AN ASSESSMENT OF RHIZOBIAL INFECTION, METABOLITE RELEASE AND GROWTH RESPONSE IN AGRICULTURALLY-IMPORTANT LEGUME AND CEREAL CROPS

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Declaration

I hereby declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the Department of Molecular and Cell Biology, University of Cape Town. It has not been submitted for any degree or examination at any other university

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Abstract

Reports on the natural and laboratory infection of cereals by rhizobium provided the impetus to embark on research using African landraces of sorghum and millet to study their interaction with rhizobia. Seven strains of root-nodule bacteria (namely *Rhizobium* GHR2, *Bradyrhizobium japonicum* Tal 110, *Sinorhizobium meliloti* strain 1, *Rhizobium leguminosarum* bv. *viceae* Cn6, *R. leguminosarum* bv. *viceae* strain 30, *Rhizobium* NGR234 and *Azorhizobium caulinodans* ORS571, hereafter referred to as "rhizobia") that fix N_2, were used to study rhizobial effects on sorghum and millet seedlings grown aseptically in Leonard jars with ½ strength Hoagland nutrient solution containing 1 mM KNO_3.

The infection process was studied using light, scanning and transmission electron microscopy on 10 or 94-d-old plants. These examinations revealed the presence of all 6 test strains on root epidermal surfaces as well as inside the tissues of inoculated, but not uninoculated, sorghum and millet roots. Large numbers of bacteria were clearly visible around cracks on the root epidermis, suggesting that these cracks served as the route of entry and localization by bacteria in interior tissues.

Koch's postulates were tested in a bioassay by applying root macerate prepared from sterile inoculated sorghum plants and this macerate successfully induced nodule formation and N_2 fixation in soybean seedlings, thus authenticating these internally located root tissue bacteria as rhizobia.

Inoculation of sorghum seedlings with 4 rhizobial test strains (i.e. *B. japonicum* Tal 110, *S. meliloti* strain 1, *R. l.* bv. *viceae* Cn6 and *R.l.* bv. *viceae* strain 30) significantly ($P \leq 0.05$) promoted sorghum shoot growth by 11-51% on fresh weight basis and 8-55% on dry weight basis. There was also 21-32% increase in root length of inoculated sorghum plants compared to uninoculated control. Additionally, root tissue concentrations of P and K were markedly ($P \leq 0.05$) increased by 17-250% in inoculated sorghum roots

relative to uninoculated plants, while in shoots Zn and Cu were significantly ($P \leq 0.05$) decreased. Bioassays of the test strains for indole acetic acid (IAA) showed that they produced biologically active concentrations of this growth-promoting molecule, ranging from 0.18 to 2.26 µg IAA per mL culture filtrate. However, the results of glasshouse studies involving the inoculation of unsterile potted soil with 3 strains from the Leonard jar experiment and 2 new rhizobial strains (*Rhizobium* NGR234 and *Azorhizobium caulinodans* ORS571) or the sugarcane endophyte, *Gluconoacetobacter diazotrophicus* PAL5, at 0.2 OD_{600} cell numbers, showed no positive effect on sorghum growth at 94 d after planting. This suggests that the active substance promoting plant growth was either released in high concentrations by indigenous soil rhizobia, or that the conditions of soil culture adversely affected its synthesis and release by the introduced strains.

Taken together, the light, SEM, TEM, plant nodulation, and plant growth data demonstrate that rhizobia can infect roots of sorghum and millet plants and increase growth via improved P and K nutrition and also that soil populations of rhizobia such as those used in this study can potentially promote plant growth in landraces of important African cereal crops such as sorghum and millet under certain conditions in the rhizosphere.

Lumichrome and riboflavin were shown to be widely produced by four rhizobial genera whose representatives were examined namely *Rhizobium, Bradyrhizobium, Mesorhizobium* and *Sinorhizobium*. Of the 14 rhizobial species tested, all of them produced measurable amounts ranging from 13.36 ng mL^{-1} to 37.65 ng mL^{-1} lumichrome and 3.36 ng mL^{-1} to 9.67 ng mL^{-1} riboflavin. It may be inferred from these data that lumichrome production is a common phenomenon among rhizobial bacteria.

The stimulatory role of lumichrome, a rhizobial metabolite, on the growth of legume and cereal seedling was also assessed in this study. At very low nanomolar concentrations (5 nM), lumichrome elicited growth promotion in cowpea, soybean, sorghum, millet and maize, but not in common bean, Bambara groundnut and Sudan grass. In soybean and cowpea, the supply of 5 nM lumichrome caused early initiation of trifoliate leaf

development, an expansion in unifoliate and trifoliate leaves, an increase in stem elongation and as a result, an increase in shoot and plant total biomass relative to control. The provision of 5 nM lumichrome also increased leaf area in maize and sorghum and thus raised shoot and total biomass. With sorghum and millet, however, root growth was also stimulated by the supply of 5 nM lumichrome. In contrast to these observations, the application of a higher dose (50 nM) of lumichrome depressed the development of the unifoliate leaves in soybean, the second trifoliate leaf in cowpea, and shoot biomass in soybean. The 50 nM concentration also consistently decreased root development in cowpea and millet. These data show that lumichrome is a novel signal affecting seedling development in both monocots and dicots.

Providing soybean and cowpea plants with their respective homologous rhizobia and/or purified lumichrome increased xylem concentration and leaf accumulation of this metabolite. Relative to control, rhizobial inoculation and lumichrome application significantly increased root respiration in maize, decreased it in lupin, but had no effect in cowpea, soybean, Bambara groundnut, pea and sorghum. Growing field plants of cowpea up to 63 d with 5 or 50 nM lumichrome decreased stomatal conductance which might have affected CO_2 intake and reduction by Rubisco. Applying lumichrome (10 nM), infective rhizobial cells (10 mL 0.2 OD_{600}) or ABA (10 nM) to plants for 44 h in growth chambers altered leaf stomatal conductance and transpiration in cowpea, lupin, soybean, Bambara groundnut and maize, but not in sorghum or pea. Where stomatal conductance was increased by lumichrome, it led to an increase in leaf transpiration relative to control plants. The effect of rhizobial inoculation closely mirrored that of lumichrome application, indicating that rhizobial effects on these physiological activities were more likely due to lumichrome released into the rhizosphere. Treating plant leaves with ABA produced stomatal effects similar to those of lumichrome. Taken together, these findings show that lumichrome modulates stomatal function, and thus controls plant water relations and photosynthetic rates.

Applying 5 or 50 nM lumichrome to field grown maize, sorghum, cowpea and soybean plants had no effect on plant growth. For the legume species, N nutrition and symbiotic

performance as measured by nodule weight, nodule number, $\delta^{15}N$, %N and nodule N were all unaffected by lumichrome application. In maize, % carbon in shoots and roots decreased significantly ($P \leq 0.001$) in response to lumichrome application and consequently, the C/N ratio in shoots and seeds decreased. By contrast, in sorghum C/N ratio of the seed increased in plants treated with 5 nM lumichrome.

Lumichrome application effected significant changes in the mineral nutrient concentrations of the four test species. In sorghum shoot, P, K, Cu, Ca, Fe and Al all increased significantly ($P \leq 0.05$) in response to 5 or 50 nM lumichrome while in sorghum root, P decreased in response to 50 nM application. Meanwhile in maize, only K in the shoot decreased in response to lumichrome. In cowpea, Al, Ca, Mg, Zn, Cu and B all decreased in the shoot of plants treated with 50 nM lumichrome, while in cowpea seeds, S decreased. In cowpea nodules K and Cu increased. In soybean though, only S decreased in the seeds of plants treated with 50 nM lumichrome, all the other mineral nutrients remaining unaltered. It therefore appears that lumichrome has a major influence on nutrient uptake of crops through as yet undetermined mechanisms.

Finally *Gluconoacetobacter diazotrophicus* was shown to occur in coffee, tea and banana plants in numbers ranging from 10^4 to 10^6 per gram fresh weight of root. Analysis of the Restriction Fragment Length Polymorphism (RFLP) pattern of the *nif*HDK genes and of the 16S-23S rRNA intergenic region did not reveal any differences between the strains.

Overall, this study demonstrated that rhizobia can infect roots of landraces of important African cereal crop like sorghum and millet,increase their growth and change their mineral nutrient concentration in tissues. Additionally, the rhizobial metabolite lumichrome was shown to influence the growth and development of different plants and alter their mineral nutrient concentration in organs. Soil populations of rhizobia such as those used in this study can therefore potentially promote plant growth in these crops.

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To Kiraba Nguru

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Plants and microorganisms interact in various ways that results in plants obtaining the nutrients and other substances which they need from their environments for optimum growth. Most of these reactions are mediated through various molecules, either from the plants or from the microbe. Sometimes the plants and microbes communicate via signal molecules to effect the above. This is true also of plants and diazotrophic (i.e. N_2 fixing) bacteria. This review focuses on the interaction between diazotrophic bacteria especially rhizobia and crop plants, and how these interactions improve plant growth and development.

One of the major nutrients needed for the production of food and feed is nitrogen. For most leguminous species, nitrogen is provided through symbiotic fixation of atmospheric nitrogen (Vincent, 1982). Biological nitrogen fixation (BNF) accounts for 65% of the nitrogen currently utilized in agriculture and will be increasingly important in future crop productivity especially for sustainable systems. In Africa, grain legumes fix about 15-210 kg N ha^{-1} seasonally and therefore play a big role in the cropping systems of traditional farmers. Tree legumes also fix about 43-581 kg N ha^{-1} y^{-1} making leaf prunings an important component of sustainability in agroforestry and alley cropping systems (Dakora and Keya, 1997). The N_2 fixed by rhizobia in leguminous plants in many cases also benefits associated non-legumes like cereals (Eaglesham *et al.*, 1981), and/or subsequent crops rotated with symbiotic legumes (Dakora and Keya, 1997). In many low imput grassland systems, the grasses depend on the N_2 fixed by the legume counterparts for their N nutrition and protein synthesis which is much needed for forage quality in livestock (Paynel *et al.*, 2001).

The extension of nitrogen-fixing symbiosis into important crop plants such as cereals has been a long standing goal in the field of BNF. Making cereals and other non-fixing crop plants self-sufficient in N nutrition would be of great benefit to resource-poor farmers in Africa. One approach for achieving this goal has involved the isolation and characterization of N_2-fixing bacteria from a variety of wild and cultivated crops

(Stoltzfuz *et al.*, 1997), an exercise which has produced a wide array of diazotrophs from plant organs including roots and stems. Some of those microbes so far identified from non-legumes include *Gluconoacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) from sugarcane (Cavalcante and Dobereiner, 1988; Gillis *et al.*, 1989; Fuentes-Ramirez *et al.*, 1993; Caballero-Mellado, 1994; James *et al.*, 1994; Sevilla *et al.*, 1998, 2001; Reis *et al.*, 2001; Riggs *et al.*, 2001; Muthukumarasamy *et al.*, 2002). Strains of *G. diazotrophicus* have also been isolated from roots and stems of coffee (Jimenez-Salgado *et al.*, 1997). *Azospirillum* is another much studied diazotroph, especially the species *lipoferum* and *brasilense,* which have been shown to infect a number of cereal plants including wheat, maize and sorghum (Reynders and Vlassak, 1982; Pacovsky *et al.*, 1985; Dobereiner and Boddey, 1981; Kapulnik *et al.*, 1983; Christansen and Vanderleyden, 1993, Fallik and Okon 1996, Malik *et al.*, 1997; Weber *et al.*, 1999; Dobbelaere *et al.*, 2001). Other known diazotrophs include *Herbaspirillum seropedicae* (Dobereiner *et al.*, 1993; Weber *et al.*, 1999; Riggs *et al.*, 2001), *Klebsiella pneumoniae* and *Panotoea agglomerans* (Riggs *et al.*, 2001), *Enterobacter sp*, *Klebsiella oxytoca*, *Azotobacter, Arthrobacter*, *Azoarcus*, *Bacillus* and *Zooglea* (Mirza *et al.*, 2001).

Information on N_2-fixation by these associative diazotrophs is rather scanty and amounts fixed disappointingly low, except for *Gluconoacetobacter diazotrophicus* which fixes ecnomical amounts of N_2 in sugarcane (Boddey *et al.*, 1988; Dobereiner *et al.*, 1993; Sevilla *et al.*, 1998; 2001). However, growth promotion has been observed with many of these diazotrophs even where N_2-fixation could not be demonstrated. In general, these diazotrophs are reported to improve root growth and function, often leading to increased uptake of water and mineral nutrients. Plant inoculation with *Azospirilum brasilense,* for example, promoted greater uptake of NO_3^-, K^+, and $H_2PO_4^-$ in corn, sorghum, wheat and setaria (Lin *et al.*, 1983; Okon and Kapulnik, 1986; Murty and Ladha, 1988; Zavalin *et al.*, 1998; Saubidet *et al.*, 2000), leading to higher crop yields. Because rhizobia also produce various metabolites such as auxins, cytokinins, riboflavin and vitamins (Phillips and Torrey, 1970; Dakora, 2003), their invasion of legume and non-legume plant roots should promote an increase in plant growth.

1.2 Rhizobia and legumes

Rhizobia comprises the species of *Rhizobium, Mesorhizobium, Bradyrhizobium, Allorhizobium, Azorhizobium* and *Sinorhizobium.* These organisms form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as signals by the legume host. These plant compounds induce the expression of nodulation (*nod, nol* and *noe*) genes in rhizobia which in turn produce lipo-chito-oligosaccharides (LCO) signals that trigger mitotic cell division in roots, leading to nodule formation (Dakora, 1995; Lhuissier *et al.*, 2001). Nitrogen is fixed within these structures. Although rhizobia principally infect legumes, there are reports of them doing so in non-legumes.

1.3 Rhizobial infection of non-legumes

The first report of non-legumes forming a symbiotic relationship with rhizobia was by Trinick (1979) in the non-legume species *Parasponia.* Effective nodulation has also been observed in *Parasponia andersonii*, following *Bradyrhizobium* inoculation of plantlets from calii (Davey *et al.*, 1993). The nodulation of *Parasponia* by both *Rhizobium* and *Bradyrhizobium* strains provided encouragement that rhizobial infection and nodule formation in non-legume crops is a possibility in the future and these observations have increased the search for rhizobium nodulation of non-legume plants such as cereals.

Experimentally, a number of workers have demonstrated the ability of rhizobia to colonize roots of non-legumes and localize internally in the tissues including the xylem (Spencer *et al.*, 1994). Following such success, several attempts have been made to extend nodulation and N_2-fixing ability to non-legume crops (Ah-Mallah *et al.*, 1990; Gough *et al.*, 1997a; Antoun *et al.*, 1998; Stone, 2001). Some of the early experiments successfully induced nodulation in oilseed rape, though only after treating the seedling roots with enzymes followed by inoculation with rhizobia (Ah-Mallah *et al.*, 1990). Applying *Bradyrhizobium japonicum* to radish significantly increased plant dry matter by 15%, but without nodulation (Antoun *et al.*, 1998). *Azorhizobium caulinodans* ORS571, which induces stem and root nodules in the tropical legume *Sesbania rostrata*, has also been shown to colonize the internal tissues of *Arabidopsis thaliana* through cracks at

poins of lateral root emergence (Gough *et al.*, 1997a; 1997b). The co-application of *A. caulinodans* and flavonoids such as naringenin and daidzein, even at very low concentration (5 x 10^{-5} M) significantly enhanced microsymbiont colonization of roots and promoted localization in the xylem of *Arabidopsis thaliana* (Stone, 2001). Other studies have demonstrated the ability of *Rhizobium leguminosarum* bv. *phaseoli* to colonize roots of lettuce plants (Chabot *et al.*, 1996).

1.4 Rhizobia and cereals crops

Naturally occurring rhizobia, isolated from nodules of *Parasponia* and some tropical legumes, have also been shown to infect roots of many agricultural species such as rice, wheat and maize via cracks made by emerging lateral roots (Webster *et al.*, 1997), although it is also possible that rhizobia and other diazotrophs can gain entry into the plant roots by other means like producing cellulase and pectinase which could aid in infection (Kovtunovych *et al.*, 1999, Verma *et al.*, 2001; McCulley, 2001). However, heavily suberized and/or lignified cell layers would still present a barrier for such bacteria (McCulley, 2001).

Inoculation of rice and wheat with *A. caulinodans* strain ORS571 carrying a *lac* Z reporter gene showed that a high proportion of the internal plant colonization occurred from lateral root cracks. Supplying the flavonone naringenin at 10^{-4} or 10^{-5} M concentration increased rhizobial entry via cracks and promoted intercellular localization in wheat roots (Webster *et al.*, 1997; 1998). However the establishment of *A. caulinodans* in wheat roots is possible without the addition of flavonones (Sabry *et al.*, 1997). There has also been an intriguing report that the *nod* D1 gene product of *Rhizobium* strain NGR234 responds to activation by phenolic compounds isolated from wheat extracts (le Strange *et al.*, 1990). Strains of *Rhizobium leguminosarum* bv. *phaseoli* harbouring *lux* genes were used to visualize in situ colonization of roots by rhizobia in maize as well as to assess the efficiency with which these bacteria infected maize roots. Their observations were consistent with findings on maize root colonization and infection by rhizobia (Schloter *et al.*, 1997; Gutierrez-Zamora and Martinez-Romero, 2001; Yanni *et al.*, 2001)

Being a major food crop, rice has attracted a lot of interest and more nodulation studies with rhizobia have been conducted with rice compoared to any other non-legume speices (Chaintreuil *et al.*, 2000; Yanni *et al.*, 1997; 2001). Al-Mallah *et al.* (1989) were the first to successfully induce nodular structures on rice roots after treating 2-d-old seedling roots with a cell wall-degrading enzyme mixture followed by rhizobial inoculation in the presence of polyethylene glycol. In a later study, Weber *et al.* (1999) detected an increase in rhizobial infection of rice roots with the application of low concentration of naringenin (10^{-4} and 10^{-5} M). Later, the same flavonone was shown to enhance the colonization in the xylem by *Azorhizobium caulinodans* strain ORS571 (Gopalaswamy *et al.*, 2000). Similar rice-rhizobial interactions have been reported by de Bruijn (1995) and Ladha *et al.* (1997).

Observations in the laboratory studies that cereal roots could be infected with rhizobia led to the hypothesis that during legume-cereal rotations and/or mixed intercropping, rhizobia are brought into close contact with cereal roots, and this probably results in non-legume root infection by native rhizobial population in soil. Attempts have therefore been made to determine if rhizobia naturally infect roots of cereals and other major food plants. The study by Yanni *et al.* (1997) was the first to isolate *Rhizbobium leguminosarum* bv. *trifolii* as a natural endophyte from roots of rice in the Nile delta. Because rice has been grown in rotation with berseem clover for about seven centuries in the Nile delta, this practice probably promoted closer affinity for this cereal as a "host plant". This hypothesis is re-enforced by the fact that the clover-nodulating rhizobia isolated from rice could occur up to 2.5 x 10^{-7} cells g^{-1} fresh weight of root, concentrations similar to those obtained for bacteroids in legume root nodules. Chaintreiul *et al.* (2000) similarly isolated photosynthetic bradyrhizobia from roots of the African brown rice, *Oryza glaberrima*, which generally grows in the same wetlands as *Aeschynomene sensitive*, a stem-nodulated legume associated with photosynthetic strains of *Bradyrhizobium*. Again this may well suggest a co-evolution of *Aeschynomene* bradyrhizobia and wild genotypes of African brown rice.

Apart from rice, rhizobia have also been isolated as natural endophytes from roots of other non-legume species such as cotton, sweet corn (McInroy and Kloepper, 1995), maize (Martinez-Romero *et al.*, 2000), wheat (Biederbeck *et al.*, 2000) and canola (Lupwayi *et al.*, 2000) either grown in rotation with legumes or in mixed cropping systems involving symbiotic legumes.

In many cases, diazotrophic bacteria including rhizobia promote the growth of their host plants by a variety of mechanisms. Unfortunately, in many studies reported in literature, the mode of action of growth promotion is not addressed. Ultimately, understanding of the mode of action at the physiological and genetic level will be most useful. In the cases where the mode of action for growth promotion has been identified, these can be categorized broadly as improving the nutrient supplies of the plant and then there are cases where the diazotroph produces certain molecules which enhance plant growth in one way or another.

1.5 Rhizobial endophytes as mediators of plant nutrient supply

Many diazotrophs improve plant growth by acting as biofertilizers which refers to the use of soil micro-organisms to increase the availability and uptake of mineral nutrients for plants (Vessey, 2003). Among the nutrients whose uptake has been enhanced by such diazotrophs include N, P and Fe. Perhaps the most important of these is N which is required in huge amounts by plants. Nitrogen is required for cellular synthesis of enzymes, proteins, chlorophyll, DNA and RNA, and is therefore important in plant growth and the production of food and feed. It is therefore no wonder that the most studied and longest exploited plant growth promoting bacteria are rhizobia because of their ability to fix N_2 in their legume hosts. Indeed commercial rhizobia inoculants have been available since the 1890's (Vessey, 2003).

It is interesting though, that although many plant-growth promoting diazotrophs have the ability to fix N_2, rarely is their mode of action for the stimulation of plant growth credited to BNF or nitrogenase activity. This is also the case of interactions between non-legumes and *Rhizobium* sp. (Antoun *et al.*, 1998; Yanni *et al.*, 2001). Indeed, there is still little

evidence of inoculation of non-legumes with rhizobia leading to agronomically significant levels of BNF in most crops (Vessey, 2003).

In some of the associative relationships, although there has been no evidence of N-fixation, there has sometimes been increased N uptake most probably from improved root architecture due to the presence of the rhizobia. Such observations were made by Murty and Ladha (1988) on rice while Yanni *et al.* (2001) recently reported that inoculating rice with strains of *R. leguminosarum* bv. *trifolii* improved the agronomic fertilizer N-use of Giza 175 hybrid rice in a field experiment.

Phosphorus is second only to nitrogen in mineral nutrients most commonly limiting the growth of terrestrial plants (Vessey, 2003). The low availability of P to plants is because the vast majority of soil P is found in insoluble forms and plants can only absorb P in two soluble forms; monobasic $H_2PO_4^-$ and the dibasic HPO_4^{2-} ions. Because of the relative immobility of this element and its very low concentration in soil solutions, substantial amounts of phosphate fertilizers are applied to agricultural soils (Antoun *et al.*, 1998). Large quantities of phosphorus therefore accumulate in the soil of which 20-80% is in the organic form. Availability of this phosphorus depends largely on microbial activity. Phosphate solubilizing bacteria are common in the rhizosphere and secretion of organic acids and phosphatases are the most common method of facilitating the conversion of insoluble forms of P to plant-available forms (Kim *et al.*, 1998). The solubilization of P in the rhizosphere is the most common mode of action implicated in plant growth promoting rhizobacteria (PGPR) that increase nutrient availability to the host plant (Richardson, 2001). Inoculation of plants with phosphate solubilizing microorganisms frequently stimulates plant growth by increasing uptake (Chabot *et al.*, 1993; Antoun *et al.*, 1998). A large number of rhizobia and bradyrhizobia are able to solubilize inorganic phosphate. In a field study by Chabot *et al.* (1996), it was observed that phosphate solubilization by strains of *R. leguminosarum* bv. *phaseoli* was the most important mechanism of maize and lettuce growth promotion in moderately to very fertile soils. Of 266 strains of plant growth promoting bacteria examined by Antoun *et al.* (1998), 54% were found to solubilize phosphorus. In this study, TAL 629 of *B. japonicum*

significantly increased (15%) the dry matter content. In another study, strains of *R. leguminosarum* bv *phaseoli* effectively colonized maize and lettuce roots. These strains were selected in vitro for their phosphate solubilizing abilities (Chabot *et al.*, 1996).

Another important element microorganisms assist in the acquisition of is iron. Although iron is the fourth most abundant element in the earth's crust, in the soil, the solubility of Fe is primarily controlled by Fe oxide. Nevertheless, the extreme insolubility of ferric hydroxide limits free Fe at pH 7 or higher in an aerobic aqueous environment at an equilibrium concentration of approximately 10^{-18} M (Guerinot, 1991; Loper and Buyer, 1991). Minimal concentrations of iron required for normal plant growth range from 10^{-9} to 10^{-4}, depending on other nutritional factors. Iron deficiency if severe can lead to reduction or even complete failure in crop yield (Chen *et al.*, 1998).

Most microorganisms use siderophores and corresponding membrane receptors for iron acquisition. Many reports have shown that Fe, chelated by microbial siderophores can also be utilized by plants (Chen *et al.*, 1998). Many nodule producing bacteria have also been shown to produce siderophores. In a study by Antoun (1998), of 266 such strains tested, 83% were found to produce siderophores. Indeed, iron-containing proteins feature prominently in the nitrogen-fixing symbioses between rhizobia and their respective host plants.

Yanni *et al.* (2001) found that inoculating rice with strains of *R. leguminosarum* bv *trifolii* improved the acquisition several mineral nutrients namely N, P, K, Mg, Ca, Zn, Na, and Mo.

1.6 Classical phytohormones from rhizobia as plant growth enhancers

Many actual and putative PGPR produce phytohormones that are believed to be related to their ability to stimulate plant growth. In most cases, these phytohormones are believed to cause changes in assimilate partitioning patterns in plants and affect growth patterns in roots resulting in bigger roots, more branched and/or plants with greater surface area (Vessey, 2003).

Some of the traditional phytohormones - traditional in the sense that they have been known for a long time – that have been reported to be produced by bacteria include auxins, cytokinnins, and gibberellins. These phytohornomes synthesized by bacteria influence the host root respiration rate, metabolism, root proliferation and hence better mineral and water uptake by the plants harbouring them (Tien *et al.*, 1979; Okon and Itzigsohn, 1995). In the tropics, many soils are extremely low in nutrients and this is often a great contraint on the growth and development of plants. Since most mineral nutrients are absorbed by plants through the rhizosphere, the roots play a crucial role in the uptake of these mineral nutrients (Dakora and Phillips, 2002). Hence any substance secreted by rhizobacteria that enhances root architecture is likely to improve mineral uptake.

In the rhizobium-legume interaction, plant hormones have been known to be part of the nodulation process since Thimann (1936) reported that pea nodules contain elevated levels of auxins. Rhizobia produce both auxins and cytokinins (Phillips and Torrey 1970; 1972; Reddy *et al.*, 1997; Hirsch *et al*, 1997; Antoun *et al.*, 1998). However, mutants defective in IAA synthesis have been described but none of them are Nod$^-$, suggesting that auxin production by rhizobia is not essential for nodule morphogenesis (Hirsch *et al*, 1994). In addition, certain early nodulin genes in legumes have been found to be induced by cytokinins, which may be one of the functions of the rhizobial produced cytokinins in these symbioses.

Indole-3-acetic acid (IAA) is a phytohormone which is known to be involved in root initiation, cell division and cell enlargement is commonly produced by PGPR (Barazani and Friedman, 1999). Law and Strijdom (1989) reported the production of IAA in yeast-mannitol medium by each of two isolates of indigenous *Bradyrhizobium* sp. Evidence of stimulation of wheat and cowpea root growth on agar by *Bradyrhizobium* strain CB756, commonly used as a commercial inoculant was obtained. IAA has also been detected in culture filtrates of *Rhizobium* and in relatively high amounts in root nodules (Prinsen *et al.*, 1991), while a study using a selected rhizobial strain (E11) of *R. leguminosarum* bv.

trifolii, results indicated that it produced auxin (IAA) and gibberellins. When inoculated into rice roots, this strain produced promotion of growth (Yanni *et al.*, 2001).

Ethylene is the only gaseous hormone and its production in the plant can be induced by wounding or chemical stress (Vessey, 2003). Among its myriad effects on plant growth and development, ethylene can cause inhibition of root growth. Glick *et al.* (1998), put forward the theory that the mode of action of some PGPR was the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme which could cleave ACC the immediate precursor to ethylene in the biosynthetic pathway for ethylene in plants. They submitted that ACC deaminase activity would decrease ethylene production in the roots of host plants and result in root lengthening. In some cases, the growth promotion effects of ACC-deaminase-producing PGPR appear to be best expressed in stressful situations such as flooded (Grichko and Glick, 2001) or heavy metal-contaminated soils (Burd *et al.*, 1998).

The discovery that PGPR produce phytohormones other than IAA and cytokinins has opened the possibility that even more plant-growth regulating substances may be produced by these bacteria and may influence plant growth. Of late, several such molecules have been described.

1.7 Novel rhizobial molecules as plant growth enhancers

In recent times, certain bacterial molecules which were not previously recognized as having a growth-promoting effect on plants have been shown to exert a powerful influence on various aspects of plant growth and development. Some of these molecules have been known for some time but their role in plant growth promotion had not been previously determined. However, evidence is mounting that more and more bacterial molecules have a growth promoting effect on plants. Most of this work has been carried out on rhizobia (Dakora, 2003). All legumes that form symbiotic relationships with microsymbionts use signal molecules to induce the expression of nodulation genes in their respective homologous microsymbionts during nodule formation (Dakora, 2002). Signal exchange begins with the secretion of phenolic compounds, flavonoids and

isoflavonoids by host plants. These induce the *nod* genes, resulting in the production of bacteria-to-plant signal molecules (Lhuissier *et al.*, 2001).

One such group of molecules are the lipo-chito-oligosaccharides (LCOs). These are bacteria-to-plant signal molecules essential for the establishment of the rhizobia-legume symbioses. LCOs invoke a number of physiological changes in the host plants These include root hair deformation, cortical cell division and ontogeny of complete nodule structures (Prithiviraj *et al.*, 2000). They also mediate host specificity. In experiments using *R.leguminosarum* bv. *viciae*, it was found that a *nod*E-determined highly unsaturated fatty acid and a *nod*L-determined O-acetyl substitute are essential for the ability of the signal to induce nodule meristem on the host plant (Spaink *et al.*, 1991). It has been observed that within minutes, nod factors change cell organization by acting on the actin cytoskeleton, enhancing tip cell wall deposition so that root hairs become longer for their species (Luissier *et al.*, 2001). Schlaman *et al.* (1997) showed that rhizobial LCOs can induce cortical cell division. They used ballistic microtargeting as a novel way of delivering the LCO of *R.leguminosarum* bv. *viciae* in *Vicia sativa* plants. Data have also been presented suggesting that at least part of the physiological role of the nod factors may be linked to molecular events involved in the control of the cell cycle. They showed that treatment of *Medicago microcallus* suspension with the cognate *Rhizobium meliloti* Nod signal molecule Nod Rm-IVCC 16: 2, S can modify gene expression both qualitatively and quantitatively. At concentrations of 10^{-6} – 10^{-9} M, this host specific plant morphen, but not the inactive non-sulphated molecule, stimulated cell cycle progression (Savoure *et al.*, 1994). de Jong *et al.* (1993), also found Nod factors to be effective in rescuing the formation of ts11 embryos. At temperatures that inhibit the development of these temperature sensitive carrot cell mutants, the presence of *Rhizobium* LCOs was found to enable these embryos, whose development was arrested, to proceed beyond the globular embryo stage. The role of LCO signal molecules in the development of root nodules indicates that they play an important role in organogenesis of plants (Spaink and Lugtenberg, 1994).

LCOs have also been reported to enhance the germination of various crops. At submicromolar concentrations of LCO, *nod* Bjv (C18: 1: MeFeu) isolated from genistein induced cultures of *Bradyrhizobium japonicum* 532C enhanced germination and early growth of *Zea mays*, *Glycine max, Phaseolus vulgaris, Beta vulgaris, Cucumis sativus, Gossypium sp.*, and *Letuca sativa* under laboratory, greenhouse and field conditions. In *Cucumis sativus* and *Brassica napus*, the LCO enhanced germination at 15°C, a temperature that is stressfully low for these crops. Irrigation of maize seedlings with a solution of the LCO (10^{-9} – 10^{-7}M) doubled such variables as leaf area, plant height, root and shoot dry weight and root length (Prithiviraj *et al.*, 2000).

