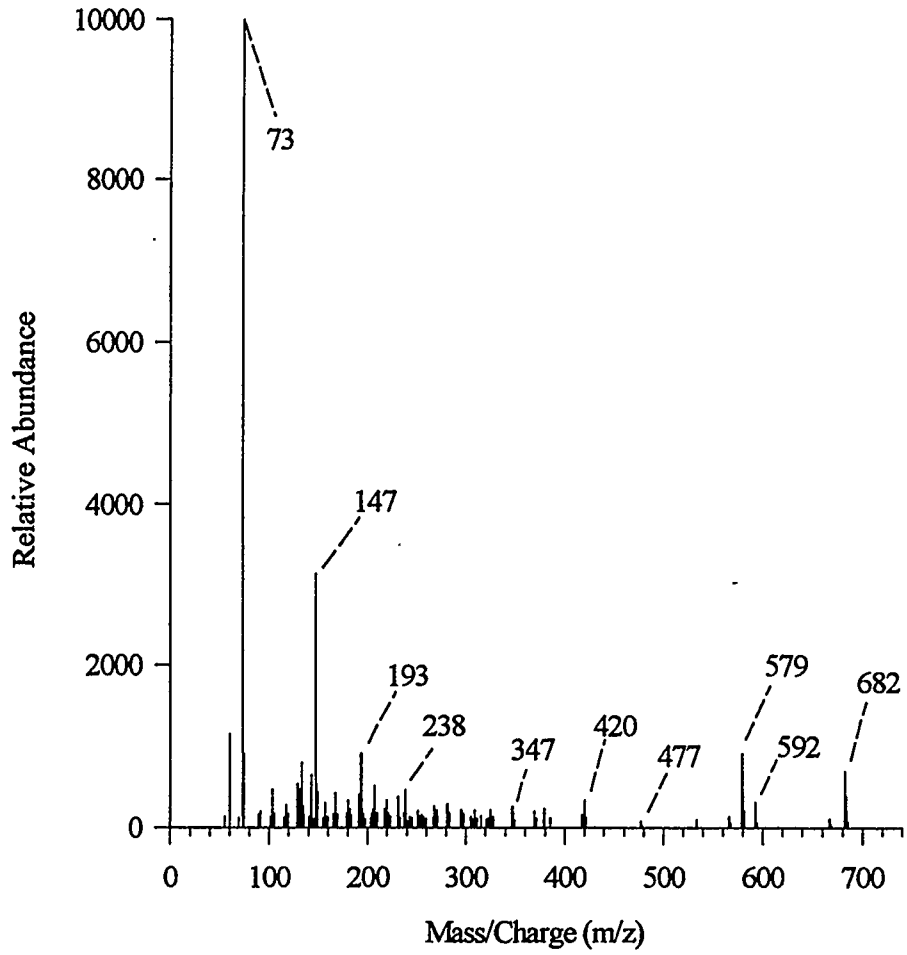


Figure 3-6. Mass spectrum of gibberellin A_{89} (MeTMSi), a deactivated form of GA_{85}, identified in an extract from very young canola cv. Tobin seedlings. The spectrum (ion range from *m/z* 50 to *m/z* 740) was determined by GC-MS (EIMS at 70 eV).

Identification of a new deactivated GA, (GA_{89})

Cotyledons of young canola seedlings also yielded a new tetrahydroxy GA. The Rt of this new GA on C_{18} reversed phase HPLC (10 μm, 10 cm x 8 mm i.d.) was about 2 min earlier than GA_8 when eluted with MeOH-H_2O-HOAc (19:89:1) at ca 500 psi (Sheng *et al.*, 1992b). The mass spectrum of the putative new GA (in MeTMSi form) had a M^+ at *m/z* 682 with a prominent ion at *m/z* 579 [M^+ - 103]. This is indicative of a tetrahydroxy GA with hydroxyls at C-12 and C-13 positions on the C ring (Sheng et al., 1992a; Nakayama et al., 1990; Chu and Mander, 1988) and no double bond in any of the A, B, or C rings. Since high levels of biologically active GA_{85} were found in the same tissues, and the putative tetrahydroxy GA was from a fraction more polar than GA_{85}, and without appreciable biological activity on the dwarf rice micro-drop assay, it was expected that the new GA would be an inactivated form of GA_{85}, e.g., 2β-hydroxy-GA_{85}. Thus, authentic 2β-hydroxy-GA_{85} methyl ester was prepared by Prof. L.N. Mander's group (Research School of Chemistry, The Australian National University, Canberra, Australia). A comparison of the putative new GA's full scan mass spectrum and KRI with those of the authentic standard (MeTMSi) confirmed it to be 2β-hydroxy-GA_{85}, and it has been officially assigned the trivial name gibberellin A_{89} (Sheng *et al.*, 1992b). The molecular structure of GA_{89} is given in Figure 3-4, and its mass spectrum (MeTMSi) is shown in Figure 3-6. The relative abundance of principal ions to the molecular ion (M^+, 682) and KRI of the putative GA_{89} (MeTMSi) are summarized in Table 3-1 in comparison with those of the authentic compound (MeTMSi). ^{1}HNMR (300MHz, $CDCl_3$) data of GA_{89}

methyl ester are as following: δ 1.16 (3H, *s*, 4-Me), 1.45 (6H, *s*, $OC(Me)_2O$), 2.70 (1H, *d*, $J_{6\alpha,5\beta}$ = 11.0 Hz, H-6α), 3.10 (3H, *s*, SO_2Me), 3.14 (1H, *d*, $J_{5\beta,6\alpha}$ = 11.0 Hz, H-5β), 3.71 (3H, *s*, OMe), 3.87 (1H, *t*, 7.6 Hz, H-12β), 4.73 (1H, *br. s*, H-3α), 5.02 (1H, *br. s*, H-17), 5.30 (1H, *br. s*, H-17') (Sheng *et al.*, 1992b)

Identification of two new GA analogues from canola

1. 16α,17-diOH gibberellin A_{34} From the aqueous MeOH extract of young canola cotyledons, another tetrahydroxy gibberellin was identified using GC-MS analysis by comparing its full scan mass spectrum and KRI with those of authentic 16α,17-dihydrodihydroxy GA_{34} (also synthesized by Prof. L.N. Mander). The new canola GA eluted together with GA_8 on both of C_{18} μBondapak and $N(CH_3)_2$ HPLC. As a 2β-hydroxylated GA, the putative 16α,17-diOH GA_{34} did not show any biological activity on the dwarf rice micro-drop assay. The molecular structure and the full mass spectrum of the putative canola 16α,17-diOH GA_{34} are shown in Figure 3-4 and 3-7, respectively. Its KRI and the relative abundance of principal ions to the molecular ion (M^+, 684, as MeTMSi) are also summarized in Table 3-1. The GC-MS identification of 16α,17-diOH GA_{34}, has been officially recognized (J. MacMillan, personal communication to R.P. Pharis), but this GA has not yet been assigned a trivial number.

2. C/D ring rearranged GA_8 Figure 3-8 shows a full-scan mass spectrum of another GA-like compound (MeTMSi) obtained from an extract of canola seedlings. It eluted from reversed phase C_{18} μBondapak HPLC just prior to GA_8. The molecular ion (M^+,

522) was the base peak for this mass spectrum. Principal ions (*m/z*) of the putative new GA-like compound (MeTMSi) and their relation to M^+ were *m/z* 507 (M^+ - 15, 2%), 490 (M^+ - 32, 4%), 447 (M^+ - 75, 6%), 432 (M^+ - 90, 3%), 405 (M^+ - 117, 4%), 403 (M^+ - 119, 4%), 388 (M^+ - 134, 6%), 373 (M^+ - 149, 8%), 350 (M^+ - 172, 11%) and 289 (M^+ - 233, 10%). According to published data (Gaskin and MacMillan, 1991), the mass spectrum of the putative GA-like compound from canola was a good match to that of C/D ring rearranged GA_8 (MeTMSi). An authentic C/D rearranged GA_8 was thus synthesized by Prof. L.N. Mander at our request. After comparing the GA-like compound with the authentic standard on GC-MS analysis for mass spectrum, KRI and relative abundance of major ions, it was confirmed that the putative GA was indeed C/D rearranged GA_8. Since the C/D rearranged class of GAs could theoretically arise as artifacts during extraction and purification, no new GA trivial number has been assigned to the C/D rearranged GAs. The molecular structure is shown in Figure 3-4, and its mass spectrum (MeTMSi) is presented in Figure 3-8.

Tentatively identified GA-like compounds from canola extracts

1. 12β-hydroxy-GA_1, a C-12 *epi* isomer of GA_{85} in extracts from canola cotyledons is also probably present in very young canola seedlings. A tentative identification of 12β-hydroxy GA_1 was achieved by GC-MS-SIM analysis. Unfortunately, the content of this putative new GA in canola tissue was very low and it was difficult to separate it from GA_{85} on both the reversed phase C_{18} HPLC and the Nucleosil $N(CH_3)_2$ HPLC columns.

The major differences between GA_{85} (12α-OH-GA_1) and the putative 12β-OH-GA_1 are that the former's mass spectrum (MeTMSi) contains an ion of *m/z* 375 with an abundance as high as its M^+, but the latter does not (Fig. 3-9). The two epimers of C-12 hydroxy GA_1 (in MeTMSi form) were barely separated on a DB1-20HS fused silica capillary GC column (e.g. only a 7-unit difference in KRI value); The α-hydroxy isomer (GA_{85}) eluted from the GC column about 0.1 min later than the C-12β hydroxy structure (Fig. 3-10). As I failed to purify enough 12β-OH GA_1 from the canola extracts for a full GC-MS scan, no clear mass spectrum of the putative new GA has been obtained. However, the SIM result does indicate its existence in an extract from young canola seedlings (Fig. 3-10). Some of the characteristic m/z ions for this putative new bioactive GA are presented in Table 3-1, and its molecular structure is shown in Figure 3-4.

2. A number of full scan mass spectra characteristic of other likely GAs (MeTMSi) were also obtained from extracts of canola tissues. As I either did not have enough information to conjecture, or had no authentic standards to confirm their molecular structures with confidence, those GA-like substances still remain unidentified. Speculatively, these are likely unknown monohydroxylated derivatives of GA_{12}, GA_{15}, GA_{24}, GA_{25}, GA_{37} and GA_{53}, and two dihydroxy GAs with mass spectra very similar to GA_1 and GA_{29}, respectively, but with KRIs and HPLC Rts different from the two known GAs (data not shown). Some of the unknown putative GAs could be C-12 hydroxylated. If so, this implies the presence of a separate GA biosynthetic pathway (early 12α-hydroxy) leading to the production of GA_{85}. It is also possible that there is an early 12β-hydroxyl pathway leading to the putative 12β-OH GA_1. However, this speculation is for reference only.

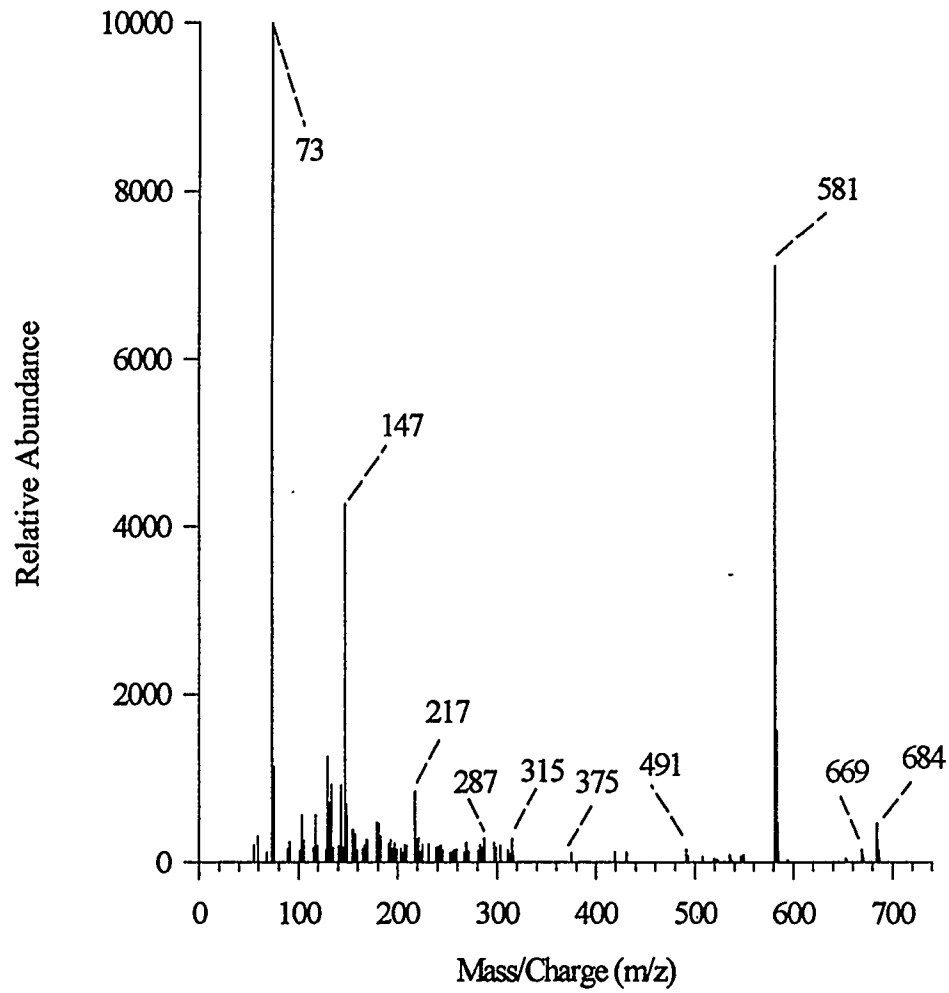


Figure 3-7. Mass spectrum of 16α,17-diOH GA_{34} (MeTMSi) identified from very young canola cv. Tobin seedlings. The spectrum (ion range from *m/z* 50 to *m/z* 740) was determined by GC-MS (EIMS at 70 eV).

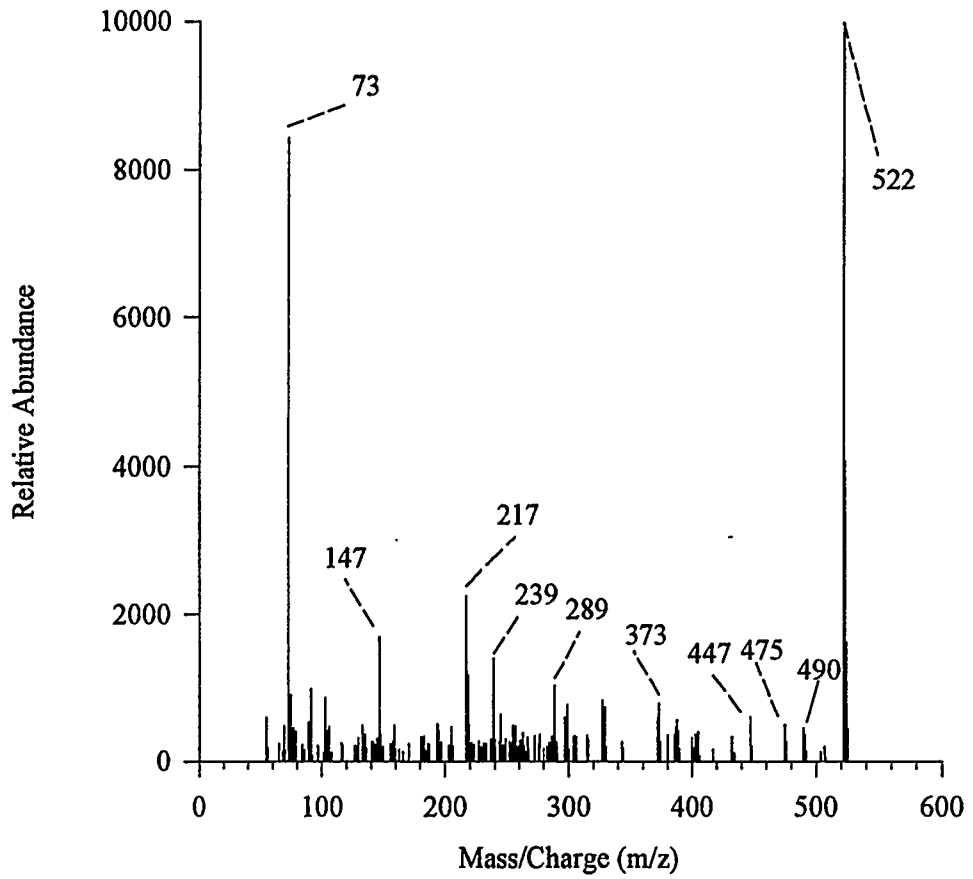


Figure 3-8. Mass spectrum of C/D ring rearranged GA_8 (MeTMSi) identified from very young canola cv. Tobin seedlings. The spectrum (ion range from *m/z* 50 to *m/z* 600) was determined by GC-MS (EIMS at 70 eV).

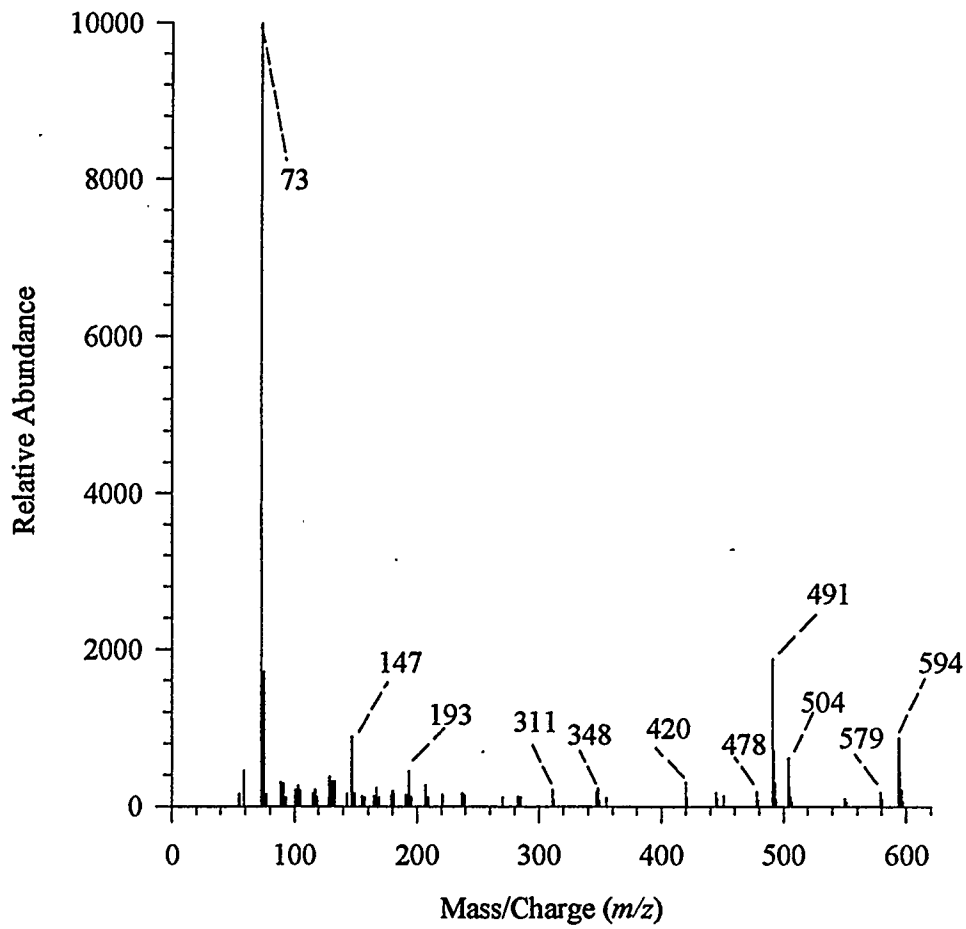


Figure 3-9. Mass spectrum of authentic 12β-hydroxy GA_1 provided by Prof. L.N. Mander (Australian National University, Australia) as a methyl ester. The spectrum (in MeTMSi form) was determined by GC-MS (EIMS at 70 eV) with an ion range from *m/z* 50 to *m/z* 650. This GA has been tentatively identified from canola cv. Tobin seedlings in this study.

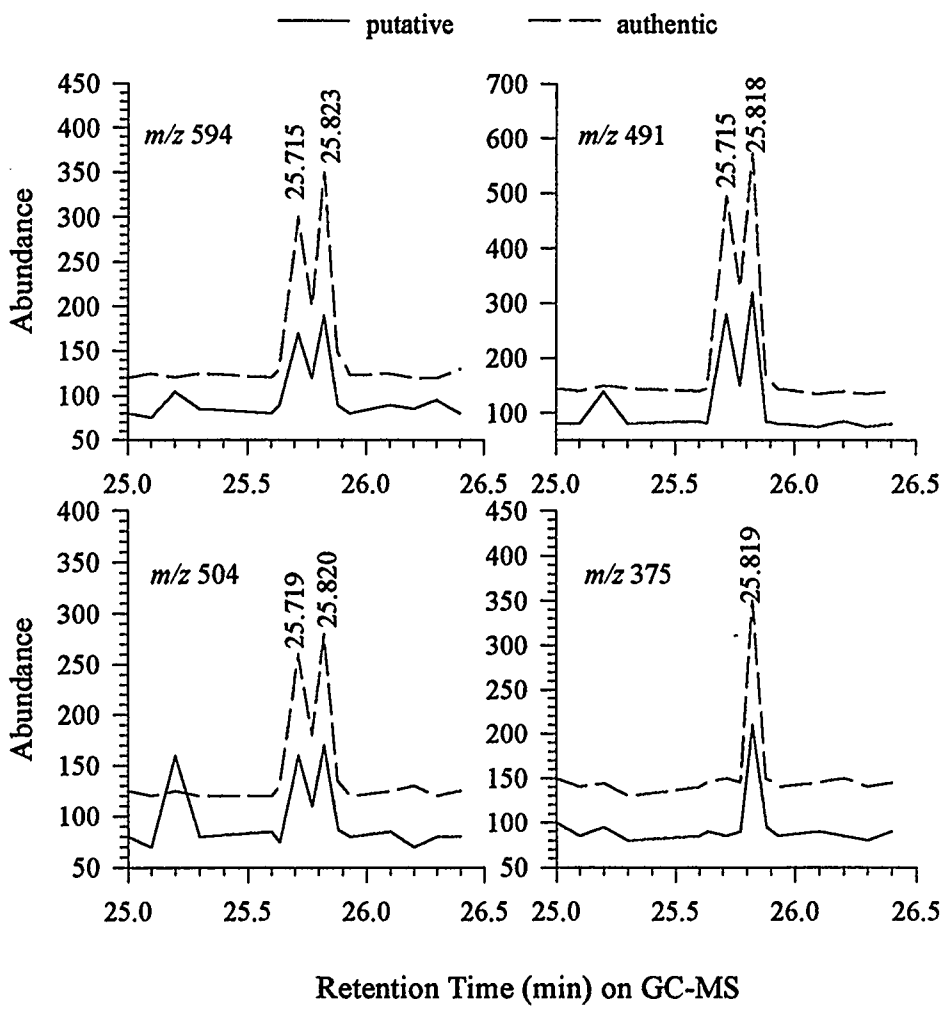


Figure 3-10. GC-MS-SIM traces of GA_{85} (12α-OH GA_1, right side) and 12β-OH GA_1 (left side), showing separation and differences of the two epimers (as MeTMSi), and also showing the comparison of the putative natural 12β-OH GA_1 with an authentic standard on Rt and relative abundance of the major selected ions.

Identification of 12 known GAs from canola seedlings by GC-MS

Based on comparisons of mass spectra and KRIs of putative GAs (MeTMSi) from canola extracts with those of the corresponding authentic GA standards (also provided by Prof. L.N. Mander), 12 known GAs were identified from the 7-d-old canola seedlings by GC-MS. They were GA_1, GA_3, GA_4, GA_7, GA_8, GA_9, GA_{19}, GA_{20}, GA_{24}, GA_{29}, GA_{44} and iso-GA_3. Figure 3-11 gives their molecular structures.

Discussion

Gibberellins identified in young seedlings of *Brassica campestris* cv. Tobin thus include: GA_1, GA_3, iso-GA_3, GA_4, GA_7, GA_8, GA_9, GA_{19}, GA_{20}, GA_{24}, GA_{29}, GA_{44}, GA_{85}, GA_{89}, 16,17-diOH GA_{34} and C/D ring rearranged GA_8 (Based on full mass spectra + KRIs), plus 12β-OH GA_1 (based on SIM + KRI). To these should be added the tentatively identified GAs, thereby bringing the total number of naturally occurring GAs in very young canola seedlings to over 20. Based on the GAs identified to date from canola extracts, it appears that young canola seedlings likely have at least three GA biosynthetic pathways (Figure 3-12), the early non-hydroxylation pathway, the early C-13 hydroxylation pathway, and the putative (early) C-12α-hydroxylation pathway. Older *Brassica napus* plants have also yielded GA_{36} and GA_{37} (Nishijima, T., personal communication). Thus, *Brassica* spp. can be considered to also have the early C-3β-hydroxylation pathway starting from GA_{12} (to GA_{14}), GA_{15} (to GA_{37}) or GA_{24} (to GA_{36}) (see Sponsel, 1988). Of the identified GAs,

GA_1, GA_3, GA_{85} and the putative 12β-hydroxy GA_1 can be considered as "effectors" of growth in canola seedlings. Gibberellin A_4 may also be an effector (Talon *et al.*, 1990; Nakayama *et al.*, 1991; Zeevaart *et al.*, 1991), and both GA_4 and GA_{20} are potential precursors of GA_1 (see Graebe, 1987; Talon *et al.*, 1990). Gibberellin A_3 is probably biosynthesized from GA_{20} via GA_5 (Albone *et al.*, 1989; Fujioka *et al.*, 1990; Smith *et al.*, 1991), or could be from GA_7, a pathway that has so far been confirmed only in *Gibberella fujikuroi* (Beader *et al.*, 1975). Gibberellins A_9, A_{24} and A_{19}, A_{44} are intermediates in the early non-hydroxylation and early C-13 hydroxylation pathways, respectively. The presence of iso-GA_3 in canola tissue may be an artifact made during work-up (Pryce, 1973; Moore *et al.*, 1982). Gibberellins A_8, A_{29}, A_{34} and A_{89} are all C-2β-hydroxylated, and can be considered as "inactivated forms" of GA_1, GA_{20}, GA_4 and GA_{85}, respectively. This indicates that very young canola seedlings may utilize 2β-hydroxylation to regulate levels of biologically active GAs during post-germination growth. The pathway leading to the highly bioactive canola new GA (GA_{85}) is not yet clear. There may be a new early C-12 hydroxylation pathway present in *Brassica* that starts from GA_{12} or GA_{53}, through some intermediates not yet known, and ends with production of GA_{85}, which can be then deactivated to GA_{89}. Alternatively, GA_{85} may come from GA_{20} via GA_1 or GA_{77} (12α-OH GA_{20}, Nakayama *et al.*, 1990), or from GA_4 via GA_1 or GA_{58} (12α-OH GA_4, Graebe *at al.*,1980). However, no definitive evidence for these "late" hydroxylations presently exists in the literature. The presumed metabolic pathways of GAs in young canola seedlings are summarized in Figure 3-12.

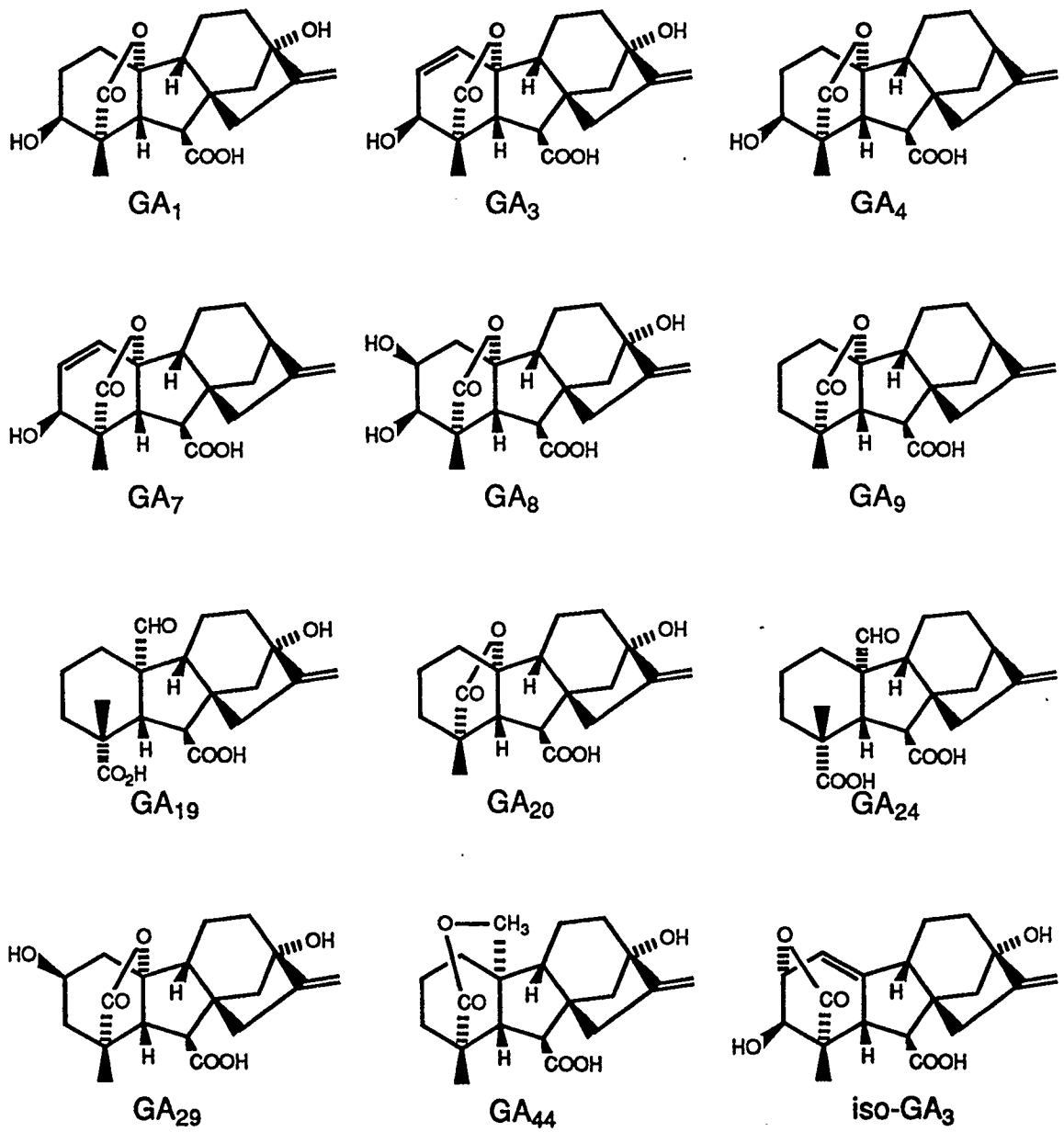


Figure 3-11. Molecular structures of 12 known GAs identified from very young canola cv. Tobin seedlings by their KRIs and full mass spectra. See also Fig. 3-4 for the structures of other, novel GAs.

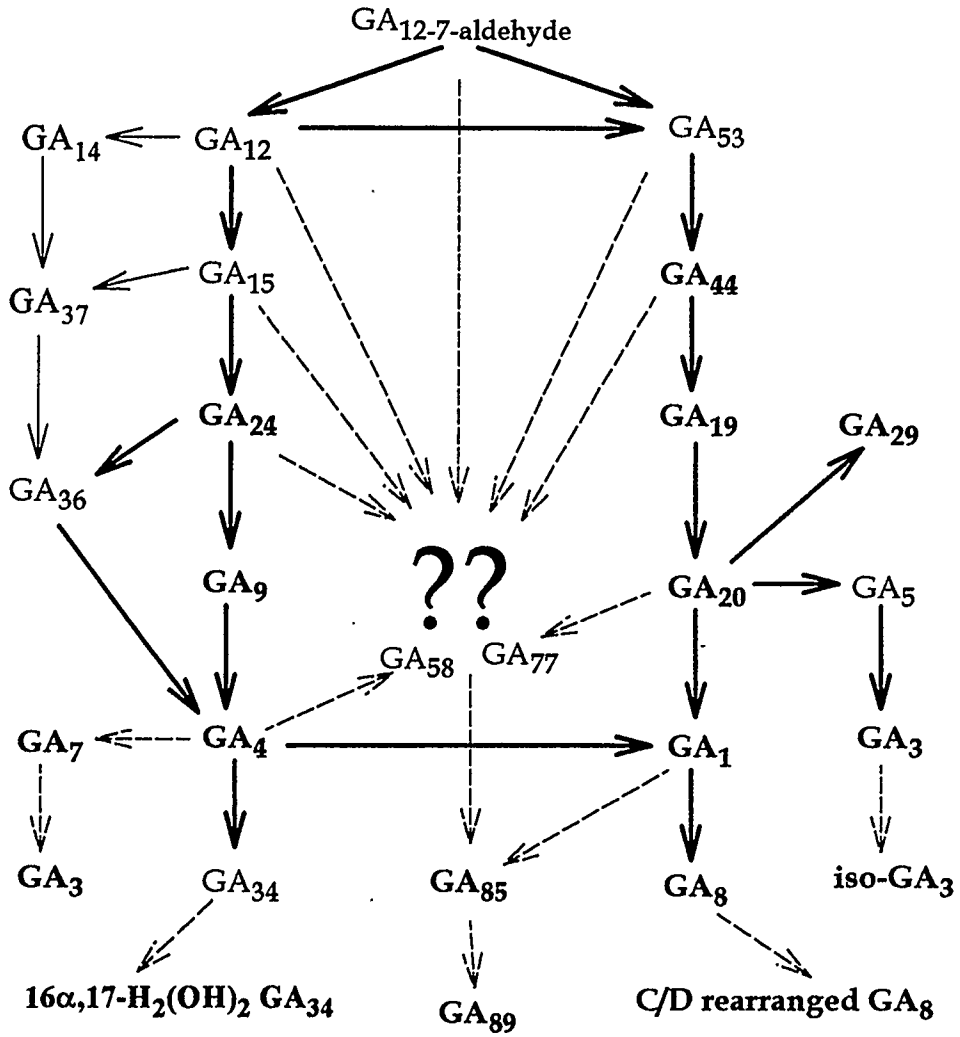
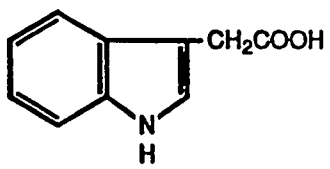
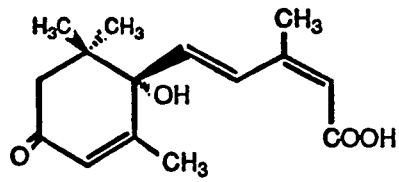


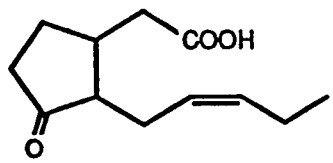
Figure 3-12. Hypothetical metabolic pathways of GAs in young canola seedlings. The GAs in bold have been identified from canola cv. Tobin by full mass spectra and KRIs in this study. Others are intermediates, but have not definitively identified. Solid line arrows represent the pathways that have been confirmed in higher plants. Dotted line arrows represent possible pathways in plants. (Other variations are possible).



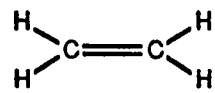
Indole-3-acetic acid
(IAA)



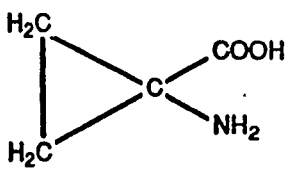
s-(+)-Abscisic acid
(ABA)



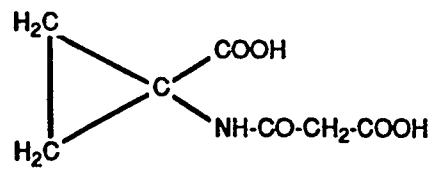
Jasmonic acid
(JA)



Ethylene



1-Aminocyclopropane-
1-carboxylic acid
(ACC)



1-Malonyl-1-aminocyclopropane-
1-carboxylic acid
(MACC)

Figure 3-13. Molecular structures of IAA, ABA, JA, ACC, MACC and ethylene--PGRs other than GAs that have been quantitatively analyzed in very young cv. Tobin canola tissues in this study.

3-2. Tissue specificity of hormone distribution in rapidly-growing young canola seedlings

This section covers quantitative analysis of certain known plant hormones and their precursors or analogues, including ABA, JA, IAA, ACC, MACC, ethylene and several GAs in rapidly growing young canola cv. Tobin seedlings by GC-MS-SIM using stable isotope-labelled internal standards for quantitation. Molecular structures of these hormones are given in Figure 3-11 (GAs) and Figure 3-13 (other PGRs), respectively.

Materials and Methods

Quantitation of ABA, JA, IAA, ACC, MACC and certain GAs from canola tissues

Plant materials Much of the tissue utilized (*Brassica campestris* cv. Tobin) was grown and harvested by Esso Ag Chemicals (Saskatoon, Saskatchewan, Canada) as described in Section 3-1. The freeze-dried root, hypocotyl and cotyledon tissue was then shipped to the University of Calgary. Before use for hormone analysis, the freeze-dried tissue was stored at -25° C in double zip-lock plastic bags with blue silica gel for absorption of moisture.

Extraction and Pre-purification of hormones For GC-MS-SIM analysis of IAA, ABA, JA, ACC, MACC and certain GAs, 100 to 300 mg tissue of freeze-dried roots, hypocotyls

or cotyledons of 7-d-old canola seedlings were ground in liquid N_2 with a small amount of acid-washed sea sand in a mortar and pestle. The powdered tissue was then homogenized in pre-cooled 80% MeOH. Prior to extraction, stable isotope-labelled internal standards were added to each sample as internal standards as follows: [$^{13}C_6$]IAA, [$^{2}H_6$]ABA and [$^{2}H_6$]JA, 100 ng for each; [$^{2}H_4$]ACC and [$^{2}H_4$]MACC, 200 ng for each; [$^{2}H_2$]GA_1, [$^{2}H_2$]GA_3, [$^{2}H_2$]GA_4, [$^{2}H_2$]GA_7, [$^{2}H_2$]GA_8, [$^{2}H_2$]GA_9, [$^{2}H_2$]GA_{20}, [$^{2}H_2$]GA_{19}, [$^{2}H_2$]GA_{29} and [$^{2}H_2$]GA_{85} (from Prof. L.N. Mander, Australian National University, Canberra, Australia), 5 ng for each. Radioactive standards of 1,000 Bq of high specific activity [^{3}H]IAA, [^{3}H]ABA, [^{3}H]GA_1, [^{3}H]GA_4 (Amersham) and [^{3}H]GA_9 (Crozier) were added to each sample at the same time. The extraction was carried out at room temperature for 30 min. After filtration through Whatman #1 filter paper, the plant residue was re-extracted 3x with 80% MeOH at room temperature. The filtrates of the 80% MeOH extract were combined (30 ml in total), and passed through a C_{18} PC column as described above (Section 3-2).

The MeOH in the extract was removed *in vacuo* at 35° C. The aqueous portion (ca. 5 ml) was adjusted to pH 2.5 with 1 M HOAc and then passed through a C_{18}-PC column. The column was made of 2 g of C_{18} material in a 10 ml syringe barrel, pre-eluted with 5 ml MeOH, and conditioned with 15-20 ml of 1% HOAc. After loading the sample, the C_{18}-PC was eluted with 10 ml solution of 6% MeOH in 1% HOAc, followed by 20 ml of 80% MeOH in water. The first two acidic fractions (e.g. 5 ml aqueous and 10 ml 6% MeOH in 1% HOAc) were collected for ACC and MACC. The next fraction (20 ml 80% MeOH in water) was collected for ABA, IAA, JA and the GAs.

Purification of ABA, IAA, JA and the GAs The 80% MeOH fraction from C_{18}-PC was taken to dryness *in vacuo* at 35° C, redissolved in 1 ml of 1% HOAc plus 9 ml of EtOAc. The EtOAc was pre-saturated with 0.5 M formic acid. The upper layer (EtOAc fraction) was loaded onto a SiO_2 partition column, which was eluted with hexane/EtOAc (5/95, v/v, saturated with 0.5 M formate) as described above in the Section 3-2. The hexane/EtOAc fraction from the SiO_2 column was evaporated *in vacuo* and subjected to reversed phase C_{18} HPLC on a Radial Pak Liquid Chromatography Cartridge (10 cm x 8 mm i.d., Waters Associates Inc., U.S.A.). The gradient used was 10% MeOH in 1% HOAc for the first 10 min, then a linear gradient to 73% MeOH/1% HOAc over 30 min with a flow rate of 2 ml per min. The fractions from C_{18} HPLC were grouped into five, e.g. group #1: GA_8 and GA_{85}; group #2: GA_1, GA_3 and IAA; group #3: GA_{20}, ABA and JA; group #4: GA_4 and GA_7; and group #5, GA_9, mainly according to Rts of the [^{3}H]-labelled standards added to the sample at the beginning of extraction.

The grouped hormone fractions were taken to dryness *in vacuo* at 35° C. The individual hormones in each group were then isolated from each other on a Nucleosil $N(CH_3)_2$ HPLC column, which was isocratically eluted with 0.1% HOAc in MeOH at 1 ml/min (Sheng *et al.*, 1992a). Fractions from $N(CH_3)_2$ HPLC usually gave purity sufficient for GC-MS analysis of ABA, JA, IAA and GAs therein.

Purification of ACC and MACC The mixture of ACC and MACC in the 15 ml fraction of ca. 4% MeOH in 1% HOAc from the above C_{18}-PC was isolated and purified for GC-SIM analysis, mainly based on the method of McGaw *et al.* (1985). Separation of ACC from

MACC was accomplished on a cation exchange short column made from a 10 ml syringe barrel with 3 ml slurry of AG 50W X8 resin (H^+ form, Bio-Rad Laboratories, U.S.A.) and conditioned with 15 ml 1% HOAc (pH ca. 2.8, 5x column bed volume). After the sample was applied in this 15 ml of solvent, the column was washed with an additional 15 ml of 1% HOAc. The 30 ml of washings were pooled together for MACC, but may have also contained possible conjugates of ACC and/or MACC. The column was then washed with 20 ml d.d. water followed by 20 ml of 2 N NH_4OH in 50% MeOH/H_2O (made of 28-30% NH_4OH/70%MeOH = 28/72, v/v) at a flow rate of 6-8 ml per min. The 20 ml of basic aqueous MeOH eluate was collected and reduced to dryness *in vacuo* at 40° C for ACC.

The MACC fraction from the cation short column was adjusted to pH 9 with 5 N NH_4OH and subjected to an anion exchange column to isolate MACC from possible conjugates of ACC and/or MACC in the extract. The anion exchange column was made of 3 ml of AG 1-X8 resin in a 10 ml syringe barrel (CH_3COO- form, Bio-Rad Lab. U.S.A.), and pre-conditioned with 20 ml of 0.1 N NH_4OH. After application of the sample, the short column was sequentially washed with 20 ml d.d. water, 20 ml MeOH and finally 20 ml of 2% HOAc in MeOH at 6-8 ml per min. The putative ACC/MACC conjugates that should elute with the first three washings were discarded. The acidic MeOH fraction containing MACC was reduced to dryness *in vacuo* at 40° C. The MACC residue was then dissolved in 1 ml of 4N HCl and chemically hydrolysed to ACC by heating at 120° C for 2 h. The hydrolysed MACC sample was evaporated to dryness in vacuo at 40° C, and was subsequently treated as an ACC sample during further purification.

Purification of the ACC samples was begun by redissolving them in 2 ml glacial HOAc containing extra resublimed phthalic anhydride (10 to 40 mg, according to purity of the samples). The derivatization took place at 100° C for about 100 min. Following addition of 1 ml d.d. water, the derivatized sample was partitioned twice against H_2O-saturated diethyl ether (1/1, v/v). The ether fraction was evaporated just to dryness (caution: sublimation of phthalimido-ACC can occur *in vacuo* at 40° C), and redissolved in 1 ml of 37% MeOH in 1% HOAc.

The phthalimido-ACC sample in 1 ml of acidic aqueous MeOH was applied to a reversed phase C_{18} HPLC column (15 cm x 4.6 mm i.d., 5 μm particle size, Alltech Associates Inc., U.S.A.). The sample was then gradiently eluted with 37 to 64% MeOH in 1% HOAc over 20 min at 1 ml per min. Retention time of phthalimido-ACC on the C_{18} column was around 9.5 min based on UV detection at 291 nm. The collected fractions containing phthalimido-ACC were evaporated to dryness *in vacuo* at 40° C, and then methylated with freshly-made ethereal diazomethane using the normal procedure as for other plant hormones (e.g., room temperature, 30 min). The methylated phthalimido ACC was dissolved in 1 ml of 40% MeOH/H_2O and chromatographed on the same C_{18} HPLC column used for the phthalimido-ACC sample, but with a gradient elution program of 40% to 82% MeOH/H_2O over 20 min at a flow rate of 1.5 ml per min. The phthalimido-ACC methyl ester (phthalimido-ACC-Me) was washed out of the column at about 9 to 10 min as monitored by a UV detector at 291 nm. The phthalimido-ACC-Me fraction was dried *in vacuo* at 40° C, again with care not to sublime the compound, prior to GC-SIM analysis.

GC-SIM Analysis of GAs The GA samples from $N(CH_3)_2$ HPLC were methylated and then trimethylsilylated, except for GA_9 that was only methylated. The GC-MS working conditions were as described previously (Sheng et al., 1992a, 1992b, Section 3-2), with exceptions noted below. A DB1-15N fused silica capillary column was utilized to chromatography the GA samples (as MeTMSi). The temperature program was the same as used for identification of GAs in Section 3-1, although instead of scanning for all *m/z* ions in the range from 50 to 740 amu, selected ion monitoring (SIM) was employed to determine the quantity of the individual endogenous GAs in the plant tissue extracts. Three pairs of ions were monitored for each GA and its associated $[^2H_2]$ internal standard. The monitored ions were: $GA_1/[^2H_2]GA_1$ (MeTMSi), *m/z* 447/449, 491/493 and 506/508; $GA_3/[^2H_2]GA_3$ (MeTMSi), *m/z* 445/447, 489/491 and 504/506; $GA_4/[^2H_2]GA_4$ (MeTMSi), *m/z* 284/286, 328/330 and 418/420; $GA_7/[^2H_2]GA_7$ (MeTMSi), *m/z* 282/284, 326/328 and 416/418; $GA_8/[^2H_2]GA_8$ (MeTMSi), *m/z* 375/377, 448/450 and 594/596; $GA_9/[^2H_2]GA_9$ (Me), *m/z* 270/272, 298/300 and 330/332; $GA_{19}/[^2H_2]GA_{19}$ (MeTMSi), *m/z* 402/404, 434/436 and 462/464; $GA_{20}/[^2H_2]GA_{20}$ (MeTMSi), *m/z* 375/377, 403/405 and 418/420; $GA_{29}/[^2H_2]GA_{29}$ (MeTMSi), m/z 447/449, 491/493 and 506/508; and $GA_{85}/[^2H_2]GA_{85}$ (MeTMSi), *m/z* 375/377, 491/493 and 594/596.

GC-SIM Analysis of IAA, ABA, JA, ACC and MACC Purified samples of IAA, ABA and JA were methylated with ethereal diazomethane as normal. The IAA samples were then trimethylsilylated to the MeTMSi form. The ACC samples (including those from the hydrolysed MACC) were run on GC-SIM as the phthalimido-ACC methyl ester. One

μl aliquots from each derivatized sample in EtOAc were introduced by cool on-column injection onto a DB1701-15N fused silica capillary column (15 m X 0.25 mm i.d., 0.25 μm methyl silicone film; J & W Scientific, Folsom, CA USA) installed in the HP GC-MS equipment as described earlier (Section 3-2). The temperature program was set for: 50° C (0.1 min) then elevated to 190° C at 20° C/min, followed by 5° C/min to 260° C. Helium flow was 40 cm/sec at 60° C, programmed for constant flow. Injector pressure was 15 psi at 50° C and increased as the temperature went up. Ionizing voltage was 70 eV. Other working conditions were similar to those used for GA analysis. Three pairs of *m/z* ions were selected to monitor each compound: ABA/[$^2H_6$]ABA (methyl ester), *m/z* 134/138, 162/166 and 190/194; JA/[$^2H_6$]JA (methyl ester), *m/z* 151/157, 193/199 and 224/230; IAA/[$^{13}C_6$]IAA (methyl ester, TMSi ether), *m/z* 145/151, 202/208 and 261/267; and ACC/[$^2H_4$]ACC (phthalimido, methyl ester), *m/z* 186/190, 213/216, and 245/249.

Identification of each hormone in the samples was based on comparison of its GC Rt (or KRI in case of GAs) and the relative abundance of the monitored *m/z* ions on GC-SIM with those of the authentic compound. Estimate of each hormone was based on SIM trace areas of its M^+ (in most cases) or base-peak ion plus two more characteristic ions for reference. With the isotope-dilution method, the peak area of the selected *m/z* ion from an endogenous hormone was divided by that of its heavier isotope internal standard to get the ratio of the peak areas. The level of the endogenous hormone was then calculated according to the known amount of the added internal standard, the ratio of the peak areas and the dry weight of the sample. Figure 3-14 summarizes the working procedure from extraction through purification to GC-MS-SIM analysis of the various plant hormones.

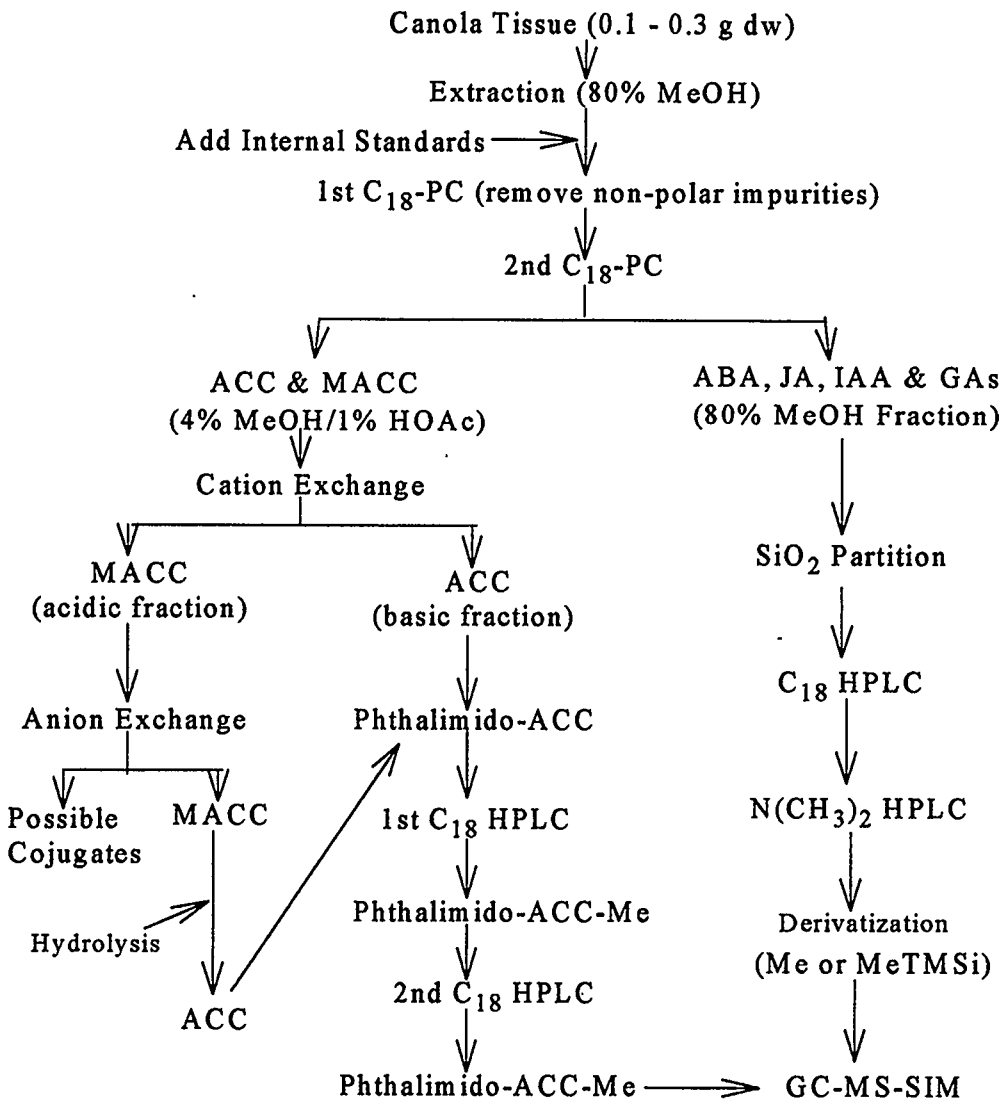


Figure 3-14. Working procedure for extraction, separation, and purification of ABA, IAA, JA, ACC, MACC and GAs from one plant extract prior to quantitation by GC-MS-SIM analysis using stable isotope-labelled internal standards.

Measurement of ethylene evolution from tissues of young canola and lettuce seedlings

Plant Materials Seeds of canola cv. Tobin were prepared as described in Chapter 2. The gnotobiotic pouches were incubated at 23° C in cool-white fluorescent light (see Section 2-2 for light intensity and spectral quality) or in darkness for 90 h, at which time seedlings were harvested and quickly dissected into roots, hypocotyls and cotyledons.

Ethylene Sampling The separated tissues were quickly placed in a 3 ml gas-tight syringe with the plungers adjusted to 1.5 ml. Each syringe contained five to eight roots, hypocotyls or cotyledons, and five syringes were used for each of the 3 dissected seedling parts. Ethylene evolving from the tissues was collected for 15 min, during which period of time the hypocotyls were kept in a vertical position in the syringes. The roots were too long to stand straight in a syringe, and were placed around the syringe barrels with the tips down. One ml from each of the collected ethylene samples was withdrawn to a second gas-tight syringe through a three-way valve (Finlayson *et al.*, 1991).

GC-FID analysis of ethylene The 1-ml ethylene samples were analyzed for ethylene content using a Varian 3700 gas chromatography (Varian Instrument Division, Walnut Creek, CA, USA). The GC column was a Porapak R (1.5 M x 2 mm i.d., Waters Associates, Inc., Milford, MA, USA) which was led to a flame ionization detector (FID). Nitrogen carrier flow was set at 30 ml/min and the temperature parameters were: injector 40° C, detector 120° C and oven 40° C.

The fw of plant tissue for each sample was determined immediately after ethylene collection. Ethylene evolution rate for each sample was calculated as p mol per g fw tissue per h, based on an abundance/content working curve that was established with standard ethylene samples run before and after the tissue-evolved samples.

Results and Discussion

Almost all of the analyzed hormones, including precursors (such as ACC), metabolites (such as MACC) were found in all the three tissues, e.g., roots, hypocotyls and cotyledons, of the canola seedlings. Only JA was too low in concentration in extracts from canola roots and hypocotyls to be unequivocally identified (JA in cotyledons was not analyzed). The levels of each hormone were quite different among the different tissues.

Gibberellins

Most of the biologically "effective" GAs, such as GA_1, GA_3 and GA_4, were highest in roots and lowest in cotyledons (Fig. 3-15). The level of GA_7 was also low in cotyledons and high in roots and hypocotyls (about the same level as in roots). In contrast, the level of GA_8 was much higher in cotyledons than in other tissues. This may indicate that the metabolism of GA_1 to GA_8 takes place more slowly in the roots and hypocotyls than in the cotyledons. Or, GA_8 glucosyl conjugate formation may be very rapid in roots and hypocotyls. In cotyledons GA_{85} was high, but in roots and hypocotyls it was relatively

low. The levels of GA_9 and GA_{20} were highest in the hypocotyls and lowest in the roots. This suggests that conversion of GA_{20} to $GA_{1 \text{ and } 3}$ and possibly of GA_9 to $GA_{4 \text{ and } 7}$ in the roots occurs more rapidly than in the hypocotyls. Alteratively, the high levels of $GA_{1, 3, 4 \text{ and } 7}$ in the roots may be transported from the hypocotyls, where conversion of $GA_{9 \text{ and } 20}$ to $GA_{1, 3, 4 \text{ and } 7}$ is rapid. However, as the level GA_{19} in the roots was about 10 times higher than in hypocotyls and cotyledons (Fig. 3-15). It is possible that roots of young canola seedlings are a site of GA biosynthesis. If so, then, the high levels of "growth-effective" GAs in the roots could be derived without transport from other tissues. Although levels of most biologically active GAs (not including GA_{85}) in the cotyledons were low, the cotyledons contained the largest amount of total GAs due to their much higher tissue dw and very high levels of GA_8 and GA_{29}. The level and abundance of GA_{85} are highest in the cotyledons (e.g. 12.5 ng/g dw). However, the level of GA_{85} in the roots and hypocotyls is also fairly high (e.g. 4-7 ng/g dw), relative to other bioactive GAs. Even though GA_{85} is highly bioactive, its distribution in canola tissues is very different from other "growth-effective" GAs (Fig. 3-15). Thus, possible physiological roles of GA_{85} in very young canola seedlings during germination and post-germination growth is as yet unknown, and may differ from GA_1, GA_3 and/or GA_4.

IAA, ABA and JA

Table 3-3 shows the levels of IAA and ABA in canola tissues. The IAA levels were twice as high in roots as in hypocotyls or cotyledons. This high level of IAA in the roots

of very young canola seedlings may be due to polar transport from the cotyledons (Rubery, 1988), as during seed germination there is (in maize at least) usually no net increase in total amount of seedling IAA (Reinecke and Bandurski, 1988).

The level of ABA in cotyledons of young canola seedlings was low in comparison with that in roots and hypocotyls. The ABA level in roots (608 ng/g dw) was more than two times higher than in cotyledons, as was the level of ABA in the hypocotyls (476 ng/g dw) (Table 3-3). Abscisic acid is commonly referred to as a seed germination inhibitor (Durley *et al.*, 1976; Ho, 1982). However, the role for ABA during post-germination growth is not clear (see Khan, 1982; Mayer and Poljakof-Mayber, 1989). Most research on the relationship between seed germination and ABA effects is based on treating seeds with exogenous ABA, which may not truly reflect the physiological function of ABA in different tissues of the seed and through different germination stages. One explanation for this tissue-specific distribution of ABA might be that during the post-germination stage, cotyledons serve as the source of energy and construction materials to the rapid growing seedlings. Hence, a low endogenous level of ABA may be needed to ensure the formation or activation of "growth-related" enzymes for a high metabolic activity in the tissue. The effect of ABA on seedling growth of young canola will be discussed later in Chapter 7. Jasmonic acid, a physiological analogue of ABA, was not detectable in roots and hypocotyls. If present, the levels of JA is likely to be very low, and thus may not play a significant role in regulation of root and hypocotyl elongation of young canola seedlings.

Ethylene and ACC/MACC

Ethylene evolution The rate of ethylene evolution from roots was significantly ($P \leq 0.01$) higher than from hypocotyls and cotyledons of the very young canola seedlings grown in gnotobiotic pouches when assessed at 90 h ASI. Roots of both light-grown and dark-grown canola cv. Tobin seedlings produced a level of ethylene twice as greater as the hypocotyls (Fig. 3-16). The effect of light on ethylene evolution from both roots and hypocotyls was profound. Plants grown in fluorescent light evolved a significantly higher levels of ethylene ($P = 0.001$ for the root and $P = 0.012$ for the hypocotyl, respectively) than those grown in the dark (Fig. 3-16). However, the differences in the evolution rate of root ethylene between canola seedlings grown in the light vs dark was not correlated with their root elongation (see Fig. 2-2 in Chapter 2), which showed no significant difference between light and dark conditions. However, the light effect on hypocotyl ethylene evolution is coincident with the growth data presented in Figure 2-4 (Section 2-3). That is, higher ethylene production by hypocotyls of light-grown canola seedlings is associated with a much slower elongation of the tissue. Ethylene production in cotyledons was very low, much lower than that in the roots and hypocotyls, with no detectable difference between the light vs dark treatments (data not shown).

The situation of plant seed germination and seedling growth in response to environmental ethylene is highly dependent on ethylene concentration, and on plant tissue type and species (Konings and Jackson, 1979; Jackson, 1985, see Abeles *et al.*, 1992). It has been reported previously that most of the ethylene produced by germinating seeds is a product of the embryonic axis rather than the cotyledons (Corbineau and Rudnicki, 1989). The rate of ethylene evolution usually increases during seed germination and seedling growth (Dunlap

and Morgan, 1977; Corbineau and Rudnicki, 1989). Even though a promotive action of ethylene on seed germination appears to be applicable to many plant species, the role of endogenous ethylene during seed germination is difficult to determine (see Abeles *et al.*, 1992). This is mainly because it is hard to distinguish if a germination stimulator acts by increasing embryonic ethylene production, or alternatively, if the increased ethylene production is the result of stimulated germination. Typically, root growth is stimulated by ethylene at a very low concentration and inhibited at higher concentrations (Jackson, 1982; Reid, 1988). However, this general conclusion is mainly based on results from exogenously applied ethylene. The role of endogenously produced ethylene on root growth *per se* is not yet clear.

ACC and MACC Levels Like ethylene evolution rates from tissues of the 7-d-old canola cv. Tobin seedlings, the levels of ACC and MACC were higher in roots, lower in hypocotyls and very low in cotyledons. This may indicate that the tissue-specific distribution of ethylene production in young canola seedlings is controlled before or at the level of ACC production. Generally, levels of MACC were much higher than ACC in roots, cotyledons and hypocotyls (Table 3-4). A similar trend is also found in young sunflower seedlings (S. Finlayson, personal communication). The plant tissues used for ACC and MACC quantitation were all grown under dim fluorescent light (kindly prepared by S. Young, Esso Ag Chemicals, Saskatoon, Saskatchewan, Canada). Therefore, no comparison was made for the levels of ACC and MACC in canola seedlings between the light and dark regimes.

Table 3-3. Distribution of endogenous IAA and ABA in roots, hypocotyls and cotyledons of 7-d-old canola seedlings as estimated by GC-SIM using stable isotope-labelled standards.

Hormone	Replicate	Level of hormone (ng/g dw) in tissue		
		Root	Hypocotyl	Cotyledon
IAA	#1	1930	870	590
	#2	2370	1560	920
	#3	2420	1340	780
	mean ± s.e.	2240 ± 156	1257 ± 203	763 ± 96
ABA	#1	660	200	350
	#2	557	649	186
	#3	--	580	160
	mean ± s.e.	609 ± 52	476 ± 140	232 ± 59

Note: --, data missing due to sample loss during purification; s.e, standard error.

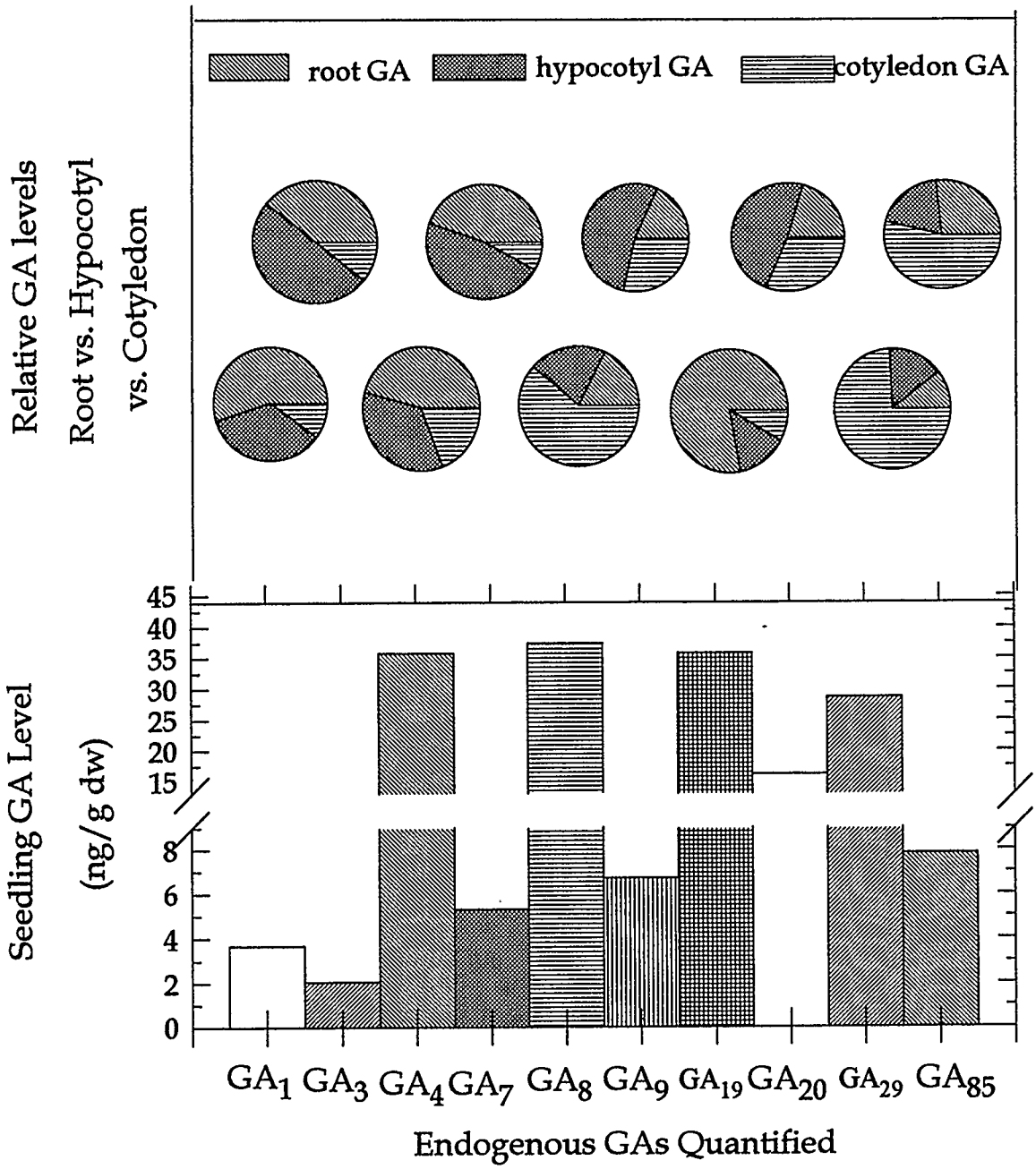


Figure 3-15. Levels of endogenous GAs in seedlings (bottom) and their distribution (relative levels) in roots vs. hypocotyls vs. cotyledons (upper) of young canola plants. Quantitation of the GAs is based on GC-SIM using stable isotope-labelled internal standards.

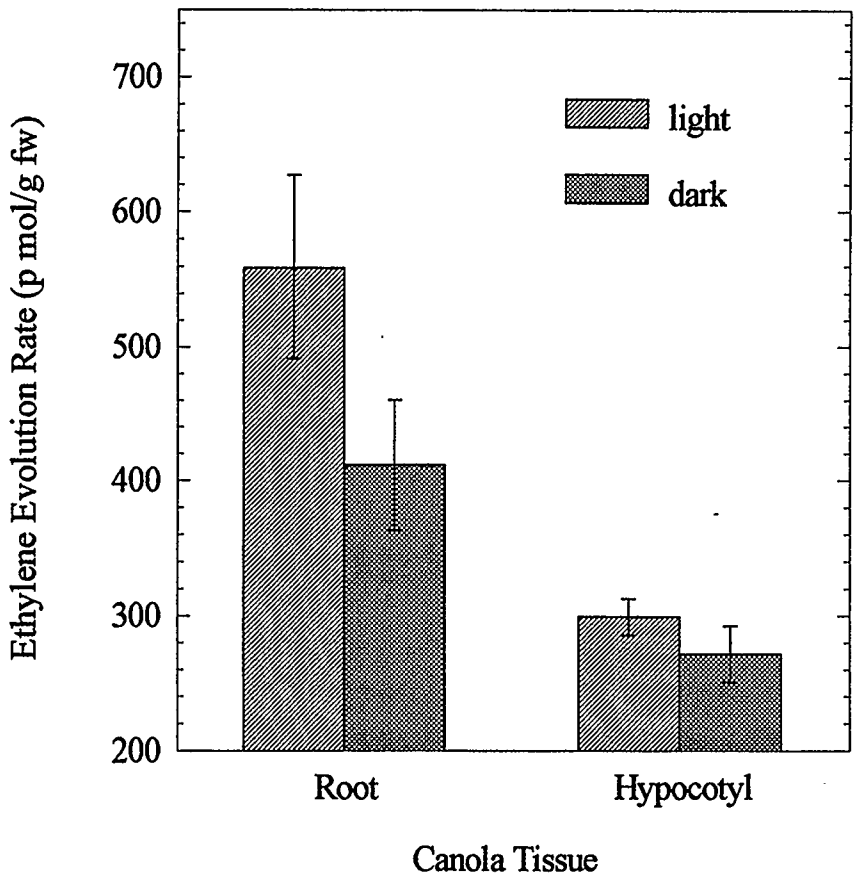


Figure 3-16. Ethylene evolution from roots and hypocotyls of 90-h-old canola cv. Tobin seedlings grown in gnotobiotic pouches, light vs. dark. Values represent the mean of 5 measurements of ethylene production by 5 to 8 seedlings, analysis was by GC-FID. Error bars represent 95% confidence intervals.

Table 3-4. Tissue-specific distribution of ACC and MACC in 7-d-old canola cv. Tobin seedlings grown under dim fluorescent light at room temperature (23° C). Estimates of ACC and MACC were based on GC-SIM using [$^2H_4$]ACC and [$^2H_4$]MACC as internal standards.

Plant Tissue	ACC Level (ng/g dw)		MACC Level (ng/g dw)	
	Mean	s.e.	Mean	s.e.
Root	6780	1120	21340	3650
hypocotyl	4350	680	15350	1320
Cotyledon	680	125	5560	720

note: data based on 3 replicates; s.e., standard error.