Another effect of these Nod factors is on flavonoid levels, which in turn affect the amount of Nod factors released by rhizobia. Treating *G. max* seedlings with pure Nod factor of *B. japonicum* in nano molar concentrations has been shown to increase the levels of flavonoids leading to enhanced levels of daidzein, coumestrol and genistein (Schmidt *et al.*, 1994).

Photosynthesis is yet another physiological aspect that has been reported to be enhanced by Nod factors. Spray application of LCOs at submicromolar concentrations improved photosynthesis rates of such diverse crops as soybean, maize, rice, bean, canola, apples and grape. On average, a 10-20% increase in the photosynthetic rate was observed and this was concomitant with an increase in stomatal conductivity and constant or decreased leaf internal CO_2 concentration. Under field conditions, spray application of LCO at concentrations of 10^{-6}, 10^{-8} and 10^{-10} M resulted in increased soybean grain yield of up to 40%. These results with LCOs suggest that there could be a possible use of these molecules in improving crop production (Smith *et al.*, 2002).

Apart from LCOs, another bacterial molecule to which plant growth promoting functions have recently been attributed is hydrogen. H_2 is produced by many legume nodules as a by-product of N_2 fixation. It is an obligate by-product of the N_2 fixing enzyme, nitrogenase, claiming about 33% of the reducing power and ATP that flows to the enzyme. Many N_2 fixing legume nodules evolve H_2 due to the absence or low abilitiy of

the uptake hydrogenase (HUP^-). In a HUP^- symbiosis, large amounts of H_2 can diffuse out of the nodule and into the soil (Dong and Layzell, 2002). In order to study the fate of this H_2 in the soil, a H_2 treatment system was developed. With increasing pH_2, a 5 fold increase was observed in O_2 uptake and CO_2 evolution declined such that net CO_2 fixation was observed in treatments of 680 ppm H_2 or more. At the exposure rate used to pretreat the soil, 60% of the electrons from H_2 were passed to O_2 and 40% were used to support CO_2 fixation. This effect of H_2 on the energy and carbon metabolism of soil may account for the well-known effect of legumes in promoting soil carbon deposition (Dong and Layzell, 2001).

Interestingly, more than 75% of the rhizobial strains isolated from major soybean production areas in the United States, as well as all clover and alfalfa symbiosis are HUP^-. It has been shown that there is a a high microbial biomass of H_2 oxidizing bacteria close to the nodule and this decreased exponentially with distance from the nodule.
Since evolution and crop breeding programs have not favoured HUP^+ symbioses over HUP^- ones, it has been suggested that H_2 evolution by nodules is beneficial to the growth and yield of the symbiosis. To test this hypothesis, Dong and Layzell (2002) carried out field trials in which the soils were pretreated with H_2. In seven week old barley and spring wheat plant, tiller numbers per plant increased compared with the air treated controls. In some instances, yield increases were also observed. The data indicated that soil H_2 fertilization may play a significant role in contributing to the benefit that cereals derive when in rotation with legume crops. The mechanism by which this is achieved has yet to be elucidated but it probably involves the enhanced growth of H_2 oxidizing microorganisms in the soil, which in turn may improve the nutrient status of the soil or act as plant growth promoting rhizobacteria enhancing growth regulator balance. This study helps to explain the evolutionary questions surrounding why HUP^- symbioses have thrived when there are genes, in many cases within the same genus and species, for the more energetically efficient HUP^+ symbioses (Dong and Layzell, 2002).

Lumichrome and riboflavin have also been recently identified as molecules that enhance plant growth. Phillips et al(1999), reported that *Sinorhizobium meliloti* produces a signal

molecule that enhances root respiration in alfalfa (*Medicago sativa* L.) and which also triggers a compensatory increase in whole-plant net carbon assimilation. Lumichrome was identified as the molecule, a common breakdown product of riboflavin under many physiological conditions as well as being released by rhizosphere bacteria. Treating alfalfa roots with 3 nM lumichrome increased root respiration 21% ($P \leq 0.05$) within 48 h. A closely linked increase in net carbon assimilation by the shoot compensated for the enhanced root respiration. Applying 5 nM lumichrome to young alfalfa roots increased plant growth by 8% ($P \leq 0.01$) over the same period. In both cases, significant growth enhancement was evident only in the shoot. Since *S. meliloti* requires CO_2 for growth, it may benefit directly from the enhanced root respiration that is triggered by lumichrome, favouring an early association between microsymbiont and legume. Since riboflavin is rapidly degraded to lumichrome in many physiological conditions, and many rhizosphere bacteria release riboflavin, plant growth promotion by lumichrome may be widespread in cropping systems.

Yang *et al.*, 2002 have also reported that *Sinorhizobium meliloti* cells carrying extra copies of riboflavin genes (*rib* BA) and which released 10 to 20% more riboflavin than a control colonized roots to densities that were 55% higher than that of the control strain. Results supported the importance of extracellular riboflavin for alfalfa root colonization by *S. meliloti* and are consistent with the hypothesis that this molecule benefits bacteria indirectly through an effect on the plant.

1.8 Legume molecules as plant and bacterial growth promoters

Legume plants produce various molecules that have an effect on the growth and development of other plants around them and/or microbes in the soil which in turn affect them. Some of these molecules are from seeds while many others are in the root exudates. Some function as signal molecules to soil microbes and other organisms while others are involved in mineral procurement in a bid to optimize growth conditions (Dakora, 2003). Various such compounds already identified in root exudates include organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions, gaseous molecules, enzymes and root border cells. Legume

crops also produce phenolics such as flavonoids and isoflavonoids and aldonic acids exuded by roots of N_2 fixing legumes which serve as major signals to Rhizobiaceae bacteria which form root nodules where N_2 is reduced to ammonia (Gagnon and Ibrahim, 1998; Dakora 2000; Dakora and Phillips, 2002). The type of flavonoid and its specific sequence are some of the factors partly responsible for the host specificity of the legume-rhizobia interaction (Long, 1989), which in turn induces nod genes, resulting in the production of bacteria to plant signals (Leon-Barrios *et al.*, 1993; Luissier *et al.*, 2001). Legume seeds also produce flavonoids and nitrogenous metabolites such as alkaloids, terpenoids, peptides and amino acids, conjugated forms of which are soluble in water and are therefore easily released as chemical signals following imbibition (Ndakidemi and Dakora, 2003). Luteolin, a flavonoid, is an active inducer in alfalfa seed extracts, while in pea, naringenin, hesperetin and luteolin are the major compounds in root exudates that induce nod genes of *R. leguminosarum* bv *viceae* at very low concentrations ranging from $10^{-6} - 10^{-7}$ (Maxwell *et al.*, 1989). In soybean, genistein is the most effective plant- to-bacteria signal (McDermott and Graham, 1990; Zhang and Smith, 1995; Zhang and Smith 1996; 1997; Pan and Smith, 2000; Belkheir *et al.*, 2001).

Chemotaxis enables the microsymbiont to move towards the legume host and this too is influence by molecules from the plant. Hydroxycinnamic acids, which are universally present in higher plants where they function as intermediates in the isoflavonoid biosynthesis, have been found to be strong chemoattractants, while genistein, diadzein and coumestrol also induce some response (Kape *et al.*, 1999).

In order for plants to develop optimally, they need an adequate supply of mineral nutrients from the soil. In the tropics, many soils are extremely low in nutrients and this is often a constraint on the growth and development of plants (Dakora and Phillips, 2002). Roots play an active role in the acquisition of these and in some cases are able to bring about an increase in the concentration of these nutrients in the soils solution (Kirk, 2002; Rengel, 2002). The root exudates may solubilize unavailable mineral nutrients, alter the pH in the rhizosphere so as to make them more available or increase availability through chelation (Dakora and Phillips, 2002)

Legumes make N available to other plants in the vicinity in intercrop systems or to subsequent crops during rotation (Eaglesham *et al.*, 1981; Dakora 2003; Paynel *et al.*, 2001). Phosphorus is normally the most limiting nutrient for the growth of leguminous crops in the tropics and subtropics especially in soils of high iron or aluminium oxide content where P is strongly bound and largely unavailable to plants (Ae *et al.*, 1990). However, certain plants like pigeon pea have root exudates with compounds such as the organic acids citric, malic, melonic, succinic as well as psidic acid and its derivatives which release P from Fe-P by chelating Fe^{3+}. The mineral status of the plant seems to dictate the types and amount or organic acid released. Such pigeon pea plants benefit others in the vicinity and subsequent crops (Ishikawa *et al.*, 2002).

Additionally, roots from P-deficient plants have a significantly greater acid phosphatase activity (Gilbert *et al.*, 1999). Citric acid and malate have also been reported produced from roots to release unavailable forms of phosphorus (Neuman *et al.*, 1999; Schulze, 2002).

Another very important plant nutrient is iron (Fe). However, its concentration in the soil is usually below what is required for adequate plant growth since it exists predominantly in the insoluble $Fe(OH)_3$ form. Lack or iron leads to chlorosis and ultimately to plant death (Guerinot and Yi, 1994; Bernards *et al.*, 2002). Some plants, when confronted by iron deficiency, extrude protons which lower the rhizosphere pH and solubilize iron (Guerinot and Yi, 1994; Masaoka *et al.*, 1993).

Yet another example of how plants deal with mineral nutrient problems is the way aluminum toxicity is handled by certain plants that show tolerance to it. In acid soils (pH < 5.0), Al is found as Al^{3+} ions which are phytotoxic, inhibiting root growth and leading to mineral deficiencies and water stress (Degenhardt *et al.*, 1998). Various plants raise the pH value; e.g. *Aspalanthus linearis* was shown utilize OH^- and HCO_3^- ions to increase alkalinity. In this wasy, this plant is able to overcome the adverse effects of low pH while enhancing nutrient acquisition and reducing trace element toxicity (Muofhe and Dakora, 1999).

The study of this thesis was conducted to test the following hypotheses

1. Rhizobia can infect roots of African landraces of sorghum and millet and colonize internal tissues and bring about growth promotion in these crops.
2. Lumichrome, a rhizobial metabolite so far only reported from *Sinorhizobium meliloti* is produced by other rhizobial speices
3. Lumichrome, though only reported to effect growth promotion in alfalfa, the host of *Sinorhizobium meliloti* can enhance growth in various other crop plants as well.
4. *Gluconoacetobacter diazotrophicus* can be found in more crops than the ones already reported.

CHAPTER TWO

RHIZOBIAL INFECTION OF AFRICAN LANDRACES OF SORGHUM AND MILLET PROMOTES PLANT GROWTH AND ALTERS NUTRIENT CONCENTRATION IN ORGANS

2.1 Introduction

Rhizosphere interactions between plants and microbes play a vital role in plant development, with benefits ranging from nutrient acquisition to hormonal stimulation of growth. The symbioses between root nodule bacteria (i.e. rhizobia) and legumes represent a cheap source of N supply to agricultural crops via N_2 fixation in nodules. While many earlier studies have addressed rhizobial inoculation of legumes for increased yields and N_2 fixation (Beck and Materon 1988), recent experiments have started to explore the use of rhizobia as plant growth-promoting rhizobacteria in non-legume plants as well as their effects on grain yields of agriculturally important crops (Noel *et al.*, 1996; Schloter *et al.*, 1997; Antoun *et al.*, 1998). Various plant species have been used in inoculation studies with rhizobia, and these include wheat, maize, rice, potato, lettuce, radish, canola, and oilseed rape (Law and Strijdom 1989; Al-Mallah *et al.*, 1989, 1990; Spencer *et al.*, 1994; Chabot *et al.*, 1996; Reddy *et al.*, 1997; Yanni *et al.*, 1997). Many of these species have presumably been bred for specific traits that could affect their rhizosphere response to rhizobial interaction. These reports nevertheless show that studies on rhizobia are rapidly extending beyond the traditional role of these microbes as N_2 fixers in symbiotic legumes to include growth stimulation of non-legume plants. Because some studies (McInroy and Kloepper 1995; Yanni *et al.*, 1997) have identified rhizobia as natural endophytes of cotton, sweet corn and rice, a wider use of unbred plant material such as landraces is likely to provide new insights into the benefits of rhizobial inoculation with various crop species.

Like other soil bacteria, rhizobia are a source of phytohormones and other growth-promoting molecules in the rhizosphere (Lynch and Clark, 1984; Loper and Schroth, 1986; Law and Strijdom, 1989) can potentially influence plant development. Law and Strijdom (1989) have reported the ability of *Bradyrhizobium* strain CB756 to promote root growth in seedlings of cowpea (a legume) and wheat (a cereal) when cultured in Leonard jars or agar medium. In contrast, they also found that two local isolates of *Bradyrhizobium* sp., which produced higher concentrations of indole acetic acid (IAA), inhibited root development in the two species, indicating strain differences in root growth stimulation. A number of studies have also shown that rhizobia can infect roots of non-

legume crop plants via cracks in the epidermis or at points of lateral root emergence (Spencer *et al.*, 1994; de Bruijn *et al.*, 1995; Reddy *et al.*, 1997; Webster *et al.*, 1997). Endophytic localization of rhizobia in intercellular spaces (Gough *et al.*, 1997; Reddy *et al.*, 1997) and the xylem (O'Callaghan *et al.*, 1997) of legume and non-legume plant roots has been observed, and this presumably enhances the growth-promoting effects of these bacteria on the host plants. Another report (Yanni *et al.*, 1997) also suggests that clover rhizobia are natural endophytes or rice plants grown in the Nile delta. The findings of all those studies (Law and Strijdom, 1989; Gough *et al.*, 1997; Reddy *et al.*, 1997; O'Callaghan *et al.*, 1997; Webster *et al.*, 1997; Yanni *et al.*, 1997) indicate that rhizobia probably influence plant growth in more ways than just N supply via N_2 fixation.

The aim of this study was 1) to assess the effects of rhizobial inoculation on plant growth and mineral nutrition of a sorghum landrace, and 2) to study rhizobia colonization of host-plant roots, including endophytic localization of the bacteria in internal tissues of African sorghum and millet landraces, and 3) to establish using Koch's postulates whether the bacteria visualized inside cereal roots with TEM are indeed N_2-fixing rhizobia.

2.2 Material and Methods

2.2.1 Studies of millet and sorghum root infection by 6 rhizobial strains

2.2.2 Plant culture

Seeds of African landraces of millet and sorghum (*Sorgum bicolor* (L.) obtained from Ruiru market, Kenya, were used in this study. The seeds were surface-sterilized by soaking in 75% ethanol for 2 min, in 10% sodium hypochlorite (bleach) solution for 10 min, and rinsing 5 times with sterile de-ionised water. Autoclaved Leonard jars were then sown to the surface-sterilized seed of millet and sorghum, and seedlings thinned out to 2 plants per jar at 10 d after planting. The Leonard jars containing acid-washed sand and were assembled as described by Vincent (1970). The Hoagland nutrient solution consisted of $0.4NH_4H_2PO_4$; $1.6Ca(NO_3)_2$; 0.8 $MgSO_4$; 0.1 Fe as Fe-chelate; 0.023 B as

$B(OH)_3$; 0.0045 Mn as $MnCl_2$; 0.0003 Cu as $CuCl_2$; 0.0015 Zn as $ZnCl_2$; 0.0001 Mo as MoO_3 or $(NH_4)6Mo_7O_{24}$; Cl as chlorides of Mn, Zn, and Cu. All concentrations are in units of mM/litre. This molar concentration was adjusted to ½ strength nutrient solution containing 1 mM KNO_3.

The rhizobia used in this study included *Bradyrhizobium japonicum* Tal 110, *Sinorhizobium meliloti* strain 1, *Rhizobium leguminosarum* bv. *viceae* strain 30, *R. leguminosarum* bv. *viceae* strain Cn6, *Bradyrhizobium* CB756, as well as strains with broad host range such as *Rhizobium* NGR234 and *Rhizobium* GHR2. Broth cultures were prepared from each of the 6 rhizobial strains by growing the strains in yeast extract mannitol broth medium (Vincent 1970) for 72 h, followed by measurement of the optical density at OD_{600}. Cell turbidity was adjusted to 0.2 OD_{600} units, and 3 mL of the broth culture of each strain used to inoculate seedlings of sorghum and millet. Uninoculated control palnts received 3 mL of sterile yeast extrace mannitol broth medium without rhizobia. The Leonard jars were then covered with sterile non-wettable cotton wool as anti-contamination mulch, and the plants placed in a growth chamber with 16 h light per day, 70% relative humidity, and 28°C/16°C day/night cycle. For each plant species, 4 replicate Leonard jars were used per rhizobial strain.

2.2.3 Microscopic studies of sorghum and millet root infection by rhizobia

2.2.4 Scanning electron microscopy

At 94 d after planting, root material (1 g fresh weight) of inoculated and uninoculated plants were harvested per replicate to study rhizobial colonization and infection of sorghum and millet roots using scanning electron microscopy (SEM). The 1 g root tissues from each replicate jar was chopped into small pieces (2-3 mm) and fixed in 2.5% glutaraldehyde in 10 mM phosphate buffer saline solution (PBS), pH 7.4, for 16 h. After washing in PBS, the specimens were post-fixed in 1% osmium tetroxide (OsO4) for 1 h, washed again in PBS, and the samples dehydrated by passing through a series of increasing concentration of ethanol (30 - 100%). The samples were then dried in a Balzer's critical point dryer (Model CPD020, Leichtenstein, Germany), mounted on

aluminium stubs, gold-palladium coated, and viewed for bacterial colonization of root surface using a scanning electron microscope (Leica Stereoscan Model 440, Cambridge, UK). The root specimens were also sectioned and the inner tissues viewed for endophytic colonization by rhizobia using SEM techniques.

2.2.5 Light microscopy

The samples used for light microscopy were treated as described for SEM and tissue dehydrated in ethanol. After dehydration, they were embedded in Spurr's resin (Spurr, 1969), and polymerized at 60°C for 24 h. Ultra-thin sections were cut from the embedded root tissue using Reichert ultracut microtome system (Reichert-Jung, Austria), fitted with glass knives. The sections were treated with chloroform vapour in order to stretch them, stained with Toluidine blue, mounted in a drop of water, covered with a cover slip, and then observed under the light microscope at the high and oil emersion powers.

2.2.6 Transmission electron microscopy

Root samples used for transmission electron microscopy (TEM) were also treated as described for SEM, and the tissues dehydrated in ethanol. Following dehydration, they were similarly embedded in Spurr's resin (Spurr, 1969), and polymerized at 60°C for 42 h. Thin sections were cut from the embedded root tissue using Reichert ultracut microtome system (Reichert-Jung, Austria) fitted from glass knives. These sections were treated with chloroform vapour to stretch them. They were then placed on 100-mesh, Formvar-coated copper grid, and stained with 2% (w/v) uranyl acetate in 70% ethanol for 10 minutes followed by 0.02% (w/v) lead citrate for 10 minutes. Each grid was washed in 6 drops of sterile distilled water and the excess blotted away before transfer to a drop of lead citrate. The lead citrate was kept in closed petri dishes to avoid reaction with excessive atmospheric carbon dioxide. Concentrated sodium hydroxide was used to remove any carbon dioxide that could cause formation of lead carbonate on the grid. The grids were then washed many times in a series of water drops and dried by blotting with clean filter paper. After staining with uranyl acetate followed by lead citrate, areas of ultra-thin sections were viewed and photographed with a transmission electron

microscope (200CX, Germany) using a magnification of 10,000. Photographic prints were then made of the bacteria inside root tissues.

2.2.7 Leonard jar experiments on rhizobial inoculation of sorghum and millet and its effects on plant growth and mineral nutrition.

Plant culture and growth analysis in Leonard jars with rhizobial inoculation

One inoculation experiment was conducted with sorghum and millet using Leonard jars in growth chambers. As described above, seedlings of sorghum and millet landraces were raised in Leonard jars from surface-sterilized seed material, and inoculated with 10 mL of the broth cultures of 4 rhizobial strains, namely *B. japonicum* Tal 110, *S. meliloti* strain 1, *R.l.* bv. *viceae* Cn6 and *R. l.* bv. *viceae* strain 30. Uninoculated controls received an equal volume of sterile yeast extract mannitol broth medium without rhizobia. Prior to sterilization, the nutrient solution in Leonard jars was adjusted to contain 1.0 mM NO_3 for meeting the N requirements of the two cereal plants. In all, 4 replicate jars were used for each rhizobial strain. After inoculation, the plants were left to grow in a growth chamber under similar light and temperature conditions as indicated previously and harvested for growth analysis at 94 d after planting. Root length was measured, and the plants separated into shoots and roots for fresh weight determination. All samples were then oven-dried at 80oC for 48 h, weighed and ground into very fine powder for nutrient analysis.

2.2.8 Measurement of macro- and micro- nutrients in organs of inoculated sorghum plants in Leonard jars

The preparation of plant samples for the determination of macro-nutrient (P, P, Ca, Mg and Na) and micro-nutrients (Fe, Cu, Zn, Mn and B) in organs was done by dry-ashing, followed by acid digestion. A weighed amount (1 g dry matter) of plant material from each of the 4 replicates was ashed overnight in a crucible at 550°C in a muffle furnace, and the ash digested in 5 mL of 6 M HCl at 50°C for 30 minutes and filtered. The concentrations of nutrient elements were then determined after appropriate dilution, by

direct aspiration on a calibrated simultaneous ICP spectrophotometer (IRIS/AP HR DUO Thermo Electron Corporation, Franklin, Massachusettes, USA).

2.2.9 Potted soil experiments and plant growth analysis with rhizobial inoculation

A pot experiment was carried out in the glasshouse with sorghum using unsterile soil. Surface-sterilized seeds were sown in free-draining potted soil, and after germination, the seedlings were thinned out to 2 plants per pot. Broth cultures of *Bradyrhizobium japonicum* Tal 110, *Sinorhizobium meliloti* strain 1, *Rhizobium leguminosarum* bv. *viceae* strain 30, *R. leguminosarum* bv. *viceae* strain Cn6, *Rhizobium* NGR234, *Azorhizobium caulinodans* ORS571 and the non-rhizobial bacterium *Gluconoacetobacter diazotrophicus* PAL5 were adjusted to 0.2 OD_{600} units and 10 mL of each strain used to inoculate sorghum seedlings grown under glasshouse conditions. Here, 10 replicate pots were used per strain, and plants irrigated with sterile de-ionized water thrice a week and harvested at 94 d after planting. At harvest, the plants from each replicate pot were separated into shoots and roots, oven dried at 80°C and dry matter determined.

2.2.10 Soil chemical analysis

The soil used in this study was collected from 0-15 cm depth at the Agricultural Research Council (ARC) Nietvrobjie field station in Stellenbosch, a site that had not been cultivated for several years. The soil samples were taken to the laboratory, sieved (2 mm) and analyzed for plant-available mineral nutrients using the technique described above for plant samples. Triplicate samples were analysed and averaged.

2.2.11 Bioassay for indole acetic acid (IAA) production by rhizobial strains

The rhizobial strains used in these experiments were analyzed for IAA production. For rapid quantitative estimation in broth culture, the colorimetric method of Gordon and Weber (1951) was used. The cultures were grown in the dark for 7 d, centrifuged at 15, 000 x g for 10 min, and IAA assayed in duplicate supernatant samples. The presence of IAA in each supernatant was measured colorimetrically by adding two parts of 0.01 M $FeCl_3$ in 35% $HClO_4$ to one part supernatant followed by reading the optical density at 530 nm after 25 min. The recorded absorbances were read off a standard curve prepared

from pure indole acetic acid. Three separate assays were performed, and their average used for estimating IAA formation.

2.2.12 Nodulation bioassay of soybean seedlings using sterile sorghum root macerate

In order to confirm that the bacteria observed microscopically inside sorghum roots were indeed rhizobia, root tissue from sorghum plants inoculated with *B. japonicum* Tal 110 was surface-sterilized, and 1 g of material macerated under aseptic conditions for testing Koch's postulates. In each case, 15 mL of sterile distilled water was added to the macerate, thoroughly mixed, and 5 mL of the homogenate used to inoculate 5-d-old soybean seedlings grown aseptically in Leonard jars. Three replicate jars were used for each treatment including the control. The treatments used consisted of uninoculated soybean control (minus shorghum root macerate), uninoculated soybean (plus macerate of uninoculated sorghum root), and inoculated soybean (plus macerate of sterile inoculated sorghum root). The surface of each jar was covered with sterile cotton wool as anti-contamination mulch and maintained in the glasshouse. At 4 weeks after inoculation, the plants were harvested, checked for nodulation and photographed. The soybean plants from each Leonard jar were then separated into nodules, roots and shoots and oven-dried at 65°C for determination of dry matter. Ground plant samples were used for N analysis and fixed-N calculated as the difference between nodulated and non-nodulated control.

2.3 Results

2.3.1 Soil characterization

The soil used in this study was analyzed in triplicate for pH and mineral nutrients, and the following data were obtained: pH (CaCl2) 6.2; C, 0.99%; P (Citrate acid), 44.6 mg/kg; S, 3.4 mg/kg; Ca, 3.6 (cmol +)/kg; Mg 0.88 (cmol +)/kg; K, 79.6 mg/kg; Na, 70.8 mg/kg; Fe, 124.5 mg/kg; Mn, 15.4 mg/kg; Zn, 3.1 mg/kg; B, 0.51 mg/kg; and Cu, 7.0 mg/kg.

2.3.2 Rhizobial colonization and infection of sorghum and millet roots

2.3.3 Light microscopy

After inoculating sorghum seedlings with *B. japonicum* Tal 110, bacterial cells were clearly visible inside sorghum root tissue using light microscopy (Fig 2.1A and B). However, attempts to visualize bacteria inside roots of millet plants proved difficult.

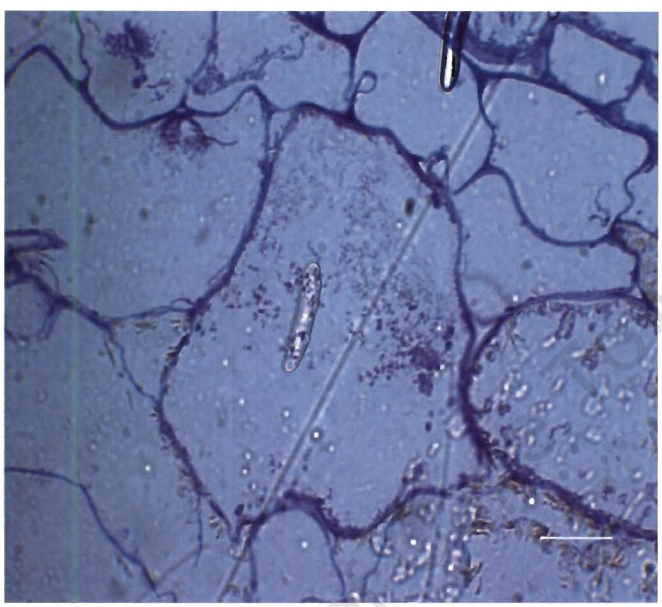


Fig 2.1. Light micrograph showing *Bradyrhizobium japonicum* Tal 110 bacteria located in epidermal cells of 10-d-old sorghum roots. Scale = 1000 µm.

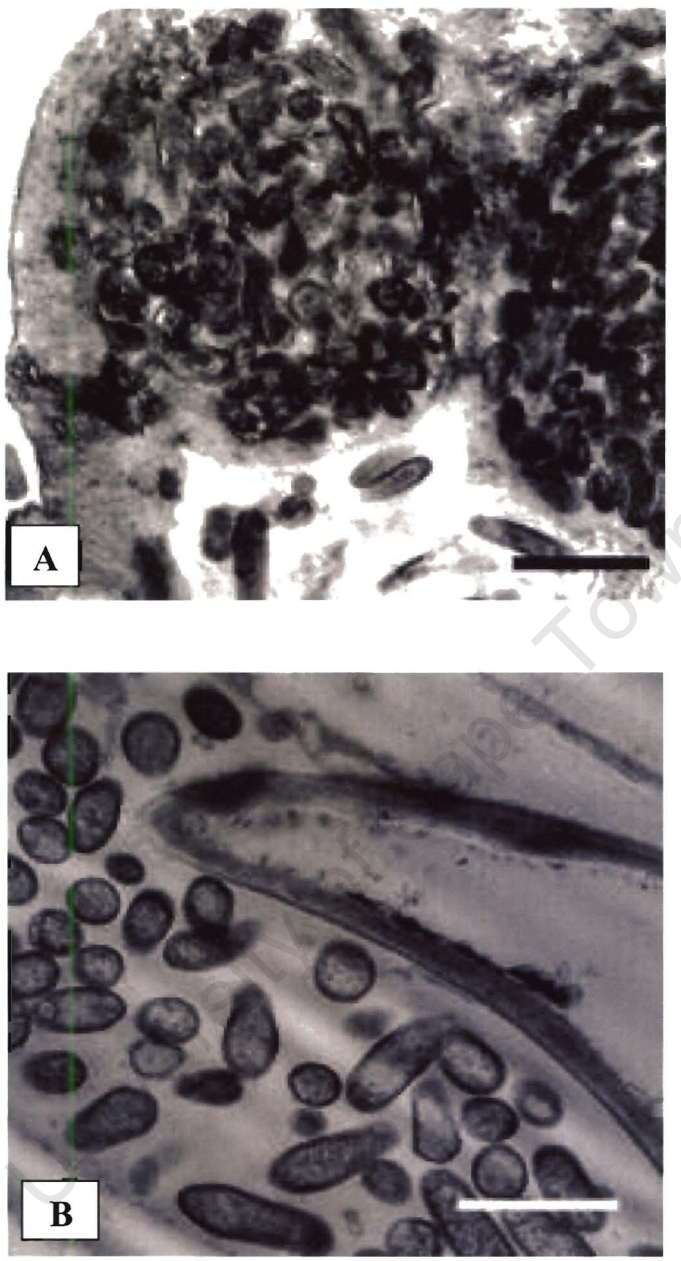


Fig 2.2. Transmission electron micrograph of A) *Bradyrhizobium japonicum* Tal 110 inside 10-d-old sorghum seedling root tissue, B) *Bradyrhizobium japonicum* Tal 110 inside 10-d-old millet seedling root tissue. Bar = 20 µm.

2.3.4 Transmission electron microscopy

The use of transmission electron microscopy also revealed the presence of *B. japonicum* Tal 110 cells inside the roots of both sorghum and millet plants (Fig 2.2 A and B).

2.3.5 Scanning electron microscopy

Scanning electron microscopy of sorghum roots showed a large number of rhizobial cells on the surface of both main and lateral roots of inoculated sorghum plants (Fig 2.3A and B), but not on those of uninoculated controls. Millet showed similar results. Attempts to locate rhizobial cells on the inside of sectioned root tissue using SEM showed clumps of *B. japonicum* Tal 110 and single cells of *Rhizobium* GHR2 in the root (Fig 2.4A and B). Other rhizobial strains including *Azorhizobium caulinodans* ORS571, *Rhizobium* NGR234 and *Rhizobium* GHR2 applied to sorghum and millet plants could be similarly observed inside root tissue using SEM techniques.