CHAPTER 4

THE DIRECT EFFECT OF *PSEUDOMONAS* PGPR ON EARLY GROWTH OF HIGHER PLANTS

4-1. Growth of *P. putida* GR12-2

Pseudomonas putida GR12-2 is a diazotrophic bacterial strain isolated from roots of an unidentified native grass in the Canadian High Arctic by Lifshitz *et al.* (1986). It was also shown that GR12-2 is a free-living bacterium and a competitive root-colonizer in the rhizosphere (Lifshitz *et al.*, 1986). The gram-negative rod cells of *P. putida* GR12-2 occur most often in pairs, but may also be present singly or occasionally in chains. This bacterium can grow over a wide range of temperatures from 4 to 30° C, with the optimum at 25° C (Hong *et al.*, 1990). Colonies of GR12-2 are smooth and can produce a fluorescent green pigment on Bacto-*Pseudomonas* Agar F (Hong *et al.*, 1990). Persistence and the capacity of the bacterium to colonize plant roots in the soil has been investigated since its identification as a PGPR (Lifshitz, *et al.*, 1986; Tang, 1992).

Materials and Methods

Purification and storage of the bacterium

Pseudomonas putida GR12-2 was provided by Esso Ag Chemicals (Saskatoon,

Saskatchewan, Canada), along with its putative Tn5 transposon mutant 42318 (Lifshitz *et al.*, 1987). The bacterial strains were streaked on Bacto-P*seudomonas* isolation agar (PIA) and Bacto-*Pseudomonas* agar F (PAF) (Difco Laboratories, Detroit, Michigan, USA) for subsequent re-isolation. The PAF-selected colonies were then tested on cv. Tobin using the gnotobiotic growth pouch assay system. One colony from each of the two strains was selected and then multiplied up in 5 ml tryptic soy broth (TSB) (Maknur Laboratories, Houston, TX USA) at 23° C for 48 h. To keep the re-purified bacterial strains for routine lab use, one ml of the cultured TSB from each strain was mixed with an equal volume of autoclaved 60% glycerol. The bacterial suspensions in 30% glycerol were quickly frozen in liquid N_2 and then stored at -75° C.

Growth of GR12-2 in TSB and PP, a glycerol C source medium

Cells of GR12-2 from the -75° C stock were scraped and streaked on PAF plates. The plates were incubated at room temperature (23° C) for 2 to 4 days until colonies formed. One colony was used to inoculate 5 ml TBS, which was then shaken at 23° C, 200 rpm overnight. One ml of the overnight-incubated bacterial culture was transferred to 500 ml flasks containing either 100 ml of enriched medium (TSB) or 100 ml of minimal medium (PP). The PP medium (designed in this study for culturing *P. putida*) was made up with 0.01 M K_2HPO_4/NaH_2PO_4 buffer containing 1.0 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$ and 30 ml glycerol per litre. The bacterial-inoculated flasks of TSB and PP were incubated at 23° C with shaking at 200 rpm. Optical density (OD) of the two bacterial cultures was

measured every 2 h from the beginning of incubation to 45 h on a spectrophotometer (Spectronic® 2000, Bausch & Lomb, Rochster, NY USA) at 780 nm to determine the population growth of *P. putida* strain GR12-2 in the minimal PP medium and enriched TSB medium.

Results and Discussion

Neither GR12-2 nor 42318 could survive on PIA plates

Both *Pseudomonas putida* strain GR12-2 and its mutant 42318 were expected to grow on PIA (*Pseudomonas* isolation agar) plates. However, from three experiments no colonies of either GR12-2 or 42318 were found on the surface of PIA plates, even after one week of incubation at 23° C. The PIA medium is supposed to be suitable for isolation and purification of *Pseudomonas* spp. The reason that *P. putida* strains GR12-2 and 42318 could not grow on this agar in our laboratory is unknown.

However, GR12-2 and 42318 grew well on PAF. Colonies of both strains appeared on PAF plates after about 50 h of incubation at room temperature (23° C) and looked round and smooth as Hong *et al.* (1990) noted. Mucilage was produced sometimes by GR12-2 and 42318 colonies if the plates were incubated for longer periods of time, such as up to one week.

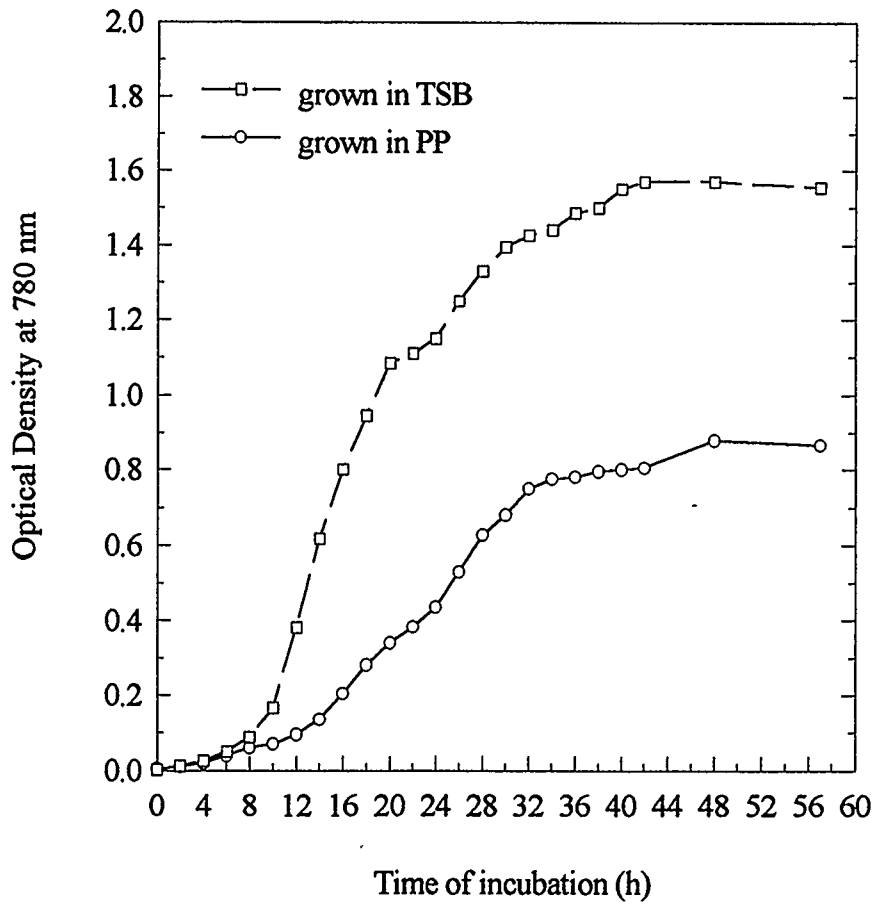


Figure 4-1. Population growth of *Pseudomonas putida* strain GR12-2 in 100 ml of enriched TSB medium or minimal PP medium (e.g. using glycerol as the only carbon source) at room temperature. Estimates of cell population in each culture are based on OD at 780 nm.

Population growth of GR12-2 in TSB medium (enriched)

GR12-2 grew well in both TSB and PP media under the above culture conditions. However, the growth rate of the bacterium was considerably faster in enriched TSB medium than in PP (Fig. 4-1). Based on the OD measurements at 780 nm with time following inoculation, the population of GR12-2 in 100 ml TSB culture began to increase very quickly after inoculation with 1 ml of cells from the overnight culture. The trend of increase in cell density lasted for more than 40 h (Fig. 4-1). There was no lag phase, as the original cells used for the inoculum had been grown in the same medium under the same environmental conditions (Boyd, 1988). After 8 h of accelerated growth the logarithmic phase began, and this lasted for 12 h with an increase of one unit OD_{780} value (from ca. 0.1 to ca. 1.1 OD). After the exponential phase, GR12-2 population growth in TSB reached a plateau for ca. 4 h and then began a slow increase until the carrying capacity of the culture environment was reached (e.g. an OD_{780} value around 1.6 after 40 h of incubation) (Fig. 4-1). From the population growth curve it is clear that the best culture stage for harvest of a maximal number of vigorous *P. putida* GR12-2 cells for use as an inoculum is when the culture OD_{780} is about 0.5 unit. Even so, a range from OD_{780} 0.2 to 1.0 is acceptable.

Population growth of GR12-2 in PP medium (minimal)

The PP medium has glycerol as its only carbon source. Even though PP was less

optimal than TSB for GR12-2, the strain did survive and reproduce in the medium (Fig. 4-1). The lag phase for GR12-12 to establish in PP was short (ca. 2-3 h), possibly due to the fact that the original inoculum came from TSB enriched medium (see Boyd, 1988). The acceleration phase covered a period from 3 to 11 h after inoculation, about the same as for the bacterium grown in TSB. Log phase of GR12-2 population growth in the 100 ml of PP started 12 h after inoculation and ended 32 h after inoculation, with a small plateau in the middle of the phase. The carrying capacity of the PP medium is much smaller than that of TSB. Population growth of GR12-2 in PP medium almost ceased when OD_{780} of the culture neared 0.8 unit (Fig. 4-1). The growth curve indicated that if PP-cultured GR12-2 is to be used as an inoculum, the cells should be harvested within a range of 0.3 to 0.7 units of the culture OD at 780 nm. Since PP has glycerol as the only carbon source, it should be a useful medium for studies of hormone production/metabolism by the bacterium. It seems unlikely that glycerol will contain significant levels of plant hormones which may interfere with research on microbe-origin PGRs. Additionally, since not many microbes can grow on glycerol, PP may also be a good medium to culture *P. putida* GR12-2 with reduced chances of contamination.

4-2. Creation of spontaneous mutants from GR12-2 with a genetic marker rifampicin

The original *P. putida* GR12-2 strain from Esso Ag Chemicals did not possess any genetic markers. Without such a selective marker it is difficult to monitor and handle the

microorganism during its purification, maintenance and propagation. Extreme caution had to be paid to prevent contamination of the PGPR strain in all lab and practical use routines. There is, however, a potentially simple and effective method to mark a bacterial strain genetically (e.g. spontaneous antibiotic resistance) without interfering with its major inherent characters, such as the ability to survive and colonize a normal environment (Turco *et al.*, 1986). However, a spontaneous antibiotic resistance mutant may lose some characteristics of its parent strain upon gaining the mutation (Turco *et al.*, 1986; Lewis *et al.*, 1987; Rozak and Colwell, 1987). Compeau *et al.* (1988) isolated spontaneous Rif^R strains of *P. putida* on nutrient agar medium containing 100 μg of Rif per ml at a frequency of 10^{-8}. The fate of spontaneous chromosomal Rif^R mutants was tested in comparison with the wild-type parent. The mutant followed most, but not all growth features of the parent.

Thus, I induced and isolated Rif-resistant mutants from GR12-2 in an attempt to select new PGPR strains that would possess plant growth promotion character (e.g. PRE) plus a selective marker.

Materials and Methods

Cells of *P. putida* GR12-2 from -75° C (re-selected from an Esso Ag Chemicals culture, see Section 4-1) were introduce into 5 ml PP medium and incubated for about 30 h at room temperature. A volume of 200 μl of the bacterial culture ($OD_{780} \approx 0.35$) was spread on each of the PAF plates containing Rif (80 μg/ml). The plates were incubated

at room temperature for 4 days, when colonies of antibiotic-resistant mutants were found on the surface of the agar. Good-looking (rapidly growing) mutant colonies were selected and re-cultured in tubes of PP medium with 60 μg/ml of Rif. After incubation overnight, cells of the antibiotic-resistant mutants were harvested and stored in 30% glycerol at -75° C as described above, prior to a subsequent PGPR activity examination in the gnotobiotic growth pouch assay.

Results and Discussion

For the GR12-2-inoculated plates of PAF/Rif, 85 colonies were formed. Forty colonies were used to inoculate tubes containing 5 ml of PP medium with 60 μg/ml Rif. Nine tubes turned cloudy after overnight incubation. These were selected as spontaneous Rif^R mutants of *P. putida* GR12-2. The 9 Rif^R mutants were named as GR12-2A, GR12-2B, GR12-2C, GR12-2D, GR12-2E, GR12-2F, GR12-2G, GR12-2S and GR12-2Z, respectively. Their PGPR effect on promotion of root elongation (PRE) was examined on canola cv. Tobin and lettuce cv. Grand Rapids. These PRE test results are presented later in this Chapter.

4-3. Direct promotion of *Pseudomonas putida* GR12-2 on early growth of several higher plants under gnotobiotic conditions.

The background of promotion of plant growth and yield by beneficial soil

psuedomonads was reviewed in Chapter 1. The effects of PGPR on higher plants is now becoming an active area of rhizosphere microbiology (Kloepper *et al.*, 1989). However, very little is known about the direct aspects of the PGPR effect on PRE of host plants: 1) is the direct effect of a single PGPR strain seen with more than one plant species, and 2) how does the bacterium accomplish this direct effect on the inoculated plants? It is these two aspects which will be emphasized with *P. putida* GR12-2 in this Chapter.

Materials and Materials

Cells of GR12-2 were propagated in 10 ml PP medium until the culture reached an OD_{780} of 0.4 units. A tube containing the same amount of PP medium was co-incubated as a control.

Cells from the two cultures (there were no cells visible in the control) were harvested by centrifuging at 4,000 rpm for 10 min at room temperature. The cells were then washed twice with filter-sterilized 0.1 M $MgSO_4$ buffer and finally pelleted by centrifuging as above.

The washed, pelleted cells were re-suspended in the 0.1 M filter-sterilized $MgSO_4$ and the cell density of all bacterial solutions was adjusted to an OD value ~0.4 - 0.5 units at 780 nm, which usually gave 10^6 to 10^7 colony-forming units (cfu) per ml in the $MgSO_4$ bacterial cell suspension.

A $MgSO_4$ cell suspension of the Tn5 transposon mutant 42318 was prepared in the same way.

Dry seeds of lettuce (*Lactuca sativa* cv. Grand Rapids), canola (*Brassica campestris* cv. Tobin), radish (*Raphanus sativa* cv. Cherry Belle), cabbage (*Brassica oleracea* cv. Golden Acre), tomato (*Lycopersicum esculentum* cv. Golden Boy), tobacco (*Nicotiana tabacum* cv. Trabezond), tree-mallow (*Lavatera*) and madwort (*Allysum*) were surface-sterilized with a 1/6 dilution of bleach (Javex) as described in Chapter 2.

After being soaked in the bacterial cell suspension for 60 min, seven to 10 seeds from each of the eight plant species, depending on seed size, were spaced evenly in each gnotobiotic growth pouch as described in Chapter 2. Ten pouches were used for each treatment for each plant species. The $MgSO_4$ solution was used as the control seed soak treatment. The mutant 42318 was tested only on canola and lettuce.

The seeded pouches were positioned vertically in a sterilized tray. The tray was then incubated in the dark at room temperature (23° C) for 90 h, at which time most seedlings were harvested. However, incubation time for the slower germinating species lasted longer, e.g. tomato, madwort, tree-mallow (120 h) and tobacco (190 h), respectively.

Root and hypocotyl length of seedlings from all treatments were measured, statistically analyzed by the independent t-Test and graphed at 95% confidence intervals as described earlier in Chapter 2.

Results and Discussion

Figure 4-2 shows growth measurements of 90-h-old seedlings of canola cv. Tobin with and without seed inoculation by GR12-2 or 42318. Root elongation was highly

significantly ($P \leq 0.01$) promoted by GR12-2, but reduced by 42318 ($P \leq 0.05$), both in comparison with non-inoculated control seedlings. Bacterial inoculation did not significantly modify canola hypocotyl length or shoot morphology in this trial (Fig. 4-2). The effect of the two *P. putida* strains on root growth of lettuce seedlings was similar to that of canola, but the trend for hypocotyl growth of lettuce was very different (Fig. 4-3). Lettuce hypocotyl length growth was highly significantly ($P \leq 0.01$) promoted by the mutant 42318, but not by the wild type GR12-2, relative to that of the non-inoculated control (Fig. 4-3). Since root growth of 42318-inoculated seedlings was significantly inhibited, promotion of hypocotyl growth in these seedlings may reflect a retention by the hypocotyl of assimilates derived from the cotyledons. It may also reflect differences in endogenous hormone levels (high IAA and GAs? see discussion later in this Chapter and in Chapters 6 and 7) in roots and hypocotyls of the treatment.

The promotive effect of GR12-2 on root elongation was also significant ($P \leq 0.05$) in several other species, e.g. cabbage, radish, tomato and tree-mallow. However, GR12-2 was only slightly promotive in madwort. Tobacco was the only species where root growth was not visibly influenced by inoculation with the two *P. putida* strains (data not shown). Unlike lettuce, there were no significant effects conferred by GR12-2 seed-inoculation on hypocotyl elongation of these plant species, although a slight increase of hypocotyl growth could be seen after GR12-2 seed inoculation treatment for four of the five species (Fig. 4-4). The results shown in Figures 4-2, 4-3 and 4-5 demonstrate that the promotive effect of GR12-2 on root growth of young seedlings is probably applicable to many plant species, especially those belonging to the Brassicaceae family.

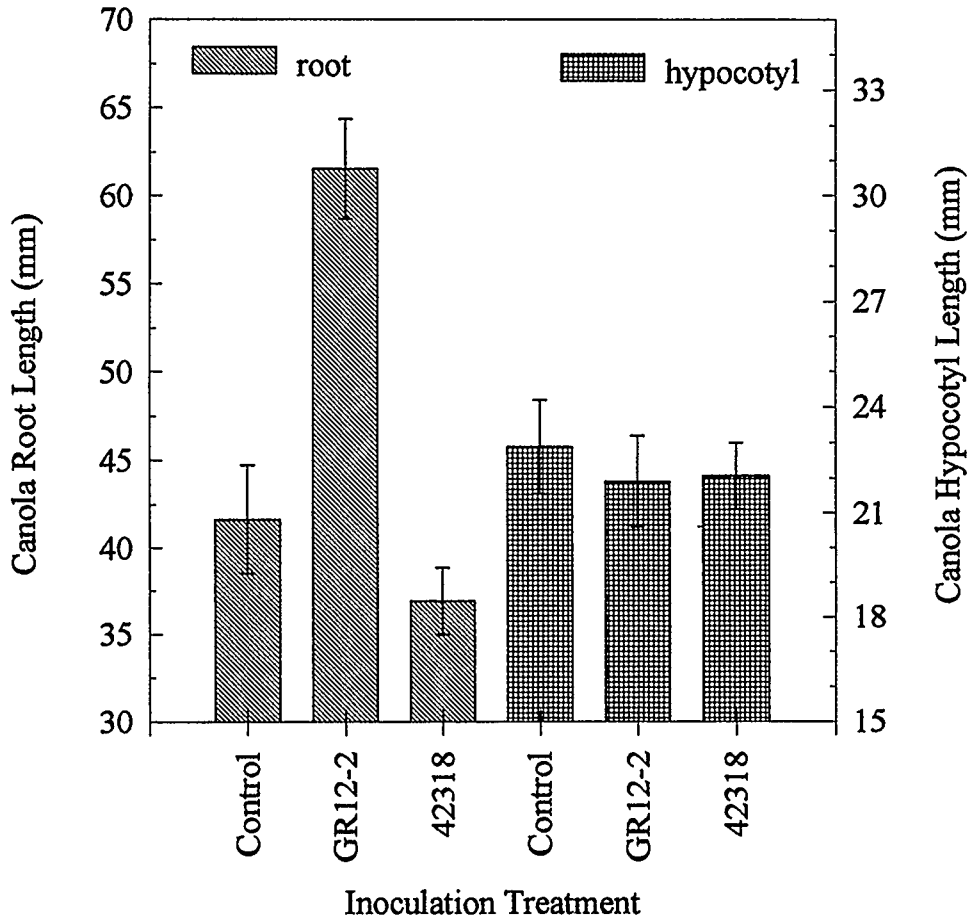


Figure 4-2. The effect of *P. putida* strains GR12-2 and 42318 on root and hypocotyl elongation of very young canola cv. Tobin seedlings grown in the dark under gnotobiotic conditions. Values represent the mean of 50 seedlings measured at 90 h ASI. Error bars represent 95% confidence intervals.

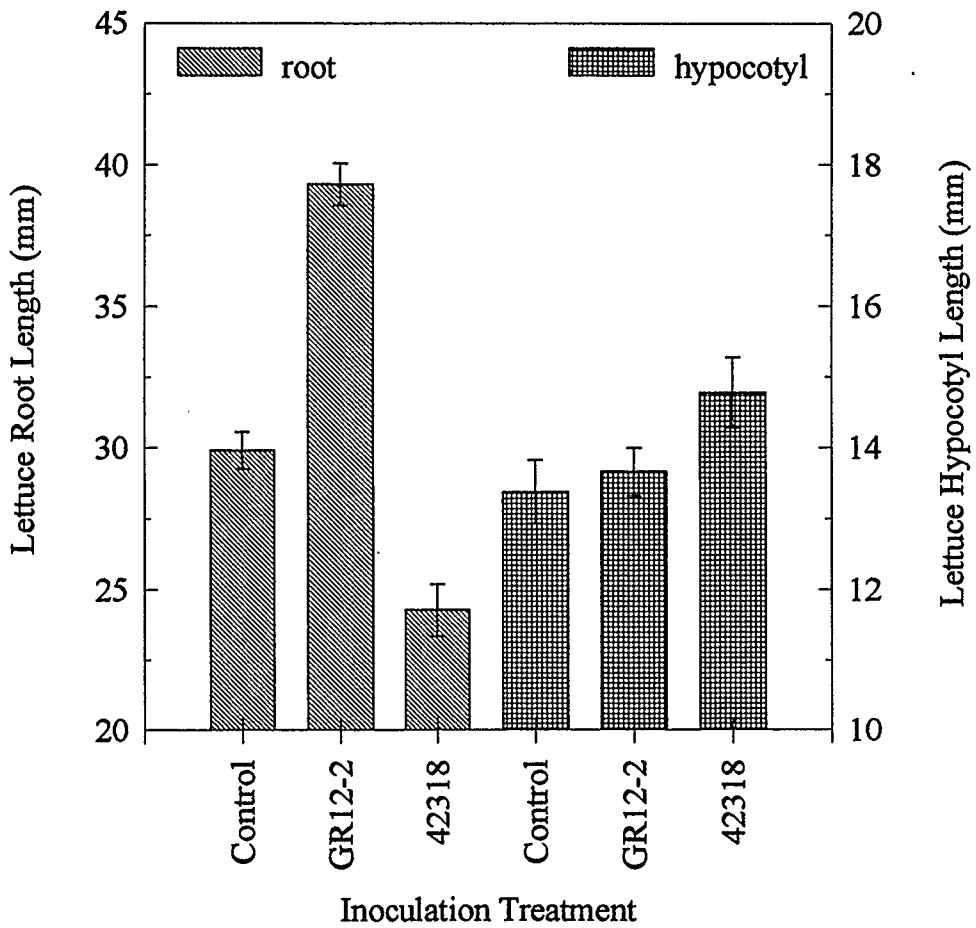


Figure 4-3. The effect of *P. putida* strains GR12-2 and 42318 on root and hypocotyl elongation of very young lettuce cv. Grand Rapids seedlings grown in the dark under gnotobiotic conditions. Values represent the mean of 50 seedlings measured at 90 h ASI. Error bars represent 95% confidence intervals.

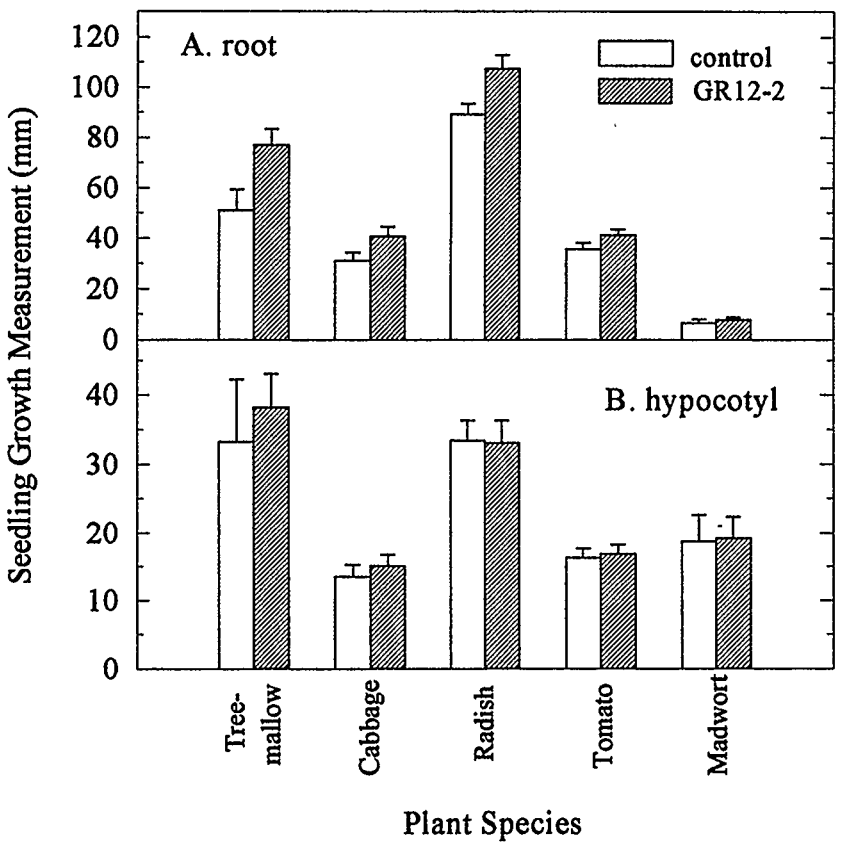


Figure 4-4. The effect of *P. putida* strains GR12-2 on root (A) and hypocotyl (B) elongation of several plant species grown in the dark under gnotobiotic conditions. Values represent the mean of 30 seedlings measured, respectively at 90 h ASI (cabbage and radish), 120 h ASI (tree-mallow and tomato), or 180 h ASI (madwort). Error bars represent 95% confidence intervals.

Under the gnotobiotic assay conditions, promotion of root elongation by GR12-2 during the post-germination growth of canola, lettuce and the several other species could not be due to antagonistic interactions between the *Pseudomonas* PGPR and pathogenic/deleterious microorganisms in the environment of the roots. Also, since the growth pouches contained only deionized H_2O, nutrient improvement should not be a promotive mechanism. It was shown earlier (Lifshitz *et al.*, 1987) that the growth-promotive effect obtained from *P. putida* inoculation is not due to bacterial N_2 fixation, even though GR12-2 is a free-living N_2 fixer. Hence, there appears to be more direct mechanism(s) by which the *Pseudomonas* PGPR promotes growth of higher plants

4-4. Potential of the spontaneous Rif-resistant mutants from *P. putida* GR12-2 on promotion of early seedling growth

The nine spontaneous Rif-resistant mutants derived from GR12-2 in Section 4-2 were tested to see if they retain all, part or none of the PRE phenotype that GR12-2 possesses on higher plants. This test was accomplished since past work has shown that a spontaneous antibiotic mutant may lose some/all of the important inherent characteristics of the parent (Turco *et al.*, 1986; Lewis *et al.*, 1987; Compeau *et al.*, 1988).

Materials and Methods

The nine spontaneous Rif-resistant mutants from *P. putida* GR12-2 (e.g. GR12-2A,

B, C, D, E, F, G, S and Z) were streaked on PAF plates containing 50 μg Rif per ml. A single colony from each mutant was then propagated in 5 ml PP medium containing 60 μg/ml Rif for 40 h. Cells from each culture were harvested, washed, and resuspended in 2 ml of 0.1 M $MgSO_4$ solution as described before.

Canola and lettuce were used to test these nine *P. putida* Rif^R mutants together with 42318 and their parent strain GR12-2 in gnotobiotic growth pouches as usual.

Results and Discussion

Figure 4-5 shows the effect of the nine spontaneous Rif^R mutants relative to the parent strain GR12-2, as well as to the Tn5 mutant strain 42318, on growth promotion of canola seedlings. Results from a similar test on lettuce seedlings are presented in Figure 4-6.

Of the nine Rif^R mutants derived from GR12-2, seven showed either higher or equal potential to promote early growth of very young (e.g. up to 90-h-old) canola and lettuce seedlings, relative to the PRE ability of the wild type parental strain, GR12-2. In fact, strain GR12-2A showed a considerably ($P \leq 0.05$) increased potential on PRE of canola and lettuce relative to GR12-2 (Figs. 4-5 & 4-6). The most desirable spontaneous Rif^R mutant tested on plant growth promotion appeared to be GR12-2S. It significantly enhanced root elongation and slightly promoted hypocotyl elongation of both lettuce and canola, relative to the parent GR12-2, which showed no effect on hypocotyl growth (Figs. 4-5 and 4-6). However, two Rif^R strains, e.g. GR12-2F and GR12-2Z had lost the PRE phenotype of GR12-2 on plant growth (Figs. 4-5 & 4-6).

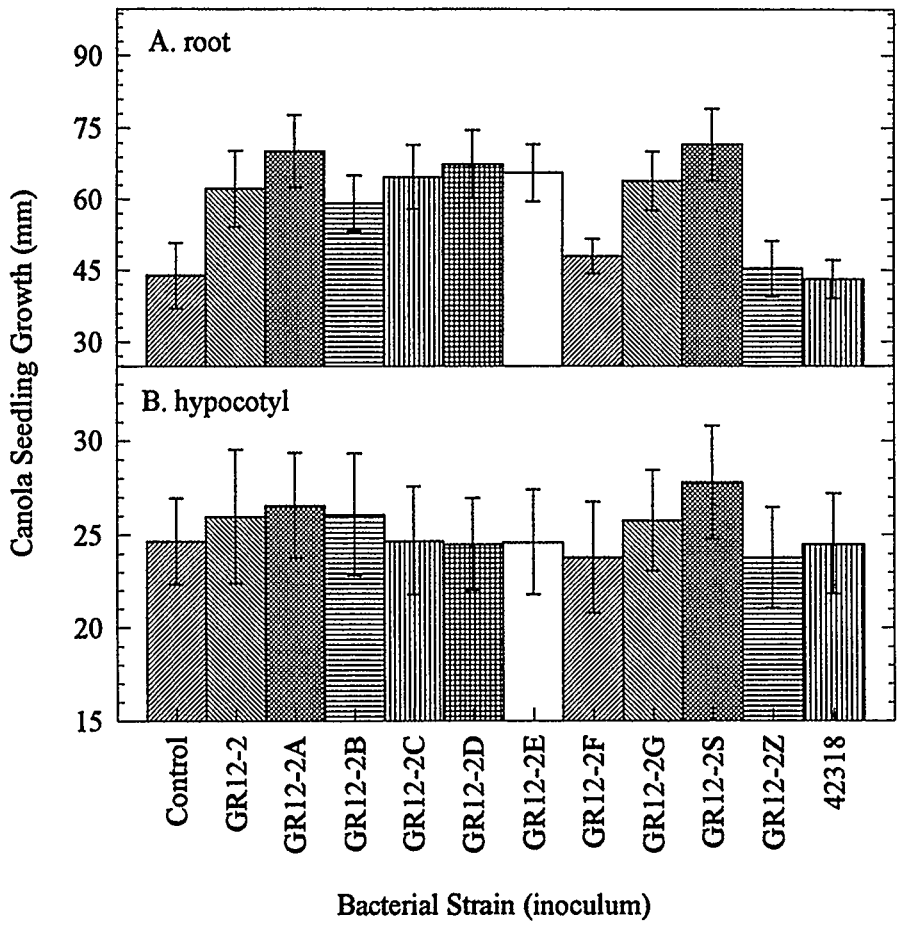


Figure 4-5. The promotive effect of the nine spontaneous Rif^R mutants derived from GR12-2, in relation to their parent strain, on elongation of roots (A) and hypocotyls (B) of young canola seedlings grown in gnotobiotic pouches in the dark. Values represent the mean of 50 measurements at 90 h ASI. Error bars represent 95% confidence intervals.

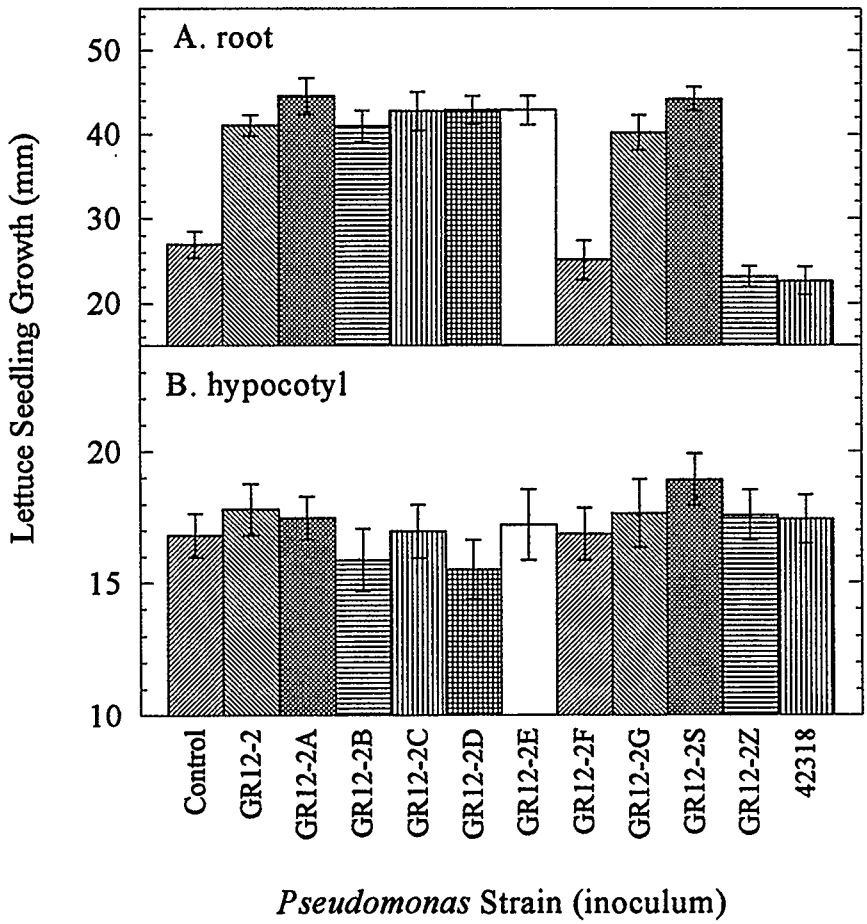


Figure 4-6. The promotive effect of the nine spontaneous Rif^R mutants derived from GR12-2, in relation to their parent strain, on elongation of roots (A) and hypocotyls (B) of young lettuce seedlings grown in gnotobiotic pouches in the dark. Values represent the mean of 50 measurements at 90 h ASI. Error bars represent 95% confidence intervals.

Thus, two of the spontaneous Rif^R mutants, GR12-2A and GR12-2S, possessed an improved PGPR potential (PRE) in the pouch assay, relative to the parent strain GR12-2. The spontaneous Rif^R mutant strains GR12-2B, GR12-2C, GR12-2D, GR12-2E and GR12-2G retained the inherent PRE ability of the parent, while strains GR12-2F and GR12-2Z had totally lost the PRE phenotype on post-germination growth of both canola and lettuce. As before (Figs. 4-2 and 4-3), the putative GR12-2:Tn5 transposon mutant strain 42318 again inhibited root elongation of canola (slightly, Fig. 4-5) and lettuce (significantly at $P \leq 0.05$, Fig. 4-6), relative to the control, non-inoculated treatment. The inhibitory effect of 42318 on root elongation noted in this study has not been reported before (Lifshitz *et al.*, 1988; Tang, 1992)

The deliberate release of beneficial microorganisms (to plant growth) into a field environment, so as to obtain gains in agriculture, horticulture and forestry, requires a way to monitor the organisms after their release. This means that the released microorganism should carry a selective marker which can be readily detected. Certain of the Rif^R mutants derived from GR12-2 would be good candidates for lab research at least since their ability to colonize the root environment and enhance plant growth is not interfered with by the genetic marker. Moreover, as strains GR12-2A and GR12-2S possess a significantly higher potential for promotion of early growth in both canola and lettuce, and are Rif resistant, their use in field trials is especially attractive. Also, the Rif^R mutants, in comparative studies with the parent, should be very useful for studying the mechanism by which PGPR promote higher plant growth.

4-5. Effect of *P. putida* GR12-2 on early growth of canola cv. *rosette* in comparison with GA_3 application

The usefulness of single gene dwarf mutants in studies of plant growth and development has been widely recognized by plant physiologists for decades. A single gene recessive dwarf mutant plant is usually associated with the loss of a primary function, e.g., shoot elongation. The direct effect of GR12-2 and its derivatives on post-germination growth of very young plant seedlings may be possible through direct or indirect regulation of expression of a specific gene or genes of the host plants. Thus, canola cv. *rosette*, a single gene (*ros/ros*) dwarf which is GA-deficient (Rood *et al.*, 1989), was tested using (i) GR12-2 seed-inoculation and (ii) GA_3 application to the seed.

Materials and Methods

Seeds of canola, *Brassica rapa*, cv. *rosette* RCBr were a gift from Prof. S.B. Rood (University of Lethbridge, Lethbridge, Alberta, Canada), and were also purchased from Carolina Biological Supply Company (Burlington, NC, USA). *Rosette* seeds were surface sterilized as described before for cv. Tobin, and then soaked for 1 h in solution of 0.1 M $MgSO_4$ alone (control) or with *P. putida* GR12-2, GR12-2S or 42318 cells. Additionally, GA_3 at concentrations from 10^{-10} to 10^{-5} mol in 0.1 M $MgSO_4$ buffer was used as a seed soak. There were 6 replicate pouches for each treatment. The gnotobiotic working procedure was as described in Chapter 2 and Section 4-1 of this Chapter. The canola cv.

rosette plants were germinated and grown in the dark at room temperature for 80 h, at which time root and hypocotyl lengths of the seedlings were measured.

In another trial the effect of light vs dark on root and hypocotyl growth of canola cv. *rosette* seedlings, with or without seed-inoculation of GR12-2, was assessed together with canola cv. Tobin using the gnotobiotic growth pouches. The assay was set up as usual, and 10 pouches for each treatment were incubated either in the dark or under cool-white fluorescent light (see Section 2-2 re light intensity and quality) for 90 h.

Results and Discussion

Effect of GR12-2 seed inoculation and application of GA_3 on *rosette* growth in the dark

As with canola cv. Tobin, seed-inoculation with GR12-2 and one of its spontaneous Rif^R mutants, GR12-2S significantly promoted root elongation of *rosette* seedlings relative to the control treatment (Fig. 4-7). A similar effect was found for GR12-2S relative to the parent strain GR12-2 (Fig. 4-7). Interestingly, the Tn5 transposon mutant 42318 acted on *rosette* as it did on cv. Tobin, e.g. it reduced root length of *rosette* seedlings, relative to the control ($P = 0.054$ on the t-Test).

Hypocotyl elongation of *rosette* tended to be reduced by bacterial inoculation, including GR12-2, GR12-2S and 42318. Although this reduction was not statistically significant, the trend shown in Figure 4-8 is quite clear.

The effect of the GA_3 seed-soak treatment on root elongation of *rosette* seedlings was

variable depending on concentrations. From 10^{-10} to 10^{-7} mol, GA_3 did not significantly alter root growth of *rosette*, though the 10^{-9} mol concentration tended to be somewhat promotive and the 10^{-7} inhibitory. However, the GA_3 seed soak at higher concentrations (e.g. 10^{-6} and 10^{-5} mol) significantly reduced root length growth of canola *rosette* seedlings harvested at 80 h ASI (Fig. 4-7). It seems that young *rosette* seedlings may already have sufficient amount of GAs in their roots for a normal elongation growth. Thus, the external applied GA_3 is unnecessary or even inhibitory (at high concentrations) to elongation of the root (Tanimoto, 1987)

The effect of applied GA_3 on *rosette* hypocotyl elongation was much greater than on the roots. Even though variation of hypocotyl length within each treatment was high, the increase of hypocotyl length by the GA_3 seed soak was still significant ($P = 0.021$) at a concentration of 10^{-8} mol and very apparent ($P = 0.065$) at 10^{-9} mol (Fig. 4-8). The lowest GA_3 concentration (e.g. 10^{-10} mol) had no detectable effect on hypocotyl growth of *rosette*. However, concentrations of GA_3 higher than 10^{-6} mol tended to be inhibitory (not significant) to hypocotyl elongation of the dark-grown *rosette* seedlings (Fig. 4-8).

It is surprising that the GA_3 seed-soak did not significantly promote hypocotyl elongation of *rosette* at concentrations higher than 10^{-7} mol. As a GA-deficient mutant, it is expected to be responsive to the external GA supply over a wider range for its shoot elongation (in the dark). In fact, Young *et al.* (1993) did significantly promote hypocotyl elongation of *B. campestris* cv. *rosette* with GA_3 at 10^{-4} M . Also, using a seed soak of canola cv. *rosette* with 10^{-5} M GA_3, I once achieved a significant promotion of hypocotyl elongation of 7-d-old canola cv. *rosette* seedlings in dim fluorescent light (data not shown).

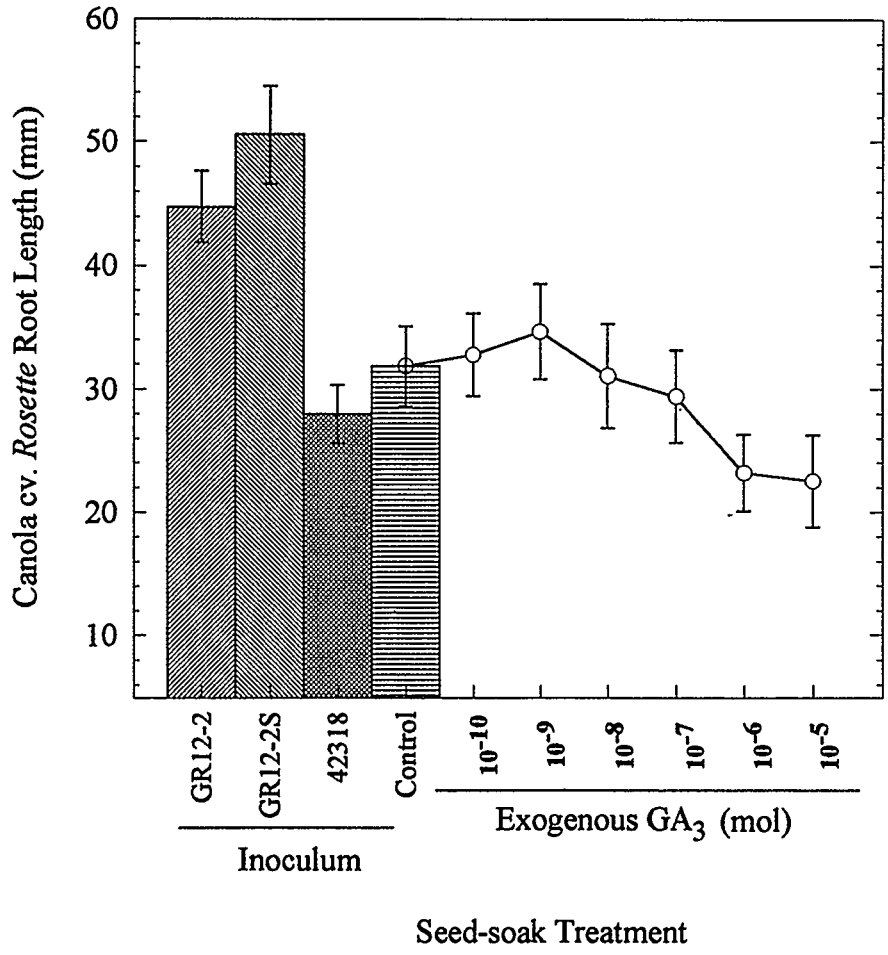


Figure 4-7. The effect of *P. putida* strains GR12-2, GR12-2S and 42318 and exogenous GA_3 (as a seed soak) on root elongation of canola cv. *rosette* seedlings grown in gnotobiotic growth pouches in the dark. Values represent the mean of 20 seedlings at 80 h ASI. Error bars represent 95% confidence intervals.

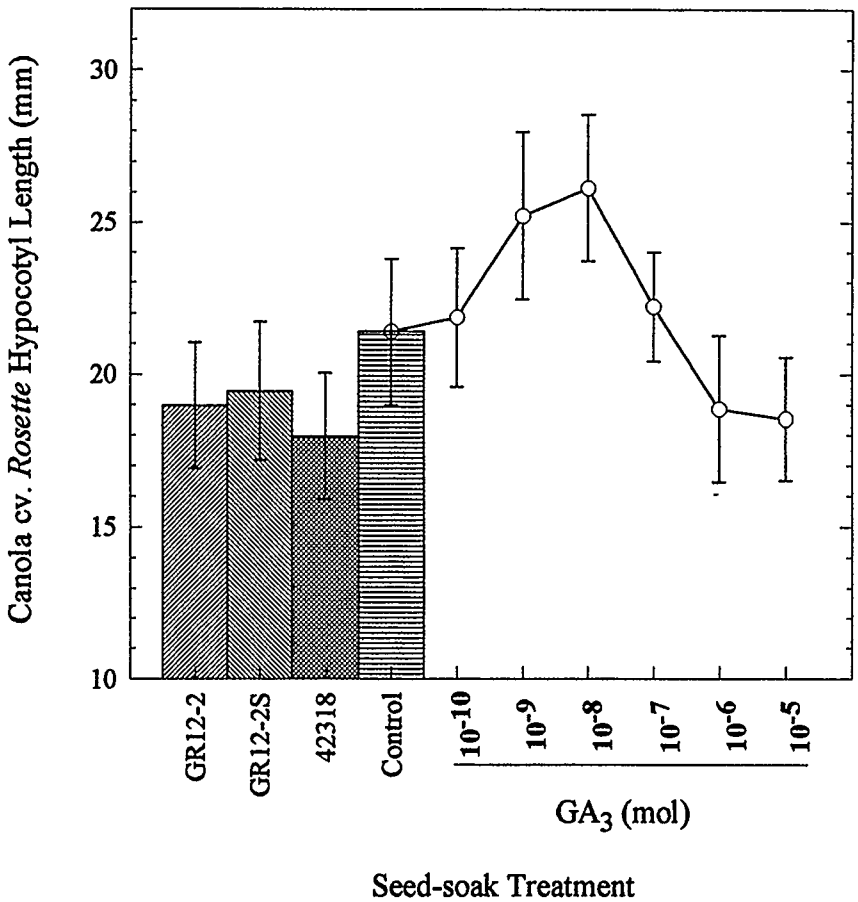


Figure 4-8. The effect of *P. putida* strains GR12-2, GR12-2S and 42318 and exogenous GA_3 (as seed soak) on hypocotyl elongation of canola cv. *rosette* seedlings grown in gnotobiotic growth pouches in the dark. Values represent the mean of 20 seedlings at 80 h ASI. Error bars represent 95% confidence intervals.

Possible reasons why GA_3 did not consistently promote *rosette* shoot growth are: i) During this very early stage of dark-etiolated growth, rosette seedlings may still obtain enough cotyledon-stored GAs for maximal hypocotyl growth. In the case of Young *et al.* (1993) and my other trial, the canola seedlings were twice as old (154-h-old) and there may have been less cotyledon-stored GAs available; ii)The level of cotyledon-stored GAs, including possibly residual GA_3 that was exogenously applied to the maternal parent plant to induce flowering and allow for normal seed development could differ from batch to batch in the commercial seeds.

Finally, it should also be borne in mind that when germination and post-germination growth took place completely in the dark, seedling etiolation occurs. The production and/or utilization of "growth-effective" GAs in *rosette* may be maximal, as appears to be the case for normal canola seedlings under low light intensity conditions where appreciable differences in GA catabolism/conjugation occur between dark-grown and light-grown plants (Rood *et al.*, 1993; Potter and Rood, 1993). This may make the light-grown *rosette* seedlings (in the 7-d trial) more GA-deficient (or GA responsive) than the dark-grown plants (in this trial, Fig. 4-8).

Effect of light and GR12-2 inoculation on post-germination growth of canola cv. *rosette*

Table 4-1 shows the growth data for 90-h-old seedlings of canola cv. *rosette* and cv. Tobin grown under cool-white fluorescent light vs dark, and with or without seed inoculation of GR12-2. The effect of bacterial inoculation on root and hypocotyl

elongation of seedlings grown in fluorescent light was very similar to that in the dark, and is independent of plant species. Strain GR12-2 highly significantly ($P \leq 0.01$) promoted root length growth of both cvs. (*rosette* and Tobin) in both light regimes. However, unlike the effect shown in Figure 4-8 on hypocotyl elongation, GR12-2 did not alter shoot growth of *rosette* in this trial (Table 4-1).

The effect of light on hypocotyl elongation was very pronounced for seedlings of both canola cvs. with or without the GR12-2 treatment. The average hypocotyl length of Tobin seedlings grown in fluorescent was 0.73 (for GR12-2 inoculated), and 0.80 fold (for the control) that of the plants grown in darkness, respectively. Both reductions were significant at $P \leq 0.05$. The response of *rosette* hypocotyl elongation to light was even much greater than for Tobin. Hypocotyls of *rosette* seedlings grown in the dark averaged 21.11 mm (with GR12-2 inoculation) or 20.75 mm (without GR12-2 inoculation), which is nearly as long as cv. Tobin grown in the light, and is about 1.6 fold that of the hypocotyl length of cv. *rosette* plants grown in the fluorescent light (Table 4-1).

The promotive effect of light on root elongation of *rosette* seedlings was noticeable, even though it was not statistically significant. Root elongation of cv. Tobin plants was not significantly affected by light (relative to dark), as previously noted in Chapter 2.

Thus, optimal root growth may not need high endogenous GAs, and may even be inhibited by high GAs. Inhibition of root growth by applied GA_3 has been reported for pea (Manos, 1961), wheat (Brian *et al.*, 1954), lettuce and oat (Krekule and Ullman, 1959), onion (McManus, 1960), cucumber (Aspinall *et al.*, 1967), tomato (Tognoni *et al.*, 1967) and bean (Odhnoff, 1963; Morris and Arthur, 1985). Tanimoto (1987) tested the

effect of various concentrations of applied GA_3 on root elongation of very young lettuce seedlings. He noticed that externally applied GA_3 could significantly promote root elongation of two-day old lettuce seedlings only when endogenous GA levels were reduced by use of ancymidol, a GA biosynthesis inhibitor. Even with ancymidol treatment, any doses higher than 10^{-6} M of GA_3 always suppressed lettuce root elongation (Tanimoto, 1987). The results obtained by Tanimoto on lettuce correlated reasonably well with the response of canola cv. *rosette* to GA_3 seed-soak treatment.

Thus, cv. *rosette* is likely to be GA-deficient only in the hypocotyl/shoot, but not in the root. Young *rosette* seedlings may actually contain enough "growth-effective GAs in the root to allow for normal growth. In fact, since light-grown *rosette* seedlings possess longer roots than dark-grown plants (Table 4-1) and biosynthesis of bioactive GAs in canola can be inhibited by light (Rood et al., 1993), levels of GAs in *rosette* roots may even be superaoptimal. A fair body of evidence obtained from applied GA_3 (Fig. 4-7; and Brian *et al.*, 1954; Krekule and Ullman, 1959; McManus, 1960; Manos, 1961; Odhnoff, 1963; Aspinall *et al.*, 1967; Tognoni *et al.*, 1967; Morris and Arthur, 1985; Tanimoto,1987) also indirectly leads to this conclusion.

If the above conclusion is correct, promotion of *rosette* root elongation by GR12-2 seed-inoculation should be associated with a reduced level of endogenous root GAs, GR12-2 may lower endogenous level of "growth-effective" GAs in roots of pouch-grown *rosette* seedlings to a more optimal level for root elongation, but without apparently altering GA levels in the hypocotyl. Alternatively the effect of GR12-2 on canola cv. *rosette* root length growth may be through optimizing the balance of more than one PGR

in the roots. The results do indicate that GR12-2 is unlikely to contribute GAs of bacterial-origin to *rosette* roots or to stimulate GA biosynthesis in the host plant.

Table 4-1. The effect of *P. putida* GR12-2 seed-inoculation and light conditions on root and hypocotyl length growth of seedlings of canola cv. *rosette* and cv. Tobin grown in gnotobiotic pouches at 23° C and harvested at 90 h ASI. Values in the table represent the mean (with standard error, s.e.) of 50 seedlings.

Inoculation treatment	Light regime	Root length mean (mm)/ ± s.e.		Hypocotyl length mean (mm)/ ± s.e.	
		Tobin	rosette	Tobin	rosette
GR12-2	light	68.72 ± 3.20	64.30 ±2.73	25.00 ± 1.51	13.10 ± 1.89
	dark	70.33 ± 2.09	58.06 ± 2.93	34.25 ± 1.75	21.11 ± 1.05
Control	light	50.29 ± 2.68	43.60 ± 2.28	27.00 ± 1.17	13.20 ± 1.09
	dark	46.21 ± 3.06	40.08 ± 2.85	33.85 ± 1.09	20.75 ± 1.22

4-6. Kinetics of the effect of GR12-2 inoculation on germination and early growth of canola and lettuce.

Evidence from above (Sections 4-3, 4-4 & 4-5) and other reports (Lifshitz *et al.*, 1987; Tang, 1992; Young *et al.*, 1993) shows that *P. putida* GR12-2 is a consistent promoter of root elongation for certain higher plants. These and other published results are based on measurements of length and/or dry weight of canola seedlings ranging in age from 3 to 7 days. As germination of canola seeds usually occurs about 14 to 18 h after imbibition (see Fig. 2-2, Chapter 2), growth data from several-day-old plants tell only a part of the story. Therefore, in order to tell when the promotive effect of GR12-2 seed-inoculation actually takes place, a kinetic analysis of differences in root and hypocotyl length between inoculation treatments was assessed during seed germination and seedling post-germination growth for both canola cv. Tobin and lettuce cv. Grand Rapids.

Methods and Materials

Preparation and inoculation of seeds of canola and lettuce with GR12-2 were as described earlier. Twenty five growth pouches were used for each treatment per plant species. The growth pouch assay was set up under gnotobiotic conditions as usual. Both seed germination and seedling growth occurred under cool-white fluorescent light (see details described in Section 2-2). Germination and growth were examined every 6 to 12 h from 0 to 110 h ASI. At 110 h ASI the fw of roots and hypocotyls was determined for

individual seedling parts (in foil packages of 5 for canola or 10 for lettuce). The tissues were then oven-dried (at 80° C) for 3 days before weighing.

A similar test was also carried out for canola and lettuce grown in the dark. As the dark experiment had to be destructive, 60 pouches were used for each treatment per plant species and seedling tissue weight was not determined.

Results and discussion

Kinetics of the effect of GR12-2 on the process of germination and post-gemination growth of canola cv. Tobin in fluorescent light

Seed germination occurred in the growth pouches about 12 h ASI. There were no detectable differences in seed germination times and seed germination frequencies between the bacterial inoculation and the non-inoculation (control) treatments. Protrusion of the radicle through the testa occurred at the same time for both inoculated and non-inoculated seeds. Morphology of the very young canola seedlings was not visibly altered by GR12-2 within the first 24 h ASI (see Figs. 4-9 and 4-10). During very early growth (18 to 24 h ASI) radicles from both control and inoculation treatments elongated very slowly. However, at about 24 h ASI, root growth began to increase considerably. The difference in root elongation between GR12-2-inoculated and non-inoculated seedlings became morphologically detectable soon after root growth became rapid (Fig. 4-9). A significant ($P \leq 0.05$) increase of root length for GR12-2 seed-inoculated seedlings

appeared at 36 h ASI. However, the initial physiological effect of GR12-2 on root growth promotion could have occurred earlier. From 36 h on, root length remained significantly ($P \leq 0.01$) longer for GR12-2-inoculated seedlings than for the controls (Fig. 4-9). The final root length of GR12-2 seed-inoculated seedlings was 1.37-fold longer than that of the controls in this trial (Fig. 4-9).

Hypocotyls of canola seedlings became detectable (measurable) at 24 h ASI. No difference was noted in initial hypocotyl growth between the inoculation and control treatments (Fig. 4-10). Rapid hypocotyl elongation began at about 36 h ASI for both GR12-2-inoculated and control plants. Differences in hypocotyl length growth between the control and GR12-2-inoculated plants occurred at 48 h ASI (Fig. 4-10). The promotion of hypocotyl growth by GR12-2 seed-inoculation was significant ($P \leq 0.05$) between 60 to 84 h, but became a non-significant trend there after (Fig. 4-10). However, promotion of hypocotyl growth by GR12-2 seed-inoculation was not always repeatable. In most trials, especially when seedlings were grown in the dark, GR12-2 inoculation significantly promoted only root elongation, not hypocotyl elongation (see Figs. 4-2, 4-5 & Table 4-1). Nevertheless, consistent promotion of both root and hypocotyl growth of canola (7-d-old) by PGPR strain GR12-2 seed-inoculation has been reported by other researchers (Lifshitz *et al.*, 1987, 1988, Young *et al.*, 1993).

The fw of canola tissues from GR12-2-inoculated plants (110-h-old) were significantly (roots, $P \leq 0.05$) or slightly (hypocotyls, $P = 0.29$) higher than those from control plants (Fig. 4-11). However, root dw was much less significantly ($P = 0.088$) promoted by GR12-2, relative to the control, and there was almost no difference in hypocotyl dw

between the inoculated and control treatments. Hence, the differential behaviour between root fw and dw increase implies that the additional root growth of GR12-2 seed-inoculated canola seedlings is more correlated with an enhanced water uptake by the tissue, rather than increased assimilate movement to the roots.

Kinetics of the effect of GR12-2 on the processes of germination and post-germination growth of lettuce under cool-white fluorescent light

The effect of GR12-2 seed inoculation on germination and post-germination growth of lettuce occurred much later than for canola. However, as for canola, there was no detectable difference in seed germination (radicle protrusion) between the inoculated and control treatments. Differences in root elongation between the inoculated and control treatments were not detectable until about 60 h ASI (Fig. 4-12), whereas in canola differences could be detected by h 30. The promotive effect of GR12-2 became statistically significant ($P \leq 0.05$) at about 65 h ASI. After h 60, the differences in root length between inoculated control treatments kept increasing until the final harvest at 110 h ASI (Fig. 4-12). It is thus clear that the direct effect of GR12-2 inoculation on lettuce seed germination and seedling post-germination growth in fluorescent light is very similar to that for canola, except for a 30 h delay (Figs. 4-9 & 4-12).

Figure 4-13 shows that there was no significant difference in hypocotyl elongation between control and GR12-2-inoculated plants grown in the light as was noted for lettuce seedlings grown in the dark (Figs. 4-3 & 4-5).

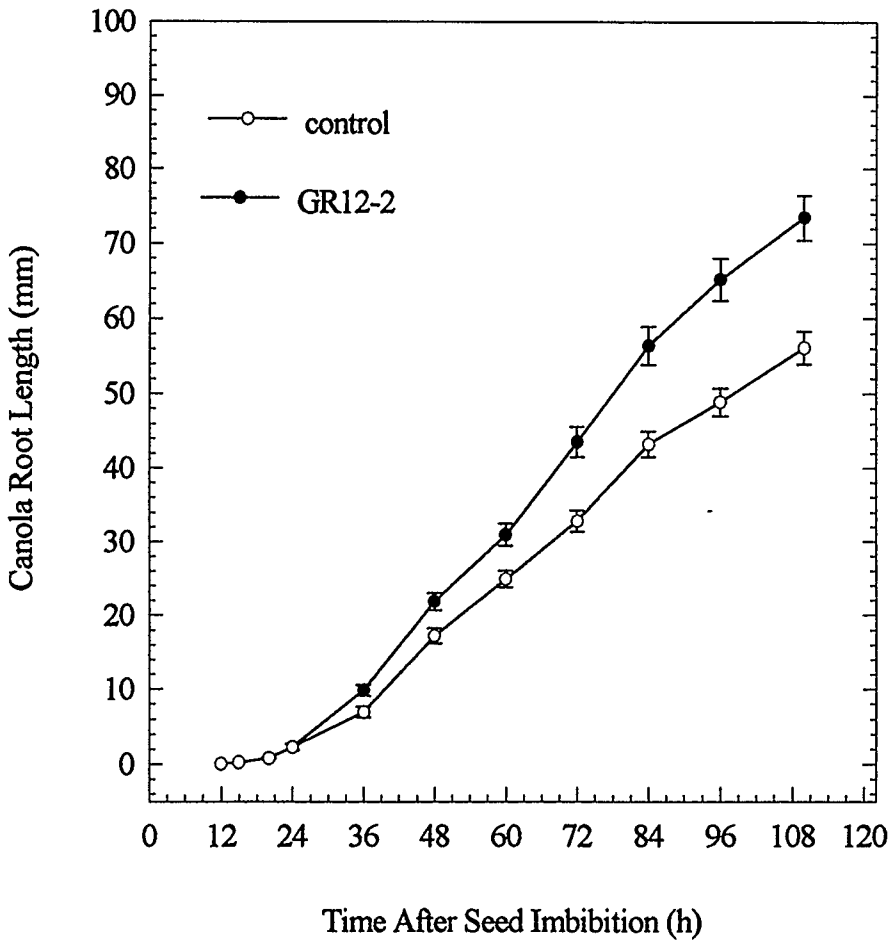


Figure 4-9. The effect of GR12-2 seed-inoculation on kinetics of canola seed germination (radicle protrusion) and seedling root elongation in gnotobiotic growth pouches under cool-white fluorescent light. Values represent the mean of 100 measurements. Error bars represent 95% confidence intervals.

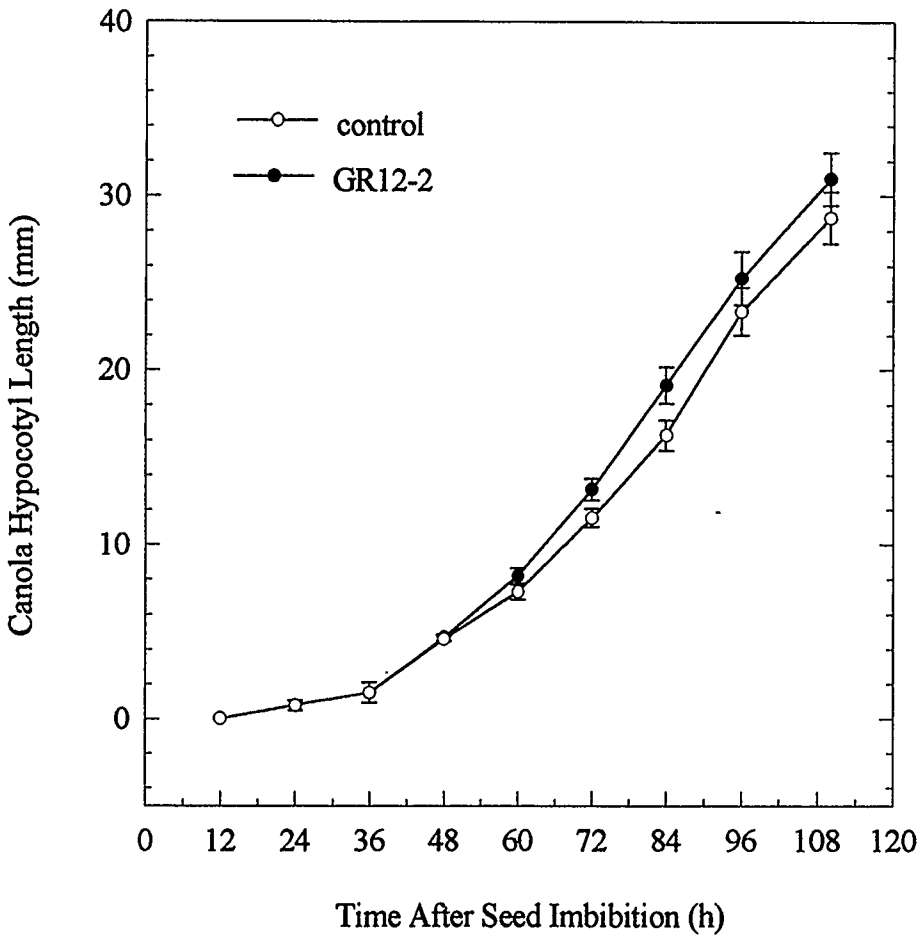


Figure 4-10. The effect of GR12-2 seed-inoculation on kinetics of hypocotyl elongation of canola seedlings grown in gnotobiotic growth pouches under cool-white fluorescent light. Values represent the mean of 100 measurements. Error bars represent 95% confidence intervals.

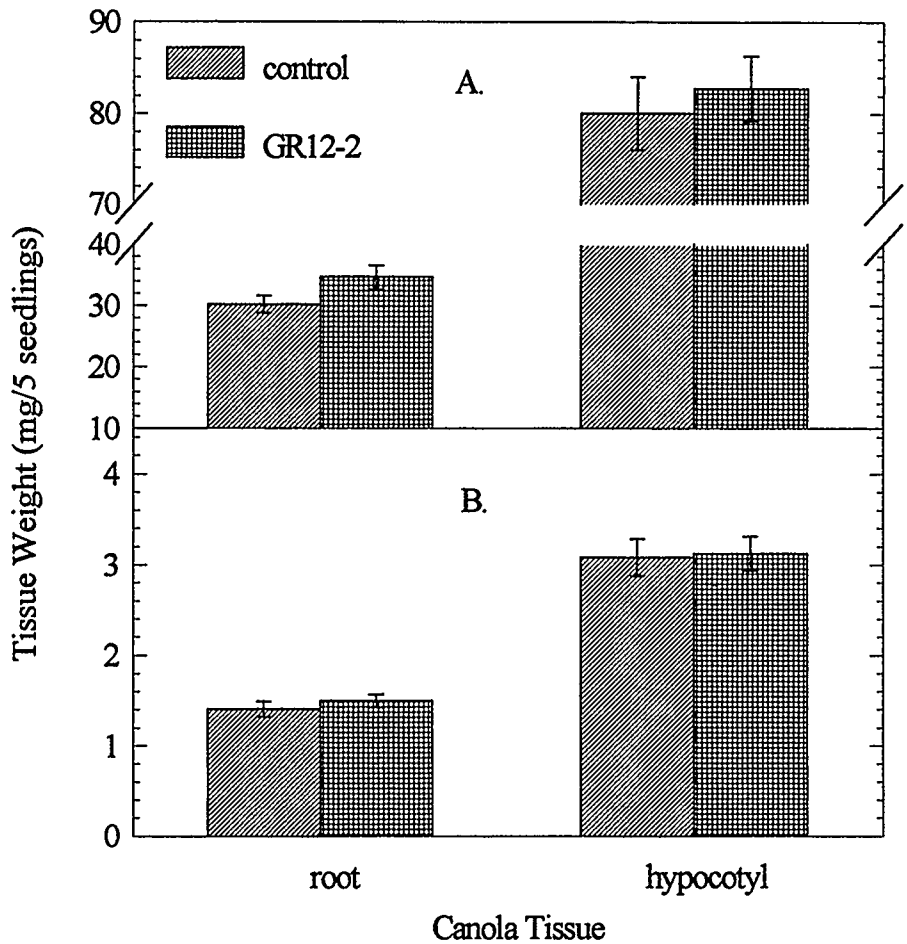


Figure 4-11. The effect of GR12-2 seed-inoculation on tissue fw (A) and dw (B) of 110-h-old canola seedlings grown in gnotobiotic pouches in cool-white fluorescent light. Values represent the mean of 20 measurements. Error bars represent 95% confidence intervals.

Root fw of the 110-h-old lettuce seedlings was significantly increased by the GR12-2 treatment, but this did not occur for hypocotyl fw (Fig. 4-14). Unlike canola, seed inoculation with GR12-2 tended to decrease the dw of both roots and hypocotyls, though this decrease was not statistically significant ($P \approx 0.3$) (Fig. 4-14). As in canola, it seems that GR12-2 inoculation promotes water uptake by the young lettuce seedlings, and this is correlated with an increased root length. However, the tendency of GR12-2 seed-inoculation to reduce the average dw of the entire lettuce seedling is unexpected. Possibly, as an inoculum, GR12-2 lives on the plant-provided organic nutrients, causing a net loss of energy and materials of the host. This could be a major negative effect of plant growth-beneficial microorganism to the plant in certain circumstances, such as when the supply of assimilates is in shortage in the host (Snellgrove *et al.*, 1982; Harris *et al.*, 1985; Douds *et al.*, 1988; and see Chapter 1) and bacterial cell numbers colonized to the host are too high. For a more complete understanding of this phenomenon, additional experiments are needed.

Kinetics of the effect of GR12-2 on the process of germination and post-germination growth of canola and lettuce grown in the dark

As under fluorescent light, GR12-2 inoculation had no significant effect on seed germination of canola and lettuce in the dark. Figure 4-15 shows the germination process of canola and lettuce in gnotobiotic growth pouches in the dark.

The effect of GR12-2 seed inoculation on post-germination growth of canola and

lettuce grown in the dark (Fig. 4-16) was very similar to that occurring in the light (Figs. 4-9 to 4-12). However, the promotive effect of GR12-2 on root elongation of dark-grown canola seedlings became visible about 5 h later than for the seedlings grown in the light. Root growth differences were also less significant during the period of 36 to 60 ASI in the dark than for light-grown seedlings (see Figs. 4-9 and 4-16). This indicates that the effect of GR12-2 on canola root growth is possibly correlated with GA metabolism. As dark-grown seedlings may contain a higher level (more supraoptimal) of growth-effective GAs, it may take longer for the PGPR strain to confer its PRE phenotype on the plant.

Kinetics of the effect of GR12-2 on root growth of lettuce grown in the dark was almost identical to that in the light, despite the fact that seed germination in the dark occurred about 6-h later than in the light (Fig. 4-16). There was no significant effect of GR12-2 seed-inoculation on hypocotyl elongation of either canola or lettuce seedlings grown in the dark in this trial (data not shown) as was for most trials in this study.

Putting together the above results, it seems clear that: i) the effect of *P. putida* GR12-2 seed inoculation on germination and post-germination growth of canola and lettuce (and possibly other species) is independent of light conditions (i.e. it occurs in both light and dark); ii) the PRE effect of this PGPR strain on canola and lettuce occurs during the post-germination growth phase. iii) the promotive effect on root length growth and root fw is repeatable, but the promotive effect on canola root dw is not as previously reported (Lifshitz *et al.*, 1987; Young *et al.*, 1993); and iv) the direct PRE effect of GR12-2 on canola (cv. Tobin and cv. rosette) and lettuce is likely mediated by PGRs within the plant, especially the levels of "growth-effective" GAs in the root.