2.3.6 Plant growth response to rhizobial inoculation in Leonard jars

With sorghum, shoot growth, measured as dry matter increased significantly (Table 2.1) by 8-54% in this species. All four rhizobial strains used in this study caused a significant ($P \leq 0.05$) increase in the shoot dry matter of sorghum (Table 2.1). Although sorghum root dry matter was unchanged with rhizobial inoculation, total biomass of plants inoculated with *Rhizobium leguminosarum* bv. *viceae* 30 and *R. leguminosarum* bv. *viceae* Cn) were significantly increased ($P < 0.05$) at whole-plant level relative to control (data not shown). Fresh weights showed a similar pattern, and were significantly ($P \leq 0.05$) increased by 11-51% with rhizobial inoculation (Table 2.2). However, the shoot root and total biomass of millet plants were unaltered by rhizobial inoculation (Table 2.2).

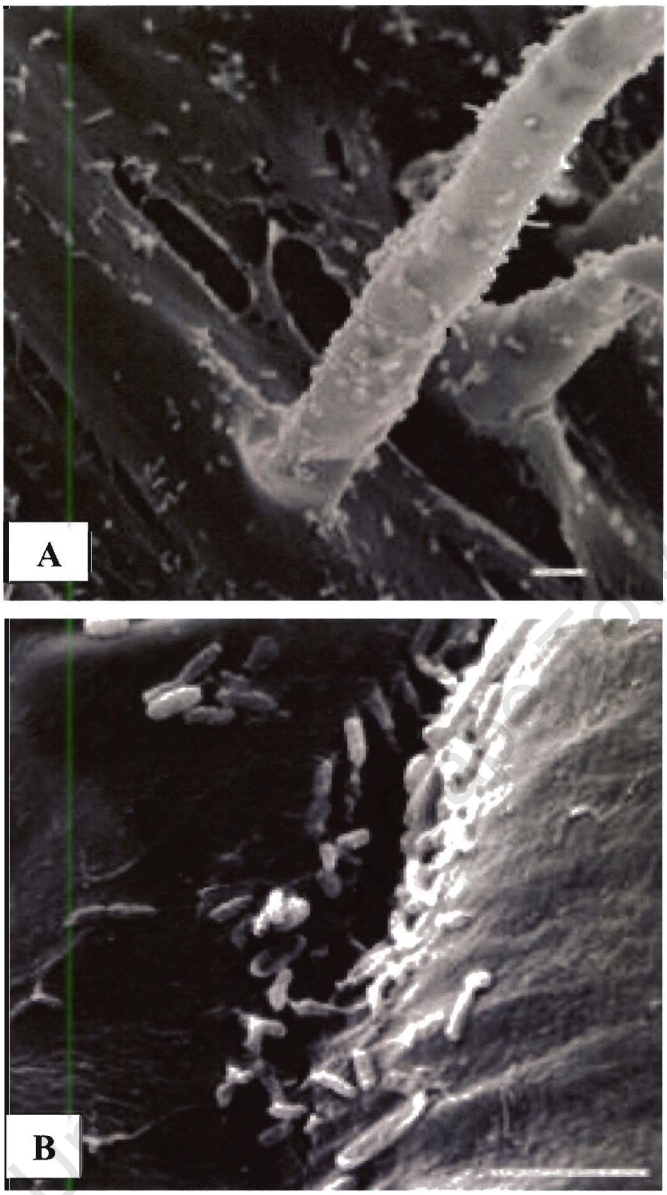


Fig 2.3. Scanning electron micrograph showing rhizobial cells on 60-d-old sorghum plant roots: A) *Azorhizobium caulinodans* ORS571, B) *Rhizobium* GHR2. Bar = 3 μm.

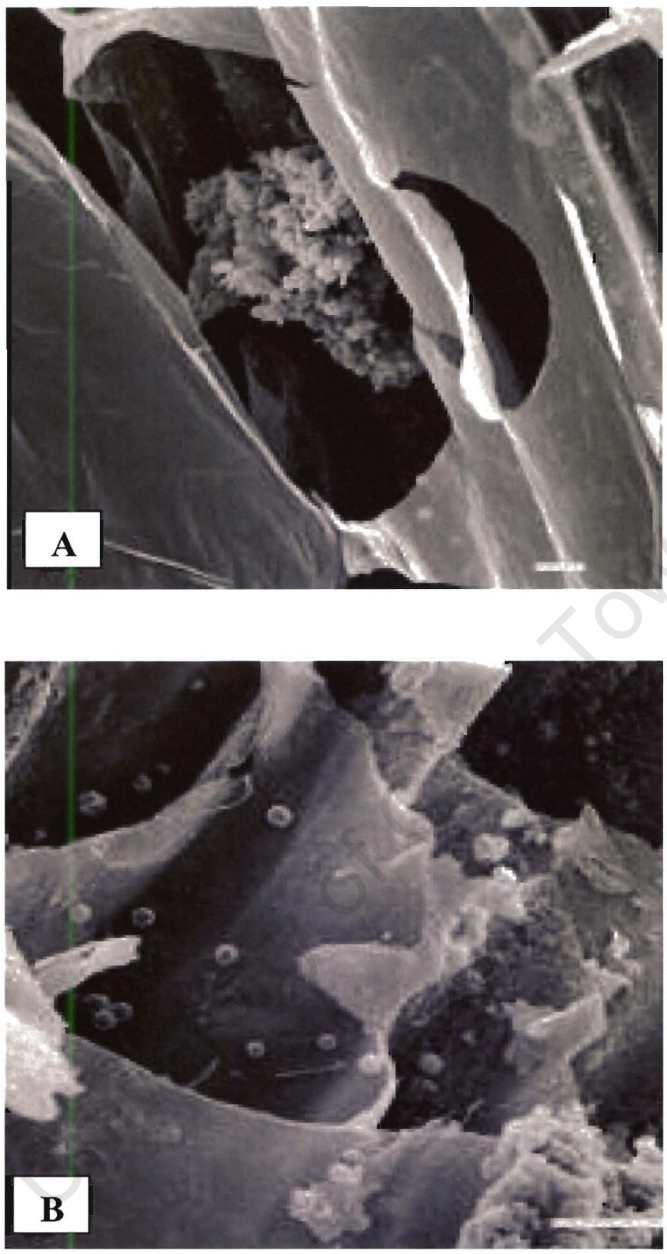


Fig. 2.4. Scanning electron micrographs showing rhizobial association within 60-d-old sorghum plant roots: A) *Bradyrhizobium japonicum* Tal 110 inside sorghum roots, B) GHR2 inside sorghum root. Bar = 3 μm.

Table 2.1. Fresh and dry matter yield of shoots and roots of sorghum grown aseptically with 1 mM NO_3 and inoculated with different rhizobial strains. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean $\pm$ S.E. (n = 4).

	Fresh weight (g/plant)		Dry weight (g/plant)	
Rhizobial strain	Shoot	Root	Shoot	Root
No inoculation	3.7 $\pm$ 0.32a	4.2 $\pm$ 0.65a	1.28 $\pm$ 0.13a	0.74 $\pm$ 0.11a
Bradyrhizobium japonicum Tal 110	4.5 $\pm$ 0.15b	4.4 $\pm$ 0.30a	1.63 $\pm$ 0.09b	0.76 $\pm$ 0.09a
Sinorhizobium meliloti strain 1	4.1 $\pm$ 0.22b	5.3 $\pm$ 0.40a	1.43 $\pm$ 0.09b	0.88 $\pm$ 0.07a
Rhizobium leguminosarum bv. *viceae* strain 30	5.0 $\pm$ 0.17b	4.9 $\pm$ 0.33a	1.65 $\pm$ 0.08b	0.85 $\pm$ 0.78a
R. leguminosarum bv. *viceae* strain Cn6	5.6 $\pm$ 0.32b	4.9 $\pm$ 0.54a	1.98 $\pm$ 0.13c	1.01 $\pm$ 0.09a

Table 2.2. Fresh and dry matter yield of shoots and roots of millet grown aseptically with 1 mM NO_3 and inoculated with different rhizobial strains. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean $\pm$ S.E. (n = 4).

	Fresh weight (g/plant)		Dry weight (g/plant)	
Rhizobial strain	Shoot	Root	Shoot	Root
No inoculation	4.0 $\pm$ 0.20a	2.8 $\pm$ 0.12a	0.86 $\pm$ 0.22a	0.45 $\pm$ 0.12a
Bradyrhizobium japonicum Tal 110	4.0 $\pm$ 0.31a	3.0 $\pm$ 0.16a	0.81 $\pm$ 0.23a	0.55 $\pm$ 0.13a
Sinorhizobium meliloti strain 1	4.5 $\pm$ 0.18a	3.4 $\pm$ 0.11a	1.00 $\pm$ 0.24a	0.50 $\pm$ 0.12a
Rhizobium leguminosarum bv. *viceae* strain 30	4.3 $\pm$ 0.15a	3.7 $\pm$ 0.11a	0.91 $\pm$ 0.19a	0.64 $\pm$ 0.11a
R. leguminosarum bv. *viceae* strain Cn6	4.3 $\pm$ 0.12a	2.8 $\pm$ 0.15a	1.16 $\pm$ 0.24a	0.46 $\pm$ 0.12a

The root lengths of sorghum plants were measured and found to increase significantly ($P \leq 0.05$) with inoculation relative to uninoculated control. All the four rhizobial strains used in this study stimulated a significant ($P \leq 0.05$) increase in root length of sorghum plants (Table 2.3). However, the root lengths of millet plants were unchanged by rhizobial inoculation (Table 2.3).

Table 2.3. IAA production and effects of rhizobial inoculation on root length of sorghum and millet plants grown aseptically with 1.0 mM NO_3. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data presented are Mean + SE (n = 4). Each IAA value is an average of three separate assays. ND = not determined.

Rhizobial strain	IAA produced ($\mu g\ mL^{-1}$)	Root length (cm)	
		Sorghum	Millet
Uninoculated control	-	63.73 ± 2.89a	71.30 ± 3.83a
Bradyrhizobium japonicum Tal 110	0.95	81.15 ± 1.54b	75.97 ± 2.09a
Sinorhizobium meliloti strain 1	1.66	84.08 ± 2.46b	69.70 ± 2.68a
Rhizobium leguminosarum bv. *viceae* strain 30	0.68	80.15 ± 7.94b	61.98 ± 1.87a
R. leguminosarum bv. *viceae* strain Cn6	0.18	76.85 ± 2.93b	64.17 ± 2.30a
Bradyrhizobium CB756	2.26	ND	ND
Rhizobium GHR2	0.45	ND	ND
Azorhizobium caulinodans ORS571	0.28	ND	ND
Rhizobium NGR234	0.63	ND	ND

2.3.7 Effects of rhizobial inoculation on the concentration of mineral nutrients in organs of sorghum plants

Because sorghum showed a marked growth response to rhizobial inoculation, the mechanism of growth promotion was studied through analysis of mineral nutrients in tissues. Unlike legumes, inoculation of sorghum with *Sinorhizobium meliloti* strain 1 and *R. leguminosarum* bv. *viceae* strain Cn6 significantly ($P \leq 0.05$) decreased N concentration in shoots (Table 2.4). Both *R. leguminosarum* bv. *viceae* strain 30 and strain Cn6 also decreased shoot Na levels (Table 2.4). With micronutrients, however, all test strains markedly ($P \leq 0.05$) reduced the levels of Zn and Cu in shoot relative to control (Table 2.5). The shoot concentration of Mn and Al were also decreased by inoculation of sorghum with *R. leguminosarum* bv. *viceae* strain 30 and strain Cn6, respectively (Table 2.5). The data for sorghum roots were equally exciting, in that P and K concentrations were significantly ($P \leq 0.05$) increased by rhizobial inoculation relative to control (Table 2.6). Except for *B. japonicum* Tal 110, inoculating sorghum with all the other test strains markedly ($P \leq 0.05$) decreased Na in roots (Table 2.6). Of the micronutrients, Al concentration in roots was markedly reduced by rhizobial inoculation of sorghum relative to uninoculated control (Table 2.7).

Table 2.4. Concentration of macronutrients in shoots of sorghum plants grown aseptically with 1 mM NO_3 and inoculated with different rhizobial strains. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean ± S.E. (n = 4)

Bacterial strain	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Na (mg/kg)
No inoculation	0.51± 0.04a	0.29 ± 0.03a	3.78 ± 0.38a	0.32 ± 0.05a	0.17 ± 0.01a	636.50 ± 103.82a
Bradyrhizobium japonicum Tal 110	0.41± 0.01ab	0.28 ± 0.02a	3.85 ± 0.22a	0.32 ± 0.01a	0.14 ± 0.00a	503.75 ± 29.47a
Sinorhizobium meliloti strain 1	0.42 ± 0.02ab	0.30 ± 0.09a	3.71 ± 0.23a	0.37 ± 0.04a	0.18 ± 0.06ab	512.75 ± 45.88a
Rhizobium leguminosarum bv. *viceae* strain 30	0.53 ± 0.06a	0.27 ± 0.01a	3.58 ± 0.13a	0.32 ± 0.03a	0.14 ± 0.00a	354.25 ± 27.23b
Rhizobium leguminosarum bv. *viceae* strain Cn6	0.39 ± 0.02b	0.32 ± 0.02a	3.39 ± 0.34a	0.32 ± 0.01a	0.15 ± 0.01a	376.50 ± 50.85b

Table 2.5. Concentration of micronutrients in shoots of sorghum plants grown aseptically with 1 mM NO_3 and inoculated with different rhizobial strains. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean ± S.E. (n = 4)

Bacterial strain	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Al (mg/kg)	B (mg/kg)
No inoculation	5.81 ± 0.53a	81.63 ± 25.93a	32.10 ± 1.71a	88.93 + 2.51a	359.25 + 78.94a	36.73 + 2.99a
Bradyrhizobium japonicum Tal 110	4.50 ± 0.07b	14.53 ± 0.75b	32.00 ± 1.06a	142.80 + 38.09a	247.00 + 10.79a	30.76 + 3.24a
Sinorhizobium meliloti strain 1	4.47 ± 0.37b	14.63 ± 1.73b	39.08 ± 6.36a	90.28 + 12.15a	301.00 + 57.46a	31.09 + 5.71a
Rhizobium leguminosarum bv. *viceae* strain 30	4.13 ± 0.11b	10.73 ± 0.71b	24.83 ± 1.18ab	108.90 + 16.86a	225.75 + 13.35a	26.48 + 2.72a
Rhizobium leguminosarum bv. *viceae* strain Cn6	4.73 ± 0.28b	11.70 ± 0.52b	27.70 ± 5.76a	110.35 + 20.46a	182.00 + 23.77b	27.74 + 4.13a

Table 2.6. Concentration of macronutrients in roots of sorghum plants grown aseptically with 1 mM NO_3 and inoculated with different rhizobial strains. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean ± S.E. (n = 4)

Bacterial strain	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Na (mg/kg)
No inoculation	0.51 ± 0.02a	0.06 ± 0.02a	0.40 ± 0.00a	0.11 ± 0.06a	0.11 ± 0.08a	1313.75 ± 169.43a
Bradyrhizobium japonicum Tal 110	0.44 ± 0.03a	0.07 ± 0.02ab	0.40 ± 0.00a	0.40 ± 0.00a	0.12 ± 0.02a	907.25 ± 63.66b
Sinorhizobium meliloti strain 1	0.48 ± 0.09a	0.18 ± 0.03b	1.17 ± 0.43b	0.13 ± 0.08a	0.15 ± 0.01a	722.75 ± 96.85b
Rhizobium leguminosarum bv. *viceae* strain 30	0.41 ± 0.02a	0.09 ± 0.09b	0.97 ± 0.32b	0.11 ± 0.03a	0.15 ± 0.01a	765.75 ± 59.21b
Rhizobium leguminosarum bv. *viceae* strain Cn6	0.38 ± 0.03a	0.21 ± 0.01b	1.17 ± 0.44b	0.10 ± 0.02a	0.12 ± 0.01a	520.75 ± 35.52c

Table 2.6. Concentration of macronutrients in roots of sorghum plants grown aseptically with 1 mM NO_3 and inoculated with different rhizobial strains. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean ± S.E. (n = 4).

Bacterial strain	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Na (mg/kg)
No inoculation	0.51 ± 0.02a	0.06 ± 0.02a	0.40 ± 0.00a	0.11 ± 0.06a	0.11 ± 0.08a	1313.75 ± 169.43a
Bradyrhizobium japonicum Tal 110	0.44 ± 0.03a	0.07 ± 0.02ab	0.40 ± 0.00a	0.40 ± 0.00a	0.12 ± 0.02a	907.25 ± 63.66b
Sinorhizobium meliloti strain 1	0.48 ± 0.09a	0.18 ± 0.03b	1.17 ± 0.43b	0.13 ± 0.08a	0.15 ± 0.01a	722.75 ± 96.85b
Rhizobium leguminosarum bv. *viceae* strain 30	0.41 ± 0.02a	0.09 ± 0.09b	0.97 ± 0.32b	0.11 ± 0.03a	0.15 ± 0.01a	765.75 ± 59.21b
Rhizobium leguminosarum bv. *viceae* strain Cn6	0.38 ± 0.03a	0.21 ± 0.01b	1.17 ± 0.44b	0.10 ± 0.02a	0.12 ± 0.01a	520.75 ± 35.52c

Table 2.7. Concentration of micronutrients in roots of sorghum plants grown aseptically with 1 mM NO_3 and inoculated with different rhizobial strains. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean ± S.E. (n = 4)

Bacterial strain	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Al (mg/kg)	B (mg/kg)
No inoculation	11.46 ± 1.08a	35.98 ± 2.35a	44.83 ± 10.81a	1018.75 ± 258.47a	1172.75± 207.73a	10.75 ± 0.91a
Bradyrhizobium japonicum Tal 110	12.24 ± 2.07a	34.70 ± 6.65a	42.35 ± 12.12a	1033.25 ± 97.01a	792.25 ± 134.44b	9.74 ± 1.72a
Sinorhizobium meliloti strain 1	14.54 ± 2.76a	38.12 ± 4.30a	33.63 ± 6.48a	1334.50 ± 99.79a	699.50 ± 96.97b	11.11 ± 0.65a
Rhizobium leguminosarum bv *viceae* strain 30	12.35 ± 0.72a	32.10 ± 1.49a	48.03 ± 4.15a	958.25 ± 103.22a	702.50 ± 70.72b	11.77 ± 2.04a
Rhizobium leguminosarum bv *viceae* strain Cn6	11.38 ± 1.54a	31.88 ± 6.09a	24.95 ± 5.22a	1188.00 ± 255.91a	492.75 ± 44.07b	13.37 ± 4.31a

2.3.8 Response of plant growth to rhizobial inoculation in potted soil

Inoculation of plants grown in unsterile potted soil in the glasshouse did not produce any significant differences in growth when compared to the uninoculated control (Table 2.8). The non-rhizobial diazotroph, *G. diazotrophicus*, which was included, did not also enhance growth of its host plant (Table 2.8).

Table 2.8. Dry matter yield of shoots, roots and whole sorghum plants grown in unsterile soil and inoculated with different rhizobial strains or the non-rhizobial diazotroph *Gluconoacetobacter diazotrophicus*. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean ± S.E. (n = 9).

Bacterial strain	Plant biomass (g/plant)		
	Shoot	Root	Total
No inoculation	6.83 ± 0.42a	7.07 ± 0.68a	13.91 ± 0.74a
Bradyrhizobium japonicum Tal 110	7.09 ± 0.64a	7.04 ± 0.53a	14.12 ± 0.88a
Sinorhizobium meliloti strain 1	7.01 ± 0.45a	6.94 ± 0.52a	13.95 ± 0.91a
Rhizobium leguminosarum bv. *viceae* strain 30	7.46 ± 0.43a	7.10 ± 0.34a	14.56 ± 0.97a
Rhizobium leguminosarum bv. *viceae* strain Cn6	7.55 ± 0.24a	7.16 ± 0.40a	14.08 ± 0.34a
Rhizobium NGR234	7.44 ± 0.47a	7.71 ± 0.95a	15.15 ± 1.03a
Azorhizobium caulinodans ORS571	6.65 ± 0.61a	6.18 ± 0.54a	12.83 ± 1.03a
Gluconoacetobacter diazotrophicus PAL 5	7.11 ± 0.32a	6.24 ± 0.44a	13.35 ± 0.46a

2.3.9 Soybean nodulation by surface-sterile sorghum root macerate

The application of 5 mL macerate prepared from sterile roots of sorghum plants which had been inoculated with *B. japonucum* Tal 110 produced nodules on soybean plants (see Fig 2.5 A, B and Table 2.9). However, the uninoculated controls as well as soybean plants treated with the macerate of uninoculated sterile sorghum root showed no evidence of nodulation (Table 2.9 and Fig 2.5 C). Because the soybean plants were effectively nodulated by the surface-sterile sorghum root macerate, the root, shoot and total dry matter of these soybean plants were significantly greater than those of non-nodulated controls (Table 2.9), and led to a measurable amount of fixed-N in the nodulated plants (Table 2.9).

Table 2.9. Nodule numbers, dry matter of shoots, roots and whole soybean plants grown aseptically in Leonard jars and inoculated with surface-sterilized macerate of sterile ininoculated root or sorghum roots inoculated with *Bradyrhizobium japonicum* Tal 110. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$ (one-way ANOVA). Data are presented as Mean $\pm$ S.E (n = 3). Fixed-N was determined as the difference between total N of inoculated and uninoculated non-nodulated soybean plants.

	Growth parameters					
Inoculation treatments	Nodules (no./plant)	Nodule dry matter (mg/plant)	Root dry matter (mg/plant)	Shoot dry matter (mg/plant)	Total dry matter (mg/plant)	Fixed N (mg/plant)
Uninoculated (minus macerate of sterile sorghum root)	0.0a	0.0a	80.7 $\pm$ 34.5a	340.0 $\pm$ 23.1a	456.7 $\pm$ 20.3a	-
Uninoculated (plus macerate of uninoculated sterile sorghum root)	0.0a	0.0a	76.7 $\pm$ 13.3a	230.0 $\pm$ 45.1a	306.7 $\pm$ 31.8a	-
Inoculated (plus macerate of sterile inoculated sorghum root)	5.3 + 0.9b	16.9b	180.0 $\pm$ 11.6b	530.0 $\pm$ 49.3b	710.0 $\pm$ 43.5c	53.1 $\pm$ 3.0

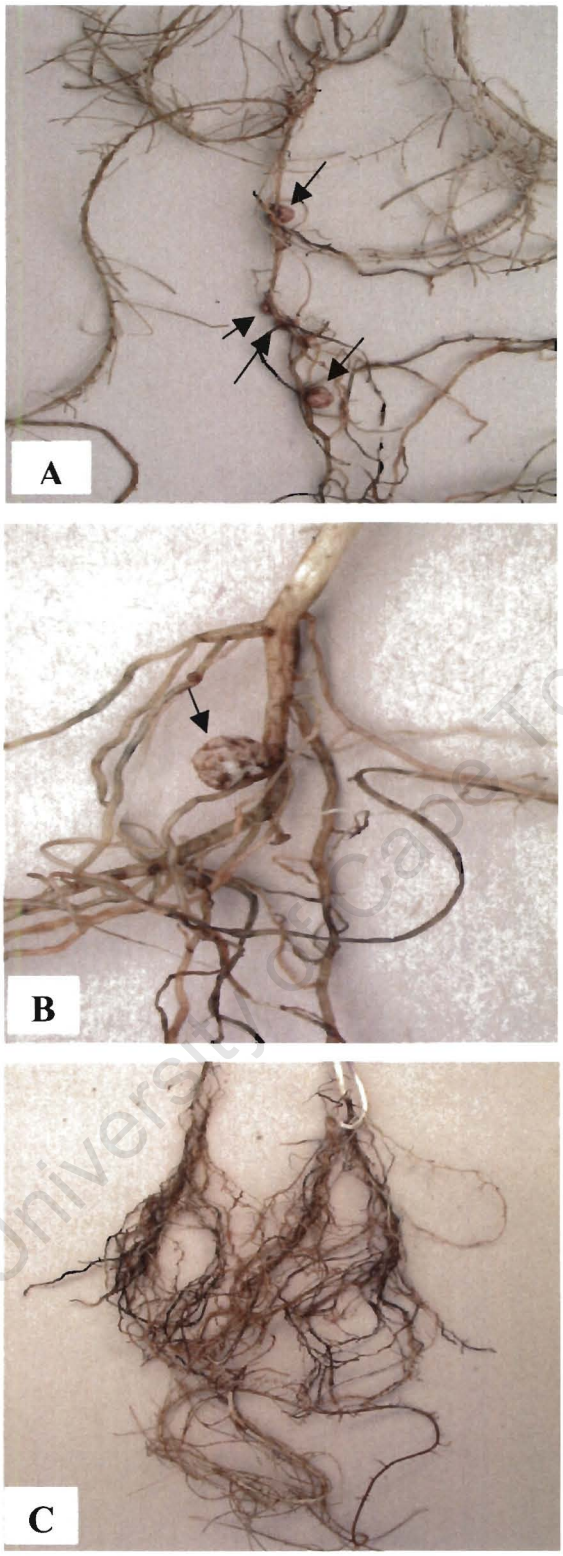


Fig 2.5. Nodulated and non-nodulated roots of soybean plants inoculated with macerate from surface-sterilized roots of sorghum which had been inoculated with *Bradyrhizobium japonicum* Tal 110. Note the nodules observed on A and B, inoculated with sterile sorghum root macerate, but not on the uninoculated control C.

2.4 Discussion

2.4.1 Rhizobial colonization and infection of sorghum and millet roots

All the rhizobial strains tested successfully colonized the roots of both sorhgum and millet plants. The use of light, scanning and transmission electron microscopy revealed the presence of high numbers of bacteria on the surface and inside of main and lateral roots of inoculated sorghum and millet plants (Figs. 2.1 – 2.4), but not on uninoculated controls (data not shown). Under the SEM, small rod-shaped bacteria, typical of rhizobial cells, were seen as colonies on the root epidermal surfaces of the sorghum and millet plants and appeared invaded by infecting rhizobial cells (Figs. 2.3). As found with rhizobial colonization of roots of non-legume plants (Spencer *et al.*,1994; de Bruijn *et al.*, 1995; Yanni *et al.*, 1997), these cracks probably served as entry points for rhizobia to invade and infect the plant's internal tissues. This is supported by the swamp of bacteria found around the cracks in a manner that suggested internal invasion of the sorghum and millet roots (Fig 2.3B). However, it is also possible that some enzymatic action by rhizobia is involved in the invasion of the internal tissues of these crops since rhizobia have been reported to produce multiple enzymes that cleave glycosidic bonds in plant cell walls (Mateos *et al.*, 1992; Jimenez-Zurdo *et al.*, 1996).

When fixed root tissues of sorghum and millet were sectioned and the surface and interior viewed using SEM techniques, the electron micrographs revealed localization of bacteria in the inner tissues. The bacteria observed in these inner tissues were assumed to be the applied rhizobial strains because uninoculated plants showed no evidence of bacteria on the root epidermins or in interior tissues. This assumption was validated when root macerate prepared from sterile inoculated sorghum plants successfully induced nodulation and N_2 fixation in soybean seedlings (Fig 2.5A, B; Table 2.9). No nodules were however found on soybean plants inoculated with sterile root homogenate prepared from uninoculated sorghum roots (Fig 2.5C). This clearly indicates that the bacteria observed microscopically inside sorghum root tissues were indeed the authentic N_2-fixing rhizobia applied to cereal plants. As shown in Fig 2.4 A, B, single cells of *R. l.* bv. *viceae* (strain 30) could be seen next to the xylem vessels of both sorghum and millet roots (Fig 2.4A, B). A similar pattern of xylem

colonization was observed for the other test strains. Not uncommon was also the observation that rhizobia could occur in clumps of cells inside the xylem of sorghum and millet roots, as shown for *R. l.* bv. *viceae* strain 30 and Cn6 or *B. japonicum* Tal 110 (Fig 2.4A). The detection of rhizobia as endophytes of inoculated sorghum and millet roots in this study, and their observed localization in the xylem, is consistent with other reports (James *et al.*, 1994; Gough *et al.*, 1997; Reddy *et al.*, 1997; O'Callaghan *et al.*, 1997; Schloter *et al.*, 1997; Yanni *et al.*, 1997; Reis *et al.*, 1999), which show that introduced rhizobia and non-rhizobial bacteria can invade and localize themselves in the xylem and intercellular spaces of their host-plant roots. However since rhizobia have also been reported to produce cellulase and polygalacturonase enzymes (Mateos *et al.*, 1992; Jimenez-Zurdo *et al.*, 1996) it is possible that these aid in its entry into root tissues of sorghum and millet.

Rhizobium invasion of symbiotic legumes is generally via root hair infection in most members of the Leguminosae. However, with nodulating tree legumes and symbiotic groundnut, rhizobial entry into roots is via cracks, wounds or points of lateral root emergence (Sen and Weaver 1984; Sprent 1989). Irrespective of the mode of entry, Rhizobium invasion of legume roots is genetically controlled by gene expression and molecular signals produced by the host plant (Dakora, 1994). Whether rhizobial entry into roots of sorghum and millet and their localization in the xylem of those organs is similarly influenced by plant factors, remains unknown. It has however been suggested that with non-legume hosts, these entry events are accidental rather than biologically programmed (Spencer *et al.,* 1994). Whatever the case, the findings of this study nevertheless demonstrate that rhizobia can naturally infect millet and sorghum roots via cracks or wounds, and localize themselves in the internal tissues of their host plants as found in studies with other plant species (Spencer *et al.*, 1994; de Bruijn *et al.*, 1995; Yanni *et al.*, 1997). But whether rhizobial colonization and infection of sorghum and millet roots yields any benefits to those plants, as occurs in symbiotic legumes, remains to be determined.

2.4.2 Rhizobial stimulation of plant growth promotion and mineral nutrition in sorghum

The inoculation of asepically-grown seedlings of an African landrace of sorghum with infective cells of rhizobial strains promoted shoot growth by 11-51% on fresh weight

basis, and 8-54% on dry weight basis. In fact, all the inoculated plants showed significantly ($P \leq 0.05$) greater shoot fresh and dry weight compared to uninoculated control, but their root masses remained unaffected (Table 2.1). These observations are consistent with a recent report (Yanni *et al.*, 1997) which showed a significant promotion of shoot growth in rice plants inoculated with endophytic strains of *R. l.* bv. *trifolii*.

Although root mass was unaffected in this study, there was a significant increase in root length of 21-32% with rhizobial inoculation of sorghum (Table 2.3), suggesting the release of a root-growth-promoting molecule by the test strains. In that regard, *Bradyrhizobium* strain CB756 was found to stimulate an increase in root length of wheat with inoculation (Law and Strijdom, 1989). Two clover rhizobia which occur as natural endophytes of rice similarly promoted root growth in that species (Yanni *et al.*, 1997). While the findings of this study and others (Law and Strijdom 1989; Yanni *et al.* 1997) could imply that soil populations of N_2-fixing rhizobia are capable of stimulating root growth (mass or length) in non-legume plants, Law and Strijdom (1989) found that, only *Bradyrhizobium* strain CB756 out of 5 *Bradyrhizobium* sp. tested, consistently induced an increase in root length of wheat plants. The others did not. It was also established that the stimulatory substance produced by *Bradyrhizobium* strain CB756 was present in both cell suspensions and cell-free culture filtrates (Law and Strijdom, 1989).

IAA is a phytohormone that stimulates root development in plants, and has been shown to be the common cause of corn growth promotion following inoculation with *Azospirillum* (Okon *et al.*, 1995). An assay for the production of this molecule in the presence of typtophan (Gordon and Weber, 1951) revealed the ability of the test strains to form IAA from oxidation of tryptophan. Although the method (Gordon and Weber, 1951) used here is less sensitive, in that it detects down to only 0.5 ug mL^{-1}, the test strains were found to produce biologically significant amounts of IAA (Table 2.3). Whether as a result of the IAA formed or some other active molecule released, rhizobial inoculation of sorghum stimulated root elongation relative to uninoculated control (Table 2.3), and increased P and K concentrations in sorghum roots, but decreased root Na and Al levels (Tables 2.5 and 2.6). Shoot N and Na concentrations were similarly decreased by some rhizobial strains such as *S. meliloti* strain 1, *R.*

leguminosarum bv. *viceae* strain 30 and Cn6 (Table 2.4). However, other interactive effects of rhizobia and sorghum roots could have also promoted P and K uptake or decreased N, Al or Na uptake, noting that interaction between rice roots and the resident population of cyanobacteria (*Anabaena* and *Nostoc* sp.) in soil increased P supply to the rice plants (Yanni and Abd El-Rahman 1993). To our knowledge, this is the first report of plant growth promotion and enhanced P and K nutrition in landraces of a major African cereal with rhizobial inoculation. Future studies using a wider collection of diverse landraces of sorghum and millet are likely to find greater benefits of rhizobial interaction with these important African crops.

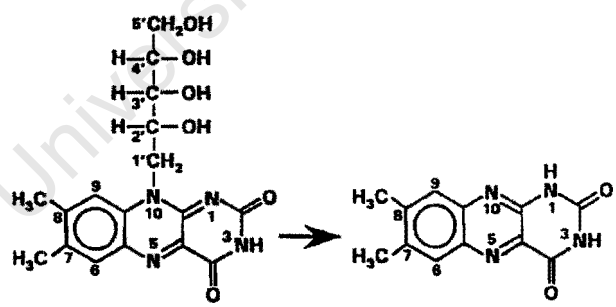
The promotion of shoot growth, root length, and P and K nutrition with the application of rhizobial to sorghum roots indicates great promise for increasing cereal yields through inoculation. This idea was tested in a pot experiment using different rhizobial strains and *G. diazotrophicus*, a non-rhizobial bacterium, on sorghum seedlings growth in unsterile soil. The results of this pot experiment with unsterile soil were in sharp contrast to those obtained with sterile Leonard jars. Neither shoot nor root growth increased when soil-grown sorghum plants were inoculated with different rhizobial strains (Table 2.8), which included those that promoted sorghum growth in Leonard jars. This finding indicates that either the resident soil bacteria produced greater amounts of the growth-promoting molecule, or that its synthesis by the introduced rhizobia was adversely affected by the conditions of soil culture. The observed lack of response is nevertheless consistent with a report (Law and Strijdom, 1989) that found *Bradyrhizobium* strain CB756 to promote wheat growth under sterile conditions in Leonard jars but not in sand culture. The findings of this study suggest that soil populations of rhizobia, such as those used in this study, can naturally stimulate growth of sorghum and millet plants under certain conditions in the rhizosphere.

CHAPTER 3

LUMICHROME, A RHIZOBIAL SIGNAL MOLECULE, ALTERS DEVELOPMENAL CHANGES IN CEREAL AND LEGUME SEEDLINGS

3.1 Introduction

Apart from their well-known role for N_2 fixation in root nodules of leguminous plants, rhizobia, (species of *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium* and *Mesorhizobium*) affect fundamental processes in plant development through the release of powerful molecules. The role of some of these compounds such as the phytohormones cytokinin (Phillips and Torrey, 1970; 1972) and IAA (Law and Strijdom, 1989; Hirsch *et al.*, 1997; Vessey, 2003) have been known for a long time. More recently, a number of novel rhizobial molecules have been identified to have similar promotive effects on seedling development and plant growth (Dakora, 2003). These rhizobial molecules include the nodulation factors lipo-chito-oligosaccharides (Lopez-Lara *et al.*, 1995; Dyachock *et al.*,2000; Prithiviraj *et al.*, 2000; Smith *et al.*, 2002) and hydrogen gas evolved as an obligate by-product of the activity of nitrogenase, the N_2-fixing enzyme present in diazotrophs (Dong and Layzell, 2002). Lumichrome is another signal compound that has been identified from culture filtrates of *Sinorhizobium meliloti* cells (Phillips *et al.*, 1999), with potency for stimulating plant growth. But lumichrome can also occur in soil as a breakdown product of riboflavin, a vitamin commonly produced by rhizobia as well as other bacterial species and plants.



Because riboflavin is easily converted enzymatically or photochemically into lumichrome (Phillips *et al.*, 1999), the presence of the latter in soil is likely to be common due to its multi-origin from bacteria, plants and the degradation of riboflavin. The study by Phillips *et al.*, (1999) showed an increase in growth of alfalfa plants with lumichrome supply; and this was attributed to enhanced root respiration and increased net C assimilation. But whether this growth response is

unique to alfalfa, is not known. The aim of this study was to test the effects of lumichrome on the growth of tropical grain legumes and cereals under glasshouse conditions.

3.2 Materials and methods

3.2.1 Glasshouse conditions

The experiments were carried out during the summer of 2001 at the University of Cape Town, under uncontrolled conditions of light, temperature and humidity in the glasshouse.

3.2.2 Plant species

The legumes used in this study were cowpea, (*Vigna unguiculata* (L.) Walp), soybean (*Glycine max* L. Merril), Bambara groundnut (*Vigna subterranean* L. Verdc) and common bean (*Phaseolus vulgaris* L.). The cereals tested included finger millet (*Eleucine coracana* L.), sorghum (*Sorghum bicolor* (L.) Moench), maize (*Zea mais* L.) and Sudan grass (*Sorghum bicolor* subsp. *sudanense* L).

3.2.3 Plant culture

In all experiments, seeds were surface-sterilized by soaking in 70% ethanol for 90 s then in 1.5 % bleach for 15 min, and then rinsed 6 times with sterile distilled water. The seeds were then sown in sterile 1.0 L pots containing vermiculite and maintained inside the glasshouse. The pots were randomized in 6 blocks, watered every second day with ½ strength modified Hoagland nutrient solution containing 1 mM NH_4NO_3 and the antibiotics ampicillin (125 mg/L) and rifampicin (10 mg/L) to control microbial contamination.

The effects of lumichrome on plants was tested by irrigating the roots of seedlings with this bacterial metabolite. Seedlings raised from surface-sterilized seeds were watered with nutrient solution adjusted to contain 0, 5 or 50 nM lumichrome as described by Phillips *et al.* (1999). For each treatment, six seeds were sown in each pot, and after germination, thinned out to 3 seedlings per pot. The pots were covered

initially with a transparent clear, plastic wrap, which was removed after 6 d. In all, 6 replicates were used per treatment. Lumichrome was only applied to the roots.

3.2.4 Plant harvest and growth analysis

Plants were harvested at various time intervals and separated into shoots and roots. In some instances, leaf areas were determined for both unifoliate and trifoliate leaves and at whole-plant level. For dry matter determination, plants were oven-dried at 65°C until constant weight.

3.2.5 Statistical anlaysis

Data collected on the effects of lumichrome on components of plant growth such as organ dry matter and leaf areas were analyzed by one-way ANOVA using STATISTICA statistical package.

3.3 Results

3.3.1 Growth of legumes supplied with lumichrome

3.3.2 Cowpea growth response to lumichrome

Culturing cowpea seedlings for 11 d and watering them with 5 nM concentration of lumichrome resulted in a significant ($P \leq 0.05$) increase in shoot dry matter and total biomass relative zero-lumichrome control (Table 3.1). This increase in total biomass was due to early initiation of trifoliate leaves and their faster growth when plants were watered with 5 nM lumichrome (Fig.3.1C). As a result, those plants produced significantly ($P \leq 0.05$) greater biomass than the control (Table 3.1). Root growth in cowpea was however not affected. But by 37 d after planting, the observed developmental changes in seedlings had evened off, except for root growth which was depressed by the application of 50 nM lumichrome (Table 3.2).

Table 3.1. Effect of lumichrome on dry matter yield of cowpea plants harvested at 11 d after planting. Values (Mean ± SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

Lumichrome conc'n (nM)	Dry matter (mg/plant)			
	Shoot	Root	Total	Trifoliate leaf
0	116 ± 12a	35 ± 7a	151 ± 15a	1.2 ± 0.3a
5	164 ± 75b	65 ± 7b	225 ± 11b	2.0 ± 0.1b
50	105 ± 17a	31 ± 4a	136 ±19a	-----

Table 3.2. Effect of lumichrome on dry matter yield of cowpea harvested at 37 d after planting. Values (Mean ± SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

Lumichrome conc'n (nM)	Dry matter (mg/plant)				
	Trifoliate leaf 1	Trifoliate leaf 2	Stem	Root	Total
0	346 ± 18a	59 ± 10a	441 ± 12a	268 ±12a	1066 ± 41a
5	380 ± 26a	66 ± 9a	484 ± 29a	258 ± 5a	1125 ± 37a
50	340 ± 22a	31 ± 3.5b	418 ± 31a	226 ± 14b	1015 ± 78a

Table 3.3. Effect of lumichrome on dry matter yield of soybean plants harvested at 37 d after planting. Values (Means ± SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$. Shoot = stem, unifoliate + trifoliate leaf.

	Dry matter (mg/plant)					
Lumichrome conc'n (nM)	Unifoliate leaf	Trifoliate leaf	Stem	Shoot	Root	Total
0	174 ± 6a	65 ± 7a	219 ± 5a	457 ± 12a	259 ± 12a	3875 ±161a
5	171 ± 9a	132 ± 29b	253 ± 13b	557 ± 131b	232 ± 14a	5054 ± 443b
50	143 ± 4c	62 ± 4a	187 ± 13a	392 ± 8c	235 ± 8a	3573 ± 114a

3.3.3 Soybean growth response to lumichrome

As with cowpea, the application of lumichrome to soybean markedly altered seedling development (Fig.3.1A,B). At 37 d after planting, the dry matter yield of the first trifoliate leaf on soybean was significantly ($P \leq 0.05$) increased in plants watered with 5 nM lumichrome (see Fig.3.1B). Similarly, stem and shoot showed a significant ($P \leq 0.05$) increase with the supply of 5 nM lumichrome, which resulted in a pronounced ($P \leq 0.05$) increase in overall biomass of soybean plants provided with 5 nM lumichrome relative to control (Table 3.3). In contrast, supplying soybean plants with a higher concentration of lumichrome (50 nM) decreased ($P \leq 0.05$) growth of unifoliate leaf and shoot relative to control and the other lumichrome treatment (Table 3.3).

When watered with 5 nM lumichrome, soybean also showed a significant ($P \leq 0.05$) increase in leaf area of unifoliate leaves and first trifoliate leaf compared to the control and other lumichrome treatment (Table 3.4). As a result, total leaf area on a per-plant basis was significantly ($P \leq 0.05$) greater in soybean plants watered with 5 nM lumichrome (Table 3.4).

The stem length of soybean plants measured at 37 d after planting also showed an increase in elongation with lumichrome supply at 5 nM concentration (Table 3.4).

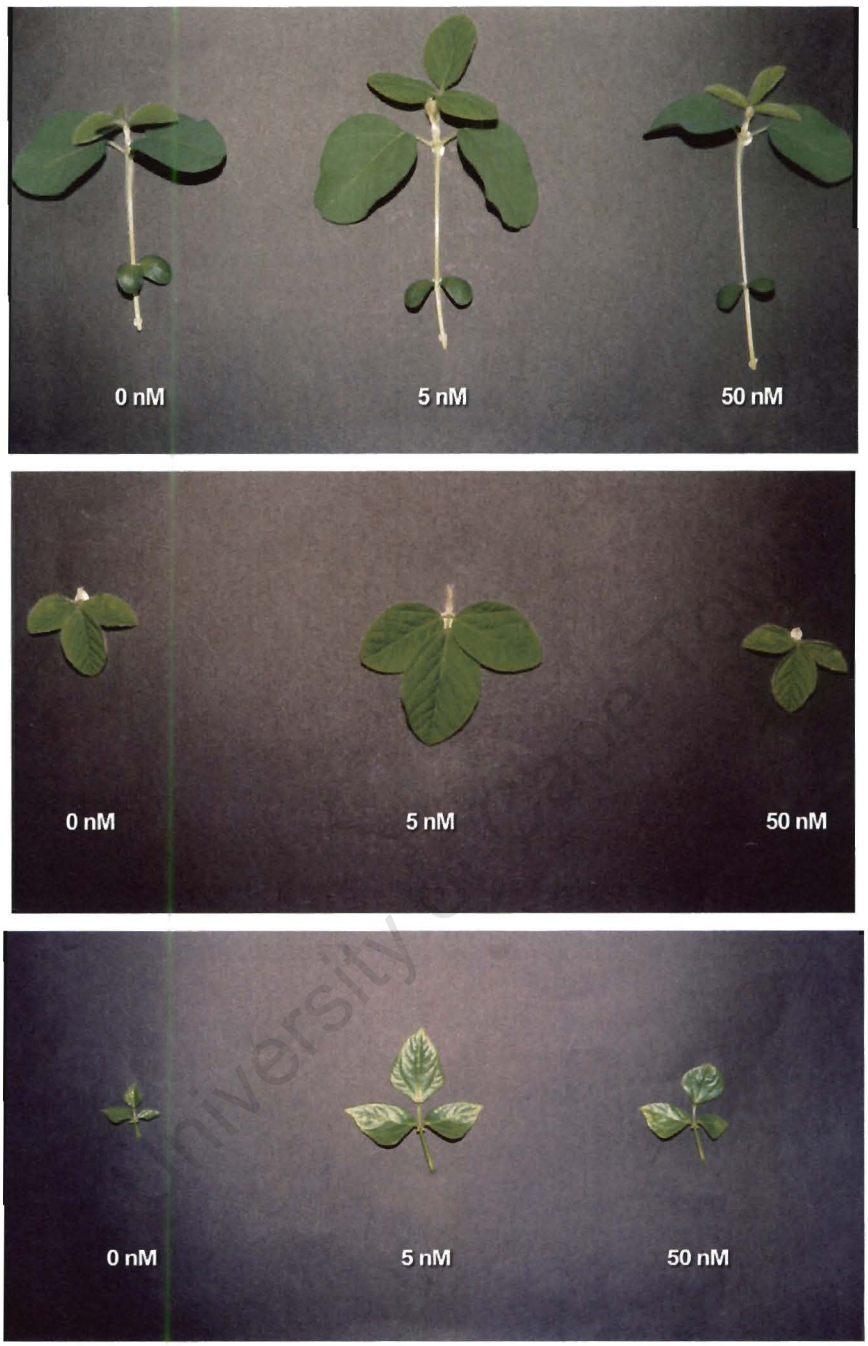


Fig. 3.1. A comparison of A) soybean seedlings watered with different concentrations of lumichrome, B) first trifoliate leaves of soybean seedlings watered with different concentrations of lumichrome, and C) first trifoliate leaves of cowpea seedlings watered with different concentrations of lumichrome.

Table 3.4. Effect of lumichrome on leaf area and stem length of soybean plants harvested at 23 (leaf area) and 37 (stem length) d after planting. Values (Mean ± SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

	Leaf area (cm^2/plant)			Stem length (cm/plant)
Lumichrome conc'n (nM)	Unifoliate	Trifoliate	Total	
0	2.97 ± 0.07a	0.91 ± 0.10a	3.88 ± 0.16a	9.10 ± 0.34a
5	3.47 ± 0.28b	1.59 ± 0.17b	5.05 ± 0.44b	10.26 ± 0.20b
50	2.69 ± 0.04a	0.89 ± 0.07a	3.57 ± 0.11a	9.20 ± 0.35a

3.3.4 Growth response of Bambara groundnut and common bean to lumichrome

Unlike cowpea and soybean, Bambara groundnut and the common bean did not show any significant growth response to the different concentrations of lumichrome (Tables 3.5 and 6).

Table 3.5. Effect of lumichrome on dry matter yield of Bambara groundnut plants harvested at 37 d after planting. Values (Mean ± SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

Lumichrome conc'n (nM)	Dry matter (mg/plant) Shoot	Root	Total
0	1.69 ± 0.11a	0.49 ± 0.018a	2.18 ± 0.17a
5	1.71 ± 0.14a	0.48 ± 0.029a	2.19 ± 0.19a
50	1.87 ± 0.20a	0.55 ± 0.037a	2.41 ± 0.15a

Table 3.6. Effect of lumichrome on dry matter yield of common bean plants harvested at 23 d after planting. Values (Means ± SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

Lumichrome conc'n (nM)	Dry matter (mg/plant)				
	Unifoliate leaf	Trifoliate leaf	Stem	Root	Total
0	492 ± 21a	92 ± 6a	373 ± 43a	395 ± 29a	1357 ± 114a
5	523 ± 39a	76 ± 5a	374 ± 28a	435 ± 22a	1412 ± 98a
50	518 ± 49a	57 ± 3a	346 ± 37a	356 ± 30a	1277 ± 87a

Table 3.7. Effects of lumichrome on dry matter yield (mg/plant) of sorghum plants harvested at either 11 or 37 d after planting. Values (Mean + SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

Lumichrome conc'n (nM)	11 DAP			37 DAP		
	Shoot	Root	Total	Shoot	Root	Total
0	8.7 ± 0.5a	9.7 ± 0.3a	18.4 ± 8a	626 ± 33a	448 ± 24a	1074 ± 48a
5	10.5 ± 0.5b	9.7 ± 0.3a	20.2 ± 6a	687 ± 46b	628 ± 24b	1316 ± 18b
50	8.9 ± 0.7a	8.7 ± 0.3a	17.8 ± 1a	583 ± 30a	513 ± 13a	1096 ± 40a

3.3.5 Growth of cereals supplied with lumichrome

3.3.6 Sorghum growth response to lumichrome

Sorghum showed growth promotion when treated with lumichrome. Exposing sorghum seedlings to 5 nM lumichrome caused a significantly ($P \leq 0.05$) increased shoot biomass relative to control and the 50 nM lumichrome concentration (Table 3.7).

Irrigating sorghum plants with 5 nM lumichrome up to 37 d after planting markedly ($P \leq 0.05$) increased shoot and root dry matter relative to control and the 50 nM lumichrome treatment (Table 3.7). As a result, plant total biomass was also significantly ($P \leq 0.05$) increased compared to the other lumichrome treatment (Table 3.7).

3.3.7 Millet growth response to lumichrome

Watering millet seedlings with 5 nM lumichrome increased ($P \leq 0.05$) root, but not shoot, dry weight at 23 d after planting (Table 3.8). In contrast, the provision of 50 nM lumichrome significantly ($P \leq 0.05$) decreased root and total plant biomass relative to control (Table 3.8). A similar decrease in root growth was observed at 37 d after planting (Table 3.8). With the provision of 5 nM lumichrome, however, both root dry matter and plant total biomass were significantly ($P \leq 0.05$) increased relative to control at 37 d after planting (Table 3.8).

Table 3.8. Effects of lumichrome on dry matter yield (mg/plant) of millet plants harvested at either 23 or 37 d after planting. Values (Mean + SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

Lumichrome conc'n (nM)	11 DAP			37 DAP		
	Shoot	Root	Total	Shoot	Root	Total
0	150 ±13a	84 ± 8a	235 ±16a	755 ±53a	334 ± 24a	1088 ±58a
5	129 ± 8a	104 ± 6b	233 ± 9a	885 ± 83a	391 ± 12b	1276 ± 75b
50	107 ± 4a	41 ± 6c	158 ± 4b	713 ± 29a	301 ± 23c	1015 ± 46a

Table 3.9. Effect of lumichrome on dry matter yield and total leaf area of maize plants harvested at 23 d after planting. Values (Mean ± SE, n = 6) followed by dissimilar letters in a column are significantly different at $P \leq 0.05$.

Lumichrome	Dry matter (mg/plant)			Total leaf area (cm^2/plant)
Concentration (nM)	First leaf	Second leaf	Total	
0	366 ± 33a	429 ± 28a	796 ± 31a	5.38 ± 0.49a
5	466 ± 45b	484 ± 34a	980 ± 46b	6.77 ± 0.50b
50	352 ± 21a	505 ± 34a	857 ± 24a	4.89 ± 0.26a

Table 3.10. Effect of lumichrome on dry matter yield of Sudan grass plants harvested at 23 d after planting. Values (Mean ± SE, n = 6) followed by dissimilar letters are significantly different at $P \leq 0.05$.

Lumichrome conc'n (nM)	Dry matter (mg/plant) Shoot	Root	Total
0	144 ± 11a	129 ± 14a	276 ± 21a
5	130 ± 10a	144 ± 12a	274 ± 19a
50	108 ± 12a	97 ± 8a	206 ± 16a

3.3.8 Growth response of maize to lumichrome

As with sorghum and millet, maize also showed a significantly ($P \leq 0.05$) positive response to lumichrome application. Relative to the control, shoot dry weight, but not root mass, was significantly ($P \leq 0.05$) increased in plants treated with 5 nM lumichrome up to 23 d after planting (Table 3.9). Unlike millet, however, maize plants did not show any decrease in shoot or root biomass at the higher 50 nM concentration of lumichrome.

Measurement of leaf areas provided further evidence of maize growth response to lumichrome (Table 3.9). Compared to control, leaf areas increased with the supply of 5 nM lumichrome to maize plants (Table 3.9). The total leaf area, measured on a per-plant basis, increased by 26% when plants were watered with 5 nM lumichrome.

3.3.9 Growth response of Sudan grass to lumichrome

Unlike sorghum, millet and maize, growth of Sudan grass was unaltered by lumichrome application (Table 3.10).

3.4 Discussion

Seedling development in plants is controlled by a number of biotic and abiotic factors, including the adequate supply of mineral nutrients, photosynthate and water. Superimposed on these basic requirements is the role of classical plant growth-promoting molecules called phytohormones. These compounds, which include auxins, cytokinins, gibberellins and abscicic acid, have been known for a long time to stimulate growth of plant organs such as leaves, roots and stems via cell division and cell expansion (Letham *et al.*, 1978; Ross *et al.*, 2002; Van der Graaff *et al.*, 2003; Campanoni *et al.*, 2003). This study has assessed the stimulatory role of luminchrome, a rhizobial metabolite, on growth of both legume and cereal seedlings. Even at very low nanomolar concentrations (5 nM) lumichrome was found to elicit growth promotion in a wide range of monocots and dicots. In both cowpea and soybean, the supply of 5 nM lumichrome caused an early initiation of trifoliate leaf development, an increase in trifoliate leaf expansion (Fig.3.1A,

B, C), and consequently greater trifoliate leaf growth (Fig.3.1B, C) which led to increased dry matter accumulation in shoot (Tables 3.1, 3.2 and 3.3). Relative to cowpea, these developmental changes were found to be more pronounced in soybean, in that, unifoliate and trifoliate leaf expansion and size increased significantly with the supply of 5 nM lumichrome (Fig. 3.1A, B, C), and thus led to greater leaf areas for photoassimilation and photosynthate production (Table 3.4).

In general, stem length of these seedlings were also markedly increased with the supply of 5 nM lumichrome (Fig. 3.1A and Table 3.5). Our findings are no doubt similar to the observed promotive effects of auxins and gibberellins on shoot growth of various plants (Letham *et al.*, 1978; Ross *et al*, 2002). However, unlike most classical studies on phytohormones, which have established correlations between endogenous hormonal activity and organ growth rate (Mirov, 1941; van der Graaff *et al.*, 2003), this study did not assess lumichrome activity in shoots (stems and leaves) of both cowpea and soybean. Like phytohormones, however, lumichrome seems to affect growth in a wide range of plant taxa and species. The effects of nanomolar concentrations of lumichrome on cowpea and soybean, the natural symbiotic partners of rhizobia, was not different from those of non-homologous cereal hosts such as sorghum, millet and maize.

Supplying 5 nM lumichrome to seedlings significantly ($P \leq 0.05$) altered developmental patterns in sorghum, millet and maize relative to control. Whether assessed at 11 or 37 d after planting, growth of sorghum shoot was significantly ($P \leq 0.05$) increased by lumichrome supply at 5 nM concentration (Tables 3.7). Shoots of maize seedlings were similarly increased by 5 nM lumichrome as a consequence of increased leaf development, which resulted in greater leaf areas (Tables 3.7). Unlike the legumes, however, root growth was considerably increased ($P \leq 0.05$) by the supply of 5 nM lumichrome to the cereals, especially sorghum and millet (Tables 3.7 and 3.8). It is possible that lumichrome supply to these species led to changes in assimilate partitioning thereby affecting growth patterns and resulting in larger root development (Vessey, 2003). However, root development in plants is known to be promoted by low concentrations of abscicic acid, but depressed by auxins and ethylene through inhibition of cell division and

DNA synthesis in apical meristems (Burg *et al.*, 1971; Chadwick and Burg, 1970). The increased root growth with 5 nM lumichrome supply to millet and its decreased development with 50 nM closely mirrors the activity of abscicic acid, which promotes root growth at low concentrations but inhibits it at higher levels (Aspinall *et al.*, 1967). Although the mechanism by which lumichrome elicits growth promotion in plants remains unknown, it must be transported in xylem from roots to shoots in order to effect changes in the growth of unifoliate and trifoliate leaves as well as stems, as observed in this study. Direct evidence is however needed to support this view. Alternatively, lumichrome could also bind to a receptor on the roots causing production and translocation of a plant-internal signal compound.

The promotive effect of lumichrome on the growth of organs and of whole plants among monocots and dicots is consistent with the finding obtained by Phillips *et al.* (1999) for alfalfa. Apart from the possibility that lumichrome probably directly affects cell division, cell expansion and cell extensibility, it has been indicated that the increased growth of alfalfa plants by lumichrome was due to enhanced root respiration that led to increased net C assimilation (Phillips *et al.*, 1999). Whatever the mechanism involved, the data obtained here have prospects for agricultural application. For example, rhizobial strains that release large quantities of lumichrome are more likely to affect growth of crop plants and possibly yield, irrespective of whether they are legumes or cereals. These results also confirm an earlier suggestion that rhizobial benefits in mixed plant cultures are likely to transcend the narrow boundaries of N_2 fixation to include the release of molecules that serve as signals for enhanced plant development (Dakora, 2003). Apart from lumichrome, lipo-chito-oligosaccharides which are bacterial nodulation factors, have been reported to trigger cell division and somatic embryogenesis in the absence of auxins and cytokinins (Dyachok *et al.*, 2000). Similar studies as done with lumichrome, further revealed that these Nod factors can promote seed germination and enhance seedling development when applied to a number of angiosperms belonging to different taxa (Zhang and Smith, 2001). As with the promotive effect of lumichrome on root growth in sorghum and millet observed in this study, the application of low concentrations of Nod factors (10^{-7} – 10^{-9} M) increased root mass and root length in soybean (Smith *et al.*,

2002). This was in addition to the fact that spraying leaves of different plant taxa (soybean, common bean, maize, rice, canola, apple and grape) with submicromolar concentrations (10^{-6} – 10^{-10} M) of Nod factors also increased photosynthate production and grain yield in field plants (Smith *et al.*, 2001). Those data and those of this study with lumichrome clearly indicate that novel molecules of bacterial origin exist that can promote plant growth to levels similar to or higher than those of classical phytohormones. Our findings with lumichrome also support the view that besides the classical phytohormones, additional signaling compounds are required to orchestrate plant development (Beveridge *et al.*, 2003).

Many molecules may therefore exist in microbes and marine biota with potency far above phytohormone activity in plant growth promotion and with some potential for increasing yields of agricultural crops. What is needed, futuristically speaking, is intensified isolation, identification and characterization of microbial extracts for molecules that have the potential to revolutionize agriculture. It must however be indicated that, in this study, the growth of some plant species was unaffected by lumichrome. For example, with the Leguminosae, growth of Bambara groundnut and common bean (Table 3.5 and 3.6) was neither increased by the provision of 5 nM lumichrome nor depressed by the higher 50 nM concentration, as observed for soybean, and, to a lesser extent, cowpea (Tables 3.1, 3.2 and 3.4). Of the monocots, development of Sudan grass was also not affected by lumichrome supply, indicating strong species differences in response to growth elicitation by lumichrome. However, just as auxin inhibition of root growth in many plant species can be overcome by antiauxins (Scott, 1972), it is possible that the lack of lumichrome effect on some species may be due to a neutralizing anti-lumichrome molecule or the effects of ions, such as silver ion, which can antidote the inhibitory effects of ethylene on *Pisum* root development (Beyer, 1976). Interestingly the application of silicate anions to symbiotic cowpea could induce an increase in ABA production and decrease zeatin ribose biosynthesis, leading to enhanced root development (Dakora and Nelwamondo, 2003). In fact, ABA is also known to mediate the regulatory effects of nitrate on lateral root development in plants (Signora *et al.*, 2001). Clearly, it seems there are many

interactive processes in plant growth involving phytohormones, nutrients ions, and lumichrome which act in concert to regulate organ growth and overall plant development.

CHAPTER FOUR

XYLEM TRANSPORT AND SHOOT ACCUMULATION OF LUMICHROME ALTERS ROOT RESPIRATION, STOMATAL CONDUCTANCE, LEAF TRANSPIRATION AND PHOTOSYNTHETIC RATES IN LEGUMES AND CEREALS

4.1 Introduction

Although bacteria are known to affect fundamental processes in plant development (Vedder-Weiss *et al.*, 1999; Vessey, 2003), the mode of action remains unknown. Recently, however, many diazotrophs, including rhizobia, have been shown to use chemical molecules to effect changes in plant development. For example, Phillips *et al.* (1999) showed that alfalfa growth was enhanced by a metabolite produced by its microsymbiont, *Sinorhizobium meliloti*.

Symbiotic nitrogen fixation relies on photosynthate supply for biochemical functioning, and labelling experiments have shown that current photosynthate is rapidly transferred to root nodules. Thus, conditions that enhance photosynthesis could stimulate symbiotic N_2 fixation (Dakora and Drake, 1999), indicating that nitrogenase activity is directly linked to C supply (Voisin, 2003).

It therefore means that any process that causes an increase in CO_2 supply and uptake is likely to increase photosynthetic rates. In fact, the growth promotion in alfalfa observed by Phillips *et al.* (1999) was apparently due to lumichrome-induced increase in root respiration, which enhanced CO_2 supply for net C assimilation.

Joseph and Phillips (2003) have also reported that stomatal conductance and transpiration of bean seedlings were both markedly increased by 10 nM homoserine lactone 42 h after its supply to the plants. This increase in transpiration could have potentially led to the movement of certain diffusion-limited minerals such as phosphorus into the root, thus benefiting the plant and its associated bacteria (Joseph and Phillips, 2003). Root respiration is also known to be enhanced by certain microbes (Norman *et al.*, 1994), possibly through the effects of chemical molecules they release into the atmosphere. Incidentally, the CO_2 so released can promote growth of symbiotic rhizobia (Lowe and Evans, 1962) and its increase in the rhizosphere from root respiration could directly benefit rhizobial growth in soil. Although bacterial products are known to stimulate plant growth (Dakora, 2003), their effect on physiological processes such as leaf stomatal conductance, transpiration and root respiration have remained unknown.

This study was conducted to determine the effect of lumichrome on root respiration, leaf stomatal conductance and transpiration in five legumes and two cereals. Because the phytohormone abscisic acid (ABA) is known to regulate stomatal opening and closure under conditions of water deficiency (Wigger *et al.*, 2002), its effects were also tested on the seven species of monocots and dicots so as to have a direct comparison with effects of lumichrome. Furthermore, because lumichrome is a secretory product of rhizobia, the direct effects of infective rhizobial cells was assessed as an additional treatment. Taken together, this study assessed whether lumichrome applied to roots of monocots and dicots was transported via xylem and accumulated in shoots. It also determined whether the application of lumichrome, ABA and rhizobial cells altered root respiration, stomatal conductance and leaf transpiration in legume and cereal plants.

4.2 Materials and Methods

4.2.1 Glasshouse experimental conditions

The experiment was conducted out in the glasshouse of the Botany Department, University of Cape Town, under uncontrolled conditions of light, humidity and temperature.