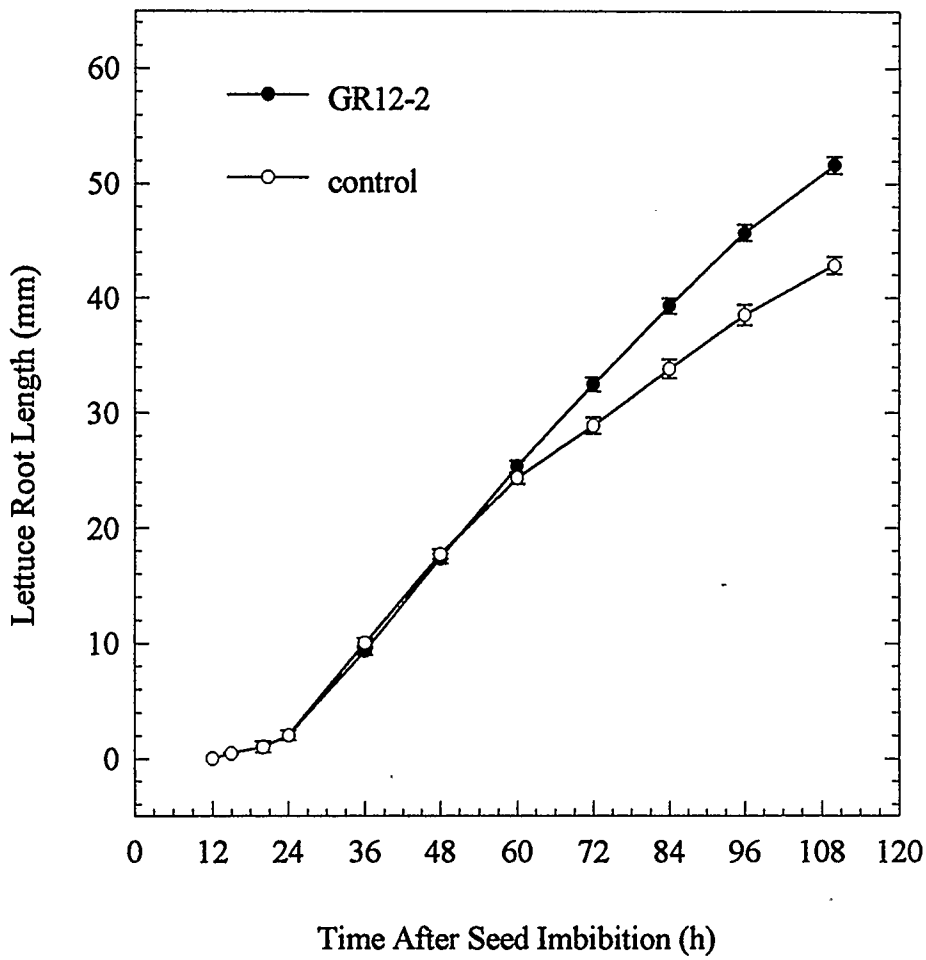


Figure 4-12. The effect of GR12-2 seed-inoculation on kinetics of lettuce seed germination and seedling root elongation in gnotobiotic growth pouches under cool-white fluorescent light. Values represent the mean of 100 measurements. Error bars represent 95% confidence intervals.

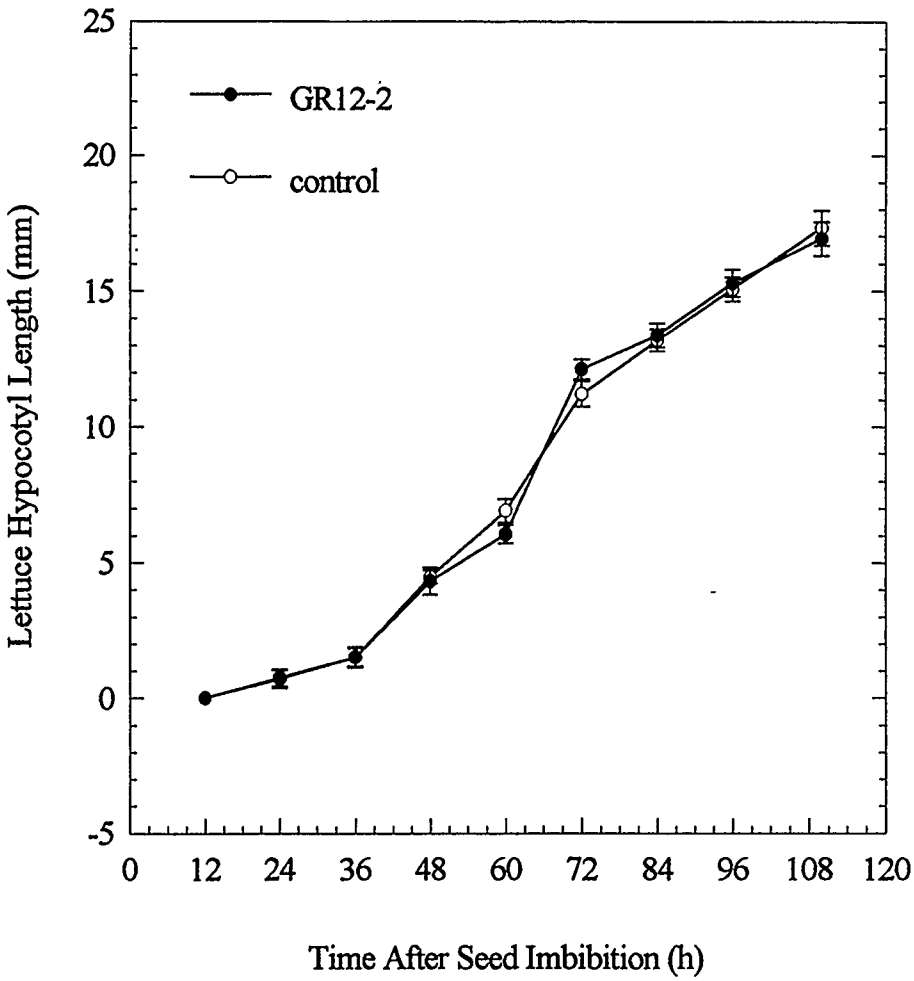


Figure 4-13. The effect of GR12-2 seed-inoculation on kinetics of hypocotyl elongation of lettuce seedlings grown in gnotobiotic growth pouches under cool-white fluorescent light. Values represent the mean of 100 measurements. Error bars represent 95% confidence intervals.

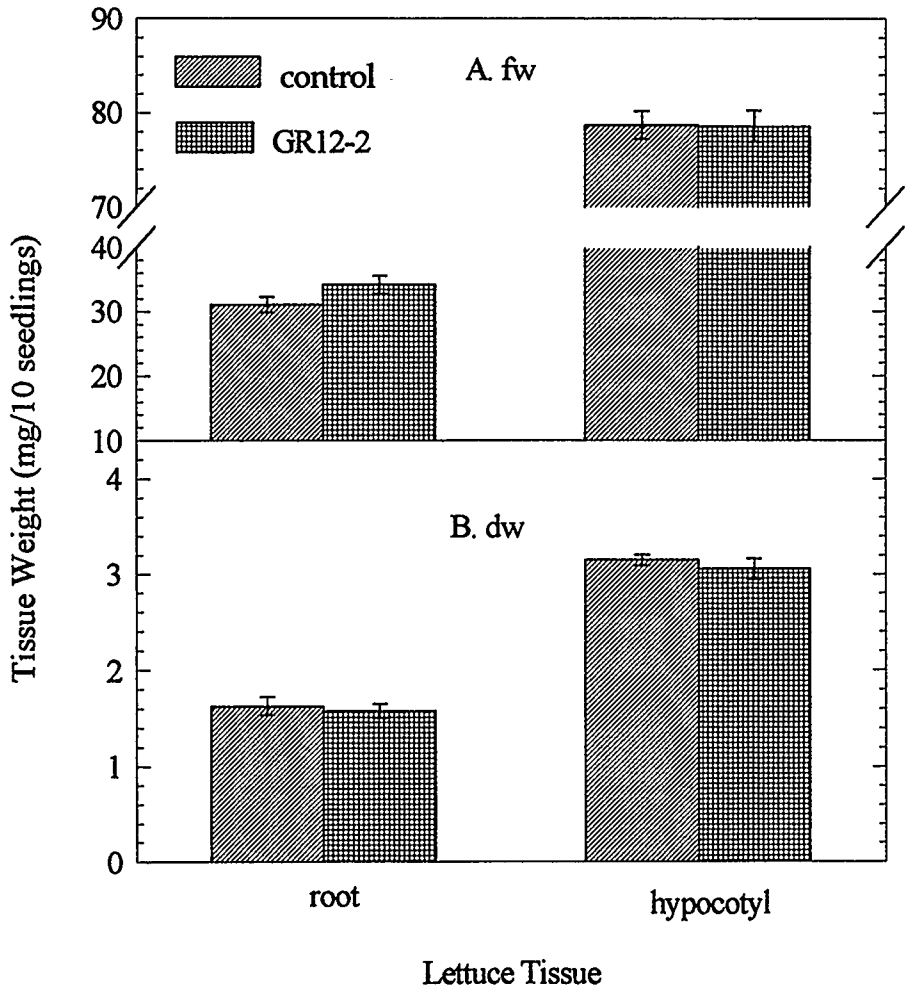


Figure 4-14. The effect of GR12-2 seed-inoculation on tissue fw (A) and dw (B) of 110-h-old lettuce seedlings grown in gnotobiotic pouches in cool-white fluorescent light. Values represent the mean of 20 measurements. Error bars represent 95% confidence intervals.

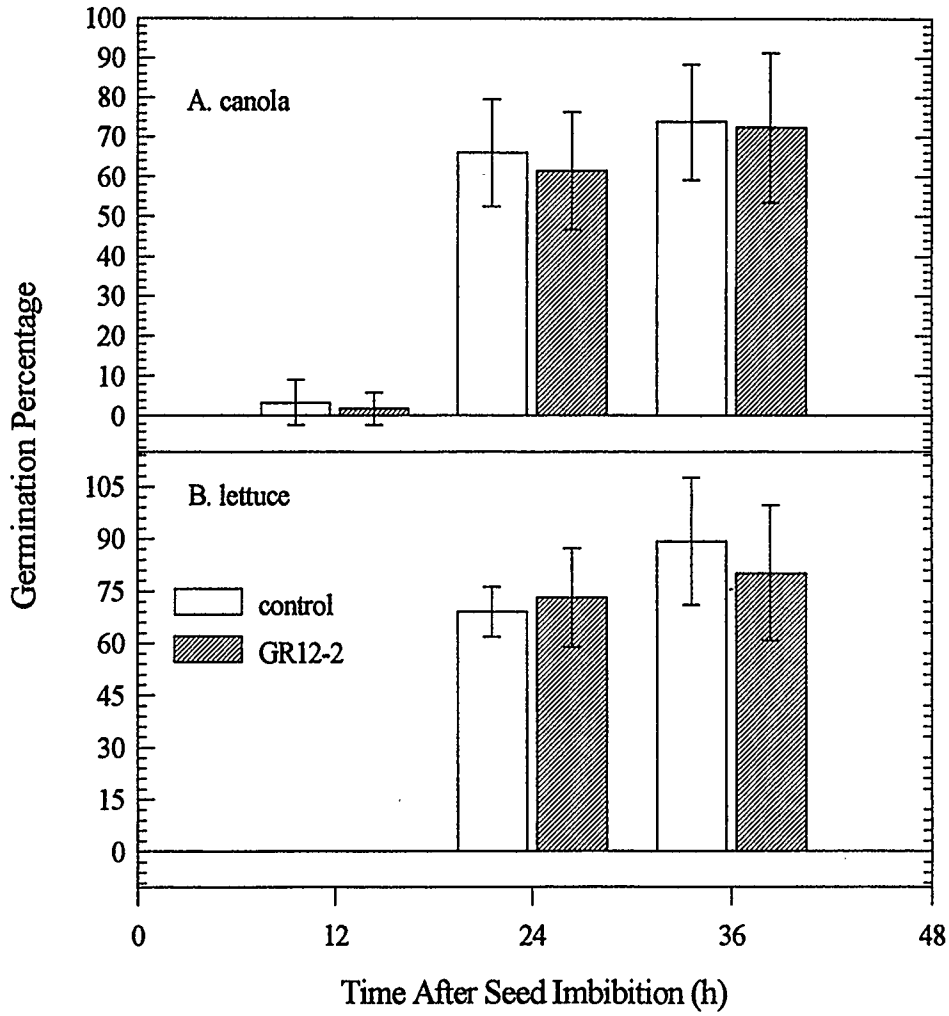


Figure 4-15. Kinetics of the effect of GR12-2 inoculation on seed germination of canola (A) and lettuce (B) in gnotobiotic growth pouches in the dark. Values represent the mean of 5 replicates of 10 seeds. Error bars represent 95% confidence intervals.

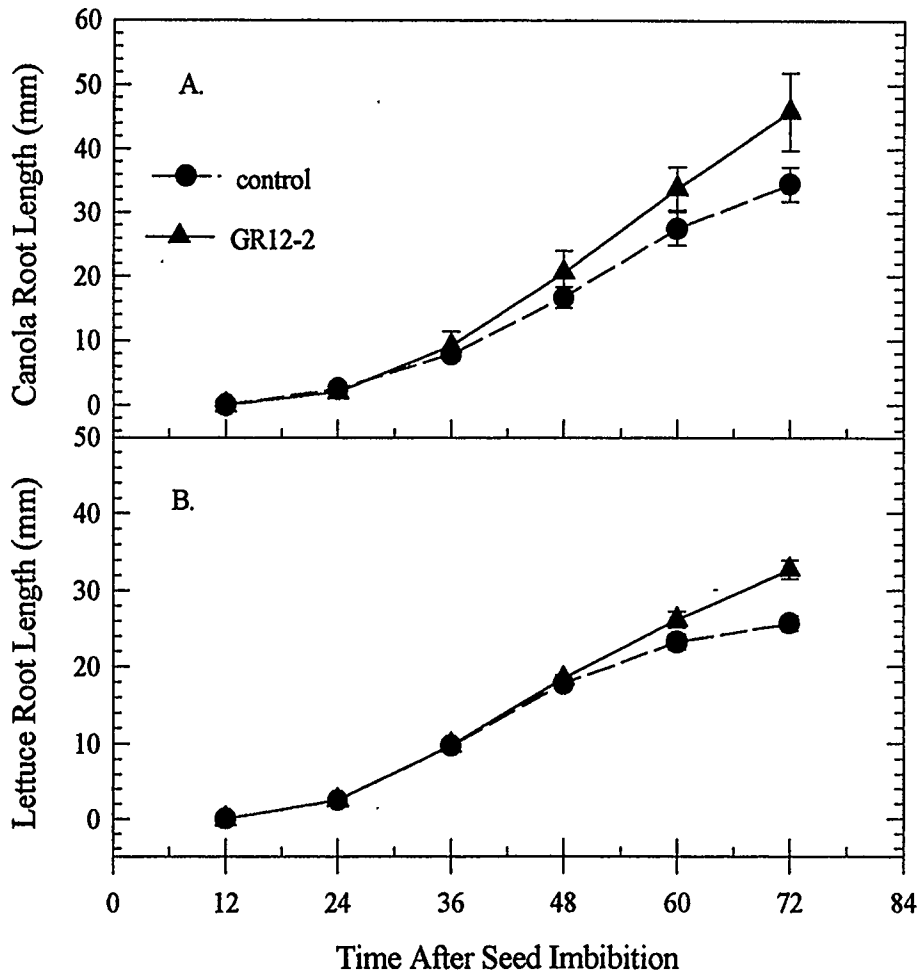


Figure 4-16. The effect of GR12-2 seed-inoculation on kinetics of root elongation of canola (A) and lettuce (B) seedlings during post-germination growth in gnotobiotic growth pouches in the dark. Values represent the mean of 25 to 35 measurements. Error bars represent 95% confidence intervals.

CHAPTER 5

THE DIRECT PROMOTIVE EFFECT OF *RHIZOBIUM* SPP. ON EARLY GROWTH OF NON-LEGUMINOUS PLANTS- DISCOVERY OF A NEW FEATURE OF RHIZOBIUM-PLANT RELATIONSHIPS

5-1. Introduction

Bacteria of the genus *Rhizobium* are able to infect leguminous plants, initiating a coordinated series of physiological and morphological events which result in the formation of root nodules, in which atmospheric N_2 is fixed. In this symbiosis *Rhizobium* provides the host plant with a reduced form of nitrogen, which in turn, effectively improves the nitrogen status of the soil in which the legume grows. Nitrogen fixation in the bacterial:plant symbiosis has been investigated for many years in hopes of finding and/or generating new *Rhizobium* strains, and to obtain a more complete understanding of the process (see Boyd, 1984; Nap and Bisseling, 1990; de Bruijn and Downie, 1991; de Bruijn *et al.*, 1992). In the *Rhizobium*-legume research area, most time and effort have been expended toward the N_2-fixation process, even though the relationship between *Rhizobium* and legumes seems far more complex (Triplett *et al.*, 1981; Bhattacharyya and Basu, 1990; Long and Staskawicz, 1993).

It was widely assumed that N_2 fixation was the sole reason why plants inoculated with *Rhizobium* had increased growth (Okon and Hadar, 1987). However, even though most of the significant and consistent increases in yield of *Rhizobium*-inoculated crops can be

attributed to N_2 fixation by the symbionts, some other mechanism(s) may be also involved (Elliott and Fredrickson, 1987). It has been suggested that PGRs produced by the microbial inoculant, or by the plant in response to inoculation, may play an additional role in this beneficial relationship between *Rhizobium* and the leguminous plant (Dullaard 1967, 1970; Phillips and Torrey, 1970; Puppo and Riguard, 1978; Wang *et al.*, 1982; Badennoch-Jones *et al.*, 1982, 1983; Atzorn *et al.*, 1988). However, most of those reports were restricted to potential functions of PGRs (produced by *Rhizobium* or by the plant in response to inoculation) in root nodule formation of legumes after bacterial inoculation.

More direct evidence was provided by Blevins' group (Evensen and Blevins, 1981; Triplett *et al.*, 1981). They found that internode growth and leaf numbers of lima bean plants (*Phaseolus lunatus* L.) were significantly increased by inoculation with a strain of *Rhizobium*, 127E14. This strain is deficient in nitrate reductase activity. This profound effect by 127E14 was repeatable on several lima bean cultivars, either grown in the greenhouse or in the growth chamber (Evensen and Blevins, 1981; Triplett *et al.*, 1981). The response of lima bean to inoculation with 127E14 appeared to be mediated by plant GAs, as applied GA_3 to the apex of uninoculated plants could mimic effect of inoculation with 127E14. Also, application of GA biosynthesis inhibitors to the root system could reverse the promotive effect of 127E14 on internode elongation (Evensen and Blevins, 1981; Triplett *et al.*, 1981). The speculated role of GAs in the promotion of lima bean shoot elongation growth was supported by analysis of endogenous GAs in the inoculated plants using the lettuce hypocotyl bioassay (Evensen and Blevins, 1981) and later by GC-MS-SIM (Dobert *et al.* 1992).

It is thus clear that *Rhizobium* may effectively promote growth of legume plants either through N_2-fixation or through other mechanisms.

Compared to the symbiotic interactions between *Rhizobium* and legumes, the relationship between *Rhizobium* and non-leguminous plants is poorly understood. It has been noted that while *Rhizobium* spp. may primarily benefit legumes, there is a beneficial effect upon companion plants and also upon succeeding non-legume crops planted in the same soil (Sharma and Bajpai, 1989), or on crops just treated with the legume soil (Fyson and Oaks, 1990).

However, to date there is no evidence that *Rhizobium* plays a direct role in promotion of growth of non-leguminous plants. Using the gnotobiotic growth pouch assay system described in Chapter 4, I decided to examine the effect of *Rhizobium* on growth of lettuce cv. Grand Rapids, and then upon canola cv. Tobin seedlings.

5-2. Growth promotion of young lettuce and canola seedlings by *Rhizobium* spp.

Materials and Methods

Rhizobium spp., its culture medium and growth

All *Rhizobium* strains used in this study were kindly provided by Dr. Michael F. Hynes. Table 5-1 lists all available information about the genetic features of the *Rhizobium* spp. that were used in this research. All *Rhizobium* strains used in this study

will be presented as code numbers through this dissertation in case a patent application is subsequently filed.

A liquid medium named PH was used for propagation of *Rhizobium* cells. The so-called PH medium is comprised of 4 g peptone, 1 g tryptone, 1 g yeast extract, 0.4 g $CaCl_2$ and 0.4 g $MgSO_4$ in 1 litre (the recipe was from Dr. M. Hynes). Growth of the *Rhizobium* strains in PH medium was relatively slower than was growth of *P. putida* GR12-2 grown in PP. When cultured at 23° C, it normally took more than 40 h to get a 10 ml PH culture (from a single colony) up to the 0.3 to 0.5 unit OD_{780} value, where cells were harvested for the growth pouch assay use. High temperatures (at or above 28° C) were not suitable for all the *Rhizobium* strains (e.g. sub-optimal cell proliferation occurred with some strains).

Preparation of the rhizobial inoculants

Four strains of *Rhizobium leguminosarum* were used in this trial. Their codes were UCS1, a strain of bv. *viciae*; UCS2, a strain of bv. *phaseoli*; UCS3, a mutant from bv. *viciae*; and UCS4, a strain of bv. *trifolii*. Three of the strains are N_2 fixers, with UCS4 uncertain (Table 5-1). One colony from each strain was inoculated into 10 ml of PH medium and cultured at 23° C for around 40 to 50 h until the value of OD_{780} reached about 0.5. Cells in each culture of *Rhizobium* were harvested and prepared as described in Chapter 4 for seed inoculation of canola and lettuce with *P. putida* GR12-2.

Table 5-1. Basic information about the genetic features of *Rhizobium* strains used in this study (provided by Dr. Hynes).

Strain	N_2 Fixing (y/n)	species	wild type (y/n)	if a mutant	
				from strain	type of mutant
UCS3	y	bv. *viciae*	n	UCS3P	spontaneous SM^R
UCS4	y/n	bv. *trifolii*	y		
UCS1	y	bv. *viciae*	y		
UCS2	y	bv. *phaseoli*	y		
UCS5	n	bv. *viciae*	n	UCS3	-plasmid "e"
UCS6	n	bv. *viciae*	n	UCS3	-plasmid "d"
UCS7	N/A	bv. *viciae*	n	UCS3	-plasmid "f"
UCS8	N/A	bv. *viciae*	n	UCS3	adenosine
UCS9	N/A	bv. *trifolii*	n	UCS4	adenosine
UCS10	N/A	bv. *viciae*	n	UCS3	tryptophan
UCS11	N/A	bv. *viciae*	n	UCS3	adenosine
UCS12	N/A	bv. *viciae*	n	UCS3	-plasmid"c"
UCS13	N/A	bv. *trifolii*	n	UCS4	tryptophan

Seeds of canola and lettuce were surface-sterilized and soaked for 1 h in 1.5 to 2 ml of 0.1 M $MgSO_4$ of the *Rhizobium* cell suspensions. The 0.1 M $MgSO_4$ buffer alone was also used to soak seeds and served as the control treatment (Section 4-3).

The *Rhizobium*-inoculated plant seeds were then placed in gnotobiotic growth pouches and germinated and grown at room temperature (23° C) in the dark for about 80 h, at which time root and hypocotyl length was determined. Data for seedling growth were analyzed and plotted as described before (Chapter 2).

Results and Discussion

Growth of lettuce cv. Grand Rapids was appreciably affected by inoculation with the *Rhizobium* strains (Fig. 5-1). Root length of 80-h-old lettuce seedlings was highly significantly ($P \leq 0.01$) enhanced by UCS2, UCS3 and UCS4. Strain UCS4 even significantly ($P \leq 0.05$) promoted root elongation relative to strains UCS2 and UCS3 (Fig. 5-1). Strain UCS1, however, had no significant effect on either root or hypocotyl elongation compared to controls. Interestingly, inoculation of lettuce seeds with strains UCS2, UCS3 and UCS4 also tended to increase hypocotyl elongation. Lettuce plants pre-treated with those *Rhizobium* strains grew significantly (UCS2) or slightly (UCS3 and UCS4) longer hypocotyls relative to the control (Fig. 5-1).

The results of *Rhizobium* inoculation on lettuce growth were also repeatable with seedlings of canola cv. Tobin. However, the variation was considerably higher within each treatment for canola than for lettuce. Figure 5-2 shows that root elongation of

canola seedlings was significantly ($P \leq 0.05$) promoted by strains UCS2, UCS3 and UCS4 at 80 h ASI, while this promotion was much less pronounced in the UCS1-inoculated plants. Among the four strains UCS4 showed the best (but not significant relative to strains UCS2 and UCS3) potential for promoting canola root growth. Hypocotyl length growth of young canola seedlings was not significantly altered by inoculation with either of the four *Rhizobium* strains in this trial.

The finding that post-germination growth of non-leguminous plant species (e.g. canola and lettuce) can be significantly promoted by *Rhizobium* spp. through seed-inoculation is both novel and interesting, and may be of potential importance to modern agriculture.

Since both lettuce and canola seedlings were only 80-h old and were grown in the dark, their growth was dependent solely on seed-derived nutrients and assimilate. Also, nodules were not formed on these non-leguminous plants, so N_2 fixation should not be a factor. Furthermore, not all of the *Rhizobium* spp. tested were root-growth promotive. Strain UCS1 was not promotive of root elongation (Fig. 5-1), although it is a N_2 fixer (see Table 5-1). Fixation of atmospheric nitrogen, therefore, is unlikely to be involved in the growth promotion seen for lettuce and canola in this gnotobiotic pouch assay. Alternatively, the promotive effect of *Rhizobium* on post-germination growth of these two non-legumes may be a direct, PGR-mediated phenomenon.

The mechanism by which these *Rhizobium* spp. affect growth of non-leguminous plants is thus examined and discussed in more details later in this Chapter.

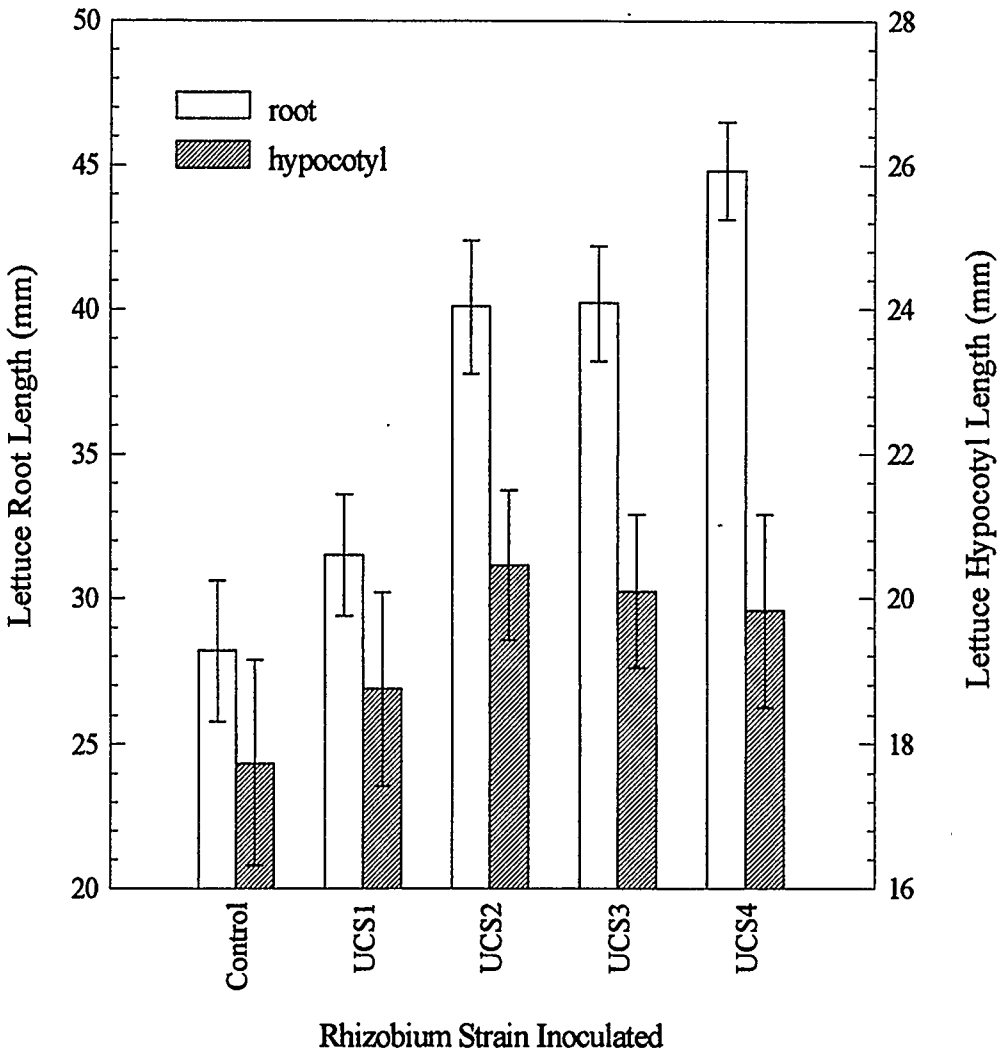


Figure 5-1. The effect of *Rhizobium* strains on growth of very young lettuce seedlings germinated and grown in gnotobiotic pouches in the dark. Values represent the mean of 40 seedlings harvested at 80 ASI. Error bars represent 95% confidence intervals.

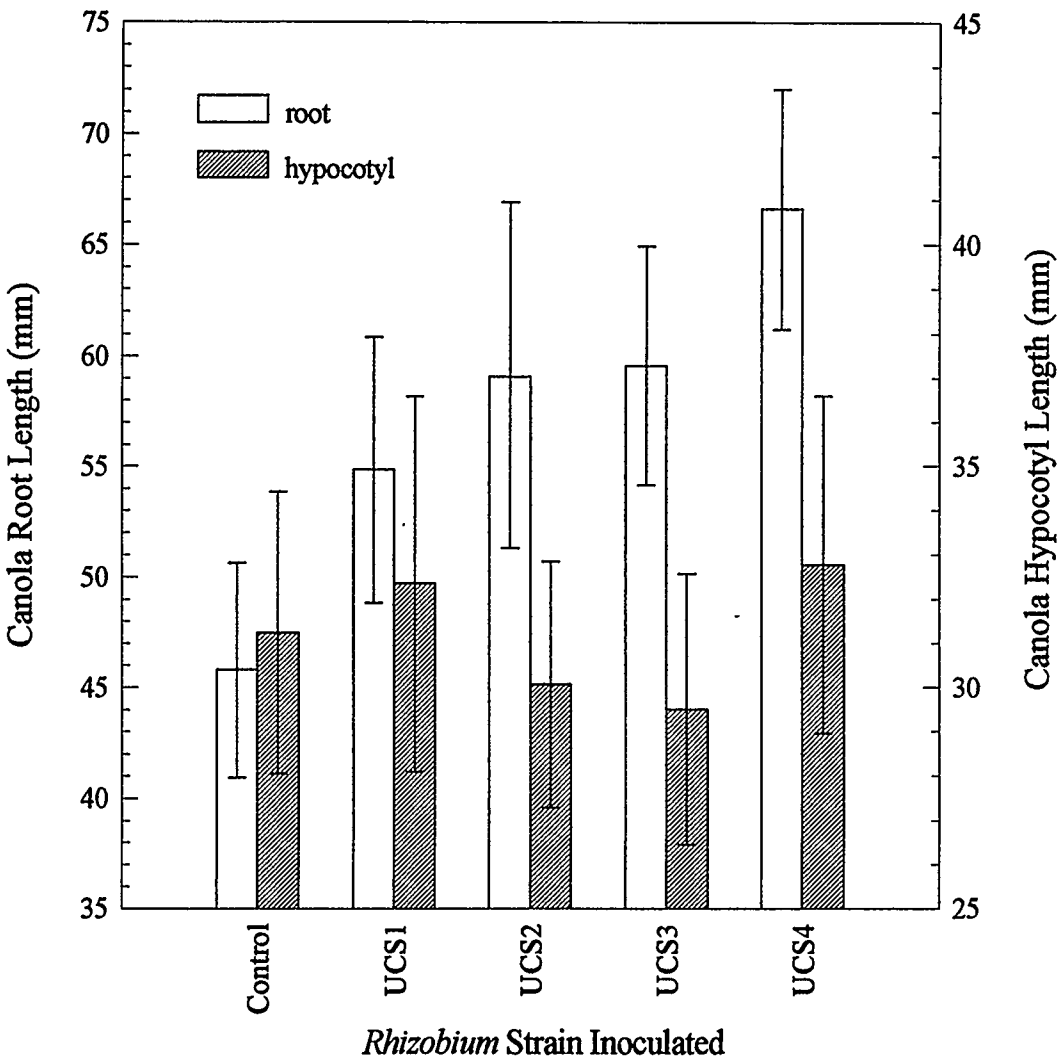


Figure 5-2. The effect of four *Rhizobium* strains on growth of canola seedlings grown in gnotobiotic growth pouches in the dark. Values represent the mean of 40 seedlings harvested at 80 h ASI. Error bars represent 95% confidence intervals.

5-3. Features of the direct effect by *Rhizobium* spp. on promotion of plant growth

It has been noted that extracellular products of *Pseudomonas putida* GR12-2 can significantly stimulate canola root elongation without involvement of bacterial cells (Tang, 1992; Lifshitz, unpublished). This indicates that there are bacterial metabolite(s) in the culture medium of GR12-2 which can mimic the PRE effect of PGPR inoculation on canola seedlings. I also found that GR12-2 culture filtrates, used as a seed soak, can significantly increase root elongation of both canola and lettuce (data not shown).

In the well-studied N_2-fixation process, it is known that for a *Rhizobium* strain to form root and stem nodules on a specific leguminous plant, it has to infect the host first (de Bruijn *et al.*, 1992). During early interactions of infection, multiple (regulatory) signals go back and forth between the bacterium and the plant to allow for a successful colonization. Without colonization bacterial N_2 fixation, and subsequently plant growth promotion, will not occur (see Nap and Bisseling, 1990; de Bruijn and Downie, 1991).

The mechanism(s) by which *Rhizobium* can directly promote the early growth of very young non-leguminous plants is not yet known. It is thus worthwhile determining if the direct effect relies on a successful infection by the bacterium (but without nodule formation). Further, it is important to determine if extracellular products (metabolites) of *Rhizobium* spp. in the culture medium can promote early growth of non-leguminous plants, as occurs for *P. putida* strain GR12-2.

Another question is whether, as in the case of free-living *Pseudomonas* spp., *Rhizobium* in association with a non-legume can fix N_2 without forming nodules. That

is, was root elongation of canola and lettuce seedlings still promoted by a *Rhizobium*-mediated N_2 fixation in the environment of the gnotobiotic growth pouches?

Materials and Methods

Growth pouch assay of effects of *Rhizobium* culture filtrates on growth of lettuce seedlings

Each of the four *Rhizobium* strains, UCS1, UCS2, UCS3 and UCS4, was inoculated in 10 ml PH medium and cultured as above. Bacterial-free filtrates of the cultures were prepared by centrifuging at 5,000 rpm for 15 min and then filtering through 0.45 μm filters. Seeds of lettuce were soaked in each culture filtrate for one h. The PH medium alone was used as the nil inoculation control of the experiment. The cell suspension of strain UCS3 was also used to soak the seeds as a positive inoculation control to the filtrate treatments. Eight pouches were used for each treatment. The gnotobiotic growth pouches were incubated at room temperature (23° C) in the dark for about 80 h, at which time lettuce seedlings were harvested and root and shoot length was determined.