4.2.2 Phytotron experimental conditions

Test plants were placed in a growth chamber with 16 h day light, 70% relative humidity, and 28°C/16°C day/night cycle. For each species, four Leonard jar replicates were used per rhizobial strain.

4.2.3 Plant species

The legumes used in this study included cowpea (*Vigna unguiculata* (L.) Walp), soybean (*Glycine max* L. Merril), Bambara groundnut (*Vigna subterranean* (L.) Verdc), pea (*Pisum sativum* L.) and lupin (*Lupinus albus* L.), while the cereals tested were sorghum (*Sorghum bicolor* (L.) Moench) and maize (*Zea mais* L.).

4.2.4 Plant culture for lumichrome identification in analysis of xylem sap and leaf tissue extracts

Cowpea and soybean plants were grown to study lumichrome transport in xylem and its accumulation in leaf tissue. Seeds were surface-sterilized by soaking in 70% ethanol for 90 s, in 1.5 % bleach for 15 min, and then rinsed 6 times with sterile distilled water. The seeds were sown in 2.5 L pots which had been filled with a mixture of sand and vermiculite (3:1) and sterilized in the autoclave.

The surface-sterilized seeds of soybean and cowpea were then inoculated with commercial inoculants of *Bradyrhizobium japonicum* strain WB74 and *Bradyrhizobium* strain CB756 respectively. The uninoculated control received sterile nutrient solution adjusted to contain 1 mM NH_4NO_3. The inoculation with rhizobia was superimposed with the application of 5 nM lumichrome. The treatments used included uninoculated zero-lumichrome (I_0L_0), uninoculated + 5 nM lumichrome (I_0L_5), inoculated + zero-lumichrome (I_1L_0) and inoculated + 5 nM lumichrome (I_1L_5). For lumichrome treatments, the seeds were surface-sterilized and soaked in 5 nM lumichrome solution for 2 h before planting, while plants receiving zero treatments were raised from seeds which had been soaked in plain sterile water. After germination, the seedlings were thinned out to 2 plants per pot. For zero-lumichrome treatments, plants were watered every second day with only sterile ½ strength modified Hoagland nutrient solution, or adjusted to contain 5 nM lumichrome for those receiving lumichrome. The plants were then maintained under uncontrolled glasshouse conditions until harvest.

4.2.5 Collection of xylem sap

About 3 weeks after planting, the plants were decapitated at the crown level and the exuding xylem sap collected into ice-cold Eppendorf tubes, and vacuum-dried before analysis by HPLC techniques.

4.2.6 Extraction of leaf tissue

At harvest, leaves of the decapitated plants were detached for each replicate treatment, pooled, thoroughly mixed, and then a 10 g sample taken for extraction. The leaves were

ground and lumichrome and riboflavin extracted in 150 mL methanol/ 1 M HCl (49:1) medium. The extract was vacuum-dried, and analyzed for lumichrome using HPLC techniques.

4.2.7 HPLC analysis of xylem sap and leaf extracts for lumichrome and riboflavin

Lumichrome and riboflavin were quantified in various samples following HPLC separation on a reverse-phase, Lichrosorb C18 column (250 x 4.6 mm; Alltech, Nicholasville, KY, USA). The leaf extracts were solubilized in 30% methanol, micro-cetrifuged, and 100 µL of each sample injected into the HPLC. The injected aliquots were separated on a gradient going from 30 to 100% methanol in 45 min. The eluting compounds were identified by UV-visible spectra (absorbance 240 – 500 nm) using a photodiode array detector (Model 996; Waters, Milford, MA, USA) and quantified at 249 or 444 nm, respectively, relative to standards separated under the same conditions (see Fig. 4.1)

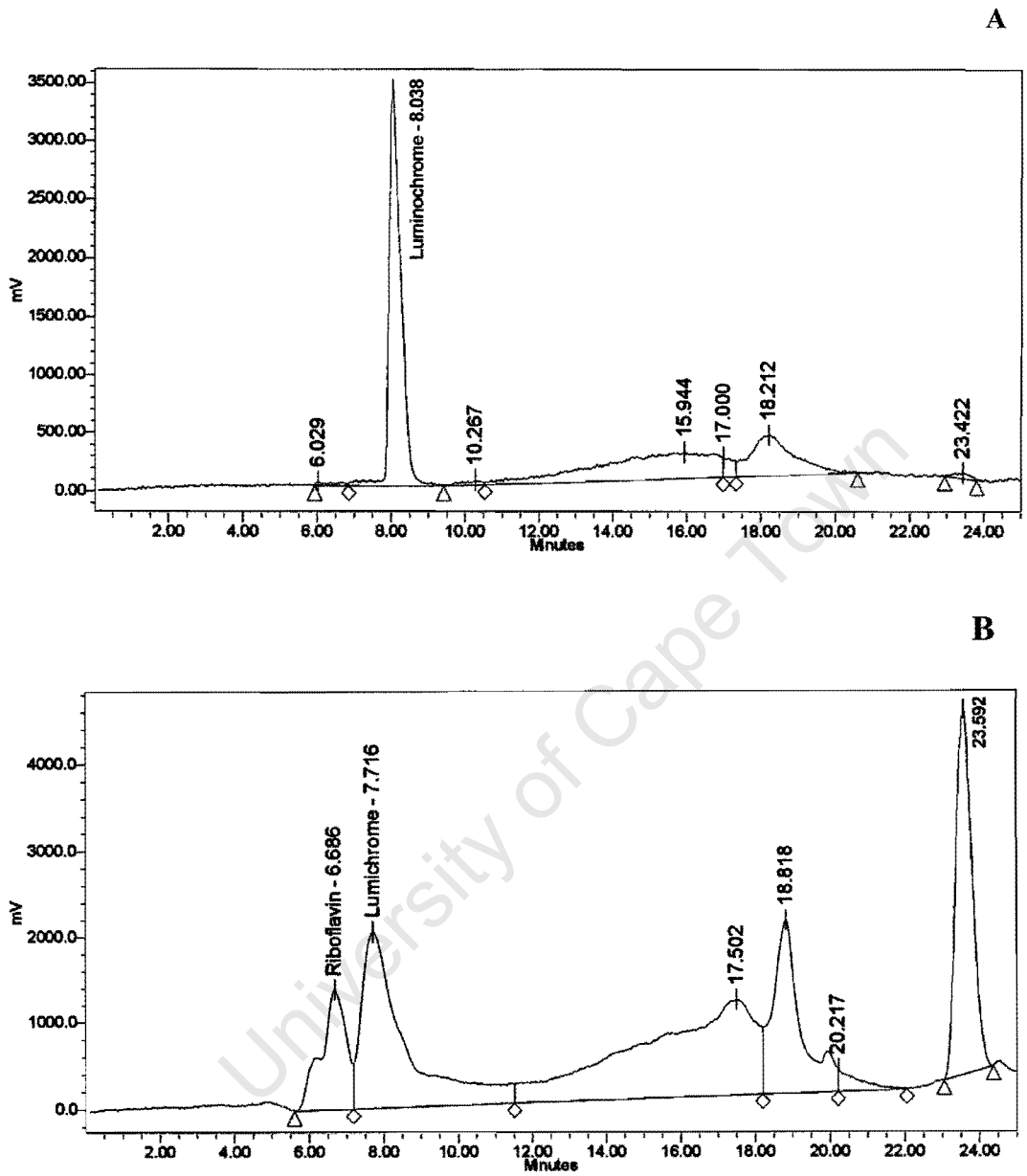


Fig. 4.1. An HPLC chromatogram showing A) a standard run of purified lumichrome and B) a run of xylem sap sample.

4.2.8 Plant culture for measurement of root respiration, stomatal conductance, and leaf transpiration in phytotron chambers

The effects of lumichrome on root respiration, stomatal conductance and leaf transpiration were measured using plants grown in liquid culture and maintained under phytotron conditions. Seeds of Bambara groundnut, soybean, cowpea, maize, lupin, sorghum and pea were germinated in sand and watered with nutrient solution for 14 d under uncontrolled glasshouse conditions. The nutrient solution was adjusted to contain 1 mM NH_4NO_3. At 14 d after planting, the sand was gently washed off roots, and seedlings with uniform size and appearance selected and transferred to ½ strength modified Hoagland nutrient solution adjusted to 1 mM NH_4NO_3 and contained in 3.5 L plastic pots.

The hydroponic culture solution was supplemented with the antibiotics rifampicin (125 mg/L) and ampicilin (10 mg/L) to avoid microbial contamination. The treatments included feeding plant roots with 10 nM lumichrome, 10 nM ABA, or 10 mL of infective rhizobial cells (0.2 OD_{600}). A zero untreated control was included for comparison with treatments.

Four seedlings were transferred to each pot and held in place on the lid with Bostik Prestic (Bostik Ltd., England). Four replicates were used per treatment. The nutrient solution was aerated by bubbling air through the system using a Shiruba aquarium air pump (Model SP 107, Japan). The experiments on root respiration, stomatal conductance, and leaf tranpiration were conducted in the phytotron chamber under the following conditions: 16 h day light, 70% relative humidity, and 28°C/16°C day/night cycle.

4.2.9 Measurement of root respiration

After 44 h of plant exposure to the lumichrome treatments in the hydroponic pots, the roots of test plants were cut off at the crown, wiped dry on absorbent paper and quickly weighed. They were then placed in the cuvette of an LCA3-type (ADC Co. Ltd., Hoddesdon, UK) infrared gas analyzer (IRGA) and the rate of respiration (i.e. volume of

CO_2 released with time) measured. The IRGA was operated in the differential mode at an ambient CO_2 concentration of 350 ppm.

4.2.10 Measurement of stomatal conductance and transpiration in phytotron

Stomatal conductance of the first trifoliate leaves of the legumes and the fourth leaves of the cereals (maize and sorghum) were measured using a sensitive and well calibrated LI-6400 IRGA (LI-COR Biosciences, Inc, Nebraska, USA) equipped with a steady-state porometer containing a small leaf cuvette. With this system, measurement of stomatal conductance automatically provided data for transpiration.

4.2.11 Measuring the effects of lumichrome application on stomatal conductance, transpiration and photosynthetic rates in field-grown legumes and cereals

Soybean, cowpea, maize and sorghum plants were grown in the field and supplied with 0, 5 or 50 nM lumichrome. At 53 d after planting, gas exchange measurements were performed on leaves of the field-grown plants to determine if this bacterial metabolite affects photosynthetic rates as reported for LCOs (Smith *et al.*, 2002). Stomatal conductance and leaf transpiration were also measured. Additionally, soybean leaves from the zero-lumichrome treatment were dipped briefly in 0, 5 or 50 nM lumichrome and photosynthesis, transpiration, and stomatal conductance measured after 0.5, 1 and 2 h.

4.2.12 Statistical analysis

All the data collected were analyzed by a one-way ANOVA using STATISTICA statistical package.

4.3 Results

4.3.1 Detection of lumichrome in xylem sap and leaf tissue

Cowpea plants inoculated with rhizobial cells at planting and supplied with 5 nM lumichrome showed significantly more ($P \leq 0.05$) lumichrome concentration in the xylem stream compared to control (Fig. 4.2). Due to the very low exudation of sap by soybean

plants, it was not possible to statistically analyze the data as done for cowpea. However, plants inoculated with infective rhizobial cells and treated with lumichrome had a mean concentration of 61.2 µmol lumichrome/mL sap, uninoculated plants receiving lumichrome had a value of 41.18 µmol lumichrome/mL sap, while the uninoculated zero-lumichrome treatment showed an average value of 26.81 µmol lumichrome/mL sap. Analysis of leaf extracts showed that the concentration of lumichrome in soybean leaves was significantly ($P \leq 0.05$) greater than that of cowpea leaf irrespective of the treatments (Fig. 4.3).

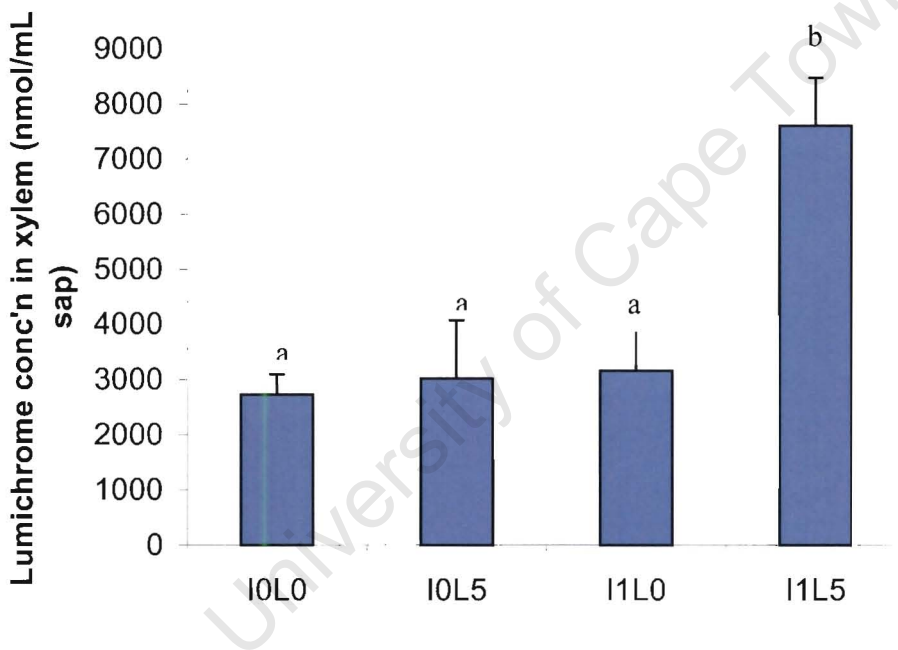


Fig. 4.2. Concentration of lumichrome in xylem sap of 44-d-old cowpea plants either inoculated or uninoculated and supplied with 5 nM lumichrome. Values are means from three replicates, each of which contained about 30 plants. Bars (± SE) with dissimilar letters are significant at $P \leq 0.05$. I_0L_0 = No inoculation and no lumichrome; I_0L_5 = No inoculation but watered with 5 nM lumichrome; I_1L_0 = Inoculated but not watered with lumichrome; I_1L5 = Inoculated and watered with 5 nM lumichrome.

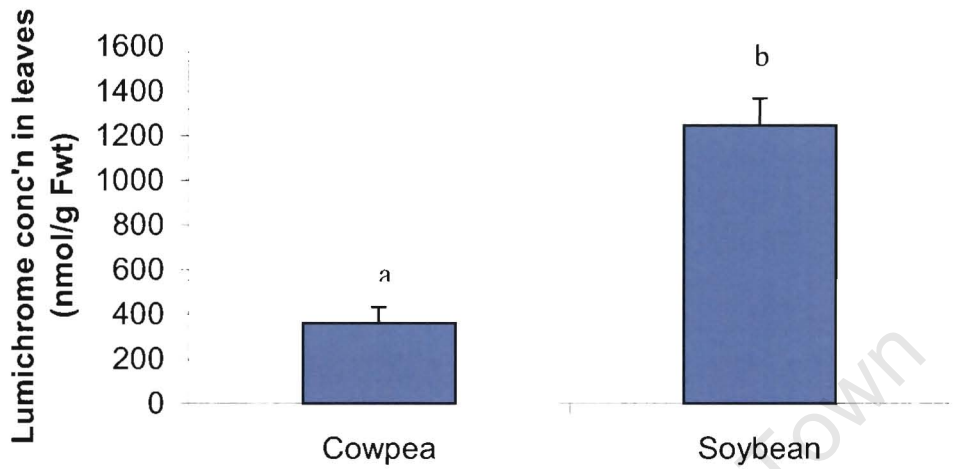


Fig. 4.3. Concentration of lumichrome in leaf tissue of 44-d-old cowpea and soybean plants supplied with 5 nM lumichrome. Values are means from three replicates, each sampled from 20 plants. Bars (± SE) with dissimilar letters are significant at $P \leq 0.05$.

4.3.2 Root respiration

Of all the legume and cereal crops tested, only lupin and maize showed a significant response to lumichrome application. With lupin, there was a significant ($P \leq 0.05$) decrease in the amount of CO_2 produced by the roots following lumichrome application. A similar result was obtained when the species was inoculated with infective rhizobial cells (Table 4.1). Maize, on the other hand, responded with a significant increase ($P \leq 0.05$) in root respiration in response to lumichorme application (Table 4.1) just as inoculating maize with rhizobia caused a marked increase in root respiration (Table 4.1). The rest of the species tested did not show any significant response to lumichrome application and inoculation (Table 4.1).

Table 4.1. Effect of applying lumichrome and rhizobial cells on root respiration of legume and cereal crop plants. CO_2 production by the roots of 14-d-old plants was measured 44 h after application. Values are means of 4 replicate pots, each containing 4 plants. Values ($\pm$ SE) with dissimilar letters in a row are significant at $P \leq 0.05$.

	Treatments		
Crop	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 mL of 0.2 OD_{600})
	μmol CO_2 produced. h^{-1}. g Fwt^{-1}		
Soybean	292.7 $\pm$ 53.6a	191.2 $\pm$ 14.0a	240.6 $\pm$ 12.4a
Cowpea	265.6 $\pm$ 14.9a	282.5 $\pm$ 10.7a	310.3 $\pm$ 13.8a
Bambara groundnut	137.4 $\pm$ 5.25a	138.3 $\pm$ 9.50a	149.4 $\pm$ 4.85a
Pea	260.5 $\pm$ 36.1a	292.9 $\pm$ 19.2a	210.8 $\pm$ 12.8a
Lupin	198.0 $\pm$ 19.2a	142.8 $\pm$ 13.0b	149.0 $\pm$ 11.9b
Sorghum	154.5 $\pm$ 10.6a	156.0 $\pm$ 50.3a	153.3 $\pm$ 8.6a
Maize	201.4 $\pm$ 10.1a	241.5 $\pm$ 12.9b	238.6 $\pm$ 19.7b

4.3.3 Stomatal conductance and transpiration in growth chamber plants

Plants grown hydroponically in growth chambers were treated to either lumichrome, ABA or rhizobial inoculation and their effects on stomatal conductance and leaf transpiration assessed relative control. The data showed species differences in response to these treatments. Transpiration increased significantly ($P \leq 0.05$) in cowpea in response to lumichrome, rhizobial inoculation and ABA application. Stomatal conductance was also increased but was significant for only ABA and inoculation treatments, but not lumichrome supply (Table 4.2). Transpiration in lupin similarly showed a significant ($P \leq 0.05$) increase in response to lumichrome and ABA, but not to root inoculation with rhizobial cells (Table 4.3).

With soybean (Table 4.4) and Bambara groundnut (Table 4.5), both stomatal conductance and transpiration were significantly ($P \leq 0.05$) decreased in plants exposed to all three treatments, relative to control. However, the effect of ABA on transpiration in soybean was not significantly ($P \leq 0.05$) different from the untreated control (Table 4.4). Of the five legumes, pea was the only species that remained unchanged in transpiration and stomatal conductance in response to lumichrome, ABA, and rhizobial inoculation (Table 4.6).

Of the two cereal species tested, only maize showed a significant reponse to the applied treatments. Transpiration in maize was significantly decreased ($P \leq 0.05$) by the application of lumichrome and rhizobial cells to roots, but not by ABA (Table 4.7).

Like pea, sorghum did not also show any significant response to the applied lumichrome treatments (Table 4.8).

Table 4.2. Stomatal conductance and transpiration of 14-d-old cowpea plants supplied with lumichrome, abscicic acid or rhizobial cells and measured 44 h after treatments. Values (Mean ± SE, n = 4, with each replicate having 4 plants) followed by dissimilar letters in a row are significantly different at $P \leq 0.05$.

	Treatment			
Physiological activity	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 mL of 0.2 OD_{600})	Abscicic acid (10 nM)
Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)	0.32 ± 0.02a	0.36 ± 0.02a	0.39 ± 0.01b	0.44 ± 0.02b
Transpiration ($mmol\ m^{-2}\ s^{-1}$)	4.28 ± 0.002a	4.95 ± 0.002b	4.99 ± 0.001b	5.86 ± 0.002c

Table 4.3. Stomatal conductance and transpiration of 14-d-old lupin plants supplied with lumichrome, abscicic acid or rhizobial cells and measured 44 h after treatments. Values (Mean ± SE, n = 4, with each replicate having 4 plants) followed by dissimilar letters in a row are significantly different at $P \leq 0.05$.

	Treatment			
Physiological activity	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 mL of 0.2 OD_{600})	Abscicic acid (10 nM)
Stomatal conductance (mmol m^{-2} s^{-1})	0.14 ± 0.006a	0.20 ± 0.01a	0.17 ± 0.04a	0.23 ± 0.03a
Transpiration (mmol m^{-2} s^{-1})	2.27 ± 0.001a	3.42 ± 0.002b	2.38 ± 0.003a	3.93 ± 0.004b

Table 4.4. Stomatal conductance and transpiration of 14-d-old soybean plants supplied with lumichrome, abscicic acid or rhizobial cells and measured 44 h after treatments. Values (Mean ± SE, n = 4, with each replicate having 4 plants) followed by dissimilar letters in a row are significantly different at $P \leq 0.05$.

	Treatment			
Physiological activity	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 mL of 0.2 OD_{600})	Abscicic acid (10 nM)
Stomatal conductance (mmol m^{-2} s^{-1})	0.77 ± 0.05a	0.54 ± 0.03b	0.37 ± 0.03c	0.57 ± 0.04b
Transpiration (mmol m^{-2} s^{-1})	8.49 ± 0.004a	6.83 ± 0.003b	4.63 ± 0.004c	7.34 ± 0.005a

Table 4.5. Stomatal conductance and transpiration of 14-d-old Bambara groundnut plants supplied with lumichrome, abscicic acid or rhizobial cells and measured 44 h after treatments. Values (Mean ± SE, n = 4, with each replicate having 4 plants) followed by dissimilar letters in a row are significantly different at $P \leq 0.05$.

	Treatment			
Physiological activity	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 mL of 0.2 OD_{600})	Abscicic acid (10 nM)
Stomatal conductance (mmol m^{-2} s^{-1})	0.39 ± 0.07a	0.18 ± 0.03b	0.26 ± 0.03b	0.25 ± 0.04b
Transpiration (mmol m^{-2} s^{-1})	4.58 ± 0.007a	1.88 ± 0.003b	2.96 ± 0.003b	2.70 ± 0.004b

Table 4.6. Stomatal conductance and transpiration of 14-d-old pea plants supplied with lumichrome, abscicic acid or rhizobial cells and measured 44 h after treatments. Values (Mean ± SE, n = 4, with each replicate having 4 plants) followed by dissimilar letters in a row are significantly different at $P \leq 0.05$.

	Treatment			
Physiological activity	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 ml of 0.2 OD_{600})	Abscicic acid (10 nM)
Stomatal conductance (mmol m^{-2} s^{-1})	0.19 ± 0.05a	0.10 ± 0.008a	0.09 ± 0.01a	0.10 ± 0.01a
Transpiration (mmol m^{-2} s^{-1})	2.27 ± 0.56a	1.68 ± 0.14a	1.37 ± 0.18a	1.56 ± 0.18a

Table 4.7. Stomatal conductance and transpiration of 14-d-old maize plants supplied with lumichrome, abscicic acid or rhizobial cells and measured 44 h after treatments. Values (Mean ± SE, n = 4, with each replicate having 4 plants) followed by dissimilar letters in a row are significantly different at $P \leq 0.05$.

	Treatment			
Physiological activity	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 mL of 0.2 OD_{600})	Abscicic acid (10 nM)
Stomatal conductance (mmol m^{-2} s^{-1})	0.26 ± 0.02a	0.22 ± 0.01a	0.21 ± 0.01a	0.22 ± 0.02a
Transpiration (mmol m^{-2} s^{-1})	4.32 ± 0.002a	3.66 ± 0.002b	3.33 ± 0.002b	3.73 ± 0.003a

Table 4.8. Stomatal conductance and transpiration of 14-d-old sorghum plants supplied with lumichrome, abscicic acid or rhizobial cells and measured 44 h after treatments. Values (Mean ± SE, n = 4, with each replicate having 4 plants) followed by dissimilar letters in a row are significantly different at $P \leq 0.05$.

	Treatment			
Physiological activity	Control	Lumichrome (10 nM)	Rhizobial inoculation (10 mL of 0.2 OD_{600})	Abscicic acid (10 nM)
Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)	0.16 ± 0.02a	0.15 ± 0.02a	0.18 ± 0.02a	0.12 ± 0.01a
Transpiration ($mmol\ m^{-2}\ s^{-1}$)	2.35 ± 0.26a	2.14 ± 0.20a	2.15 ± 0.21a	1.79 ± 0.19a

4.3.4 Photosynthetic rates, leaf stomatal conductance and transpiration in field-grown plants

Sorghum, maize, cowpea and soybean plants grown in the field and supplied with 0, 5 or 50 nM lumichrome were assessed for photosynthetic rates, stomatal conductance and transpiration (Tables 4.9 and 4.10). Although field-grown maize plants showed an insignificant increase in stomatal conductance with increasing lumichrome application, it translated into a significantly increased leaf transpiration (Table 4.9). As shown in Table 4.10, only cowpea plants showed decreased photosynthetic rates when grown in the field and supplied with 5 or 50 nM lumichrome.

Soybean leaves dipped into 0, 5 or 50 nM lumichrome for 0.5, 1 and 2 h did not show any significant changes in photosynthetic rates, stomatal conductance and leaf transpiration (Table 4.11).

Table 4.9. Photosynthetic rate, stomatal conductance and transpiration rate of 63-d-old maize and sorghum plants grown with 0, 5 or 50 nM lumichrome under field conditions. Values (Mean ± SE, n = 4) with dissimilar letters in a column are significant at $P \leq 0.05$.

	Maize			Sorghum		
Lumichrome concentration mM	Photosynthesis rate $\mu mol\ m^{-2}\ s^{-1}$	Stomatal conductance $mmol\ m^{-2}\ s^{-1}$	Transpiration rate $mmol\ m^{-2}\ s^{-1}$	Photosynthesis rate $\mu mol\ m^{-2}\ s^{-1}$	Stomatal conductance $mmol\ m^{-2}\ s^{-1}$	Transpiration rate $mmol\ m^{-2}\ s^{-1}$
0	16.88 + 0.84a	0.15 + 0.01a	4.66 + 0.22a	22.50 + 1.71a	0.25 + 0.03a	6.22 + 0.49a
5	18.29 + 1.04a	0.17 + 0.01a	5.53 + 0.29b	21.27 + 1.30a	0.24 + 0.02a	5.76 + 0.42a
50	16.81 + 0.97a	0.18 + 0.01a	5.65 + 0.27b	20.29 + 1.46a	0.22 + 0.02a	5.43 + 0.41a

Table 4.10. Photosynthetic rate, stomatal conductance and transpiration rate of 63-d-old cowpea and soybean plants grown with 0, 5 or 50 nM lumichrome under field conditions. Values (Mean ± SE, n = 4) with dissimilar letters in a column are significant at $P \leq 0.05$.

	Cowpea			Soybean		
Lumichrome concentration mM	Photosynthesis rate µmol m^{-2} s^{-1}	Stomatal conductance mmol m^{-2} s^{-1}	Transpiration rate mmol m^{-2} s^{-1}	Photosynthesis rate µmol m^{-2} s^{-1}	Stomatal conductance mmol m^{-2} s^{-1}	Transpiration rate mmol m^{-2} s^{-1}
0	21.44 + 0.78a	1.32 + 0.11a	14.24 + 0.90a	14.56 + 0.78a	1.57 + 0.87a	10.27 + 0.71a
5	17.61 + 1.28b	1.04 + 0.09b	13.98 + 0.59a	13.66 + 1.04a	0.65 + 0.10a	9.84 + 0.62a
50	17.14 + 0.92b	1.15 + 0.06a	13.60 + 0.88a	14.95 + 0.70a	0.74 + 0.07a	10.76 + 0.66a

Table 4.11. Photosynthesis rate, stomatal conductance and transpiration rate of 63-d-old soybean plants growing in the field and having their leaves dipped in 0 nM, 5 nM or 50 nM lumichrome for 0.5 h, 1.0 h or 2.0 h. Values (Mean $\pm$ SE, n = 4) with dissimilar letters in a column are significant at $P \leq 0.05$.

	0.5 h			1 h			2 h		
Lumichrome Concentration nM	Photosyntheis rate $\mu mol\ m^{-2}\ s^{-1}$	Stomatal conductance $mmol\ m^{-2}\ s^{-1}$	Transpiration rate $mmol\ m^{-2}\ s^{-1}$	Photosyntheis rate $\mu mol\ m^{-2}\ s^{-1}$	Stomatal conductance $mmol\ m^{-2}\ s^{-1}$	Transpiration rate $mmol\ m^{-2}\ s^{-1}$	Photosyntheis rate $\mu mol\ m^{-2}\ s^{-1}$	Stomatal conductance $mmol\ m^{-2}\ s^{-1}$	Transpiration rate $mmol\ m^{-2}\ s^{-1}$
0	16.53 + 0.78a	1.20 + 0.11a	19.29 + 0.64a	17.78 + 0.81a	0.87 + 0.07	18.14 + 0.51a	16.95 + 0.73a	0.78 + 0.09a	17.33 + 0.74a
5	17.03 + 0.58a	1.08 + 0.10a	19.31 + 0.63a	17.96 + 0.76a	0.85 + 0.05a	18.75 + 0.43a	17.17 + 1.05a	0.74 + 0.08a	17.80 + 0.92a
50	17.66 + 0.56a	1.33 + 0.12a	19.81 + 0.54a	18.26 + 0.81a	0.84 + 0.08a	17.06 + 1.45a	18.20 + 0.59a	0.86 + 0.07a	18.51 + 0.48a

4.4 Discussion

4.4.1 Xylem transport and *in situ* accumulation in leaves: A functional model for lumichrome as a signal molecule

The findings of this study show that soybean and cowpea plants can produce and transport lumichrome in their xylem stream. For example, uninoculated soybean plants receiving no exogenous lumichrome transported about 26.8 μmol lumichrome/mL xylem sap, while with cowpea xylem loading of lumichrome was 2.6 μmol/mL. Because rhizobial bacteria also produce lumichrome (Phillips *et al.*, 1999), providing soybean and cowpea with their homologous rhizobial cells or purified lumichrome increased the concentration of this molecule in xylem. An earlier study (Phillips *et al.*, 1999) has shown that lumichrome applied to the rhizosphere of alfalfa increased root respiration and whole-plant net carbon assimilation, and led to enhanced plant growth and biomass accumulation.

Although Phillips *et al.* (1999) attributed the increase in alfalfa growth to net C assimilation, it is possible that lumichrome also directly affects cell division, cell expansion and cell extensibility. As shown elsewhere (Chapter 3), supplying as low as 5 nM lumichrome to plants elicited an expansion in unifoliate and trifoliate leaves, caused early initiation of trifoliate leaf development, and increased stem elongation, leading to enhanced plant growth and dry matter accumulation. Based on these findings, lumichrome could be viewed as a novel signal molecule, and if so, then it has to be transported from roots to shoots where it effects the observed developmental changes. Some plant signals such as ABA are similarly transported from root to shoot in the xylem stream (Chen 2002; Lin *et al.*, 2003; Maurel *et al.*, 2004). Apart from establishing the presence of lumichrome in xylem, the HPLC analysis has also demonstrated its accumulation in leaves (Figs. 4.2 and 4.3). Interestingly, the lower concentration of lumichrome in cowpea xylem was reflected in its reduced accumulation in leaves relative to soybean, which showed higher xylem concentrations and a correspondingly increased lumichrome accumulation in leaves (Fig. 4.3). This species difference was similarly reflected in the magnitude of developmental changes observed for the two legumes, with

soybean exhibiting more dramatic changes with lumichrome supply compared to cowpea. Like salicylic acid, which acts as a signal in systemic disease resistance (Ryals *et al.*, 1994), lumichrome enters the xylem stream and gets transported to the shoot where it accumulates in leaves. It is hypothesize here that the presence and accumulation of lumichrome in leaf tissue then triggers events which promote cell division and leaf expansion. It is possible that this might be another model by which lumichrome causes plant growth promotion, in addition to the observed net C assimilation from lumichrome-induced root respiration (Phillips *et al.*, 1999). However direct evidence is needed to show that, like salicylic acid, lumichrome can cause gene expression and increase leaf expansion and stem elongation, which together result in increased dry matter accumulation.

4.4.2 Effects of lumichrome on root respiration

Because an earlier study (Phillips et al. 1999) showed that applying lumichrome to alfalfa increased root respiration and net C assimilation, we tested a range of monocots and dicots to determine whether the observation by Phillips *et al.* (1999) is common to all plant species. As shown in Table 4.1, lumichrome significantly increased root respiration in maize, a finding consistent with the study by Phillips *et al.* (1999). However, root respiration was markedly decreased by lumichrome application to lupin, and unaffected in cowpea, soybean, Bambara groundnut, pea and sorghum (Table 4.1). These findings suggest that there are species differences in plant response to lumichrome application. Supplying infective rhizobial cells to roots of the seven test species also stimulated CO_2 release that closely mirrored the lumichrome effect, in that, root respiration was significantly increased in maize, but decreased in lupin (Table 4.1). As with the application of exogenous lumichrome, the supply of infective rhizobial cells did not also alter root respiration in cowpea, soybean, Bambara groundnut, pea and sorghum.

Although it is still unclear how the CO_2 production is elicited, both symbiotic and pathogenic microbes are reported to induce CO_2 release following host-plant infection (Sarig *et al.*, 1992; Norman *et al.*, 1994; Volpin and Phillips 1998; Vedder-Weiss *et al.*, 1999). Interestingly, the CO_2 so produced from root stimulation by lumichrome or

rhizobial cells can promote growth of rhizobia and AM fungi in soil (Lowe and Evans 1962; Becard and Piche 1989; Becard *et al.,* 1992) with potential for improved N and P nutrition in symbiotic legumes.

4.4.3 Lumichrome effects on transpiration, stomatal and photosynthetic activity in plants

Growing field plants up to 63 d with 5 or 50 nM lumichrome decreased photosynthetic rates in cowpea relative to control (Table 4.10), possibly as a result of the decreased stomatal conductance which affected CO_2 intake and reduction by Rubisco. Although photosynthesis in sorghum was also decreased by lumichrome, this was not significant. This photosynthetic effect of lumichrome parallels the data by Smith *et al.* (2001) which showed an increase in photosynthetic rates with the application of lipo-chito-oligosaccharide Nod factors to leaves of both dicot and monocot species. Whether this difference lies in the fact that lumichrome was applied to roots in this study and Nod factors to leaves in that study, remains to be resolved, just as the mechanism effecting photosynthetic change also requires further studies.

Applying either lumichrome (10 nM), infective rhizobial cells (10 mL 0.2 OD_{600}) or ABA (10 nM) to plants for 44-h in growth chambers altered leaf stomatal conductance and transpiration in cowpea, lupin, soybean, Bambara groundnut and maize, but not in pea or sorghum. In all these instances, where stomatal conductance was increased by lumichrome, it also led to an increase in leaf transpiration relative to control plants (Tables 4.2, 4.3, 4.4, 4.5 and 4.6). Because lumichrome is produced and released by rhizobia (Phillips *et al.*, 1999), root inoculation with infective rhizobial cells was compared with the direct effects of exogenous lumichrome on leaf transpiration and stomatal conductance. As shown in Tables 4.2 to 4.6, the effect of bacterial inoculation closely mirrored that of 10 nM lumichrome application, indicating that the rhizobial effects on these physiological activities were more likely due to lumichrome released into the rhizosphere. It is therefore also probable to now interpret the findings by Figueiredo *et al.* (1999) that rhizobial inoculation alleviates the effects of water stress in symbiotic legumes to mean that lumichrome released by these strains decreased stomatal

conductance, and thus reduced water loss by the host plant. As observed by Figueiredo *et al.* (1999), rhizobial inoculation in this study also decreased stomatal conductance in soybean, Bambara groundnut and to some extent maize, leading to decreased transpiration rates (Tables 4.4, 4.5 and 4.6). In contrast, inoculated cowpea plants showed increased stomatal conductance with a concomitant increase in transpiration rates (Table 4.2), while with lupin, pea and sorghum there was no response to bacterial inoculation.

Treating plant leaves with ABA in this study produced results that were species-dependent (Tables 4.2 to 4.6). Stomatal conductance was significantly decreased in soybean and Bambara groundnut by ABA with close similarity to that found for *Phaseolus vulguaris* supplied with 20 µM ABA (Nemecek-Marshall *et al.*, 1995). Several studies have in fact shown that ABA causes stomatal closure (Blake and Ferrell, 1977; Comstock *et al.*, 2002; Wigger *et al.*, 2002; Wilknson and Davis, 2002), with a concomitant decrease in stomatal conductance. However, leaf stomatal conductance was significantly increased in cowpea but unaffected in lupin, pea, maize and sorghum with ABA application. This indicates that plant species differ in their response to stomatal contol by ABA.

Taken together, the findings of this study show that lumichrome released into the rhizosphere can modulate stomatal function and thus control water relations in host and non-host plants as well as affect photosynthetic process through an unknown mechanism.

CHAPTER FIVE

GROWTH, NITROGEN NUTRITION AND YIELD RESPONSE OF FOOD GRAIN LEGUMES AND CEREALS TO FIELD APPLICATION OF NANOMOLAR CONCENTRATIONS OF LUMICHROME, A SYMBIOTIC SIGNAL MOLECULE FROM RHIZOBIAL BACTERIA

5.1 Introduction

Rhizobia naturally produce chemical molecules that affect plant growth and development. These compounds include auxins, gibberrelins, cytokinins, abscisic acid, lipo-chito-oligosaccharides (LCOs), vitamins, lumichrome and riboflavin (Phillips and Torrey, 1972; Dart, 1974; Lynch and Clark, 1984; Loper and Schroth, 1986; Law and Strijdom, 1989; Phillips *et al.*, 1999; Dakora, 2003). All of these molecules influence plant growth and fundamental processes in plant growth. It is likely therefore that their release into soil could promote plant growth and increase yields in cropping systems. This is in addition to the fact that certain microbial metabolites enhance nutrient availability to plants (Dakora and Phillips, 2002). Rhizobia also release lipo-chito-oligosaccharide molecules (LCOs) that stimulate seed germination and seedling growth in a wide range of angiosperm species by a still unknown mechanism. Even where growth promotion was effected by growth-promoting bacteria, the mechanisms in many instances remain unclear (Vessey, 2003). This is also true of rhizobia where reports of growth promotion on non-leguminous crops like rice and maize have been made (Yanni *et al.*, 1997; Gutierrez-Zamora and Martinez-Romero, 2001; Chaintreuil *et al.*, 2000), but growth mechanism remains uncertain. Recent findings show that lumichrome and LCOs released by rhizobia stimulate growth of crops (Phillips *et al.*, 1999; Zhang *et al.*, 2002; Dakora, 2003). The following study was carried out to investigate the response of sorghum, maize, cowpea and soybean to 0, 5 and 50 nM lumichrome application under field conditions.

5.2 Materials and methods

5.2.1 Study area

The experimental site was the Agricultural Research Council (ARC) in Nietvrobji field station (33° 54'S, 18° 14'E in Stellenbosch in the Western Cape, South Africa.

5.2.2 Experimental design

A two-factorial design with 4 replicates was used. The plot size was 2.4 x 2 m^2 for soybean and cowpea and 2.7 x 2 m^2 for maize and sorghum. Both cowpea and soybean were sown 20 cm within rows and 60 cm between rows, while the spacing for the maize and sorghum was 40 cm within rows and 90 cm between rows.

5.2.3 Plant culture

For this experiment, cowpea, soybean, maize and sorghum were used. The cowpea and soybean were inoculated at planting using the commercial inoculants, *Bradyrhizobium japonicum* strain WB74 for soybean and *Bradyrhizobium sp* (*Vigna*) strain CB756 for cowpea. No other inputs were added to the field except for the three lumichrome treatments. The experimental treatments included 0, 5, and 50 nM lumichrome. Each lumichrome concentration was applied at the rate of 100 mL per hill per week for the first 8 weeks after planting. The plants were irrigated for 30 minutes every 3 d by overhead sprinkler.

5.2.4 Plant sampling

At 53 days-after-planting (DAP), during flowering for cowpea and soybean, and vegetative growth for maize and sorghum. Four plants per plot were sampled randomly for all the crop species under investigation. All of the plant was dug out and separated into shoots and roots. Soil was carefully washed off the root samples and the nodules plucked off the cowpea and soybean roots. Nodules per plant were counted and together with other plant organs were dried for 48 h in the oven at 65°C until constant weight. The roots, shoots and nodules were ground into a fine powder for nutrient analysis

5.2.5 ^{15}N isotope analysis and determination of Ndfa

Concentration of N in the plant organs (shoots, roots and nodules) was determined as % N using a Carla Erba NA 1500 elemental analyzer (Fisons instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT 252 mass spectrophotometer (Bremen, Germany) via a conflo II open-split device. Amount of N per organ was estimated from

the product of % N and dry mass. The natural abundance technique was used to estimate N fixed from the following equation:

$$\% \text{ N derived from fixation (Ndfa)} = \frac{\delta\ ^{15}\text{N (reference plant)} - \delta\ ^{15}\text{N (legume)}}{\delta^{15}\text{N (reference plant)} - \text{B}} \text{ X } 100$$

Where B is the $\delta^{15}N$ value of the legume species relying entirely on biological N_2 fixation for its N nutrition. B values used were as follows: soybean, shoot –1.152; root 0.66. Cowpea, shoot – 1.759; root –0.94. Sorghum was used as the reference plant.

5.2.6 Nutrient analysis in plant and soil material

Plant tissue was analysed for elemental composition. Preparation of samples for the determination of macronutrients (P, K, Ca, Mg and Na) and micronutrients (Fe, Cu, Zn, Mn, and B) in plant tissues was done by dry-ashing, followed by acid digestion, and measurement using spectrophotometer. A weighed amount (1 g dry matter) of plant material was ashed overnight inside a crucible at 550°C in a muffle furnace. The ash residue was digested in 5 mL of 6 M HCl at 50°C for 30 minutes, and filtered. The concentrations of nutrient elements were then determined after appropriate dilution, by direct aspiration on a calibrated simultaneous ICP spectrophotometer (IRIS/AP HR DUO Thermal Electron corporation Franklin, Massachusettes, USA).

5.2.7 Plant harvest

At physiological maturity, all the plants were harvested and separated into pods in the case of soybean and cowpea, or into cobs and heads for maize and sorghum. These were then air-dried and shelled for determination of grain yield and mineral nutrients.

5.2.8 Statistical analysis

Statistical analysis was done using a two-way ANOVA and STATISTICA PACKAGE.

5.3 Results

5.3.1 Soil chemical characteristics

The chemical characterization of soil used in this study produced the following data: pH ($CaCl_2$) 6.2; C, 0.99%; P, (Citrate acid) 44.6 mg/kg; S, 3.4 mg/kg; Ca, (cmol) 3.6 (+)/kg; Mg (cmol) 0.88 (+)/kg; K, 79.6 mg/kg; Na, 70.8 mg/kg; Fe, 124.5 mg/kg; Mn, 15.4 mg/kg; Zn, 3.1 mg/kg; B, 0.51 mg/kg; Cu, 7.0 mg/kg.

5.3.2 Plant growth

There was no significant response to lumichrome application by any of the four crop species whether on the basis of shoot, root or whole-plant dry matter (Table 5.1). Both grain yield and 100-seeds weight per species were unaltered compared to the 0 nM lumichrome control (Table 5.1).

5.3.3 N nutrition and symbiotic performance of legume species

Neither %N nor $\delta^{15}N$ of shoots, roots and seed were altered by the application of lumichrome to the four plant species. Similarly, the total N fixed and Ndfa in organs and whole-plants remained unchanged with lumichrome application (Tables 5.2 and 5.3). Futhermore, nodule weight, nodule number, $\delta^{15}N$, %N and nodule N were all unaffected by lumichrome application to cowpea or soybean plants (Table 5.4).

Table 5.1. Effects of lumichrome application on dry matter yield of cowpea, soybean, maize and sorghum plants at 53 DAP. Values followed by dissimilar letters in a column are significantly different at *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 presented in bold type and separated by different letters.

Treatment		Shoot (g/plant)	Root (g/plant)	Total (g/plant)	Grain yield (g/crop)	Weight of 100 seed (g)
Main effects:						
	Cowpea	**8.27c**	**1.08a**	**9.74c**	**869.88d**	**12.50a**
	Soybean	**8.91c**	**1.83a**	**11.29c**	**2284.67a**	**19.88b**
	Maize	**21.98a**	**6.21b**	**28.19a**	**1049.90c**	**29.87c**
	Sorghum	**13.9b**	**5.75b**	**19.66b**	**1217.04bc**	**2.11d**
	0 nM	13.72	4.06	18.01	1348.50	16.19
	5 nM	13.18	3.84	17.27	1358.44	16.04
	50 nM	12.90	3.26	16.38	1359.18	16.04
Interactions:						
Cowpea	0 nM	6.20	0.91	7.46	840.83	12.71
	5 nM	9.71	1.26	11.37	910.58	12.86
	50 nM	8.92	1.06	10.39	858.25	11.94
Soybean	0 nM	9.16	2.11	11.84	2253.50	20.04
	5 nM	9.39	1.85	11.81	2295.25	19.85
	50 nM	8.20	1.53	10.21	2305.25	19.76
Maize	0 nM	20.83	6.27	27.10	970.95	29.87
	5 nM	23.26	6.91	30.17	1021.80	29.35
	50 nM	21.83	5.46	27.29	1156.95	30.41
Sorghum	0 nM	18.69a	6.94	25.63	1328.73	2.16
	5 nM	10.37a	5.36	15.73	1206.15	2.11
	50 nM	12.64a	4.97	17.61	1116.25	2.04
F ratio:						
Crops (d.f=3)		**14.79*** **	**21.76*** **	**16.23*** **	**53.71*** **	**1573.81*** **
Lum (d.f=2)		0.08	0.72	0.20	0.0063	0.12
Interaction d.f=6)		0.94	0.36	0.82	0.331	0.66

Table 5.2. Effects of lumichrome on $\delta^{15}N$, % N and total N in shoot roots seeds and whole plant of cowpea, soybean, maize and sorghum at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		$\delta^{15}N$ (0/00)			%N			Total N (mg/plant)			
		Shoot	Root	Seed	Shoot	Root	Seed	Shoot	Root	Seed	Plant
Main effects:											
	Cowpea	**2.39a**	**4.04a**	**-1.08a**	**3.17a**	1.27	**3.98a**	26.24	**1.34a**	**0.50a**	**29.89a**
	Soybean	**1.77a**	**3.76a**	**0.65b**	**3.58b**	1.18	**6.95b**	31.59	**2.14a**	**1.38b**	**36.39a**
	Maize	**6.05b**	**6.82b**	**0.04c**	**1.48c**	1.05	**1.17c**	32.37	**6.45b**	**0.35c**	**38.82a**
	Sorghum	**6.19b**	**6.54b**	**3.14d**	**2.29d**	1.22	**1.41d**	30.41	**6.74b**	**0.03d**	**37.15a**
	0 nM Lum	4.16	5.34	0.54	2.57	1.18	3.35	30.31	4.41	0.56	36.02
	5 nM Lum	3.91	5.29	0.83	2.58	1.13	3.42	29.22	4.15	0.58	34.68
	50 nM Lum	4.23	5.24	0.69	2.74	1.24	3.38	30.93	3.93	0.55	35.99
Interactions:											
Cowpea	0 nM Lum	2.50	4.30	-1.04	3.21	1.33	3.93	19.88	1.22	0.50	23.28
	5 nM Lum	2.15	3.92	-0.99	3.14	1.26	3.97	30.01	1.53	0.51	33.94
	50 nM	2.53	3.88	-1.22	3.15	1.22	4.07	28.83	1.25	0.49	32.44
Soybean	0 nM Lum	2.11	3.85	0.62	3.55	1.21	6.98	32.54	2.58	1.40	38.09
	5 nM Lum	1.95	3.76	0.64	3.31	1.03	7.11	30.33	1.88	1.41	35.04
	50 nM Lum	1.24	3.67	0.68	3.89	1.30	6.78	31.01	1.98	1.34	36.05
Maize	0 nM Lum	5.60	6.42	-0.64	1.40	0.99	1.09	29.84	6.10	0.33	35.94
	5 nM Lum	5.50	7.12	0.61	1.39	1.00	1.30	31.98	6.98	0.38	38.96
	50 nM Lum	7.07	6.92	0.17	1.67	1.15	1.13	35.31	6.27	0.34	41.58
Sorghum	0 nM Lum	6.45	6.79	3.21	2.12	1.16	1.38	38.99	7.76	0.03	46.75
	5 nM Lum	6.03	6.35	3.06	2.49	1.21	1.31	24.55	6.22	0.03	30.77
	50 nM Lum	6.09	6.49	3.14	2.25	1.30	1.55	27.67	6.23	0.03	33.01
F ratio:											
Crops (d.f = 3)		**106.97*** **	**124.73*** **	**137.05*** **	**84.87*** **	1.92	**1726.00*** **	0.88	**24.67*** **	**2340.79*** **	1.37
Lum (d.f = 2)		0.77	0.16	1.23	1.18	0.942	0.42	0.12	0.24	2.645	0.07
Interact'n (d.f = 6)		2.10	1.25	1.60	1.22	0.512	1.44	1.24	0.32	1.02	1.12

Table 5.3. Effects of lumichrome on symbiotic performance of cowpea and soybean, harvested at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		Ndfa (%)		N-fixed (mg/plant)			
		Shoot	Root	Shoot	Root	Total	N fixed (kg/ha)
Main effects:							
	Cowpea	**0.49a**	**0.35a**	**13.20a**	**0.48a**	**13.69a**	**22.81a**
	Soybean	**0.61b**	**0.49b**	**19.39b**	**1.05b**	**20.45b**	**34.08b**
	0 nM Lum	0.52	0.40	14.39	0.83	15.22	25.36
	5 nM Lum	0.54	0.43	16.41	0.72	17.14	28.56
	50 nM Lum	0.58	0.44	18.10	0.75	18.85	31.41
Interactions:							
Cowpea	0 nM Lum	0.48	0.33	9.80	0.41	10.21	17.00
	5 nM Lum	0.52	0.36	15.77	0.57	16.34	27.23
	50 nM Lum	0.47	0.37	14.04	0.48	14.52	24.20
Soybean	0 nM Lum	0.57	0.48	19.00	1.25	20.23	33.72
	5 nM Lum	0.57	0.49	17.06	0.88	17.94	29.90
	50 nM Lum	0.69	0.51	22.15	1.02	23.17	38.62
F ratio:							
Crops (d.f = 3)		**8.27***	**38.73*****	**7.16***	**28.69*****	**8.13*****	**8.13***
Lum (d.f = 2)		0.67	1.01	0.86	0.36	0.78	0.78
Interct'n (d.f = 6)		1.55	0.10	1.14	2.08	1.21	1.21

Table 5.4. Effects of lumichrome on symbiotic performance of cowpea and soybean plants harvested at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		Nodule weight (per plant)	Nodule number (per plant)	δ15N nodule	% N nodule	Total N nodule	% C nodule	C/N nodule
Main effects:								
	Cowpea	**0.39a**	**19.09a**	9.38	6.00	2.32	**37.48a**	**6.32a**
	Soybean	**0.54b**	**110.39b**	8.63	5.16	2.65	**40.85b**	**8.10b**
	0 nM Lum	0.46	74.80	9.18	5.74	2.58	39.34	7.00
	5 nM Lum	0.49	64.02	9.63	5.48	2.61	39.54	7.37
	50 nM Lum	0.44	55.39	8.20	5.52	2.26	38.64	7.26
Interactions:								
Cowpea	0 nM Lum	0.35	21.05	8.99	6.21	2.19	37.53	6.10
	5 nM Lum	0.40	21.69	9.60	6.00	2.40	38.20	6.42
	50 nM Lum	0.40	14.53	9.55	5.79	2.36	36.74	6.44
Soybean	0 nM Lum	0.57	128.56	9.37	5.26	2.98	41.15	7.90
	5 nM Lum	0.57	106.35	9.66	4.96	2.82	40.87	8.31
	50 nM Lum	0.48	96.26	6.85	5.26	2.16	40.54	8.07
F ratio:								
Crops (d.f = 3)		**5.07***	**30.84*****	1.17	1.33	0.71	**41.12*****	**153.57*****
Lum (d.f = 2)		0.12	0.47	1.47	0.05	0.32	1.08	2.35
Interct'n (d.f = 6)		0.35	0.25	1.97	0.05	0.52	0.45	0.29

5.3.4 Carbon

The % C in the shoots maize treated with 50 nM lumichrome decreased significantly ($P \leq 0.001$) as shown in Table 5.5. Those of the other three crops did not alter significantly relative to the control. Similarly, % C decreased significantly ($P \leq 0.001$) in the roots of maize plants treated with either 5 or 50 nM lumichrome (Table 5.5). The C/N ratio of maize in the shoot also decreased significantly ($P \leq 0.01$) in response to 50 nM lumichrome treatment, while in the seed, it decreased significantly ($P \leq 0.001$) in response to 5 nM lumichrome. By contrast, in sorghum the C/N ratio of the seed increased significantly ($P \leq 0.001$) in plants treated with 5 nM lumichrome (Table 5.5).

5.3.5 Macro-and micro-nutrients

The concentration of P in the shoots of sorghum increased significantly ($P \leq 0.05$) in plants treated with 5 nM lumichrome but remained unchanged in the other crops plants tested (Table 5.6) while in the root, this nutrient decreased significantly ($P \leq 0.01$) in response to 50 nM lumichrome in the sorghum but not in the other crops (Table 5.8). In the seed, none of the crops showed a significant response (Table 5.10).

The concentration K on the other hand was only affected in the shoots of cereal crops in response to application to lumichrome. In maize, there was a significantly increase ($P \leq 0.01$) in the plants treated with 50 nM lumichrome, while in sorghum, there was a significant increase ($P \leq 0.01$) in plants treated with both 5 or 50 nM (Table 5.6). In the root and seed, K concentration was unchanged relative to the control.

The concentration of Ca decreased significantly ($P \leq 0.001$) in cowpea shoots in response to 5 nM (Table 5.6). In all the other crops, it was unchanged. Similarly, the concentration of Mg in the shoots of cowpea decreased significantly ($P \leq 0.001$) in plants treated with 50 nM lumichrome but increased significantly ($P \leq 0.001$) in sorghum plants treated with 5 nM lumichrome (Table 5.6). In the roots and seed, the concentration of Mg remained unchaged in response to lumichrome treatment in all the crops (Tables 5.8 and 5.10).

Shoot Fe increased significantly ($P \leq 0.001$) in sorghum plants treated with either 5 or 50 nM lumichrome (Table 5.6), but none of the other crops showed a significant change. The concentration of Zn on the other hand decreased significantly ($P \leq 0.01$) in cowpea plants that received 50 nM lumichrome but not those treated with 5 nM lumichrome (Table 5.7).

S was unchanged in the shoots and roots of all the test crops, however, in the seeds, cowpea plants treated with 50 nM lumichrome showed a significant decrease ($P \leq 0.001$) in this element though not in those treated with 5 nM lumichrome. A similar observation was made for soybean where plants treated with 50 but not with 5 nM lumichrome showed a significant decreased ($P \leq 0.001$) in the concentration of S (Table 5.10).

The concentration of Cu changed significantly ($P \leq 0.001$) in cowpea and sorghum shoot. In cowpea, there was decrease in plants treated with 50 nM lumichrome, while in sorghum, there was an increase in plants treated with both 5 or 50 nM lumichrome (Table 5.7).

Table 5.5. Effects of lumichrome on % C, total C and C/N ratio in shoot roots seeds and whole plant of cowpea, soybean, maize and sorghum at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		%C			Total C (mg/plant)			C/N ratio (mg/mg)			
		Shoot	Root	Seed	Shoot	Root	Seed	Shoot	Root	Seed	Plant
Main effects:											
	Cowpea	**38.13b**	**39.37a**	**42.63b**	**316.45b**	**42.54c**	**5.34a**	**12.28b**	**33.67a**	10.71a	**12.15a**
	Soybean	**41.98a**	**42.75b**	**51.53a**	**374.12b**	**78.19bc**	**10.24b**	**11.87b**	**37.08a**	7.42b	**12.45b**
	Maize	**38.25b**	**24.43c**	**41.96b**	**840.77a**	**151.08a**	**12.54c**	**26.32a**	**23.66b**	36.07c	**25.81b**
	Sorghum	**24.43c**	**17.44d**	**43.19b**	**336.88b**	**96.90b**	**0.91d**	**13.42b**	**22.27b**	30.83d	**11.88b**
	0 nM Lum	**36.03a**	**33.17a**	**43.78a**	475.85	113.49	7.17	**16.89a**	31.44	21.33	15.04
	5 nM Lum	**36.33a**	**29.54b**	**45.62b**	485.63	86.53	7.35	**16.53a**	29.49	21.37	17.60
	50 nM Lum	**34.73b**	**30.29b**	**45.09b**	439.54	76.52	7.25	**14.50b**	26.58	21.07	14.08
Interactions:											
Cowpea	0 nM Lum	**37.93cb**	**39.12a**	41.95	234.93	35.49	5.33	**12.02cd**	31.98	**10.72d**	**11.70c**
	5 nM Lum	**38.98b**	**39.05a**	43.75	379.32	49.69	5.64	**12.55cd**	34.88	**11.04d**	**12.52c**
	50 nM Lum	**37.49c**	**39.96a**	42.19	334.49	42.46	5.04	**12.29cd**	34.15	**10.38d**	**12.23c**
Soybean	0 nM Lum	**42.28a**	**42.83a**	51.14	387.09	90.36	10.24	**12.09cd**	35.69	**7.33e**	**12.73c**
	5 nM Lum	**42.15a**	**42.77a**	51.74	394.75	78.98	10.28	**12.85cd**	42.14	**7.29e**	**13.33c**
	50 nM Lum	**41.50a**	**42.65a**	51.71	340.53	65.22	10.21	**10.68d**	33.39	**7.64e**	**11.28c**
Maize	0 nM Lum	**39.98b**	**33.11b**	40.88	835.86	211.55	12.21	**29.05a**	33.48	**37.42a**	**29.71a**
	5 nM Lum	**39.43b**	**19.56c**	42.67	913.65	130.85	12.54	**28.50a**	19.65	**33.18b**	**26.86a**
	50 nM Lum	**35.35b**	**20.62c**	42.32	772.80	110.86	12.87	**21.40b**	17.86	**37.60a**	**20.86ab**
Sorghum	0 nM Lum	**23.92e**	**17.60c**	41.13	445.50	116.57	0.89	**14.40c**	24.60	**29.84c**	**6.01cd**
	5 nM Lum	**24.77e**	**16.79c**	44.30	254.81	86.59	0.94	**12.21cd**	21.28	**33.98b**	**17.66bc**
	50 nM Lum	**24.59e**	**17.93c**	44.15	310.32	87.54	0.90	**13.64cd**	20.94	**28.67c**	**11.95c**
F ratio:											
Crops (d.f = 3)		**776.46*** **	**284.17*** **	**84.72*** **	**18.03*** **	**11.32*** **	**1218.18*** **	**138.15*** **	**13.58*** **	**887.27*** **	**30.48*** **
Lum (d.f = 2)		**12.57*** **	**9.571*** **	**5.02*** **	0.23	2.69	0.495	**6.36** **	2.02	0.16	2.88
Interact'n (d.f = 6)		**6.48*** **	**9.30*** **	0.74	0.59	1.15	0.84	**4.51** **	2.15	**6.79*** **	**3.08*** **

The concentration of Na did not change in any of the test plants in response to lumichrome application (Tables 5.6 and 5.8). B concentration however, decreased significantly ($P \leq 0.001$) in the shoots of cowpea plants treated with 50 nM lumichrome (Table 5.7). Similarly, shoot Al decreased significantly ($P \leq 0.05$) in plants treated with 50 but now with 5 nM lumichrome (Table 5.7). In contrast, Al concentration increased significantly ($P \leq 0.05$) in plants treated with either 5 or 50 nM lumichrome, but remained unchanged in the roots (Tables 5.7 and 5.9).

In the nodules, all the nutrients remained unchanged in concentration except for K and Cu (Table 5.11). The concentration of K increased significantly ($P \leq 0.01$) in cowpea in plants treated with either 5 or 50 nM lumichrome. With Cu, the concentration increased significantly ($P \leq 0.05$) in cowpea in response to both 5 and 50 nM lumichrome treatment. Soybean nodules did not show any significant change in nutrient concentrations in plants which had lumichrome applied to them (Table 5.11).