Growth pouch assay of the effects of *Rhizobium* on promotion of lettuce and canola seedlings grown under differing nitrogen conditions

To check the possibility that growth promotion of non-leguminous plants by *Rhizobium*

is associated with bacterial N_2-fixation (by the bacterium *per se*), the effect of *Rhizobium* strain UCS3 was examined on canola and lettuce in gnotobiotic pouches under different N conditions. Cells of *Rhizobium* strain UCS3 were prepared as usual, and seeds of canola and lettuce were inoculated with the bacteria as usual. However, instead of using only water, each growth pouch was filled with 10 ml of either de-ionized water, or a series of NH_4NO_3 concentrations, e.g. 1, 10, 100, 500, and 1,000 ppm on lettuce. The external NH_4NO_3 used on canola was at 1, 10 and 100 ppm, since nitrogen is reported to be inhibitory to canola growth at concentrations above 50 ppm (Lifshitz *et al.*, 1987). The assays were run in the dark, as described earlier (Section 5-2).

Growth pouch assay of the effects of various *Rhizobium* mutants on lettuce growth

As a further check to determine if a N_2-fixation mechanism is involved in the *Rhizobium* promotion of growth in non-leguminous plants, two non-N_2-fixing *Rhizobium* mutants were tested using the gnotobiotic growth pouch assay system, e.g. strain UCS5, a mutant of UCS3 by missing plasmid "e" (still capable of inducing nodules), and strain UCS6 (also derived from UCS3, missing plasmid "d", thus Nod^-).

Adenosine- and tryptophan- auxotrophic mutants of strains UCS3 and UCS4 were also examined on the gnotobiotic growth pouch assay. It was assumed that the adenosine mutants might not produce cytokinins (CKs), while the tryptophan mutants might not biosynthesize IAA. However, since IAA synthesis in bacteria may proceed via other pathways (Frankenberger and Brunner, 1983; Arshad and Frankenberger, 1991; Cohen and

Slovin, 1993), this assumption may or may not be valid. The three adenosine mutants were strains UCS8 and UCS11 (derived from UCS3) and strain UCS9 derived from UCS4. Two tryptophan mutants tested were strain UCS10 (derived from UCS3) and strain UCS13 that was derived from UCS4. The N_2-fixation abilities of these putative hormone biosynthesis mutants is presently unknown (Table 5-1).

All the selected mutants (deficient in N_2-fixing ability or in putative hormone biosynthesis) were cultured in PH medium (Section 5-1) and cells were harvested as described earlier for *P. putida* in Section 4-3. Lettuce seeds were treated and inoculated as per Chapter 4, with eight gnotobiotic growth pouches being used for each treatment, and germination/seedling post-germination growth occurring at room temperature (23° C) in the dark for 80 h. The length growth of the root and hypocotyl of young lettuce seedlings was recorded and analyzed statistically.

Results and Discussion

Is inoculation essential for *Rhizobium* to promote growth of young lettuce plants?

None of the four cell-free culture filtrates from the *Rhizobium* strains (e.g. UCS1, UCS2, UCS3, and UCS4) conferred any significant ($P \leq 0.05$) promotive effect on root elongation of the lettuce seedlings (Fig. 5-3) relative to the control. This was very different from the effect of the culture filtrates of *P. putida* GR12-2, which effectively promoted root elongation for both canola (Tang, 1992; Lifshitz, unpublished) and lettuce

seedlings under the same conditions (Sheng, unpublished). Interestingly, the *Rhizobium* culture filtrates had a somewhat depressing effect on lettuce hypocotyl growth, except for strain UCS3 (Fig. 5-3). The fact that culture filtrates of the *Rhizobium* strains (which are promotive to plant growth when seed-inoculated) had no significant effect on early growth of young lettuce seedlings implies that for *Rhizobium* PGPR to promote growth of non-leguminous plants, effective colonization by the bacterium is essential. However, other speculative explanations are also possible. One is that *Rhizobium* may be capable of producing root-growth promoters in different culture media, or only when inducing signals from the plant are provided.

The effect of N conditions on *Rhizobium* plant growth promotion

Root elongation was significantly reduced by NH_4NO_3 at higher concentrations (500 and 1,000 ppm) for both the control and the *Rhizobium* inoculated treatments (Fig. 5-4b). The highest N concentration (1,000 ppm) was most inhibitory. Concentrations of NH_4NO_3 at and lower than 100 ppm had no significant effect on lettuce seedling root elongation, although 10 and 100 ppm tended to be slightly promotive. However, it is very clear that under all N conditions seed inoculation of *Rhizobium* strain UCS3 was significantly promotive of lettuce root growth, relative to the control (Fig. 5-4b). Similar results were also obtained with strains UCS2 and UCS4 (data not shown), indicating that the PRE of young lettuce seedlings by *Rhizobium* inoculation is nothing to do with improvement of nitrogen conditions in plant growth environment (growth pouches).

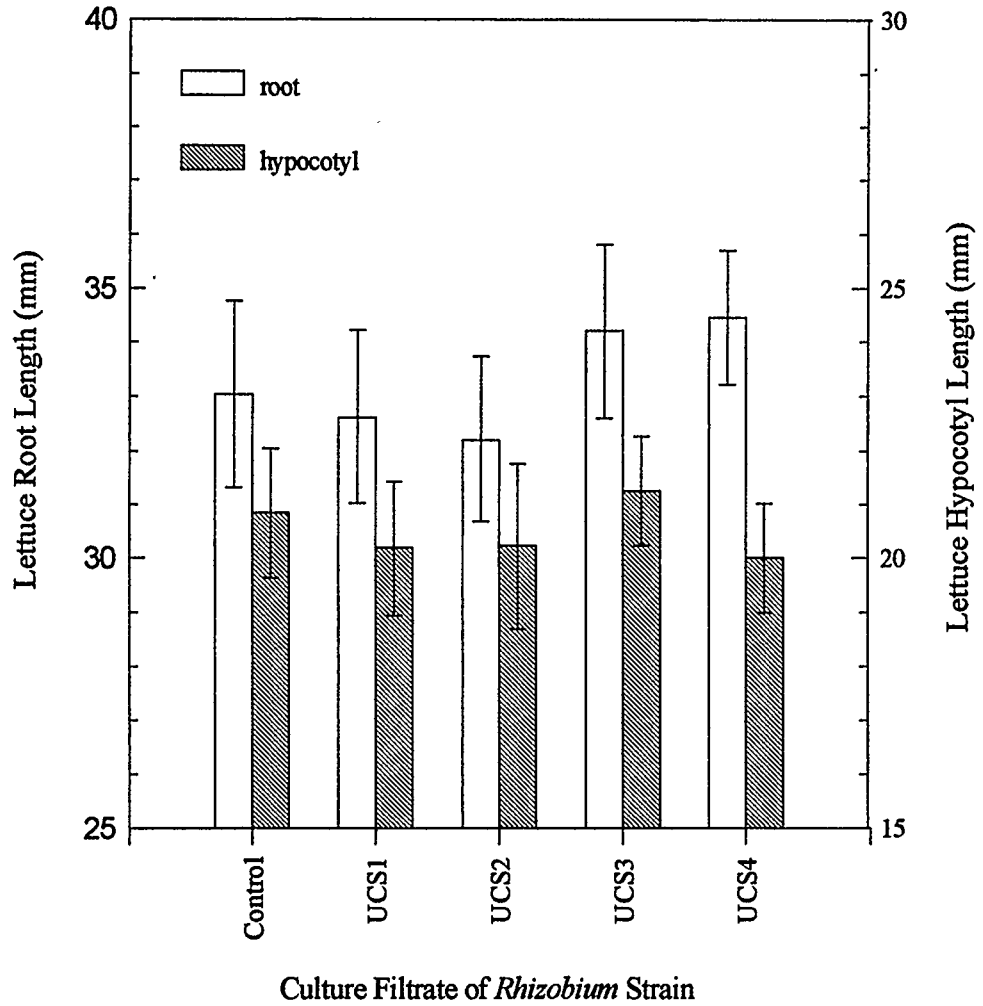


Figure 5-3. The effects of culture filtrates of four *Rhizobium* strains on growth of lettuce seedlings. Seeds were germinated and grown in gnotobiotic pouches in darkness. Values represent the mean of 50 seedlings harvested at 80 h ASI. Error bars represent 95% confidence intervals.

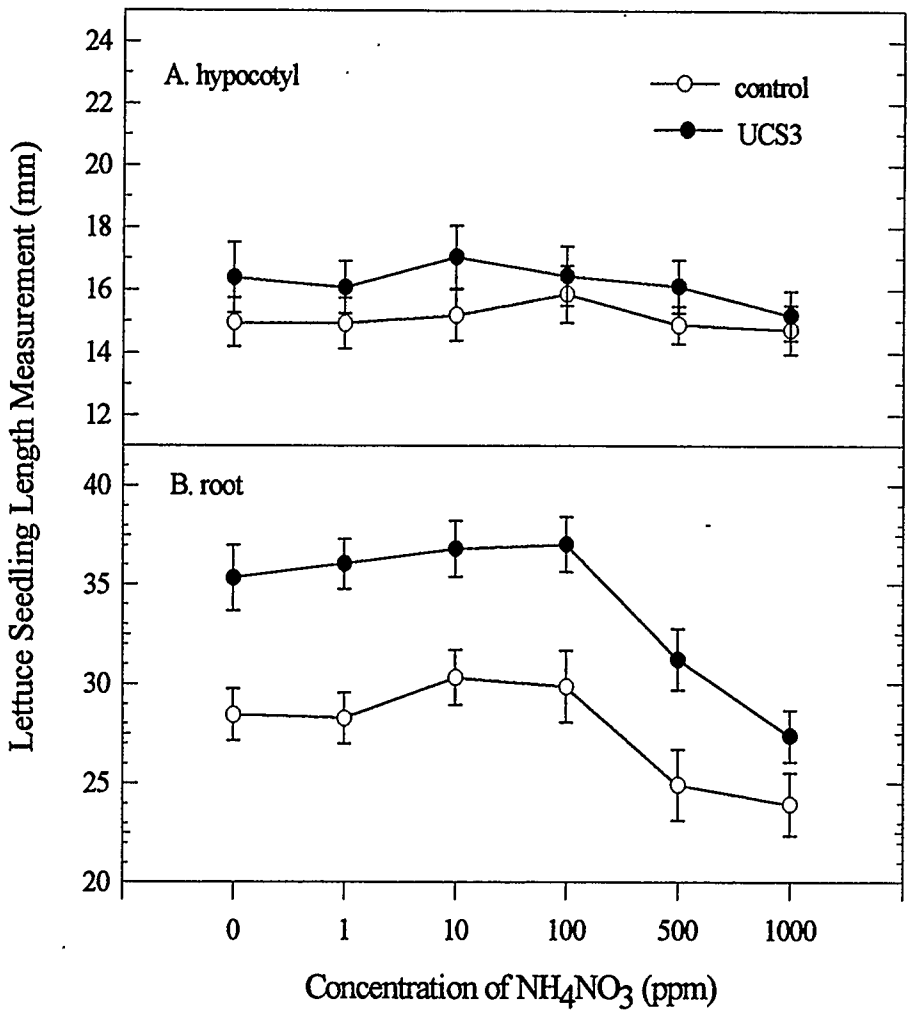


Figure 5-4. The effect of external nitrogen in pouch solution on lettuce hypocotyl (A) and root (B) elongation after seed inoculation with *Rhizobium* strain UCS3. Lettuce seeds were germinated and grown in gnotobiotic pouches in the dark at 23° C. Values represent the mean of 40 measurements at 80 h ASI. Error bars represent 95% confidence intervals.

Hypocotyl growth of the young lettuce seedlings was not significantly altered by any of the applied NH_4NO_3 concentrations in pouch solution, with or without *Rhizobium* inoculation. However, seed inoculation with UCS3 showed a (non-significant) promotive effect on lettuce hypocotyl elongation at all N concentrations tested relative to the control (Fig. 5-4a).

For canola the external NH_4NO_3 also showed no significant effect on post-germination growth of roots and hypocotyls (Fig. 5-5). However, as above for lettuce seed-inoculation with UCS3 highly significantly ($P \leq 0.01$) promoted root elongation, and tended to promote hypocotyl elongation of the 80-h-old young canola seedlings under all N conditions (Fig. 5-5).

Inhibition of root elongation of young canola seedlings (*B. campestris*) by external nitrogen has been reported before, although concentrations lower than 50 ppm had no significant effect (Lifshitz *et al.*, 1987). The results shown in Figs. 5-4 and 5-5 clearly demonstrate that seed germination and/or seedling early growth of lettuce and canola did not benefit from extra inorganic nitrogen in the growth environment (e.g. pouch solution), and the trend of general effect of the externally applied NH_4NO_3 on growth of canola and lettuce is identical to UCS3-inoculated and the non-inoculated plants (Figs. 5-4 & 5-5).

Since *Rhizobium* strain UCS3 can consistently promote root elongation of lettuce and canola seedlings at all of the tested NH_4NO_3 concentrations and there is no significant interactions between inoculation and external nitrogen treatments (based on two way ANOVA), it seems unlikely that this *Rhizobium* PGPR effect on growth promotion of non-leguminous plants by the seed-inoculation treatment is due to a N_2-fixation mechanism.

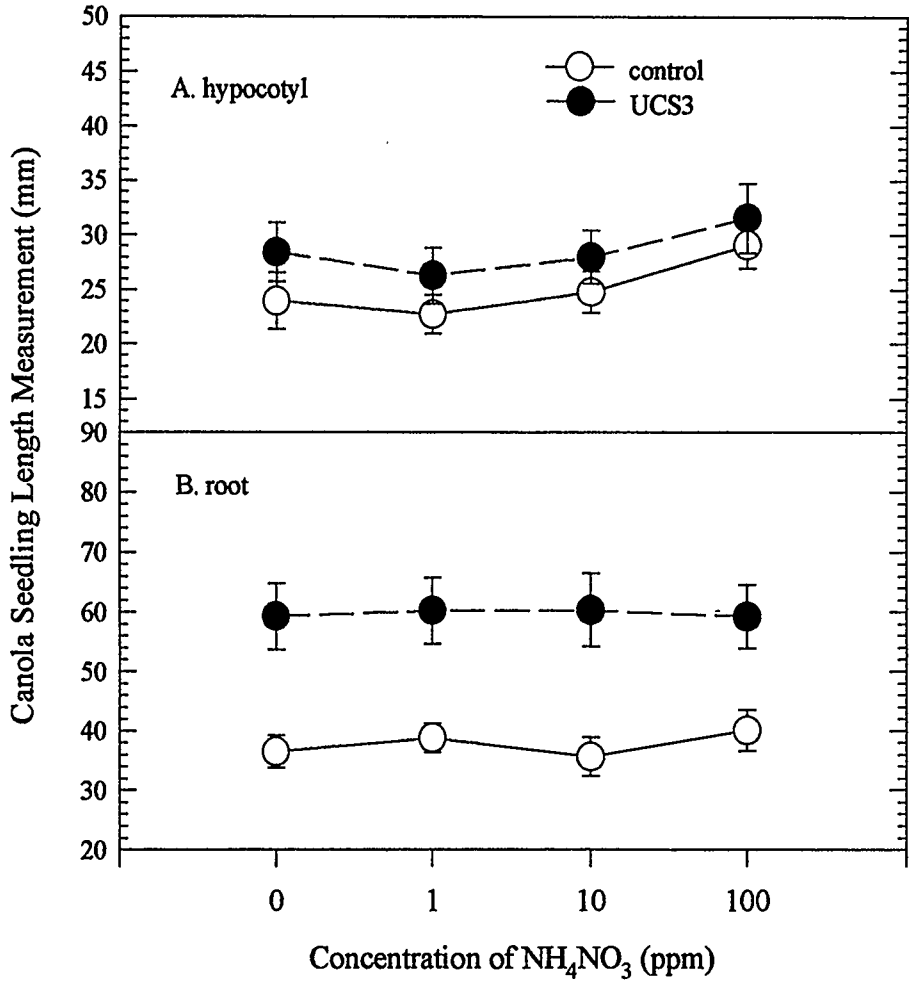


Figure 5-5. The effect of external NH_4NO_3 in pouch solution on canola hypocotyl (A) and root (B) elongation after seed-inoculation with *Rhizobium* strain UCS3. Canola seeds were germinated and grown in gnotobiotic pouches in the dark at 23° C. Values represent the mean of 40 measurements at 80 h ASI. Error bars represent 95% confidence intervals.

The effects of N_2-fixation deficient *Rhizobium* mutants on early growth of lettuce seedlings

The two *Rhizobium* mutants UCS5 and UCS6 (unable to fix N_2) did not show any significant differences from their parent strains in promotion of lettuce seedling root elongation (Fig. 5-6). Strain UCS6 promoted root elongation as effectively as the parent strain (UCS3), while strain UCS5 was slightly less promotive (relative to the parent), but still highly significantly ($P \leq 0.01$) promotive in comparison with the control (Fig. 5-6a). Like their parent, the two mutants also gave a (non-significant) promotive effect on the hypocotyl growth (Fig. 5-6b). Therefore, growth promotion of non-leguminous plants by *Rhizobium* does not require that a strain should retain its capability to fix N_2. As before, the N_2-fixing strain UCS1 had no significant effect on lettuce seedling growth, relative to the controls, implying that N_2-fixation certainly plays no role in the growth promotion of non-legume plants by *Rhizobium*.

The effect of putative hormone-deficient *Rhizobium* mutants on germination/early growth of lettuce seedlings

Interestingly, the putative hormone biosynthesis mutants of *Rhizobium* behaved quite differently from the parent strains (Fig. 5-6). The two adenosine-auxotrophic mutants, UCS8 and UCS11 (derived from UCS3), showed a highly significant ($P \leq 0.01$) reduced ability in promotion of lettuce seedling root elongation, relative to the parent strain (Fig.

5-6). Nor was hypocotyl elongation promoted by either of those two adenosine-auxotrophic mutants. Since these two mutants are presumably unable to produce cytokinins (CKs), these results suggest that CKs may play a role in the direct growth-promotive effect of *Rhizobium* on lettuce seedling growth. However, adenosine-deficient mutant UCS9 (derived from UCS4) only showed a reduced growth promotion on hypocotyl elongation, but was still very promotive of lettuce seedling root elongation relative to its parent strain (Fig. 5-6). Thus, until actual CK production levels are assessed for these three adenosine-deficient mutants, no firm conclusions can be made.

The tryptophan-deficient mutant UCS10 (derived from UCS3) had totally lost the parent strain's inherent ability to promote both root and hypocotyl growth of lettuce (Fig. 5-6). Similarly, the tryptophan mutant UCS13 (derived from UCS4) had a significantly ($P \leq 0.01$) reduced ability (relative to the parent) on the promotion of root and hypocotyl growth of lettuce seedlings, even though it did still promote root elongation, relative to the uninoculated control treatment. If it is as assumed that these two tryptophan mutants are indeed deficient in IAA production, then IAA derived from the *Rhizobium* PGPR parental strains may be causally involved in the direct growth-promotive effect. However, soil microorganisms may use precursors other than tryptophan, such as indole-3-lactic acid tryptophol and other indole compounds, to produce IAA (Frankenberger and Brunner, 1983; Arshad and Frankenberger, 1991; Cohen and Slovin, 1993). *Rhizobium* may also be capable of synthesizing auxins other than IAA. Moreover, tryptophan-auxotrophic bacterial mutants may not be IAA-deficient at all. Therefore, auxin status of the two *Rhizobium* mutants needs to be determined for any clear conclusions.

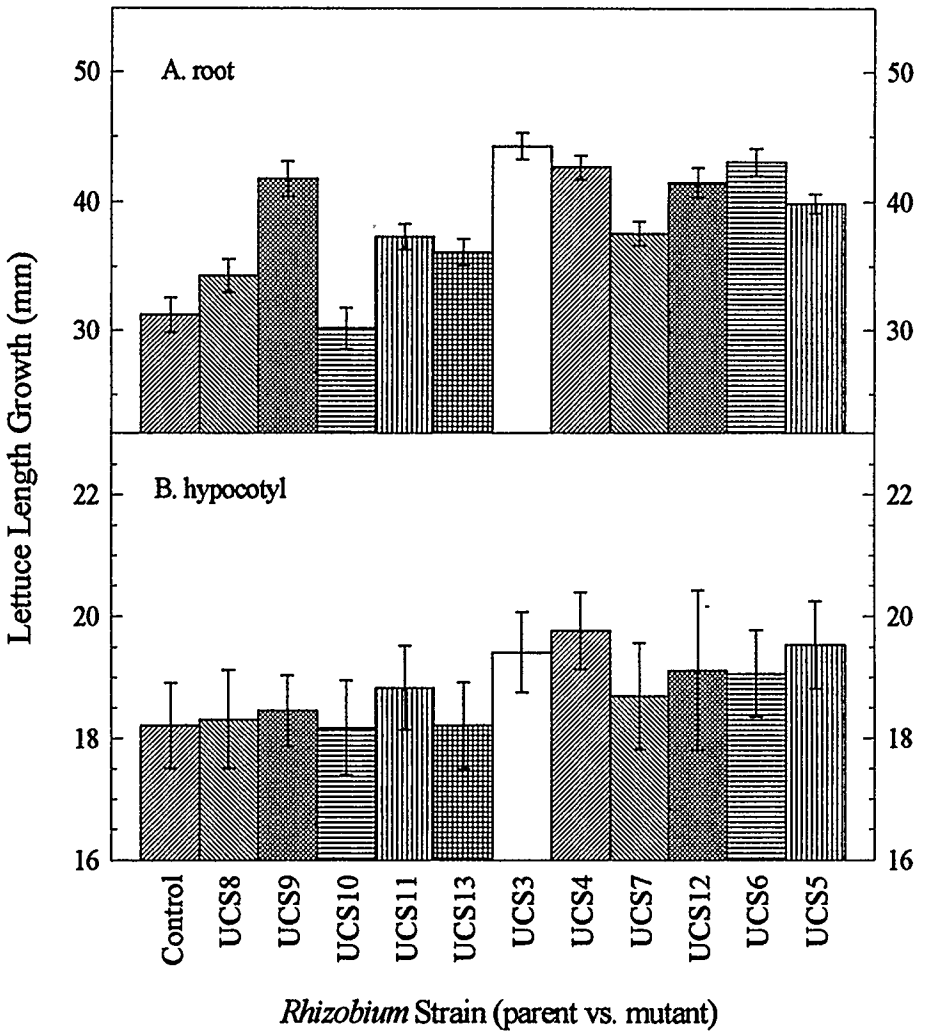


Figure 5-6. A comparison of promotive effect of *Rhizobium* strains (wild type vs. mutants) on root (A) and hypocotyl (B) growth of lettuce seedlings grown in the gnotobiotic pouches in darkness at 23° C. Values represent the mean of 50 measurements at 80 h ASI. Error bars represent 95% confidence intervals. Adenosine mutants: UCS9 (from UCS4) and UCS8 UCS11 (from UCS3); Tryptophan mutants: UCS10 (from UCS3) and UCS13 (from UCS4); N_2-fixing mutants: UCS5 & UCS6 (from UCS3).

Nevertheless, these results with the adenosine- and tryptophan-auxotrophic *Rhizobium* mutants tend to infer that plant hormones, including cytokinins and auxins may be involved in the beneficial interactions between *Rhizobium* and non-leguminous plants during stage of seedling growth.

5-4. Kinetics of the effects of *Rhizobium* on seed-germination and seedling post-germination growth of canola and lettuce

The same question applied to *P. putida* GR12-2 and its related mutants can be also asked for *Rhizobium* spp. That is, when does the direct PGPR effect occur; during seed germination or during early seedling growth? To answer this question an experiment similar to that designed for *P. putida* GR12-2 (Section 4-6) was conducted for *Rhizobium* strain UCS3.

Materials and Methods

Seeds of canola and lettuce were surface sterilized and inoculated by seed-soak in bacterial cell suspensions for one h as described in Section 4-3. The *Rhizobium* strain used in this trial was UCS3. Each plant species was subjected to two treatments, e.g. inoculation with the bacterium and an uninoculated control (seed soak with 0.1 M $MgSO_4$). The gnotobiotic growth pouch assay was used, with 20 pouches for each treatment. The growth pouches were incubated at room temperature (23° C) under the

cool-white fluorescent light (see Section 2-2) for convenience of observation. The process of seed germination and early seedling growth was assessed every 6 or 12 h.

Kinetics of the effect of *Rhizobium* strain UCS3 on canola and lettuce seed germination and on seedling post-germination growth were also assessed in the dark. Sixty pouches were used for each treatment, with five pouches being examined at every sampling time. Working procedures for these kinetics trials of the effect of *Rhizobium* on growth of non-legumes were the same as described in Chapter 4-6 for *P. putida* GR12-2.

Results and Discussion

As with *P. putida* GR12-2 (Section 4-6), inoculation with *Rhizobium* strain UCS3 did not significantly affect seed germination of canola and lettuce. Germination rate (time of radicle protrusion) was 12 h ASI for lettuce and 16 h ASI for canola, respectively, in the light. Germination frequency (percentage of seeds germinated at 24 h ASI) was about 90% for lettuce and 60% for canola, respectively. Neither germination rate nor frequency were enhanced by the bacterial seed-inoculation treatment (data not shown).

Root elongation of canola began to differ between the inoculated and uninoculated plants very soon after the protrusion of the radicle (e.g. ~24 h ASI). By 36 h ASI the difference in canola root length between the two treatments became significant ($P \leq 0.05$). From then on the root length of UCS3-inoculated seedlings was significantly longer than that of the controls (Fig. 5-7). Interestingly, in this trial the difference in root length between the two treatments at 80 h ASI was not as large as for previous experiments

where plants were grown in the dark (see Figs. 5-1 and 5-2). Thus, growth under dim fluorescent light may have (but not significantly) reduced the promotive effect of *Rhizobium* strain UCS3 on canola root elongation.

Figure 5-8 shows the effect of UCS3 on canola hypocotyl growth. The *Rhizobium*-inoculated plants began to show a significant ($P \leq 0.05$) increase in hypocotyl length (relative to the control) between 48- to 60-h ASI, and this trend lasted for about two days, then slowly diminished, and finally disappearing around 100 h ASI (Fig. 5-8). It, thus, seems that the promotion of hypocotyl length growth for canola seedlings caused by *Rhizobium* seed-inoculation may occur only within a certain period of time (e.g. from 48 to 60 h ASI) during the post-germination growth stage. However, the promotion of root elongation in canola by *Rhizobium* not only occurs much earlier and more significant, but also lasts longer than for hypocotyl elongation (Figs. 5-7 and 5-8).

Trends for lettuce seed germination and early seedling growth in the light after UCS3 seed inoculation were similar to those noted above for canola. There was no profound effect on seed germination, but a promotive effect became noticeable on root growth around 48 ASI (20 h later than for canola). Significant promotion of root elongation occurred from 60 h ASI on (Fig. 5-9). Again, promotion of root elongation by UCS3 in this trial was much reduced, compared to growth data recorded earlier for dark-grown trials (see Figs. 5-1, 5-4 and 5-6). As suggested above, this phenomenon may be due to growing the plants in the dim fluorescent light rather than in the dark. As biosynthesis of plant hormones, including GAs (Potter and Rood, 1993; Rood *et al.*, 1993; Toyomasu *et al.*, 1993; Zeevaart, 1993) and ethylene (see Abeles and Morgan, 1992 and references

therein), can be regulated by light quality and intensity, a light interaction with the direct effect by *Pseudomonas* and *Rhizobium* PGPR strains on canola and lettuce seedling growth may be causally associated with PGRs (biosynthesis and/or metabolism). Alternatively, the difference in PGPR promotion of seedling root elongation between the light and dark conditions could also be caused by differences in bacterial growth that may occur between the two light regimes.

There were no significant differences in hypocotyl elongation of lettuce between the UCS3-inoculated plants and the controls during the observed post-germination growth period (Fig. 5-10).

Kinetics of the effect of *Rhizobium* inoculation on seed germination and seedling growth of canola and lettuce in the dark

The kinetics of the effect of *Rhizobium* strain UCS3 on seed germination and post-germination growth of canola and lettuce in the dark showed no significant differences from that in the light. As with *P. putida* GR12-2, the promotive effect on root elongation of young seedlings canola and lettuce by *Rhizobium* strain UCS3 occurs during post-germination growth and is independent of light (data not shown).

Thus, the kinetics study demonstrates that the direct effect of *Rhizobium* seed inoculation on canola and lettuce occurs during the early seedling post-germination growth, but not the seed germination process, both in light and in dark.

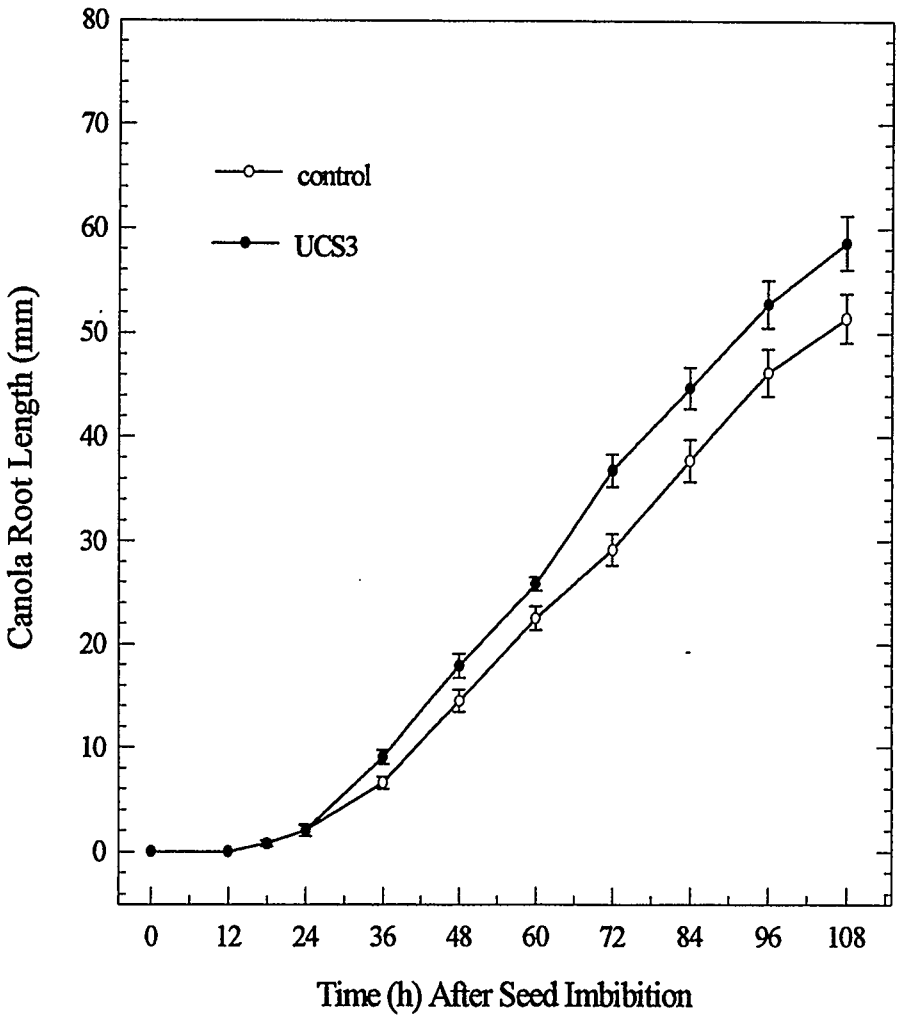


Figure 5-7. Kinetics of effects of *Rhizobium* strain UCS3 on canola cv. Tobin seed germination and root elongation during post-germination growth in gnotobiotic growth pouches under cool-white fluorescent light at 23° C. Values represent the mean of 100 plants. Error bars represent 95% confidence intervals.

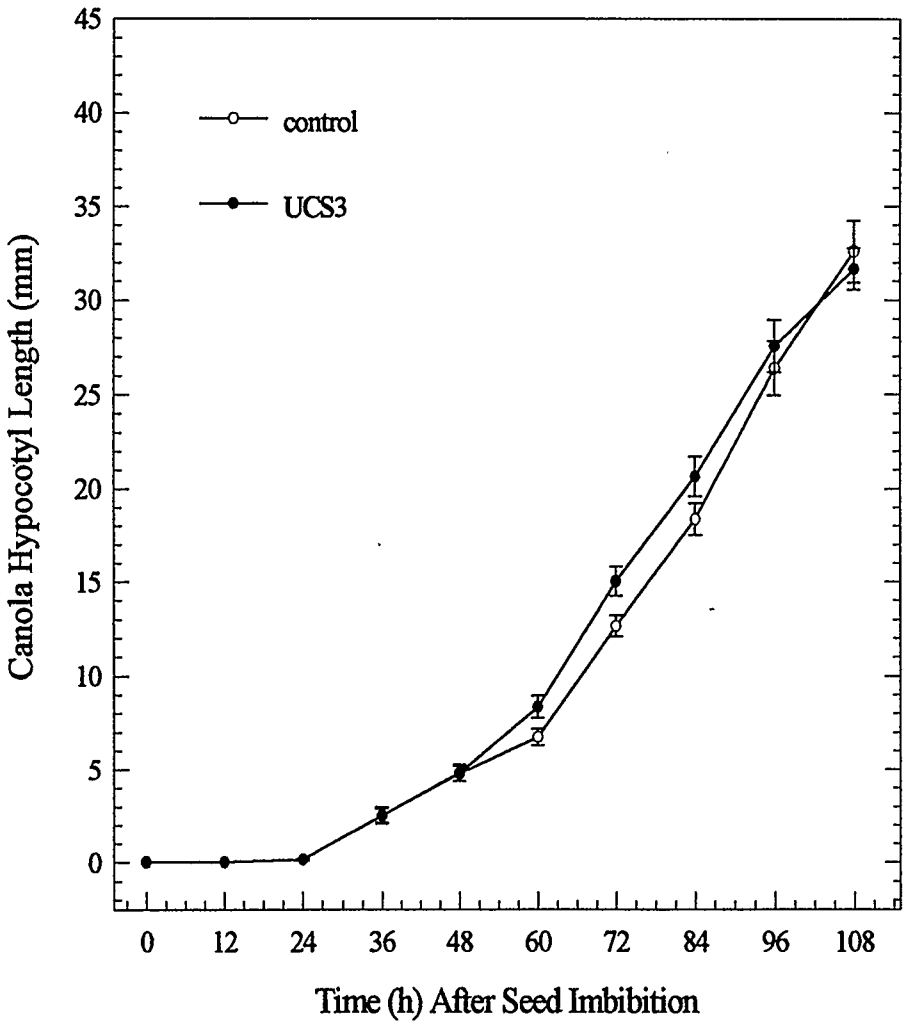


Figure 5-8. Kinetics of effects of *Rhizobium* strain UCS3 on canola cv. Tobin seed germination and hypocotyl elongation during post-germination growth in gnotobiotic growth pouches under cool-white fluorescent light at 23° C. Values represent the mean of 100 plants. Error bars represent 95% confidence intervals.