Table 5.6 Effects of lumichrome on macro nutrient concentrations of shoots of cowpea, soybean, maize and sorghum at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	Na (mg/kg)	S (mg/kg)
Main effects:							
	Cowpea	**4.63ab**	**20.63a**	**17.73a**	**3.44a**	**357.92a**	**2.34a**
	Soybean	**3.99b**	**20.17a**	**12.49b**	**3.86a**	**291.67a**	**1.35b**
	Maize	**3.42b**	**20.59a**	**2.40c**	**1.78b**	**376.92ab**	**1.32b**
	Sorghum	**5.40**	**26.06b**	**4.58d**	**2.53c**	**211.58c**	**0.99b**
	0 nM Lum	3.85	20.09	**9.68a**	2.88	294.06	1.79
	5 nM Lum	4.77	23.47	**10.20a**	3.13	340.25	1.31
	50 nM Lum	4.46	22.03	**8.01b**	2.69	294.25	1.40
Interactions:							
Cowpea	0 nM Lum	**5.45abc**	**23.78abc**	**20.58a**	**4.23a**	388.75	2.30
	5 nM Lum	**5.10abc**	**22.30abc**	**20.53a**	**3.83ab**	407.75	2.13
	50 nM Lum	**3.33cd**	**15.80c**	**12.08b**	**2.28cde**	277.25	2.60
Soybean	0 nM Lum	**3.78bcd**	**20.73bc**	**12.70b**	**3.98a**	287.75	1.38
	5 nM Lum	**4.03bcd**	**22.43abc**	**12.0b**	**3.90a**	315.00	1.38
	50 nM Lum	**4.18bcd**	**17.35bc**	**12.18b**	**3.70ab**	272.25	1.30
Maize	0 nM Lum	**2.55d**	**16.18c**	**2.03d**	**1.45e**	326.25	2.53
	5 nM Lum	**3.18cd**	**20.13bc**	**2.43d**	**1.85de**	407.50	0.68
	50 nM Lum	**4.53bcd**	**25.48ab**	**2.75cd**	**2.05cde**	397.00	0.75
Sorghum	0 nM Lum	**3.63bcd**	**19.68bc**	**3.43cd**	**1.88de**	173.50	0.98
	5 nM Lum	**6.78a**	**29.03a**	**5.25c**	**2.95bc**	230.75	1.05
	50 nM Lum	**5.80ab**	**29.48a**	**5.05c**	**2.75cd**	230.50	0.95
F ratio:							
Crops (d.f = 3)		**4.39****	**3.83*****	**221.12*****	**29.64*****	**7.53*****	**3.94***
Lum (d.f = 2)		1.76	1.86	**7.65****	2.20	1.27	1.03
Interact'n (d.f = 6)		**2.75***	**3.54****	**9.72*****	**4.97*****	0.84	1.14

Table 5.7 Effects of lumichrome on micro nutrient concentrations of shoots of cowpea, soybean, maize and sorghum at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		Cu (mg/g)	Mn (mg/g)	B (mg/g)	Fe (mg/kg)	Al (mg/kg)	Zn (mg/kg)
Main effects:							
	Cowpea	**8.43a**	**27.88a**	**43.02a**	**1098.57c**	**505.16a**	**50.40a**
	Soybean	**10.17b**	**26.17a**	**52.62b**	**833.49c**	**227.12b**	**53.72a**
	Maize	**4.63c**	**15.66b**	**5.21c**	**148.63c**	**140.05c**	**26.91b**
	Sorghum	**11.87d**	**22.46ab**	**4.68c**	**270.34b**	**307.44b**	**36.85b**
	0 nM Lum	8.14	22.95	27.76	212.91	263.71	41.69
	5 nM Lum	9.33	23.96	27.63	274.35	348.38	44.02
	50 nM Lum	8.85	22.21	23.76	246.10	272.74	40.19
Interactions:							
Cowpea	0 nM Lum	**10.48b**	34.64	**50.08a**	**358.25ab**	**523.90a**	**63.41a**
	5 nM Lum	**9.40b**	28.90	**48.73a**	**415.58a**	**628.08a**	**56.48ab**
	50 nM Lum	**5.40cd**	20.09	**30.26b**	**250.40bcd**	**363.50bc**	**31.33cd**
Soybean	0 nM Lum	**10.21b**	28.32	**53.57a**	**209.43cde**	**232.78bcd**	**55.02ab**
	5 nM Lum	**10.21b**	24.81	**50.86a**	**188.60cde**	**228.48bcd**	**54.09ab**
	50 nM Lum	**10.09b**	25.37	**53.42a**	**253.48bcd**	**220.10cd**	**52.04ab**
Maize	0 nM Lum	**3.67d**	12.00	**4.06c**	**116.83e**	**122.81d**	**19.41d**
	5 nM Lum	**4.23d**	16.07	**5.44c**	**156.15de**	**157.90d**	**22.25d**
	50 nM Lum	**5.98cd**	18.93	**6.13c**	**172.91de**	**139.45d**	**39.07bcd**
Sorghum	0 nM Lum	**8.20b**	16.85	**3.12c**	**166.33de**	**175.38d**	**28.95cd**
	5 nM Lum	**13.51a**	26.05	**5.50c**	**337.08ab**	**379.05b**	**43.26abc**
	50 nM Lum	**13.91a**	24.47	**5.24c**	**307.63abc**	**367.90bc**	**38.34bcd**
F ratio:							
Crops (d.f = 3)		**32.66*** **	**4.45** **	**267.11*** **	**13.45*** **	**30.74*** **	**11.17*** **
Lum (d.f = 2)		1.64	0.16	2.93	2.56	**3.64***	0.38
Interact'n (d.f = 6)		**6.55*** **	1.53	**5.05*** **	**3.02***	**3.17***	**3.72** **

Table 5.8 Effects of lumichrome on macro nutrient concentrations of roots of cowpea, soybean, maize and sorghum at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	Na (mg/kg)	S (mg/g)
Main effects:							
	Cowpea	**2.93a**	**20.02a**	**3.66a**	**2.91a**	**1755.17a**	**4.48a**
	Soybean	**3.04a**	**16.07a**	**3.51a**	**1.78b**	**780.50b**	**0.95b**
	Maize	**1.64b**	**13.23b**	**1.96b**	**1.52b**	**1051.58c**	**0.56bc**
	Sorghum	**1.79b**	**1.34b**	**2.53c**	**1.76b**	**573.08d**	**0.77b**
	0 nM Lum	2.40	15.71	2.93	1.93	957.81	1.58
	5 nM Lum	2.50	15.32	2.95	1.99	1070.13	1.64
	50 nM Lum	2.15	15.98	2.86	2.06	1092.31	1.84
Interactions:							
Cowpea	0 nM Lum	**2.88b**	18.95	3.70	2.83	1585.25	4.23
	5 nM Lum	**2.90b**	19.38	3.78	2.98	1701.25	4.33
	50 nM Lum	**3.00ab**	21.73	3.50	2.93	1979.00	4.88
Soybean	0 nM Lum	**3.40ab**	16.18	3.63	1.70	707.25	0.88
	5 nM Lum	**3.70a**	16.48	3.65	1.70	809.25	0.98
	50 nM Lum	**2.03c**	15.55	3.25	1.95	825.00	1.00
Maize	0 nM Lum	**1.50c**	12.83	2.00	1.55	972.00	0.48
	5 nM Lum	**1.68c**	13.58	1.90	1.53	1232.25	0.53
	50 nM Lum	**1.75c**	13.28	1.98	1.48	950.50	0.68
Sorghum	0 nM Lum	**1.83c**	14.88	2.40	1.63	566.75	0.75
	5 nM Lum	**1.73c**	11.85	2.48	1.78	537.75	0.75
	50 nM Lum	**1.83c**	13.35	2.70	1.88	614.75	0.80
F ratio:							
Crops (d.f = 3)		**28.37*** **	**36.98*** **	**30.70*** **	**48.43*** **	**55.74*** **	**361.28*** **
Lum (d.f = 2)		2.27	0.53	0.15	0.72	1.45	2.47
Interact'n (d.f = 6)		**4.03****	1.81	0.46	0.37	1.17	0.78

Table 5.9. Effects of lumichrome on macro nutrient concentrations of roots of cowpea, soybean, maize and sorghum at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		Cu (mg/g)	Mn (mg/g)	B (mg/g)	Fe (mg/kg)	Al (mg/kg)	Zn (mg/kg)
Main effects:							
	Cowpea	**13.50a**	**11.39a**	**31.97a**	**1098.57c**	**1778.43a**	**46.37b**
	Soybean	**15.03a**	**10.99a**	**14.82b**	**833.49c**	**1400.08a**	**16.44c**
	Maize	**22.08b**	**18.45b**	**10.05b**	**3758.00a**	**4611.42b**	**41.60b**
	Sorghum	**20.73b**	**22.19c**	**7.64c**	**2432.46b**	**4618.96b**	**81.76a**
	0 nM Lum	18.26	16.23	16.32	2131.35	3068.74	44.10
	5 nM Lum	17.93	15.86	15.77	1952.61	3207.07	44.19
	50 nM Lum	17.32	15.17	16.27	2007.93	3030.86	51.51
Interactions:							
Cowpea	0 nM Lum	14.94	12.61	32.14	1208.03	1747.70	48.92
	5 nM Lum	12.01	11.21	31.30	1080.75	1842.10	42.82
	50 nM Lum	13.55	10.36	32.49	1006.93	1745.50	47.39
Soybean	0 nM Lum	16.13	11.34	15.00	888.25	1569.65	16.49
	5 nM Lum	15.06	11.45	14.77	868.68	1371.80	16.97
	50 nM Lum	13.90	10.18	14.71	743.55	1258.80	15.87
Maize	0 nM Lum	22.17	18.14	10.69	4035.50	4530.50	41.50
	5 nM Lum	21.87	17.90	8.70	3139.88	4405.38	35.37
	50 nM Lum	22.21	19.31	10.75	4098.63	4898.38	47.93
Sorghum	0 nM Lum	19.78	22.84	7.46	2393.63	4427.13	69.50
	5 nM Lum	22.78	22.90	8.33	2721.13	5209.00	80.93
	50 nM Lum	19.62	20.84	7.14	2182.63	4220.75	94.87
F ratio:							
Crops (d.f = 3)		**11.91*** **	**46.59*** **	**221.26**	**19.39*** **	**67.55*** **	**19.05*** **
Lum (d.f = 2)		0.20	0.60	0.22	0.12	0.25	0.65
Interact'n (d.f = 6)		0.43	0.44	0.36	0.41	0.81	0.40

Table 5.10 Effects of lumichrome on mineral nutrient concentrations in seeds of cowpea, soybean, maize and sorghum harvested at physiological maturity. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	Na (mg/kg)	S (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Mn (mg/kg)
Main effects:											
	Cowpea	**5.61a**	**12.33b**	**1.16a**	**1.81a**	**184.75a**	**1.41a**	**67.99a**	**38.60a**	**8.28a**	**10.01a**
	Soybean	**7.03b**	**17.43a**	**1.57b**	**2.11b**	**211.42b**	**2.01b**	**86.18b**	**40.66b**	**14.17b**	**16.85b**
	Maize	**3.04c**	**2.91c**	**0.10c**	**0.92c**	**121.92c**	**0.62c**	**23.94c**	**20.53c**	**0.88c**	**4.19c**
	Sorghum	**4.73d**	**3.02**	**0.283**	**1.25d**	**252.00d**	**0.48d**	**31.36d**	**16.33d**	**3.15d**	**10.22a**
	0 nM Lum	5.04	8.90	0.78	1.53	191.38	**1.15a**	53.03	28.84	6.68	10.55
	5 nM Lum	5.09	8.93	0.76	1.51	192.13	**1.16a**	51.14	29.14	6.65	10.16
	50 nM Lum	5.17	8.93	0.79	1.53	194.06	**1.07b**	52.93	29.10	6.52	10.25
Interactions:											
Cowpea	0 nM Lum	5.58	12.25	1.15	1.80	183.75	**1.40c**	71.53	38.58	8.13	10.34
	5 nM Lum	5.70	12.43	1.18	1.80	186.75	**1.45c**	66.70	39.74	8.53	10.05
	50 nM Lum	5.55	12.30	1.15	1.83	183.75	**1.33d**	65.75	37.49	8.18	9.63
Soybean	0 nM Lum	7.10	17.43	1.60	2.18	209.25	**2.08a**	84.83	39.96	14.42	17.00
	5 nM Lum	6.98	17.43	1.50	2.08	207.25	**2.13a**	86.09	41.70	14.20	16.59
	50 nM Lum	7.00	17.45	1.60	2.08	217.75	**1.83b**	87.61	40.30	13.88	16.97
Maize	0 nM Lum	2.83	2.83	0.10	0.88	120.75	**0.63e**	24.61	19.89	0.90	4.36
	5 nM Lum	3.00	2.88	0.10	0.93	123.25	**0.60ef**	21.83	19.41	0.81	4.10
	50 nM Lum	3.30	3.03	0.10	0.95	121.75	**0.63e**	25.37	22.30	0.93	4.11
Sorghum	0 nM Lum	4.68	3.10	0.28	1.25	251.75	**0.45g**	31.14	16.96	3.29	10.50
	5 nM Lum	4.70	3.00	0.28	1.23	251.25	**0.48g**	29.96	15.69	3.05	9.89
	50 nM Lum	4.83	2.95	0.30	1.28	253.00	**0.50gf**	32.99	16.33	3.10	10.28
F ratio:											
Crops (d.f = 3)		**494.45*** **	**9645.22*** **	**1729.20*** **	**890.93*** **	**516.14*** **	**1216*** **	**353.01*** **	**519.27*** **	**945.10*** **	**611.61*** **
Lum (d.f = 2)		0.94	0.08	0.80	0.70	0.44	**8.18** **	0.60	0.11	0.26	1.31
Interact'n (d.f = 6)		0.17	0.49	1.20	1.70	0.55	**5.66*** **	0.57	1.81	0.34	0.33

Table 5.11. Effects of lumichrome on concentrations of P, K, Mg, Na, Fe, Cu, Zn, Mn, and B in nodules of cowpea and soybean, at 53 DAP. Values followed by dissimilar letters in a column are significantly different at $P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ presented in bold type and separated by different letters.

Crop/Lumichrome Treatment		P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	Na (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	B (mg/kg)	Al (mg/kg)
Main effects:												
	Cowpea	**4.63a**	**24.40a**	3.17	**4.72a**	**343.92a**	2433.42	10.04	91.30	9.88	17.35	2009
	Soybean	**4.88b**	**16.20b**	3.06	**3.97b**	**446.33b**	1512.20	9.96	77.53	9.51	15.81	1650
	0 nM Lum	4.74	20.06	3.11	4.23	401.88	1224.29	10.01	85.58	8.40	16.43	1513.05
	5 nM Lum	4.69	20.70	3.09	4.49	405.38	2498.53	9.79	84.82	9.97	16.87	2000.43
	50 nM Lum	4.85	20.14	2.99	4.31	378.13	2195.63	10.21	82.84	10.71	16.42	1976.92
Interactions:												
Cowpea	0 nM Lum	4.70	**23.25b**	3.03	4.68	367.00	1202.77	**9.60b**	99.21	7.86	17.16	1468.84
	5 nM Lum	4.58	**25.20a**	3.18	4.85	329.00	3592.45	**9.48b**	115.63	9.81	19.21	2301.75
	50 nM Lum	4.63	**24.75a**	3.00	4.63	335.75	2505.05	**11.04a**	59.06	11.96	15.67	2258.00
Soybean	0 nM Lum	4.78	**16.88c**	3.30	3.78	436.75	1245.80	**10.42ab**	71.95	8.94	15.71	1557.25
	5 nM Lum	4.80	**16.20c**	3.00	4.13	481.75	1404.60	**10.09ab**	54.01	10.13	14.53	1699.10
	50 nM Lum	5.08	**15.53c**	2.98	4.00	420.50	1886.20	**9.38b**	106.62	9.45	17.18	1695.84
F ratio:												
Crops (d.f = 3)		**7.11***	**488.24*****	0.007	**37.97*****	**10.05*****	2.51	0.05	0.52	0.21	1.91	2.84
Lum (d.f = 2)		1.05	1.17	0.604	1.61	0.28	1.75	0.53	0.01	2.91	0.07	2.22
Interact'n (d.f = 6)		1.35	**6.05****	1.065	0.44	0.62	1.29	**5.6***	2.87	1.87	2.58	1.11

5.4 Discussion

5.4.1 Effects of lumichrome on plant growth and nitrogen nutrition of field plants

The results of this study have shown that exogenous supply of lumichrome to field-grown plants has no effect on growth of various plant species, whether measured on per organ or whole-plant basis (Table 5.1). Consequently, shoot, root and grain yield were unaffected by lumichrome application (Table 5.1). Although tillering in sorghum was increased by 18% in lumichrome-treated plants, this effect was not significant (data not shown). The overall lack of response by cowpea, soybean, maize and sorghum to lumichrome is perhaps understandable since photosynthetic rates were unaltered in these species, except for cowpea (see Chapter 4). The results of this field study with lumichrome contrasts data from glasshouse experiments which showed increased growth response of cowpea, soybean, sorghum, millet and maize to the application of 5 nM lumichrome (see Chapter 3). Because riboflavin production has been reported to be a common feature of soil microbes (Carpenter 1943; Gonzalez-Lopez *et al.*, 1983; Rodelas *et al.*, 1993; Phillips *et al.*, 1999; Sierra *et al.*, 1999), and its degradation into lumichrome easily occurs photochemically and enzymatically by the action of light or enzymes from soil microorganisms and plants themselves (Yanagita, 1956; Yagi, 1962), the accumulation of lumichrome in soil from these processes could reach levels that render exogenous supply of this molecule ineffective in stimulating plant growth as observed in this study.

As with plant growth, lumichrome similarly showed no effect on the symbiotic performance and N nutrition of all four test species (Table 5.2 and 5.3). Despite this lack of growth response by the four species to lumichrome, the concentration of C in maize shoots was significantly decreased by plant supply with 50 nM lumichrome, just as root C was similarly reduced by 5 and 50 nM lumichrome (Table 5.5). Consequently, the C/N ratio in maize was decreased in shoots and seeds of plants treated with 50 nM and 5 nM lumichrome respectively. Although photosynthesis appeared to have decreased with field application of 5 or 50 nM lumichrome, the effect was significant for only cowpea (Chapter 4). So the decrease in tissue C could be due to losses from root exudation of C-

based metabolites or root respiration (Martin and Kemp, 1986; Meharg and Killham, 1988; 1989; 1990; Merckx *et al.*, 1986; Keith *et al.*, 1986; Killham and Yeomans, 2001). Although data from other studies (Phillips et al. 1999; Chapter 4) have shown that root respiration increased with lumichrome application and led to increased C assimilation, it is possible that the escape of uncaptured CO_2 can result in C loss to the plant.

5.4.2 Effects of lumichrome on mineral nutrient concentration of plant organs

Analysis of nutrient concentrations in organs of the four test species showed no effect of lumichrome application except for Ca, which decreased in shoots (Table 5.6). There were however species differences in tissue levels of both macro and micro nutrients. In general, Mg, Ca, Zn and Mn were significantly greater in shoots of the two legumes relative to the cereals. Similarly, Ca, P, K and P and, to some extent Mg and S also showed increased concentration in roots of the legumes compared to their cereal counterparts. Analysis of cowpea and soybean grain harvested at physiological maturity revealed significantly greater concentrations of both macronutrients (P, K, Ca and Mg) and micronutrients (Fe, Zn, S, Cu and Mn) compared to maize and sorghum grain. In dietary terms, consumption of grain legumes would therefore seem much healthier than grain cereals such as maize and sorghum grain.

In this study, there was a significant species x lumichrome interaction. For example, the concentrations of P, K, C, Mg, Fe, Zn and Cu in cowpea shoots were all depressed by treatment with 50 nM lumichrome relative to 0 or 5 nM level. With maize shoot, however, the levels of K, Mg, Fe, Zn and Cu increased with the application of 50 nM lumichrome relative to control. The shoot concentration of P, K, C, Mg, Fe, Zn and Cu were significantly elevated in sorghum plants supplied with 5 and 50 nM lumichrome compared to the control, while with soybean, there was no effect. Root analysis also showed decreased P concentration in soybean plants exposed to 50 nM lumichrome relative to control or the 5 nM level. Nutrient concentrations in roots of the other species were however unaltered. Interestingly, the concentration of S in the grain of the two legumes were markedly depressed by the application of 50 nM lumichrome relative to the zero or 5 nM level, but unaffected in the cereals.

Because lumichrome is a rhizobial product, inoculating cereals with infective cells of this bacterium is therefore likely to increase mineral nutrient acquisition and in *situ* tissue accumulation as observed in this study for sorghum and maize. The findings of this field study with lumichrome corroborated the results of glasshouse experiments which showed enhanced P and K nutrition following the inoculation of sorghum plants with symbiotically-effective rhizobial cells (see Chapter 2). An increased concentration of N, P, K, Mg, Ca, Zn, Na and Mo was also obtained for rice plants inoculated with infective rhizobial cells in the Nile delta (Yanni *et al.*, 2001), and this could now be interpreted to mean that lumichrome released by those bacterial strains was probably the cause of the enhanced nutrient acquisition. It must however be pointed out that besides lumichrome, plant and bacterial exudates also contain other molecules such as organic acids, amino acids, siderophores, phytosiderophores and phenolics that enhance mineral nutrition in plants (Jurkevitch *et al.*, 1986; Treeby *et al.*, 1989; Masaoka *et al.*, 1993; Dakora and Phillips, 2003).

An earlier study with groundnut also showed significantly increased concentration of mineral nutrients in organs of inoculated plants relative to uninoculated (Howell, 1987). The increased the concentration of K, Cu and to some extent, Fe and Mn observed for cowpea nodules (Table 5.10) in this study closely mirrors the effect of, rhizobial inoculation which also increased nodule concentration of K, Cu, Fe and Mn, as well as P, Ca, Mg, B, Al and Na (Howell, 1987). The similarity in nutritional response in these experiments could suggest that the effect of live bacterial cells on nutrient concentration was due to lumichrome released into the rhizosphere. Taken together, this field study has shown that lumichrome released from soil bacteria promotes the acquisition of certain nutrients in both legumes and cereals.

CHAPTER 6

QUANTITATION OF LUMICHROME AND RIBOFLAVIN RELEASED NATURALLY BY RHIZOBIAL STRAINS BELONGING TO DIFFERENT GENERA

6.1 Introduction

Apart from their role in N_2 reduction by nitrogenase, rhizobial bacteria also produce and secrete various molecules that affect seedling development and plant growth. Nod factor metabolites (lipo-chito-oligosaccharides) have been shown to be excreted into the growth medium of rhizobial bacteria (Spaink *et al.*, 1992). However the production and excretion of these compounds tend to decrease with the influence of environmental factors such as low pH, low P and low temperature (McKay and Djordjevic, 1993). Other bacteria species such as *Pseudomonas fluorescens* release B vitamins (Marek-Kozaczuk and Skorupska, 2001) that enhance clover growth and N_2 fixation by auxotrophic strains of *Rhizobium leguminosarum* bv. *trifolii* (Derylo and Skorupska, 1993; Marek-Kozaczak *et al.*, 1996). Nanomolar concentrations of homoserine lactone released by rhizosphere bacteria can increase stomatal conductance and leaf transpiration (Joseph and Phillips, 2003).

Furthermore, a study by Phillips *et al.* (1999) has shown that *Sinorhizobium meliloti* can naturally release lumichrome and riboflavin at measurable rates and the lumichrome so released was shown to promote alfalfa plant growth. It has also been shown that some auxotrophic mutants of *Rhizobium trifolii* (which nodulate clover) require riboflavin for symbiotic effectiveness (Schwinghamer, 1970). Recently Yang *et al.* (2002) studied riboflavin production and overexpression in diverse strains of rhizobia. Because riboflavin breakdown yields lumichrome, it is likely that the presence of the latter in soil can increase from rhizobial excretion and riboflavin degradation. So far, however, no detailed study has examined the levels of lumichrome and riboflavin released naturally by different rhizobial strains.

This study assess the rates and concentrations of lumichrome and riboflavin released into culture filtrate by rhizobia belonging to different genera.

6.2 Materials and Methods

6.2.1 Rhizobial strains

For this study, rhizobial strains were selected from the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium*. They included, *Rhizobium* GHR2 (host plant *Acacia cyanophylla*), *Rhizobium* NGR234 (host plant *Lablab purpureus*), *R. leguminosarum* bv. *viceae* strain 30 (host plant *Vicia faba*), *R. leguminosarum* bv. *viceae* strain Cn6 (host plant lentil), *Mesorhizobium* (wild type) xhj 7, *Bradyrhizobium* (wild type) 14b, *B. japonicum* Tal 110, *Bradyrhizobium* strain CB756, *B. japonicum* WB74, *Sinorhizobium meliloti* strain 1 (host plant *Medicago sativa*), *S. meliloti* strain RAKI, *S. fredii* strain 6217, *S. arboris* lma 14919 and *S. kostiense* 19227.

6.2.2 Culture media

The medium used for broth culture was optimized for the maximum production of lumichrome (Phillips et al, 1999) and it contained (g/liter): K_2HPO_4 (1.0), KH_2PO_4 (1.0), KNO_3 (6.0), proline (5.0), $MgSO_4$ (0.26), $FeCl_3.6H_2O$ (0.02), $CaCl_2.2H_2O$ (0.07), dextrose (10.0), and the minor components (mg/liter) thiamine (2.0), biotin (2.0), $Na_2MoO_4.2H_2O$ (0.24), H_3BO_4 (3.0), $MnSO_4.H_2O$ (1.83), $ZnSO_4.7H_2O$ (0.29), $CuSO_4.5H_20$ (0.13) and $CoCl_2.6H_2O$ (0.24). The medium was autoclaved at 121°C for 15 min and cooled to room temperature. About 1 mL of bacterial culture on agar slant was used to inoculate the broth medium and the culture incubated at 32°C with shaking for 7 d in the dark until stationary phase was reached.

6.2.3 Extraction of riboflavin and lumichrome from culture filtrate

The cultures were centrifuged at 6 K for 10 min to pellet the bacterial cells. 5 ml of the supernatant was then passed through a C18 cartridge, rinsed three times with deionized water to remove all salts, and the lumichrome and riboflavin eluted with methanol, dried down, and then resolubilized in 30 uL methanol for thin layer chromatography (TLC) as described by Phillips *et al.* (1999). All experimental procedures were conducted under low light conditions to avoid degradation of riboflavin.

6.2.4 Thin layer chromatographic separation of lumichrome and riboflavin

The separation of lumichrome and riboflavin in culture filtrates was carried out as described by Phillips *et al.* (1999). The resolubilized lumichrome and riboflavin were spotted on slica-gel-coated glass plates (Alltech 0.2 x 100 x 100 mm HPTLC silica gel 60 plates). The compounds in the lipophilic fraction were separated using chloroform/methanol/water (17.5:12.5:1.5) mixture. The TLC plates were viewed on a UV –light box (Ultra-Violet Products Ltd, Science Part, Milton Road, Cambridge, UK) and photographed with Polaroid film (Thermal paper, High density type, Kyoto, Japan).

Standards of known concentrations of riboflavin and lumichrome were also spotted, run on the TLC plates and photographed as described above. Once more, everything was done under low light conditions.

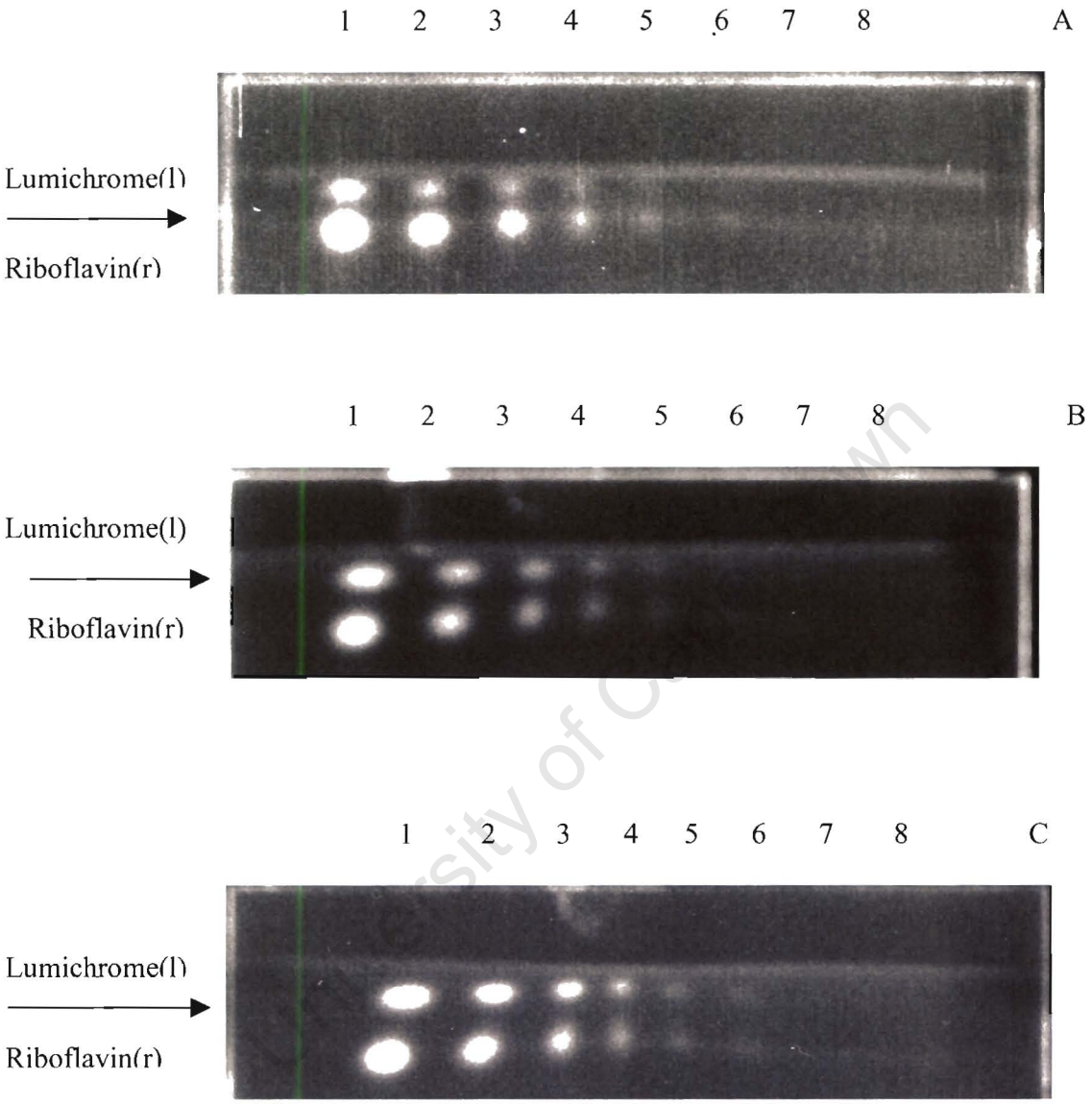


Fig 6.1 A, B and C represent standard runs in triplicate. After scraping the riboflavin and lumichrome off the plates, eluting, and measuring the absorbances, these known amounts were plotted against the absorbances to yield a standard curve of the two compounds.

Standards for lumichrome (upper spots) and riboflavin (lower spots) in triplicate: lanes 1, 48 ng l and 24 ng r; 2, 24 ng l and 12 ng r; 3, 12 ng l and 6 ng r; 5, 6 ng l and 3 ng r; 6, 3 ng l and 1.5 r; 7, 1.5 ng l and 0.75 ng; 8, 0.75 ng l and 0.375 ng r.

6.2.5 Quantification of riboflavin and lumichrome concentration and rates of release

The spots indicating the positions of the riboflavin and lumichrome were located under a UV lamp for both the standards and the compounds extracted from the bacterial culture filtrates and marked on the TLC plates. The spots were then scraped off the plates, eluted and their absorbances measured at 444 nm for riboflavin and 249 nm for lumichrome using a spectrophotometer (DU-64 Beckman Instruments Inc., Fullerton, Canada). For each strain, three independently grown bacterial broth cultures were used, with each assay being a replicate. For visual comparison, the three replicates for each strain were run side by side on the TLC plates.

6.2.6 Statistical analysis

Statistical analysis was done to compare the concentrations and rates of lumichrome and riboflavin released by rhizobia using one-way ANOVA and STATISTICA package.

6.3 Results

Standard runs with purified lumichrome and riboflavin are shown in Fig.6.1, while Figs 6.2 to 6.8 represent prints of TLC plates with the extracted lumichrome and riboflavin from various rhizobial strains. All the strains studied, which included *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium* species, produced and released both lumichrome and riboflavin.

More specifically, *B. japonicum* TAL 110, *S. fredii* strain 6217 and *S. meliloti* strain RAKI produced significantly ($P \leq 0.05$) higher amounts of riboflavin compared to the other strains (Table 6.1). All the other strains produced amounts that were not significantly different ($P \leq 0.05$). The rates of production of lumichrome and riboflavin closely mirrored the amounts produced.

R. leguminosrum bv. *viceae* strain 30, *B. japonicum* CB756 and *Rhizobium* NGR234 each produced lumichrome that was significantly ($P \leq 0.05$) lower than that released by the

other strains (Table 6.2). Again, the rate of lumichrome production followed the same pattern as the amounts released into culture filtrate.