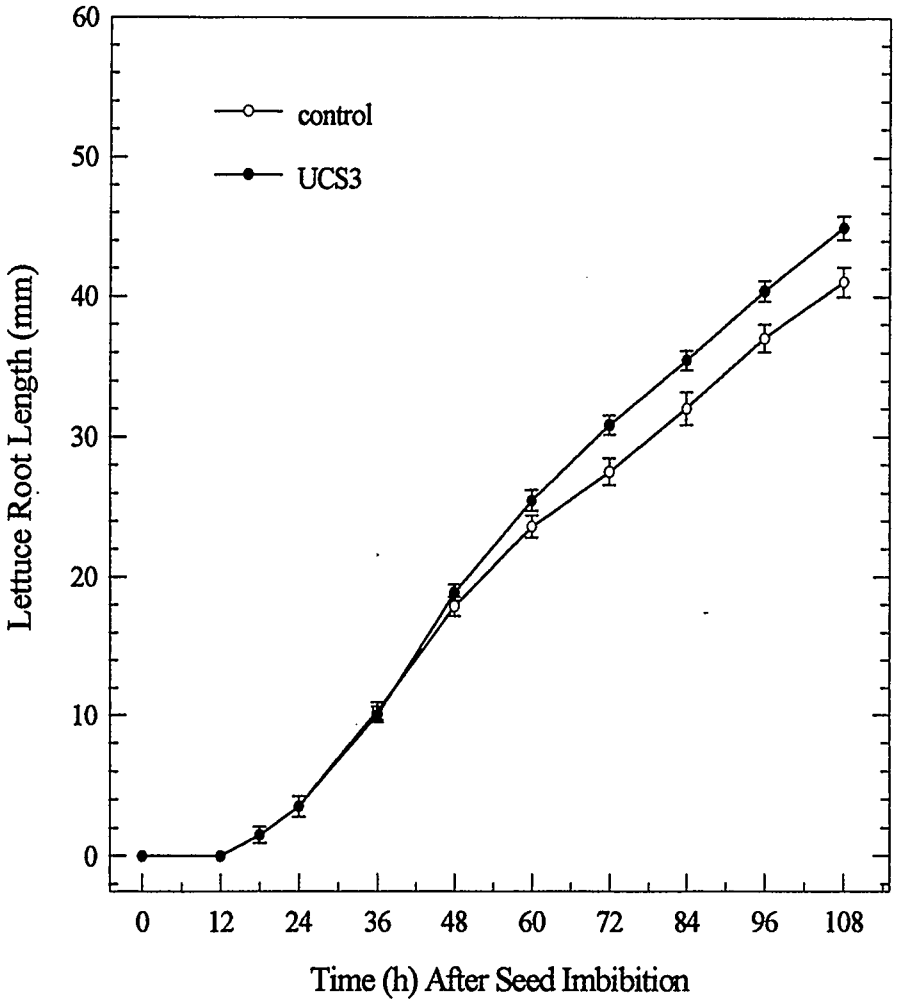


Figure 5-9. Kinetics of effects of *Rhizobium* strain UCS3 on lettuce cv. Grand Rapids seed germination and root elongation during post-germination growth in the gnotobiotic growth pouches under cool-white fluorescent light at 23° C. Values represent the mean of 100 plants. Error bars represent 95% confidence intervals.

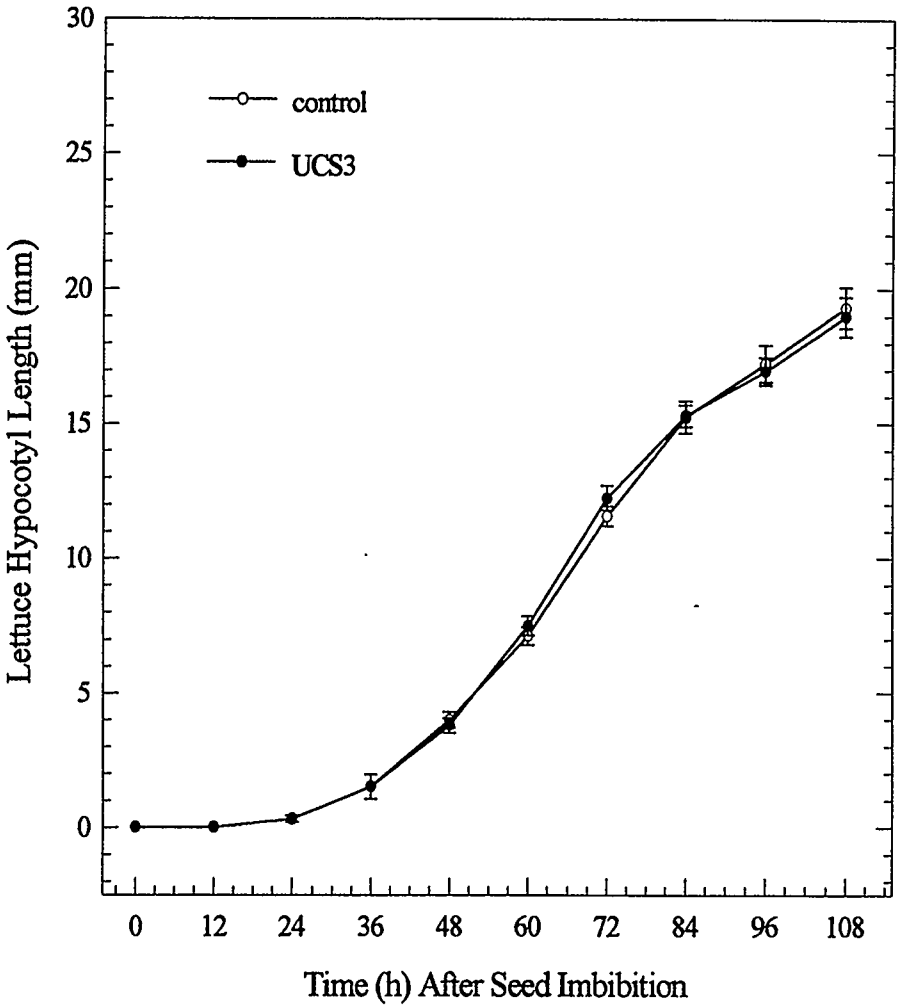


Figure 5-10. Kinetics of effects of *Rhizobium* strain UCS3 on lettuce cv. Grand Rapids seed germination and hypocotyl elongation during post-germination growth in the gnotobiotic growth pouches under cool-white fluorescent light at 23° C. Values represent the mean of 100 plants. Error bars represent 95% confidence intervals.

CHAPTER 6

ENDOGENOUS HORMONES IN VERY YOUNG HOST PLANT SEEDLINGS AFFECTED BY PGPR SEED-INOCULATION

6-1. Introduction

It was shown earlier that root elongation of very young seedlings can be significantly promoted by *P. putida* strain GR12-2 (see Chapter 4; Lifshitz *et al.*, 1987, 1988; Tang, 1992; Young *et al.*, 1993) and certain *Rhizobium* spp. (see Chapter 5) through seed inoculation. Sometimes this promotive effect by PGPR inoculation may be also applicable to hypocotyl growth (Lifshitz *et al.*, 1987; Young *et al.*, 1993; and also see Chapters 4 & 5).

As discussed in Chapters 1, 4 and 5, all proposed "indirect effect" mechanisms (e.g. biological control, N_2 fixation and improvement of nutrient uptake) are unlikely to play an important role in this beneficial effect of *Pseudomonas* and *Rhizobium* PGPR strains on higher plants in the gnotobiotic growth pouch assay system. A more direct relationship is thus apparently involved in the PGPR promotion of seedling early growth.

Previous results from this study have shown (by GC-MS) that *P. putida* strain GR12-2 and *Rhizobium* strains UCS3 and UCS4 are all IAA producers. And, these PGPR strains may also be GA producers as their culture filtrates always show certain GA-like activities on the dwarf rice micro-drop assay. Although no individual GAs have been definitively identified by GC-MS in this study, tentative identification of GA_1, GA_3, GA_4, GA_9 and

GA_{20} by GC-MS-SIM was obtained earlier for GR12-2 (Pearce, Young, and Pharis, unpublished). *Pseudomonas putida* GR12-2 is also likely a CK producer (Jameson *et al.*, 1991). Indeed, production of PGRs by plant growth beneficial soil bacteria in cultures has been reported for *Azospirillum* (Bottini *et al.*, 1989) and *Rhizobium* (Atzorn *et al.*, 1988). There is also a large body of evidence that strongly implies the involvement of PGRs produced by soil microorganisms in altering (promoting) growth of higher plants (Gruen, 1959; Brown, 1974; Tien et al., 1979; Frankenberger and Brunner, 1983; Lynch, 1985; Hussain et al., 1987; Arshad and Frankenberger, 1991).

Interestingly, as shown in Chapter 5, most putative IAA-deficient and CK-deficient mutants derived from the *Rhizobium* PGPR strains UCS3 and UCS4 have partially or totally lost the potential of their parents to promote lettuce root elongation. Also, the promotive effect by *P. putida* GR12-2 on canola root growth can be effectively mimicked by seed soak with AVG, an ACC biosynthesis inhibitor, over a wide range of applied concentrations (Young *et al.*, 1993). Cytokinins or GAs as seed soaks also appear to be effective in this growth promotion if they are used at concentrations lower than 10^{-6} M (Young *et al.*, 1993). Similar results were also found on growth promotion of 42-day-old wheat seedlings by inoculation with *Azospirillum brasilense*, where applied GA_3 could replace the effect of the bacterial inoculation in certain circumstances (Kucey, 1988).

It is thus possible that seed-inoculation with a PGPR strain may contribute its microbe-origin PGR(s) to the host, and/or regulate hormone biosynthesis processes in the host plant. Either way, the levels and/or balance of endogenous hormones in seedlings will be modified. This in turn may cause a change in morphology of seedlings during the

post-germination growth stage. However, which hormones in the plant are altered by PGPR inoculation, and how this alteration is responsible for the promotion of early seedling growth is unknown, and is thus worthy of investigation.

Ethylene is possibly the most important hormone involved in this physiological alteration of plant early growth by the PGPR strain GR12-2, as AVG (which blocks ACC biosynthesis, thus reduces ethylene production) was the only PGR tested by Young *et al.* (1993) that could fully substitute for inoculation with GR12-2 in PRE of canola seedling growth. It is documented that some soil microorganisms are capable of producing ethylene synthesis inhibitors (see Lieberman, 1979), such as AVG by *Streptomyces* (Pruess *et al.*, 1974), MVG by *Pseudomonas* (Scannell *et al.*, 1972) and rhizobitoxine by *Rhizobium* (Owens *et al.*, 1971). There is also evidence that some soil microflora inhibit plant growth, likely through ethylene production (Arshad and Frankenberger, 1988).

Endogenous plant GAs, as discussed in Chapters 1 through 4, are likely to be involved in promotion of root elongation by PGPR, and therefore need to be analyzed in rapidly growing tissues (e.g. roots and hypocotyls) of young seedlings that have been seed-inoculated with PGPR strains.

This chapter reports on the physiology of endogenous hormones in host plants in response to PGPR inoculation. The effect of GR12-2 on endogenous levels of IAA, ABA ACC, MACC and certain GAs was investigated in canola. Freeze-dried canola tissues were kindly provided by Ms. S. Young (Esso Ag Chemicals) and Dr. B.G. Glick (Waterloo University, Waterloo, Ontario, Canada). All the plant materials used in this chapter were prepared as described before (Chapter 3), as were the working protocols for

extraction, purification and GC-SIM analysis of the hormones (unless otherwise mentioned).

Also, as described in Chapter 3, ethylene evolution from roots, hypocotyls and cotyledons of very young canola and lettuce seedlings grown in gnotobiotic pouches, with or without GR12-2 seed-inoculation, was analyzed on GC-FID.

Results of the quantitative analysis of these endogenous PGRs are briefly presented in following sections.

6-2. The effect of GR12-2 seed inoculation on endogenous levels of IAA in 7-d-old canola seedlings

The GC-SIM analysis shows that endogenous levels of IAA were just slightly lower in roots ($P = 0.87$) and hypocotyls ($P = 0.53$) of 7-d-old GR12-2 seed-inoculated canola seedlings than in the non-inoculated controls, respectively (Table 6-1). The IAA level in cotyledons was also higher for the control plants (780 ng/g dw) than for the GR12-2 seed inoculated ones (694 ng/g dw). As this study was focussed on the possible hormonal control of rapidly growing canola tissues, endogenous hormones in the seedling cotyledons were only quantified in a single experiment. The 11% reduction in endogenous IAA level in cotyledons by GR12-2 inoculation is probably not physiologically significant, and will not be discussed further.

The effects of IAA in the regulation of the growth and development of roots has been well demonstrated (see reviews by Scott, 1972; Audus, 1975; Torrey, 1976; Pilet *et al.*,

1979; Pilet and Saudy, 1987; Saugy and Pilet, 1987). Exogenous IAA usually inhibits root growth (Scott, 1972; Pilet and Elliott, 1981; Pilet and Saugy, 1985; Muday, and Haworth, 1993; Young *et al.*, 1993). But, occasionally IAA at very low concentrations ($\leq 10^{-8}$ M) may promote root elongation of maize (Pilet, 1961; Pilet and Saugy, 1985, 1987). However, this did not occur with canola (Young *et al.*, 1993) or lettuce (Tanimoto and Watanabe 1986), where IAA was always inhibitory to root elongation.

According to most studies of endogenous IAA on growth of roots, it is suggested that endogenous auxin content is already near growth-inhibitory levels in maize and lettuce roots (Scott, 1972; Batra *et al.*, 1975; Pilet et al., 1979; Pilet and Saudy, 1985; Tanimoto and Watanabe, 1986). That is probably the reason why exogenously applied IAA is usually inhibitory on the root elongation (Tanimoto and Watanabe, 1986).

The canola root seems an especially rich source of IAA (Table 6-1). This high level of endogenous IAA may also be supraoptimal in the root for its rapid elongation, as suggested for maize and lettuce seedlings. Supraoptimal concentrations of IAA have been reported to cause H^+ influx (Evans *et al.*, 1980) and inhibit growth through increased ethylene production (Mulkey *et al.*, 1982a, b). Therefore, a reduced level of IAA in canola roots by GR12-2 seed-inoculation could thus be speculatively more optimal for the tissue to develop its full growth potential. However, this slight reduction (6%) associated with GR12-2 inoculation seems unlikely to be very physiologically important.

The slight difference in endogenous levels of IAA in hypocotyls between GR12-2-inoculated plants and the controls seems to be correlated with their hypocotyl elongation status, since GR12-2 usually has no or only a slightly promotive effect on canola

hypocotyl growth (see Chapter 4).

Table 6-1. The effect of GR12-2 seed inoculation on endogenous levels of IAA in roots and hypocotyls of 7-d-old canola cv. Tobin seedlings grown in dim fluorescent light. Estimates of IAA are based on GC-MS-SIM using [$^{13}C_6$]-IAA as a quantitative internal standard.

Experiment Replicate	IAA (ng/g dw) in Root		IAA (ng/g dw) in Hypocotyl	
	Control	GR12-2	Control	GR12-2
Replicate 1	4668	3937	1800	1600
Replicate 2	2420	2380	1540	1420
Replicate 3	6197	6736	1254	1150
Replicate 4	2730	2032		
The mean	4004	3771	1531	1390
± s.e.	± 884	± 1071	± 158	± 131
t value	0.167		0.69	
P value	0.87		0.53	
df	6		4	

df = degree of freedom; s.e.= standard error.

6-3. The effect of GR12-2 seed inoculation on endogenous levels of ABA in 7-d-old canola seedlings

The effect of GR12-2 seed inoculation on reducing endogenous level of ABA in roots of 7-d-old canola seedlings was noticeable (but not significant , P = 0.08) (Table 6-2).

There are not as many references about the physiological roles of ABA in plant root elongation as for IAA. Abscisic acid is generally considered to be an inhibitor of root elongation (Wain, 1977; Pilet and Saugy, 1987 and references therein). However, a promotive effect of ABA on root growth has also been reported (Gaither *et al.*, 1975; Jackson, 1983; Mulkey and Evans, 1983; Pilet and Saugy, 1987). The function of endogenous ABA during seed germination and seedling post-germination growth is actually unknown, even though the roles of this hormone in seed development and dormancy have been suggested (Wain, 1977; Ho, 1982; Bewley and Black, 1983; Hilhorst and Karssen, 1992). Endogenous ABA in drought-treated seedling roots has been investigated, there is with little information available on normally growing plants (Robertson *et al.*, 1985).

Exogenously applied ABA (by seed soak and growth solution) was found to be highly inhibitory to canola root elongation, even at a relatively low concentration (e.g. 10^{-8} M, Young *et al.*, 1993). The fact that endogenous levels of ABA in young canola roots were reduced (P = 0.08) by GR12-2 seed-inoculation (Table 6-2) is coincident with the results of external applied ABA by Young *et al.* (1993). It is possible that during early growth stages the endogenous level of ABA in canola roots is high enough to be growth

inhibitory. Thus, factors that can decrease root ABA content may promote root elongation, and *vice versa*.

Hypocotyl ABA level for GR12-2 inoculated plants was the same as for the controls. But, cotyledon ABA level was apparently reduced by GR12-2 seed inoculation (e.g. 123 ng/g dw, relative to 186 ng/g dw in the control).

Table 6-2. The effect of GR12-2 seed inoculation on endogenous levels of ABA in roots and hypocotyls of 7-d-old canola seedlings grown in dim fluorescent light. Estimates of the hormone is based on GC-MS-SIM using [$^2H_6$]-ABA as a quantitative internal standard.

Experiment	ABA (ng/g dw) in Root		ABA (ng/g dw) in Hypocotyl	
Replicate	Control	GR12-2	Control	GR12-2
Replicate 1	517	445	354	385
Replicate 2	679	508	410	432
Replicate 3	557	396	649	768
mean ± s.e.	584 ± 49	450 ± 32	471 ± 90	528 ± 121
t value	2.30		-0.38	
P value	0.08		0.72	

s.e. = standard error.

6-4. The effect of GR12-2 seed inoculation on endogenous levels of certain GAs in 7-d-old canola seedlings

Figure 6-1 shows that canola roots were rich in endogenous GAs, and levels of all the quantified GAs tended to be reduced by GR12-2 seed-inoculation. The reduction of GA_3 level in 7-d-old canola seedling roots by GR12-2 was highly significant ($P = 0.007$). The level of GA_{29} was also significantly ($P = 0.035$) reduced by this PGPR strain, relative to the control. The reduction effects of GR12-2 on the endogenous levels of GA_{19} ($P = 0.07$), GA_{85} ($P = 0.11$) and GA_7 ($P = 0.13$) were noticeable. The differences in levels of GA_4, GA_8, GA_9 and GA_{20} between GR12-2 seed-inoculated and control plants could be also physiologically meaningful, though not statistically significant due to high deviations within each treatment (Fig. 6-1). As levels GA_9, GA_{19} and GA_{20} are all higher for the non-inoculated plants it is possible that GA metabolism in young canola seedling roots is influenced by GR12-2 seed inoculation. For example, GR12-2 may slow down the GA biosynthesis (both early C-13 hydroxylation and the early non- hydroxylation pathways) at steps before formation of GA_9 and GA_{19} (see Fig. 3-13 in Chapter 3). This would lead to a final decrease of all GAs subsequent to GA_9 and/or GA_{19} in roots of the inoculated plants. A retardative effect of GR12-2 on GA biosynthesis in roots of young canola seedlings can be direct after inoculation (mechanism unknown) or indirect through production of other PGRs that can modify GA biosynthesis in plants, such as GA biosynthesis inhibitors or other known hormones, including ethylene (Pearce *et al.*, 1991) and cytokinins (R.C. Coolbaugh, personal communication to R.P. Pharis).

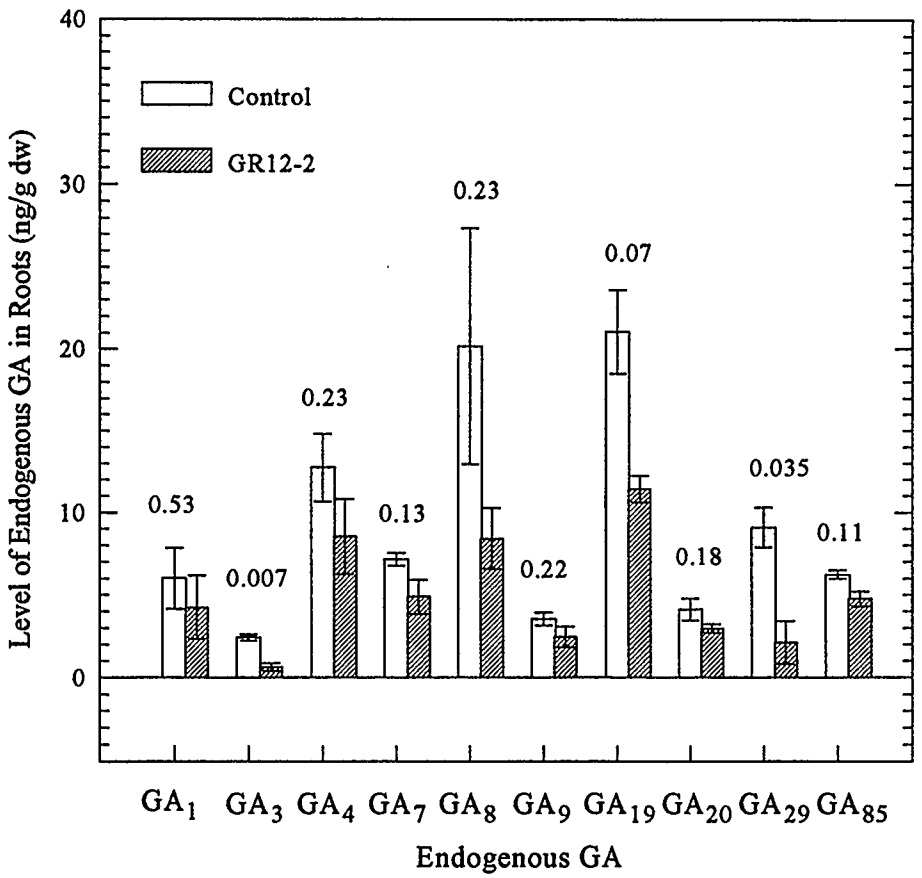


Figure 6-1. Estimates of certain endogenous GAs in roots of 7-d-old canola seedlings that were pre-treated with GR12-2 seed-inoculation vs uninoculated control. Seedlings were grown in gnotobiotic conditions under dim cool-white fluorescent light. Values represent the mean of 4 replicates. Error bars represent the standard errors of the means. Numbers on the top of each bar pair represent un-paired t-Test P values of significance of the differences between the GR12-2 inoculated and control treatments.

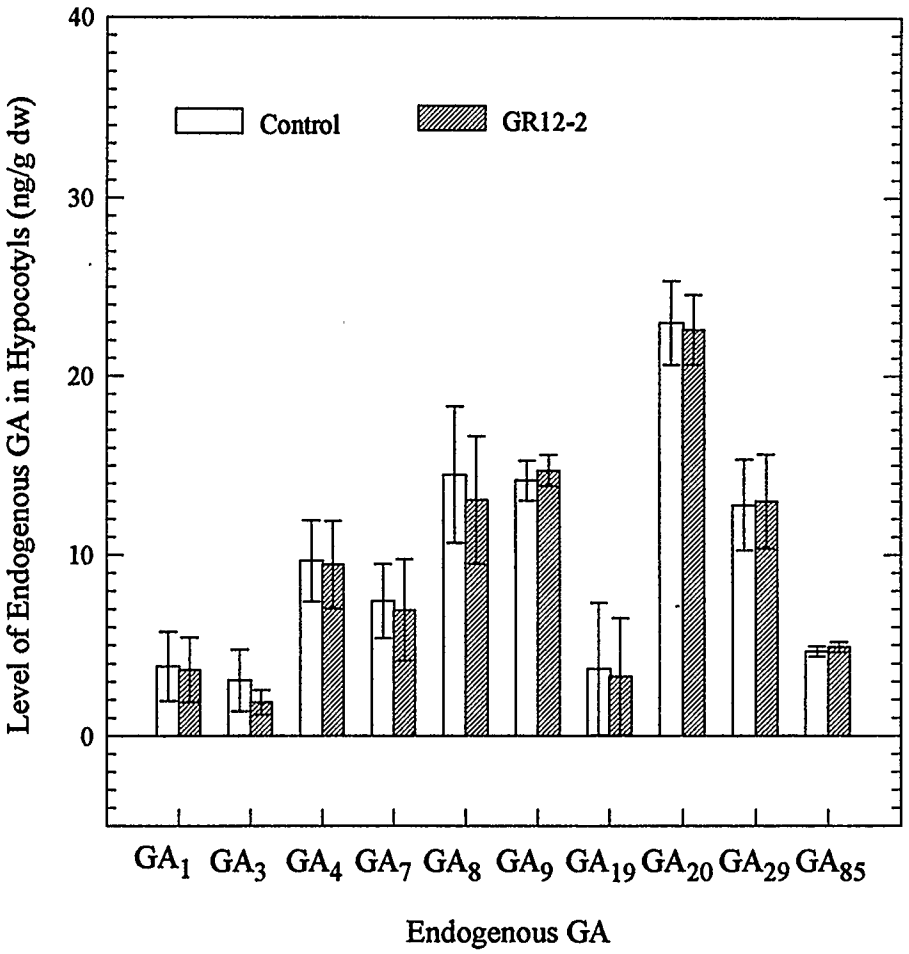


Figure 6-2. Estimates of certain endogenous GAs in hypocotyls of 7-d-old canola seedlings that were pre-treated with GR12-2 seed-inoculation vs uninoculated control. Seedlings were grown in gnotobiotic conditions under dim cool-white fluorescent light. Values represent the mean of 4 replicates. Error bars represent the standard errors of the means.

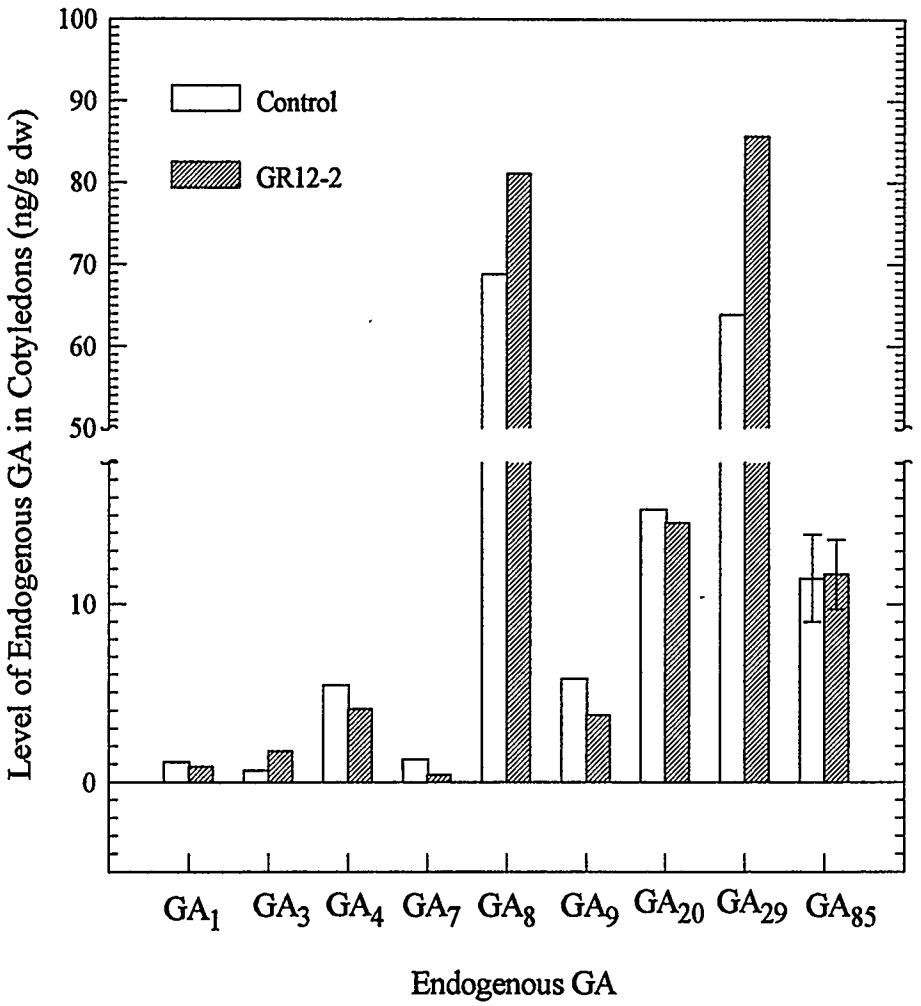


Figure 6-3. Estimates of certain endogenous GAs in cotyledons of 7-d-old canola seedlings that were pre-treated with GR12-2 seed-inoculation vs uninoculated control. Seedlings were grown in gnotobiotic conditions under dim cool-white fluorescent light. Values represent one measurement except for GA_{85} (3 replicates).

Levels of endogenous GAs in canola hypocotyls were not significantly affected by GR12-2 seed inoculation (Fig. 6-2). On the un-paired t-Test, none of the GA levels showed a significant difference, even at the level of $P \leq 0.30$ between GR12-2 inoculation and non-inoculation treatments.

The relative abundance of individual GAs in hypocotyls were obviously different from that in the roots. The levels of GA_9 and GA_{20} in hypocotyls were high relative to other GAs. In the roots, however, GA_9 and GA_{20} levels were relatively low. This implies that differences in endogenous levels of GAs in the roots between the inoculated and non-inoculated plants are probably not due to transport of GAs from the hypocotyls, but rather from the biosynthesis process in the root *per se*. The fact that the root contains considerably higher levels of GA_{19} seems also to support this suggestion.

Estimates of the levels of endogenous GAs in canola cotyledons were only based on one replicate, except for GA_{85} (3 replicates). Figure 6-3 shows that contents of all the recognized "growth-effective" GAs (e.g. $GA_{1/3/4/7}$) were low in cotyledons relative to that in roots and hypocotyls. But, that is not the case for GA_{85} which is also highly growth active. In contrast, levels of deactivated GA forms were relatively high in cotyledons. Both GA_8 (2β-hydroxylated GA_1) and GA_{29} (2β-hydroxylated GA_{20}) were found at about 60 ng per g dw, 3 to 4 times of levels in the roots or hypocotyls. This indicates that GA turnover from bioactive forms to non-active forms in canola cotyledons is quite fast during post-germination growth of the young seedlings. Alternatively, the movement of deactivated GA forms from cotyledons to hypocotyls may be much slower than for the biologically functional ones.

From Figure 6-3, it can be also seen that there may be differences in GA metabolism in the cotyledons between the GR12-2 inoculation and control treatments. The turnover of bioactive GAs into their deactivated forms appears to be faster in the GR12-2-inoculated plants, relative to the control, possibly due to the faster seedling growth rate.

The trends in endogenous GA levels in canola roots (Fig. 6-1) after seed-inoculation by *P. putida* strain GR12-2 are intriguing. Possibly, as has been suggested for auxins in roots of lettuce (Tanimoto and Watanabe, 1986) and maize (Batra *et al.*, 1979; Pilet *et al.*, 1979; Pilet and Saugy, 1985), the levels of "growth-effective" GAs in roots of very young canola seedlings are high enough to be growth-inhibitory (supraoptimal) during the post-germination stage of seedling growth. Thus, seed-inoculation with GR12-2 may lower the supraoptimal levels of endogenous GAs in canola roots (Fig. 6-1), thereby promoting root length growth of the bacterial-inoculated plants (see Chapter 4).

No dwarfism has been so far reported for root growth, despite the fact that GA deficient mutants are known to occur for stem elongation in many plant species, including canola (Rood *et al.*, 1989). Considering the fact that some (if not all) dwarf GA-deficient mutants are leaky mutants (Phinney, 1984; also see Chapter 4 for canola *rosette* grown in the dark), it seems possible that plant roots require very low levels of GAs for their normal growth and development (Tanimoto and Watanabe, 1986). Thus, all the reported shoot dwarf mutants showing GA deficiencies may still have sufficient amounts of endogenous GAs for root growth. Or, the mutation (GA block) may be tissue-specific, and thus not expressed in the root. However, these suggestions are, so far, only speculations.

As GAs are always present in saturating levels in the root, exogenously applied GAs usually show very little effect at low concentrations, or even inhibitory at high concentrations on plant root elongation (Tanimoto, 1987; also see Chapter 4). Promotion of root elongation by applied GAs can be readily achieved only when growth retardants (inhibitors of GA biosynthesis), such as ancymidol and AMO-1618 are used (Tanimoto, 1987). The amount of external GA_3 required to recover from growth retardant-inhibition (by 12 μM ancymidol) is 10^4 times lower for roots than for hypocotyls of young pea seedlings (Tanimoto, 1988). More interestingly, both ancymidol and AMO-1618 tend to promote root elongation of 2-d-old lettuce seedlings at low concentrations (≤0.4 μM for ancymidol, and ≤ 10 μM for AMO-1618). This promotive effect can be achieved even at higher concentrations (1.2 μM for ancymidol and 100 μM for AMO-1618) when 0.1 μM GA_3 is applied together with the GA biosynthesis retardants (Tanimoto, 1987).

Combining the growth data (Chapter 4) and the GA quantification result (Fig. 6-1) with previous reports (Evans, 1984; Feldman, 1984; Morris and Arthur, 1985; Tanimoto, 1987; 1988), it is possible that the 7-d-old canola seedling roots (control) may contain too high levels of endogenous GAs, whether grown in the dark or under dim fluorescent light, for an optimal root elongation. Thus, any reduction of such supraoptimal endogenous GA levels by *P. putida* GR12-2 seed inoculation might allow for more rapid root elongation growth by the young seedlings.

6-5. The effect of GR12-2 seed-inoculation on ACC and MACC levels in roots and hypocotyls of young canola seedlings

The contents of ACC in canola roots were slightly reduced (P = 0.36, unpaired t-Test) by *P. putida* GR12-2 seed-inoculation, with the level of MACC very slightly increased by same treatment (Table 6-3). The trends of ACC and MACC in roots were also found for hypocotyls of the 7-d-old canola seedlings from GR12-2-inoculated and non-inoculated seeds (Table 6-3).

Table 6-3. The effect of *P. putida* GR12-2 seed inoculation on the endogenous levels of ACC and MACC in roots and hypocotyls of 7-d-old canola seedlings grown in dim cool-white fluorescent light under gnotobiotic conditions .

Canola Tissue	Inoculation Treatment	ACC level (μg/g dw) mean ± s.e.	ACC P value	MACC level (μg/g dw) mean ± s.e.	MACC P value
root	control	7.29 ± 0.33	0.3616	22.80 ± 1.80	0.5494
	GR12-2	6.94 ± 0.36		24.56 ± 2.01	
hypocotyl	control	4.14 ± 0.17	0.2653	19.64 ± 1.88	0.6900
	GR12-2	3.85 ± 0.16		20.92 ± 2.33	

6-6. The effect of GR12-2 seed-inoculation on ethylene evolution by canola seedlings

Ethylene evolution from roots of 90-h-old canola seedlings was significantly influenced by GR12-2 seed inoculation (Fig.. 6-4). This *Pseudomonas* PGPR strain reduced root ethylene evolution rate by canola seedlings, whether grown in the dark or in cool-white fluorescent light (see Section 2-2 for light intensity and quality). However, GR12-2 seed inoculation had no significant effect on ethylene evolution from the hypocotyls (Fig. 6-4).

The effect of light on ethylene evolution, however, was significant for both roots and hypocotyls of the young canola seedlings, with or without seed inoculation by GR12-2 (Fig. 6-4). Seedlings grown in cool-white fluorescent light produced a significantly higher amount of ethylene than those grown in the dark (1.35 fold for roots and 1.93 fold for hypocotyls). The stimulative effect of light on ethylene evolution was thus independent of bacterial inoculation (Fig. 6-4).

Figure 6-5 shows the kinetics of ethylene evolution rate from roots of canola seedlings during post germination growth. The seedlings were grown in gnotobiotic pouches under fluorescent light. At 45 h ASI, there was no significant difference in root ethylene evolution rate between the GR12-2 seed-inoculated plants and the controls. The significant difference in root ethylene production occurred at 60 h ASI, lasted for about 40 h. After that differences became non significant.

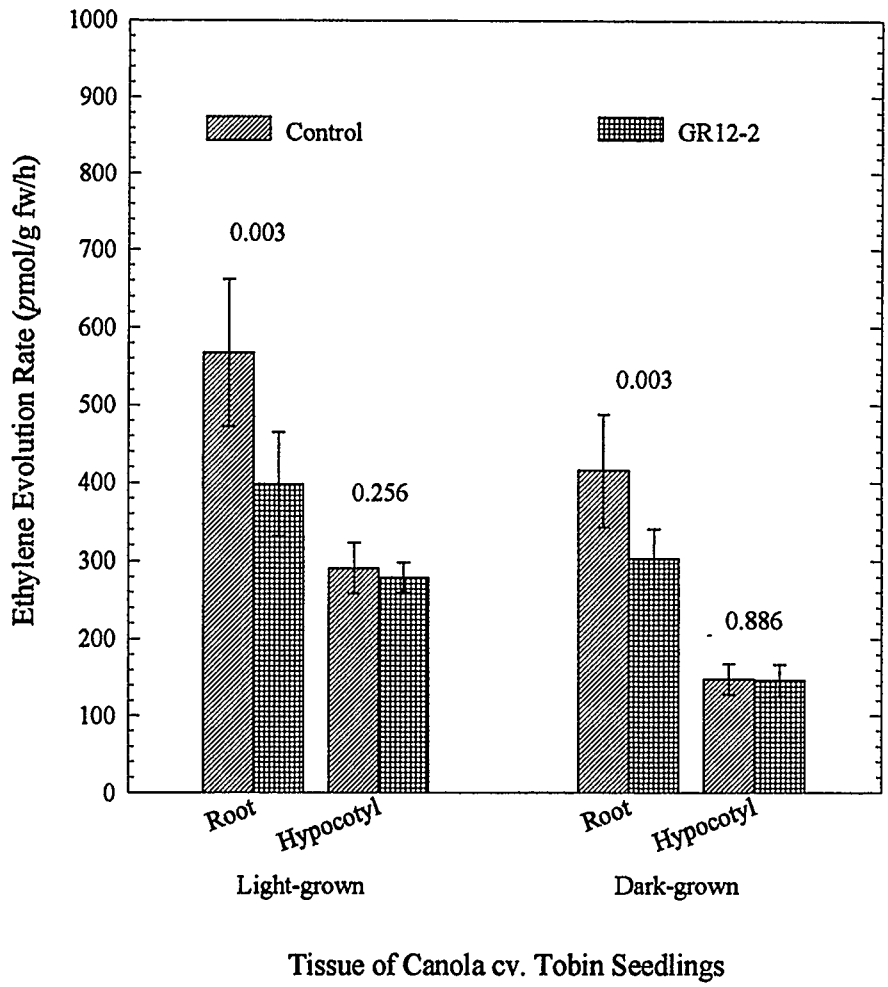


Figure 6-4. The effect of GR12-2 seed inoculation on ethylene evolution from roots and hypocotyls of 90-h-old of canola seedlings grown in gnotobiotic pouches under light vs dark conditions. Values represent the mean of 5 measurements (5 to 7 seedlings per measurement). Error bars represent 95% confidence intervals. Numbers on the top of each bar pair represent un-paired t-Test P values of significance of the differences between the GR12-2 inoculated and control treatments.

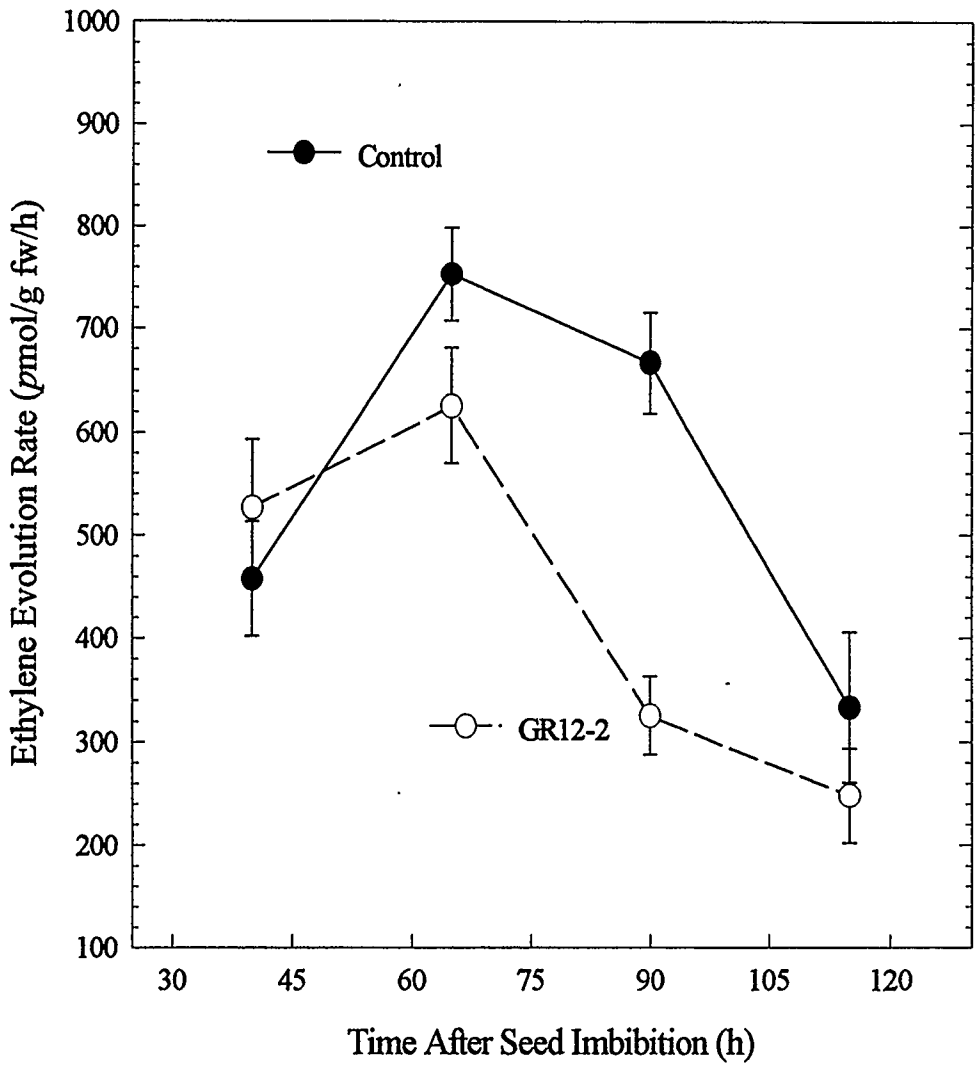


Figure 6-5. Kinetics of the effect of GR12-2 seed inoculation on ethylene evolution rate from roots of canola seedlings grown in gnotobiotic pouches under cool-white fluorescent light during the post-germination growth stage. Values represent the mean of 5 measurements (5 to 7 seedlings were used for each measurement). Error bars represent 95% confidence intervals.

6-7. The effect of GR12-2 seed-inoculation on ethylene evolution by lettuce seedlings

In a manner similar to the effect of GR12-2 seed inoculation on canola root ethylene production, this *Pseudomonas* PGPR strain highly significantly reduced ($P \leq 0.01$) ethylene evolution rate from roots of 90-h-old lettuce seedlings, grown either in the dark or under cool-white fluorescent light. However, the effect of GR12-2 on lettuce hypocotyl ethylene evolution was not significant (Fig. 6-6). This reduction of ethylene evolution rate from roots is well correlated with the highly significant promotion by the bacterium of elongation growth of the tissue (Fig. 4-3).

Although the effect of light on lettuce root ethylene evolution was not significant, it was significant for the hypocotyls (Fig. 6-6). Hypocotyls from dark- grown seedlings evolved less than half amount of ethylene relative to seedlings grown under the fluorescent light.

The kinetics of the effect of GR12-2 seed inoculation on lettuce root ethylene evolution during post-germination growth is shown in Figure 6-7. The rate of ethylene evolution from 45-h-old lettuce seedling roots was very high, e.g. ca. 1,300 pmol per g fw per h for both GR12-2-inoculated and non-inoculated plants. With time the ethylene evolution rate went down, and the effect of GR12-2 seed inoculation on ethylene production became more apparent. At about 90 h ASI the reduction effect of GR12-2 inoculation began to be significant. This trend lasted till the end of the observation period at 115 h ASI (Fig. 6-7).

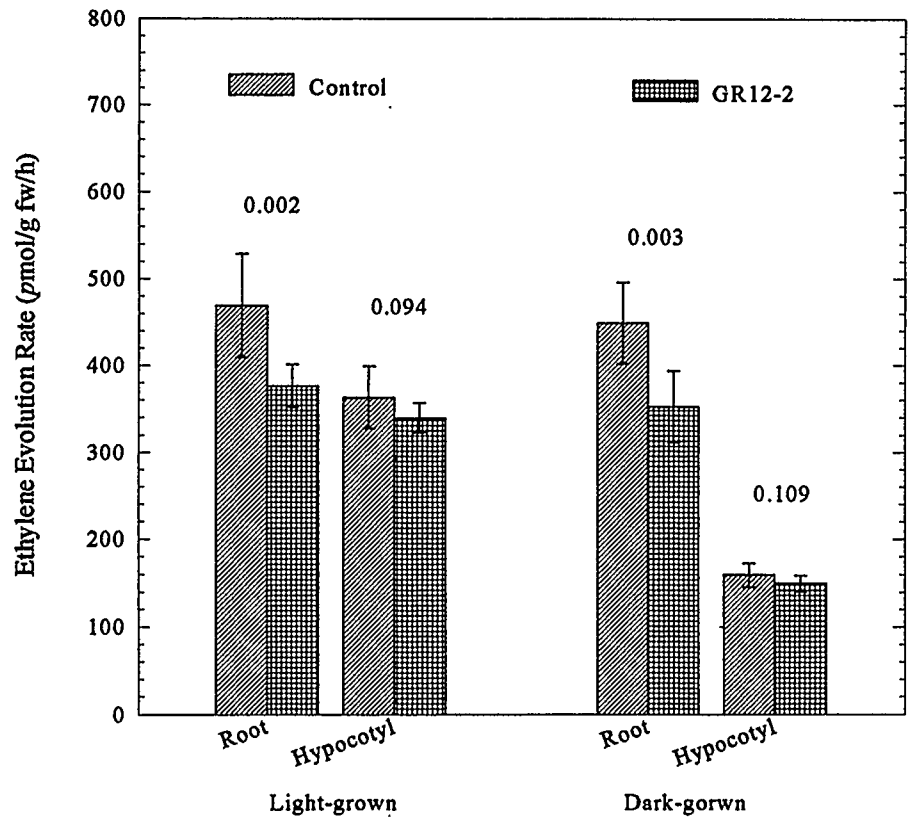


Figure 6-6. The effect of GR12-2 seed inoculation on ethylene evolution from roots and hypocotyls of 90-h-old lettuce seedlings grown in gnotobiotic pouches under light vs dark conditions. Values represent the mean of 5 measurements (5 to 7 seedlings per measurement). Error bars represent 95% confidence intervals. Numbers on the top of each bar pair represent un-paired t-Test P values of significance of the differences between the GR12-2 inoculated and control treatments.

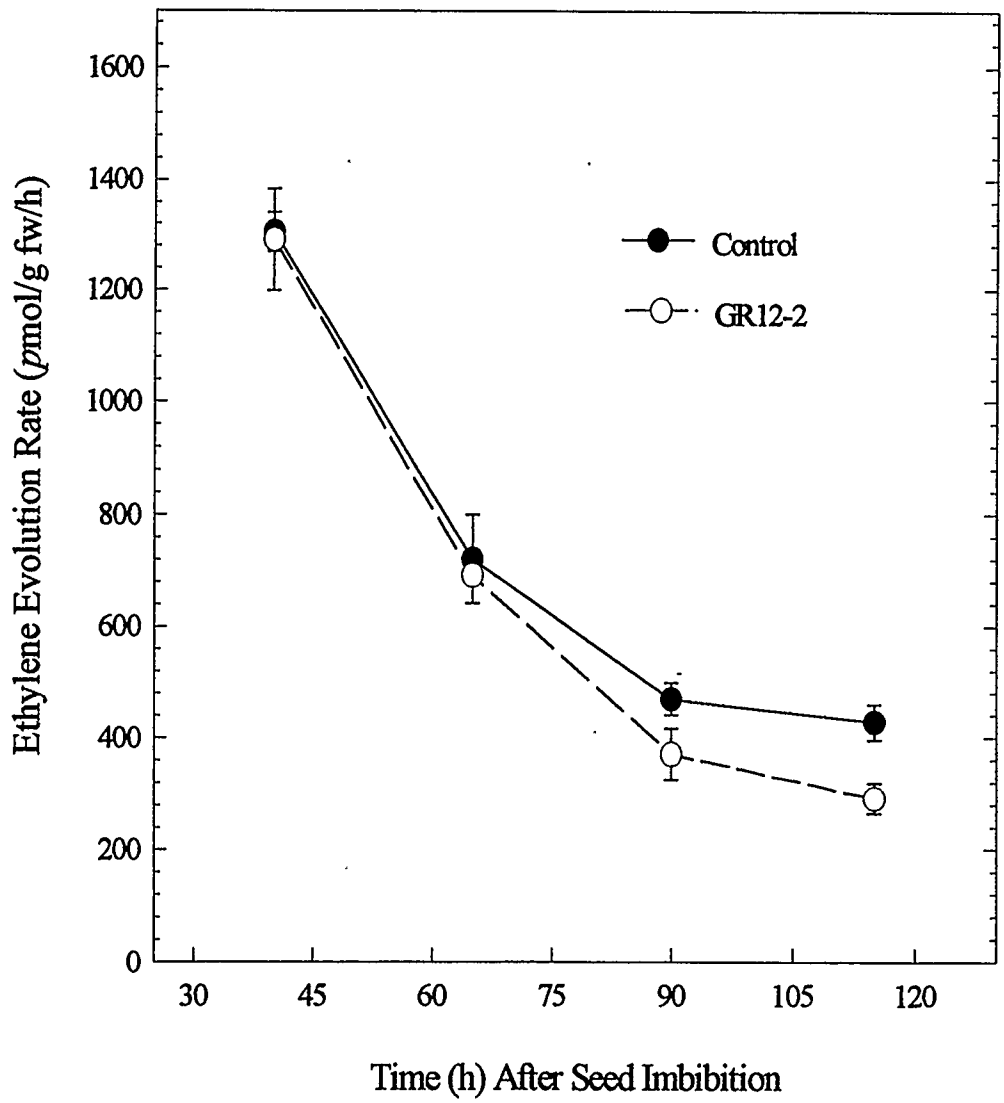


Figure 6-7. Kinetics of the effect of GR12-2 seed inoculation on ethylene evolution from roots of lettuce seedlings grown in gnotobiotic pouches under cool-white fluorescent light during the post-germination growth stage. Values represent the mean of 5 measurements (5 to 7 seedlings were used for each measurement). Error bars represent 95% confidence intervals.

Ethylene has been widely recognized as an effective inhibitor of root extension when externally applied (Andreae *et al.*, 1968; Jackson and Stead, 1983), even though promotion of root growth has also been reported for tomato, rice, rye and white mustard at lower concentrations (e.g. < 0.1 ppm) (Smith and Robertson, 1971; Konings and Jackson, 1979; Jackson and Stead, 1983, and references cited therein). The extent of root growth promotion by applied ethylene varies between species and is greater in those with lower rates of ethylene production (like rice) and less in those which produce ethylene at higher rates (like white mustard) (Konings and Jackson, 1979). In sunflower, root elongation of young seedlings can be effectively inhibited by externally applied ACC. However, ethylene evolution rate from the elongating part (root tips) is higher than from other parts of the root, and from faster-growing roots is higher than from-slower growing ones (Dr. S.A. Finlayson, personal communication).

The physiological role of endogenous ethylene in root elongation is, therefore, not yet clear. Promotion of root ethylene production from canola seedlings by light (Fig. 6-4) is correlated with a nil effect on root elongation (Fig. 2-2). On the other hand, the light-promoted lettuce seedling root growth (Fig. 2-3) is correlated with a nil effect on the ethylene evolution rate (Fig. 6-6). This may indicate that ethylene is, at least, not the only hormone responsible to the regulation of root growth of canola and lettuce seedlings.

The inhibition of stem growth by light has been recently confirmed for etiolated pea seedlings (Behringer and Davies, 1993), but the effect of light on ethylene production in shoots is quite ambiguous. It can be either promotive (Craker et al., 1973; Rohwer and Schierle, 1982) or inhibitory (Goeschl et al., 1967; Samimy, 1978). The effects of light

on ethylene production may depend on many other factors, such as plant species, particular organ, age and growth conditions (Michalczuk and Rudnicki, 1993). Nevertheless, it seems unambiguous that light is an effective stimulator of ethylene production in hypocotyls of canola and lettuce seedlings grown in gnotobiotic pouches, and it seems likely that the differences in ethylene evolution rates between plants grown in the light and dark can be related to their hypocotyl elongation.

The reductive effect of GR12-2 on ethylene production of canola and lettuce seedling roots (Figs. 6-4 and 6-6) is correlated with their increased growth (Figs. 4-2 and 4-3), especially the kinetics of root ethylene evolution (Figs. 6-5 and 6-7) and root elongation (Figs. 4-9 and 4-12). It seems possible then that the effect GR12-2 on growth of young canola and lettuce seedlings is mediated through ethylene biosynthesis in the plants.

Indirect evidence through application of AVG (Young *et al.*, 1993) implies that the regulation may be at the ACC production step. However, it is also possible that the bacterial regulation of ethylene production in roots of canola and lettuce seedlings occurs at the ethylene forming enzyme (EFE) step, as has been suggested for the effect of light on this event (Michalczuk and Rudnicki, 1993). The reason for this suggestion is that GR12-2 seed inoculation does not significantly alter ACC level in young canola seedling roots (Table 6-3), but does significantly lower ethylene evolution rate by the roots (Fig. 6-4). Thus, the regulation step by light and/or GR12-2 seed inoculation on ethylene production in canola seedling roots is not clear. It could be either at ACC synthase or EFE, or both. We need more direct evidence, e.g., gene expression data of the two enzymes, to answer this question.

CHAPTER 7

OVERVIEW AND CONCLUSIONS

It is evident from the results presented in Chapters 2 through 6 that *Pseudomonas* and *Rhizobium* PGPR strains can promote post-germination growth of higher plants in a direct way, possibly through altering endogenous hormones in the host.

The finding of a significant growth-promotive-effect of *Rhizobium* on non-leguminous plants (e.g. canola and lettuce) is very exciting, although the hormonal mechanism(s) by which *Rhizobium* spp. promote growth of non-legumes is much less studied than for *P. putida* strain GR12-2 in this work.

The bacterial-induced promotion of root elongation of very young seedlings occurs in the absence of plant pathogenic or deleterious microorganisms, and is independent of light and nutrient conditions. Thus, inoculation with PGPR strains may offer a substantial benefit to agriculture and other plant-based industries. The present study yields a better understanding of the mechanisms by which the direct promotive effect of PGPR on plant growth is brought about after seed inoculation. The results are, I believe, both theoretically and economically important.

Although root systems are less studied by plant physiologists, relative to aerial shoot systems, the involvement of hormones in root growth and development has attracted considerable attention in the past years (Elliott, 1977; Mulkey et al., 1982; Jackson and Stead, 1983; Liu et al., 1990; Nordström and Eliasson, 1993). It seems that all of the known hormones, e.g. IAA, ABA, CKs, GAs and ethylene are participating in the

regulation of root morphology and growth.

According to results of this study and results from literature discussed in previous Chapters, I suggest below possible roles for certain endogenous hormones in the direct effect of PGPR on promoting root elongation of higher plants:

The role of IAA

Root elongation promoted by *P. putida* GR12-2 seed inoculation is correlated with a slightly reduced IAA level (non significant change) in roots of young canola seedlings (Table 6-1). Such a small change in root IAA by GR12-2 seed inoculation seems unlikely to be the major reason for the PGPR-promoted root elongation. However, if auxin levels in canola roots are normally high enough so as to be growth inhibitory, as in maize and lettuce (Scott, 1972; Batra *et al.*, 1975; Pilet *et al.*, 1979; Pilet and Saudy, 1985; Tanimoto and Watanabe, 1986; Tanimoto, 1988), any reduction of IAA in canola roots by GR12-2 could be beneficial for a more rapid elongation. There is indirect evidence supporting this suggestion, namely that strain 42318, a non PRE mutant derived from GR12-2, usually produces more IAA in culture, and significantly increases IAA levels in young canola seedling roots upon seed inoculation (Sheng, C., Pearce, D. and Pharis, R, unpublished).

The role of ABA

The reduction of ABA in roots of GR12-2 seed-inoculated canola seedlings is apparent

(P = 0.08, Table 6-2). Since externally applied ABA is often inhibitory to root growth of maize and lettuce (Pilet and Saugy, 1987), and canola (Young et al., 1993), the reduced ABA level in PGPR-treated plants could be physiologically meaningful (e.g. it may allow roots to reach their full growth potential). The actual role of endogenous ABA in root elongation is not yet known. The role of ABA in the PGPR-plant interactions thus needs more research before any firm conclusions can be made, since applied ABA can also occasionally promote root elongation (Jackson, 1983; Mulkey et al., 1983).

The role of GAs

Plant roots require much lower endogenous levels of GAs for their normal elongation growth, relative to the shoots. In fact, optimal levels for root growth may be so low that the usual "normal" levels of GAs found in the roots are supraoptimal for maximal root growth (Tanimoto and Watanabe, 1986, Tanimoto, 1987; Tanimoto, 1988). Seed-inoculation with *P. putida* GR12-2 effectively lowers the levels of both total and growth-effective GAs in canola roots (Fig. 6-1). Thus PGPR-inoculation may bring the endogenous level of root GAs back to a more optimal content, thereby directly promoting root elongation growth of very young canola (and lettuce) seedlings in the gnotobiotic growth pouches. The mechanism by which *P. putida* GR12-2 influences GA biosynthesis in roots of young canola seedlings remains unknown. However, the possibility of ethylene (Pearce *et al.*, 1991) and CKs (R.C. Coolbaugh, personal communication to R.P. Pharis) produced by the bacterium inhibiting GA biosynthesis should not be ruled out.

The role of ethylene

Species from the mustard family may produce a relatively high level of ethylene from their roots, e.g. white mustard (Konings and Jackson, 1979), Such a high level of ethylene in the root is probably sufficient (Konings and Jackson, 1979), or even supraoptimal in certain circumstances, for any physiological requirement for root growth. A growth pouch is made of paper, enclosed in a plastic bag. It is possible that ethylene evolved from growing seedlings can not easily diffuse out of the pouch, and thus may accumulate to a growth-inhibitory level for seedling elongation. Therefore, factors (such as AVG) that can inhibit ethylene production in the pouch-grown seedlings may induce an enhanced root elongation growth (Young *et al.*, 1993). The PGPR seed inoculation may also reduce root ethylene (Figs. 6-4 to 6-7), and thus result in the promotion of root elongation of pouch-grown seedlings.

The hormonal physiology of PGPR on PRE of young canola seedlings is shown schematically in Figure 7-1 as a model depicting a putative mechanism for the direct effect of *Pseudomonas* PGPR strain GR12-2 on promotion of root elongation. This model may be also suitable for other PGPR (e.g. *Rhizobium*) strains, and for other plant species.

The hormonal aspects of photocontrol of canola and lettuce root elongation

There is no significant effect of cool-white fluorescent light on root growth of canola

seedlings grown in the gnotobiotic growth pouch system (Fig. 2-2). However, addition of light is highly promotive of root elongation for lettuce (Fig. 2-3). The effect of light on root ethylene production is also different between the two species, e.g. not significant on lettuce and significantly promotive on canola (Fig. 6-6 & 6-4, respectively). Based on the data of root growth and endogenous hormones, a hypothetical scheme is shown in Figure 7-2 to explain how light may influence root elongation of canola and lettuce seedlings through regulation of the biosynthesis of ethylene and GAs in roots. That fluorescent light does not promote canola root elongation may due to the fact that light significantly increases root ethylene production (Fig. 6-4), even though the endogenous growth-effective GAs have probably been reduced to more optimal levels (Garcia-Martinez-Garcia, 1993; Potter and Rood, 1993; Rood, 1993, Rood et al., 1993; Zeevaart 1993; see also discussion in Chapter 6). For lettuce seedlings (Fig. 7-2), light has no significant effect on root ethylene production. Thus light could effectively promote root elongation growth by reducing the supraoptimal endogenous level of the growth-effective GAs present in roots of the very young lettuce seedlings (Tanimoto, 1987). However, a mechanism by which cool-white fluorescent light might reduce GA biosynthesis in lettuce roots is not yet clear.

Figures 7-1 and 7-2 suggest that root elongation of canola and lettuce seedlings during post-germination growth in the gnotobiotic pouches is possibly mediated by a balance of endogenous hormones, ethylene, GAs and ABA in particular. It is now common knowledge that growth and development of plants are regulated by a coordination of various internal signals (e.g. external factors functioning through internal signals). The

direct effect of PGPR (e.g *P. putida* GR12-2 and *Rhizobium* spp.) on PRE of canola and lettuce seedlings during post-germination growth is thus most likely associated with more than one endogenous plant hormone in the root.

In summary, seed-inoculation with PGPR may allow the full growth potential of canola and lettuce roots to be realized by bringing endogenous ethylene and GA levels in root tissue to a more optimal level (e.g. reduces both). The two hormones (and also ABA & IAA) are known to be growth-inhibitory when applied to the roots (with a few exceptions). For a rapid (full potential) root elongation, all endogenous hormones should be at or near an optimal levels (see Fig. 7-1 & 7-2). Speculatively, then, the direct effect of PGPR on PRE of higher plants is to get these hormones at such an optimal level/balance (see Tables 6-1 to 6-3; Figs. 6-1 to 6-7). How PGPR species might accomplish this is, however, unknown. Inhibition of ethylene production by PGPR-produced AVG-like compounds, and/or inhibition of GA biosynthesis by PGPR-delivered PGRs (CKs?) are attractive hypotheses for future research in this area (see below).

Suggested Future Research Directions:

I. Hormonal physiology of the direct effect of PGPR on promotion of plant growth

i) Mechanisms by which PGPR influence the endogenous levels of hormones in the host plant upon seed inoculation.

ii) Effect of external PGRs on post-germination growth of the host plant under different conditions and with different combinations (PGPR x PGRs).

II. Molecular biology of the direct effect of PGPR on promotion of plant growth

i) Identify genes of PGPR and of the host plants which are responsible for the direct effect of PGPR on promotion of root elongation;

i) Mechanisms by which PGPR influence the endogenous levels of hormones in the host plant upon seed inoculation;

iii) Create/select more desirable PGPR strains (mutants) for basic research on the mechanism of action and for field use in agriculture *et at.*

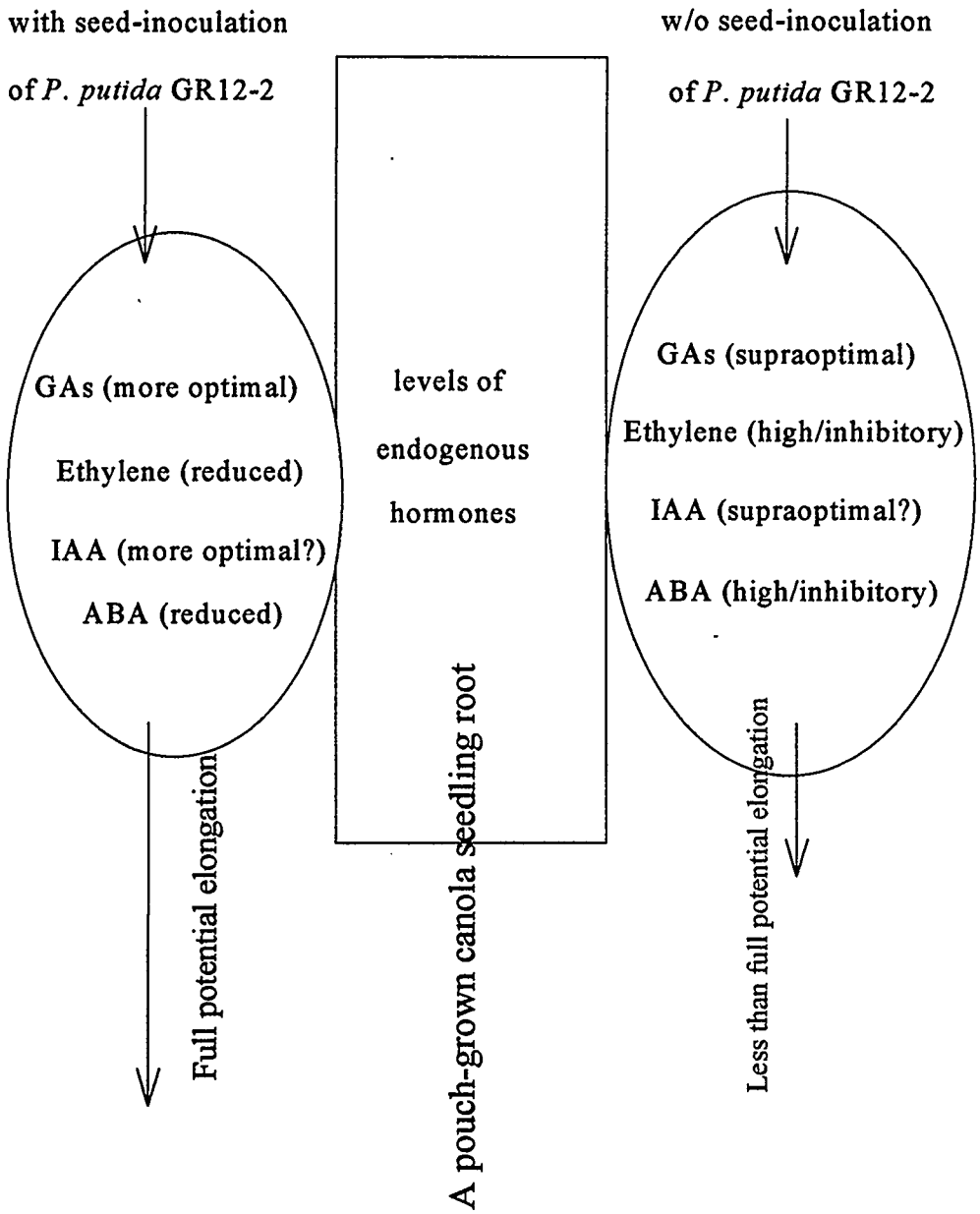


Figure 7-1. A putative working model of how *P. putida* GR12-2 might directly promote root elongation of young canola seedlings grown in gnotobiotic growth pouches. Mechanisms by which endogenous hormone levels are reduced by PGPR remains unknown.

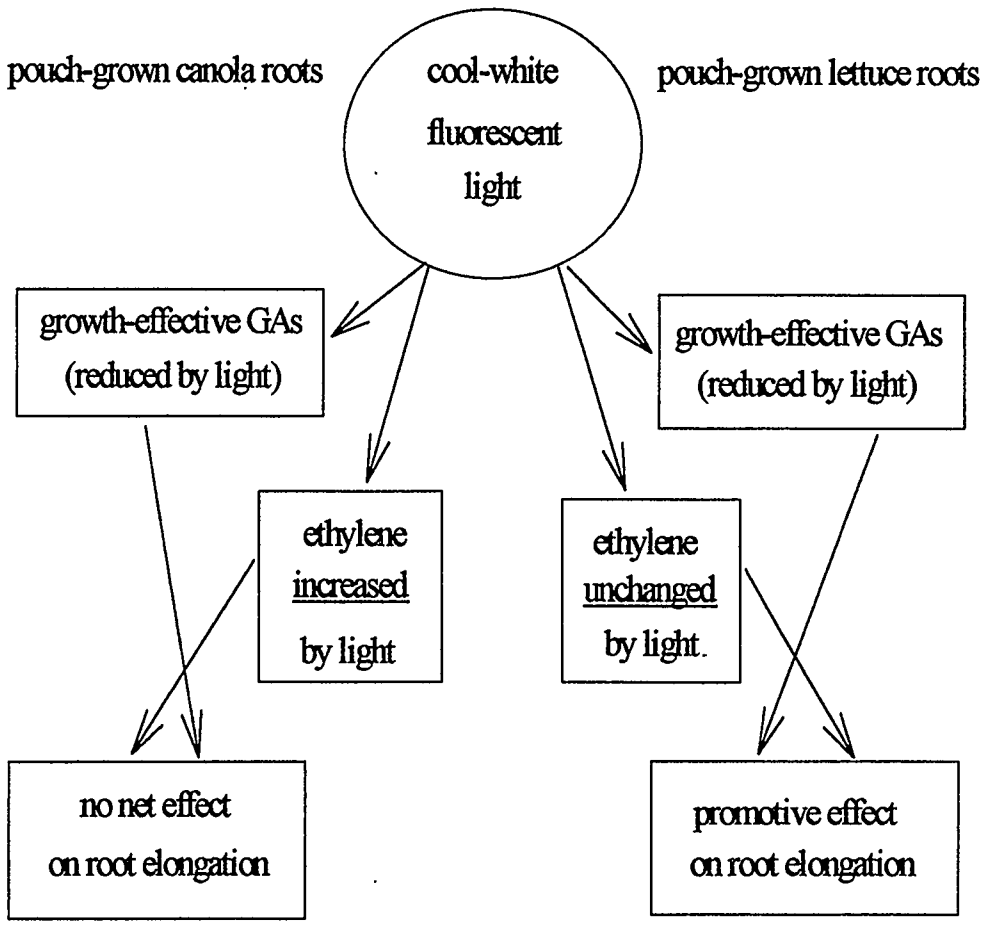


Figure 7-2. A putative model of the effect of light on root elongation of very young canola and lettuce seedlings grown in gnotobiotic pouches. This model shows possible interactions between endogenous ethylene and growth-effective GAs for regulation of root elongation, e.g. a more full potential root elongation requires both lowered levels of GAs and not too high levels of ethylene. Mechanisms by which hormone levels in roots are altered by light remains unknown.

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