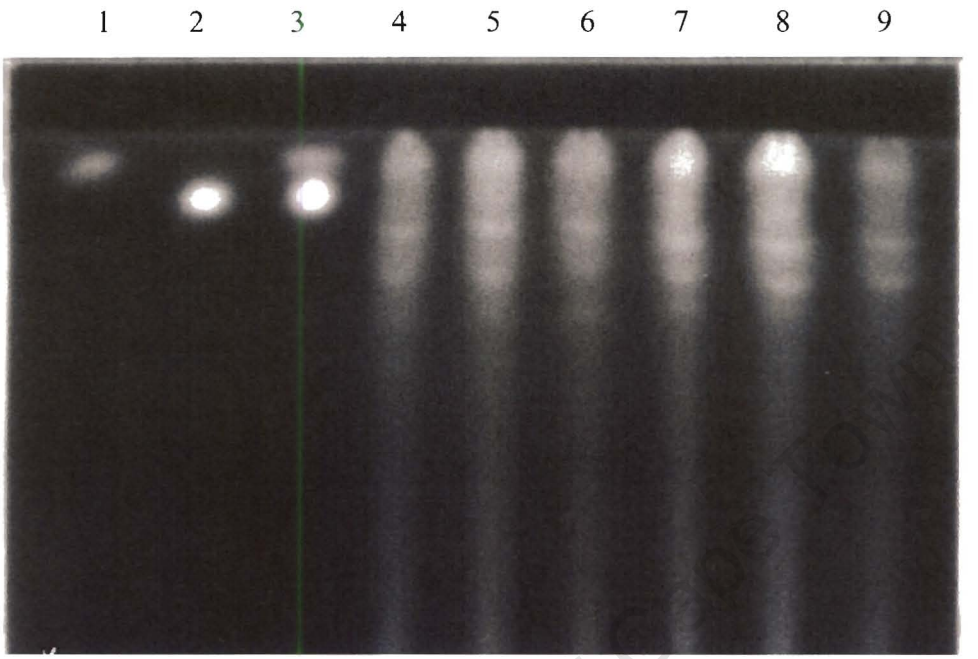


Fig 6.2. HPTLC plate showing lumichrome and riboflavin standards as well as separation of methanoic extracts from 10 ml of cell free culture filtrates of different rhizobial isolates. Lanes 1, 24 ng lumichrome; 2, 12 ng riboflavin; 3, 24 ng lumichrome (top spot) and 12 ng riboflavin (bottom spot), 4, 5 and 6, *S. meliloti* strain 1; 7,8 and 9, *B. japonicum* TAL 110

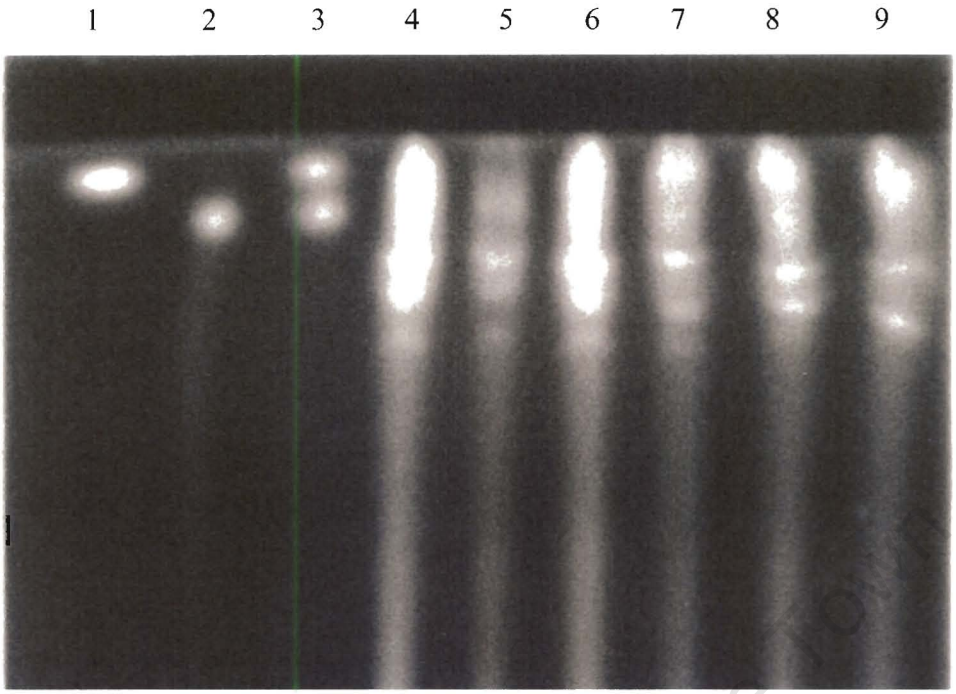


Fig 6.3. HPTLC plate showing lumichrome and riboflavin standards as well as separation of methanoic extracts from 10 ml of cell free culture filtrates of different rhizobial isolates. Lanes 1, 24 ng lumichrome; 2, 12 ng riboflavin; 3, 24 ng lumichrome (top spot) and 12 ng riboflavin (bottom spot), 4, 5 and 6, *S. meliloti* strain RAKI; Lanes 7,8 and 9, *B. japonicum* strain CB756

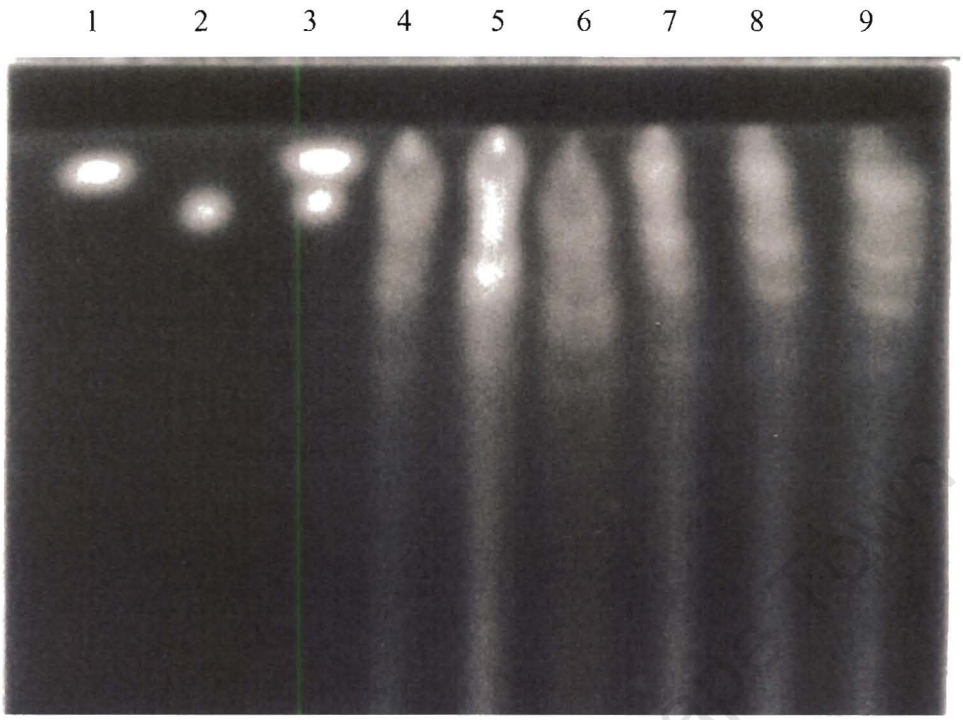


Fig 6.4. HPTLC plate showing lumichrome and riboflavin standards as well as separation of methanoic extracts from 10 ml of cell free culture filtrates of different rhizobial isolates. Lanes 1, 24 ng lumichrome; 2, 12 ng riboflavin; 3, 24 ng lumichrome (top spot) and 12 ng riboflavin (bottom spot), 4, 5 and 6, *Rhizobium* GHR2; 7,8 and 9, *S. fredii* strain lma 6217.

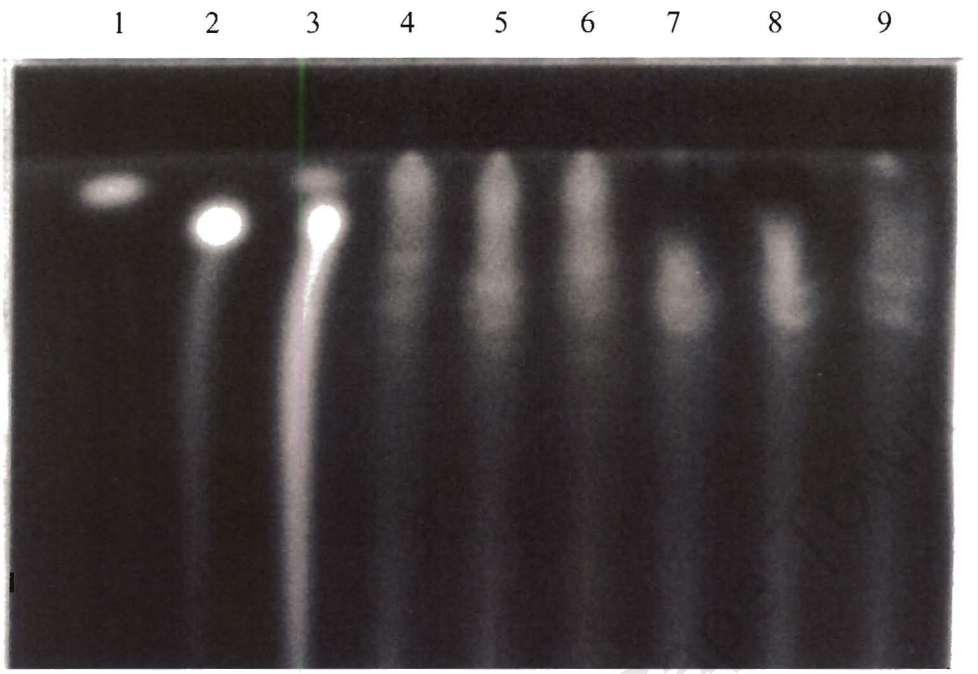


Fig 6.5. HPTLC plate showing lumichrome and riboflavin standards as well as separation of methanoic extracts from 10 ml of cell free culture filtrates of different rhizobial isolates. Lanes 1, 24 ng lumichrome; 2, 12 ng riboflavin; 3, 24 ng lumichrome (top spot) and 12 ng riboflavin (bottom spot), 4, 5 and 6, *R. leguminosarum* bv. *viceae* (lentil); 7,8 and 9, *R. leguminosarum* bv. *viceae* (*Vicia faba*).

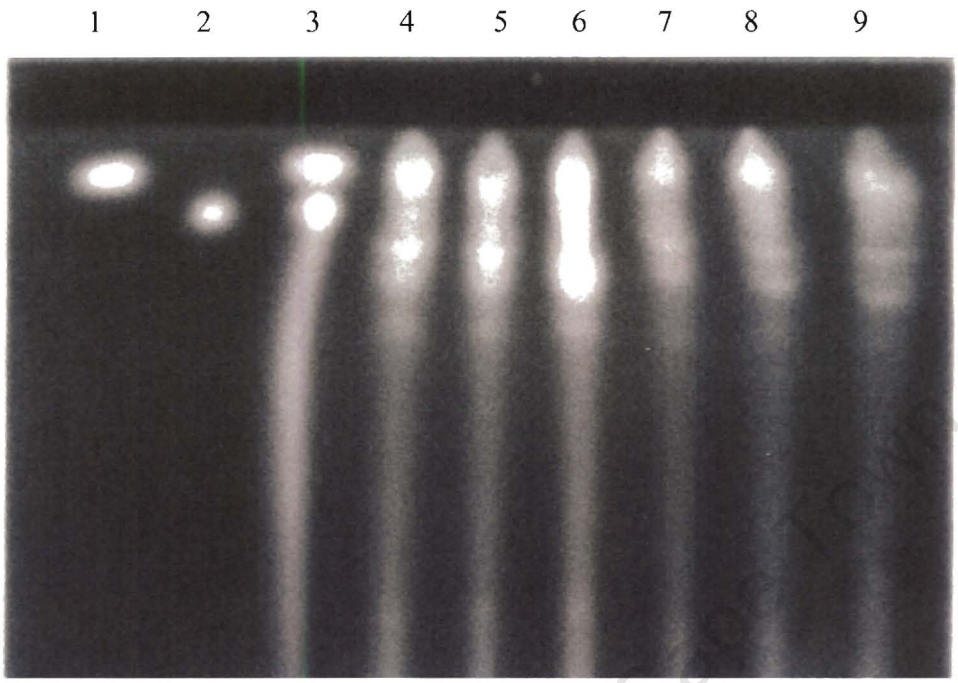


Fig 6.6. HPTLC plate showing lumichrome and riboflavin standards as well as separation of methanoic extracts from 10 ml of cell free culture filtrates of different rhizobial isolates. Lanes 1, 24 ng lumichrome; 2, 12 ng riboflavin; 3, 24 ng lumichrome (top spot) and 12 ng riboflavin (bottom spot), 4, 5 and 6, *S. arboris* strain lma 14919; 7,8 and 9, *Rhizobium* NGR234

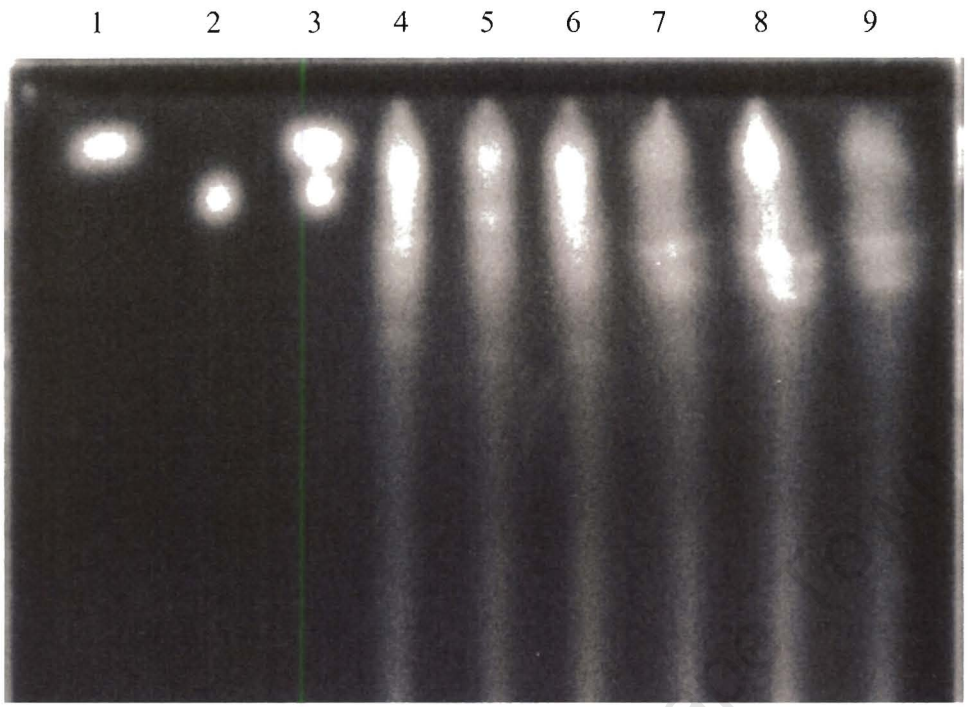


Fig 6.7. HPTLC plate showing lumichrome and riboflavin standards as well as separation of methanoic extracts from 10 ml of cell free culture filtrates of different rhizobial isolates. Lanes 1, 24 ng lumichrome; 2, 12 ng riboflavin; 3, 24 ng lumichrome (top spot) and 12 ng riboflavin (bottom spot), 4, 5 and 6, *S. kostiense* strain lma 19227; 7,8 and 9, *B. japonicum* WB74.

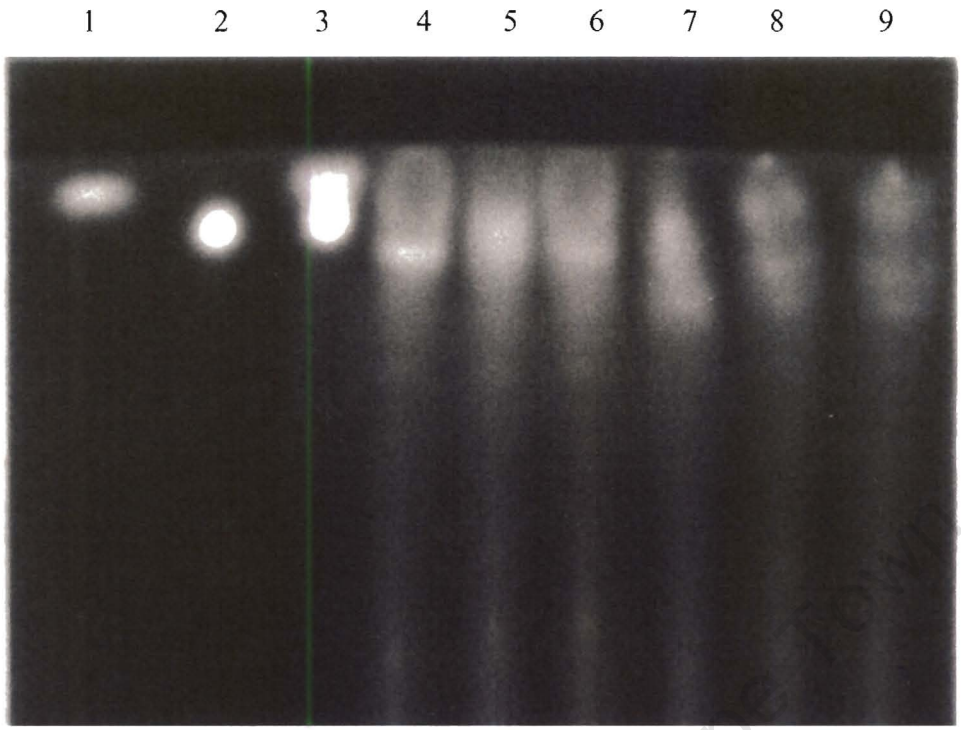


Fig 6.8. HPTLC plate showing lumichrome and riboflavin standards as well as separation of methanoic extracts from 10 ml of cell free culture filtrates of different rhizobial isolates. Lanes 1, 24 ng lumichrome; 2, 12 ng riboflavin; 3, 24 ng lumichrome (top spot) and 12 ng riboflavin (bottom spot), 4, 5 and 6, *Mesorhizobium* (wild type isolate) strain xhj 7; 7,8 and 9, *Bradyrhizobium* (wild type isolate) strain 14b

The calculated estimated values for riboflavin are shown in Table 6.1.

Table 6.1. Absorbances (at 444 nm), amounts and rates of release of riboflavin extracted from culture filtrates of different rhizobial strains. Values (Mean + S.E.) followed by dissimilar letters in a column are significantly different at $P < 0.05$.

		Riboflavin	
Rhizobial strain	Absorbance	Concentration ($ng\ mL^{-1}$)	Rate of release ($ng\ mL^{-1}\ h^{-1}$)
Rhizobium leguminosarum bv *viceae* strain 30	0.669 ± 0.157a	3.360 ± 0.789a	0.0020 ± 0.0005a
R. leguminosarum bv *viceae* strain Cn6	1.107 ± 0.066a	5.551 ± 0.336a	0.0033 ± 0.0002a
Bradyrhizobium japonicum Tal 110	1.697 ± 0.134b	8.515 ± 0.675b	0.0051 ± 0.0004b
Sinorhizobium meliloti strain 1	0.907 ± 0.196a	4.550 ± 0.982a	0.0027 ± 0.0006a
Bradyrhizobium strain CB756	1.400 ± 0.243a	7.025 ± 1.217a	0.0042 ± 0.0007a
Sinorhizobium meliloti strain RAKI	1.641 ± 0.131b	8.235 ± 0.656b	0.0049 ± 0.0004b
Sinorhizobium fredii strain 6217	1.927 ± 0.074b	9.670 ± 0.371b	0.0058 ± 0.0002b
Rhizobium GHR2	1.368 ± 0.185a	6.862 ± 0.928a	0.0041 ± 0.0006a
Rhizobium NGR234	1.310 ± 0.077a	4.564 ± 0.388a	0.0039 ± 0.0002a
Sinorhizobium arboris lma 14919	0.910 ± 0.095a	6.573 ± 0.475a	0.0027 ± 0.0003a
Bradyrhizobium japonicum WB74	1.549 ± 0.059a	7.771 ± 0.298a	0.0046 ± 0.0002a
Sinorhizobium kostiense 19227	1.425 ± 0.293a	7.148 ± 1.472a	0.0043 ± 0.0009a
Bradyrhizobium (wild type) 14b	1.193 ± 0.190a	5.986 ± 0.955a	0.0034 ± 0.0007a
Mesorhizobium (wild type) xhj 7	1.400 ± 0.062a	7.073 ± 0.312a	0.0042 ± 0.0002a

Table 6.2. Absorbances (at 249 nm), amounts and rates of production of lumichrome extracted from culture filtrates of different rhizobial strains. Values (Mean ± S.E.) followed by dissimilar letters in a column are significantly different at $P < 0.05$.

		Lumichrome	
Rhizobial strain	Absorbance	Concentration ($ng\ mL^{-1}$)	Rate of release ($ng\ mL^{-1}\ h^{-1}$)
Rhizobium leguminosarum bv *viceae* strain 30	1.441 ± 0.142b	13.356 ± 2.331b	0.0139 ± 0.0014b
R. leguminosarum bv viceae strain Cn6	1.747 ± 0.372a	28.328 ± 6.037a	0.0169 ± 0.0036a
Bradyrhizobium japonicum Tal 110	2.246 ± 0.108a	36.412 ± 1.754a	0.0217 ± 0.0011a
Sinorhizobium meliloti strain 1	2.038 ± 0.499a	33.040 ± 8.096a	0.0197 ± 0.0048a
Bradyrhizobium japonicum CB756	0.901 ± 0.332b	14.607 ± 5.386b	0.0087 ± 0.0032b
Sinorhizobium meliloti strain RAKI	1.270 ± 0.415a	20.584 ± 6.724a	0.0122 ± 0.0040a
Sinorhizobium fredii strain 6217	1.504 ± 0.240a	24.383 ± 3.898a	0.0145 ± 0.0023a
Rhizobium GHR2	1.527 ± 0.556a	24.756 ± 9.007a	0.0147 ± 0.0054a
Rhizobium NGR234	0.842 ± 0.126a	25.448 ± 2.037a	0.0081 ± 0.0012a
Sinorhizobium arboris lma 14919	1.570 ± 0.135b	13.448 ± 2.182b	0.0151 ± 0.0013b
Bradyrhizobium japonicum WB74	2.322 ± 0.244a	37.650 ± 3.957a	0.0224 ± 0.0024a
Sinorhizobium kostiense 19227	1.673 ± 0.162a	27.117 ± 2.618a	0.0161 ± 0.0016a
Bradyrhizobium (wild type) 14b	1.937 ± 0.099a	31.408 ± 1.602a	0.0187 ± 0.0010a
Mesorhizobium (wild type) xhj 7	2.020 ± 0.117a	32.754 ± 1.894a	0.0195 ± 0.0011a

6.4 Discussion

The chemical data presented here show that the production of riboflavin and lumichrome is a widespread phenomenon across the Rhizobiaceae. All the four groups sampled namely *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* all produced some amounts that ranged from 3.36 ng mL-1 to 6.67 ng mL-1 for riboflavin (Table 6.1) and from 13.56 ng mL-1 to 37.65 ng mL-1 for lumichrome (Tables 6.2). The results suggest that lumichrome production is a widespread phenomenon among the rhizobia since three representative strains were tested from each of the four groups and all of them secreted lumichrome into the culture filtrate

Phillips *et al.* (1999) were the first group to identify lumichrome as a metabolite produced by *S. meliloti* in culture. There are no reports of the production of this rhizobial signal molecule in other rhizobial groups. What is more common in literature are reports on riboflavin. For example a study (Schwinghamer, 1970) reported that for effective symbiosis of clover by an auxotrophic mutant strain of *R. trifolii*, riboflavin was required. More recently, Yang *et al.* (2002) identified the genes contributing to riboflavin production in *S. meliloti* and then constructed strains that overproduced this vitamin in order to characterize how additional riboflavin affects interactions between alfalfa and *S. meliloti*. They found riboflavin-synthesis genes in three separate linkage groups and observed that cells carrying extra copies of one of the genes, *rib*BA, colonized roots to densities that were 55% higher than that of a control strain. They concluded that this molecule benefits bacteria directly through an effect on the plant. Since riboflavin seems to play such a key role in the physiology of symbiosis of rhizobia, it is likely to be widely produced by rhizobia. Riboflavin is easily converted to lumichrome photochemically under neutral and acidic conditions (Yagi, 1956). Bacterial enzymes for example from *Pseudomonas* also bring about this conversion (Yanagita, 1956). Therefore, lumichrome is likely to be a common molecule in the rhizosphere.

Although the role of riboflavin in other legume-rhizobium symbioses remains to be demonstrated experimentally, it is possible that considering the importance of this

compound in the above mentioned symbiosis studied by Yang *et al.* (2002), that other legume-rhizobium symbioses utilize this molecule and so its production by rhizobia is likely to be widespread as demonstrated by this current study. Since lumichrome is a breakdown of riboflavin (Phillips *et al.*, 1999), it is likely to also be a commonly produced compound by these same bacteria. Lumichrome has been demonstrated to increase root respiration in alfalfa, and since exogenous CO_2 is required for the growth of rhizobia (Lowe and Evans, 1962), it is highly likely that lumichrome is widely produced by rhizobia since it is beneficial to these relationships.

Diazotrophic bacteria are known to produce various growth factors including vitamins e.g *Azotobacter* (Gonzalez-Lopez *et al.*, 1983; Dahm *et al.*, 1993) and *Azospillum* (Rodelas *et al.*, 1993). More specifically, rhizobia have been reported to produce these compounds for example *R. trifolii* has been reported to synthesize all the organic substances essential for growth from a synthetic carbohydrate-mineral salts medium of known composition (West and Wilson, 1938). Other rhizobial strains have also been reported to do this (Sierra *et al.*, 1999). These growth factors including riboflavin can be detected in the soil (Carpenter, 1943) and this means that plants could benefit from this exogenous source of growth factors. Streit *et al.* (1996) reported that bacterial vitamin production can be one of several factors affecting microbial competition for root colonization and stated that biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *R. meliloti* 1021. Additionally, *R. leguminosarum* bv. *trifolii,* an agriculturally important bacterium that forms N fixing root nodules on clover (*Trifolium repens*), occurs at a low level in soil and competes with other bacteria to colonize host plant root (Marek-Kozaczuk and Skorupska, 2001). This microsymbiont is a thiamine and pantothenic acid auxotroph and water-soluble vitamins released from plant roots or produced by soil microorganisms have been proposed to promote rhizobial growth (Derylo and Skorupska 1993; Rovira and Harris, 1961). The production of such vitamins has been demonstrated to be the basis of plant growth promotion by other non-rhizobial bacteria in mixed cultures with rhizobia, in that the production of these vitamins enhances the colonization of rhizobia. Marek-Kozaczuk and Skorupska (2001) examined the range and amount of water-soluble B vitamins produced by *Pseudomonas fluorescens* strain

267 supplemented with different C sources at different pH values. By random transposon mutagenesis, they isolated thiamine and niacin auxotrophic mutants of this strain and used them to elucidate the importance of vitamin production on clover root colonization and in plant growth promotion. Red root clover colonization decreased by about 1 order of magnitude in the case of niacin auxotrophs. The vitamin auxotrophs of *P. fluorescens* in a mixed inoculation of clover with *R. leguminosarum* bv. *trifolii* strain 24.1 showed no plant-growth promotion activity. All this points to the fact that these vitamins, including riboflavin and lumichrome, produced by microbes have a profound effect on symbioses.

This study showed that lumichrome is taken up by cowpea and soybean plants and can be detected at higher concentrations in the xylem sap and leaf tissues of plants provided with these compounds (Chapter 4). It is therefore possible that lumichrome produced rhizobia as demonstrated in this chapter, could influence plant growth and microbial interactions in the field.

CHAPTER SEVEN

ISOLATION OF *GLUCONOACETOBACTER DIAZOTROPHICUS* FROM *CAMELIA SINENSIS* L., *MUSA SPP.* L. AND *COFFEA ARABICA* L. FROM KENYA

7.1 Introduction

The N_2-fixing bacterium *Gluconoacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) which had previously been thought to occur only as an endophyte of sugarcane (Gillis *et al.*, 1989; Stephan, 1991), has since been shown to occur naturally in other crop plants including coffee, (Jimenez-Salgado *et al.*, 1997) millet, (Loganathan *et al.*, 1999) and pineapple (Tapia-Hernandez, 2000).

G. diazotrophicus is the only diazotrophic species of *Acetobacter* so far identified (Lee *et a*l., 2000). It is of special interest and has potential agronomic use because besides fixing N_2 in the presence of KNO_3, it is also active at low pH values (< 3.0; Reis *et al.*, 1994) an important fact in Africa because many soils in Africa are poor in bases, highly weathered and leached, with consequent problems of acidity (Giller, 2001). Additionally, *G. diazotrophicus* can excrete almost half of the fixed N in a form available to plants (Cojho *et al.*, 1993). This has now been confirmed by recent studies by Sevilla *et al.* (1998; 2001).

The Mexican finding *of G. diazotrophicus* in the rhizosphere and tissues of *Coffee arabica*, growing in soils of low pH in Mexico stimulated interest in the possibility that this diazotroph might be found in association with coffee and other crops growing in soils of low pH in Kenya. Coffee, tea and banana were chosen because coffee and tea are the most important crops in the Kenyan economy while banana is a staple in some parts of the country (Acland, 1980). The objectives of this study was to determine if G. diazotrophicus occurs in the above Kenyan crops and at what numbers in tissues and rhizosphere soil.

7.2 Materials and methods

7.2.1 Collection of roots and soil for isolation of *G. diazotrophicus*

Roots and rhizosphere soil were collected from coffee, tea and banana plants growing under field conditions from various locations in central Kenya within a 60 km radius. Only those growing in soil having a pH between 3.5 and 5.2 were sampled.

N-free semi-solid LGI medium supplemented with 10% brown sugar at pH 5.5 and cyclohexamide (150 mg L^{-1}) was used for enrichment culturing of N_2-fixing acetobacters. For subsequent culturing, LGI plates, supplemented with yeast extract (20 mg L^{-1}) and cyclohexamide were used. The isolates were grown in LGI liquid medium for the isolation of DNA. Incubation was at 30°C.

7.2.2 Isolation of *G. diazotrophicus*

The root samples were rinsed three times in sterile distilled water and the soil and water collected into sterile bottles. Soil samples were shaken for 1 h at room temperature to dissociate the bacteria. The washed root samples were weighed and surface sterilized using 70% ethanol for 90 seconds and 1.5% sodium hypochlorite plus a few drops of Tween 20 for 15 minutes with shaking. They were washed three times in sterile distilled water and dried. Dried, non-damaged root samples were placed on LGI solid medium and incubated at 30°C to ensure sterility. Individual roots were macerated in sterile pestle and mortar in 0.1 M $MgSO_4$ and transferred to sterile bottles.

Aliquots (100 µL) of supernatant of the rhizosphere soil and the macerated root samples were placed in vials containing 5 ml of N-free semi-solid LGI medium and incubated for seven days. Those showing a yellow surface pellicle were streaked onto LGI agar plates. Colonies showing the morphology described for *G. diazotrophicus* were streaked onto fresh LGI plates to ascertain cultural purity. PAL5 (ATCC 49039) was used as a control.

7.2.3 Determination of the RFLP pattern of the *nif*HDK genes

The restriction fragment length polymorphism (RFLP) pattern of the *G. diazotrophicus* *nif*HDK genes was determined. Each isolate was grown for 36 h in 500 ml LGI medium to an OD_{600} of 0.3 and harvested by centrifugation. Total DNA was isolated as described by Ausubel *et al.* (1987). DNA was digested with *Eco*R1 and restriction fragments were electrophoresed in 1.0% agarose gels in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA pH 8) at 40 V for 13 h. Total DNA digests were transferred from gels to nylon filters by the Southern procedure (Caballero Mellado and Martinez-Romero, 1994). The RFLP patterns of the *nif*HDK genes were determined by hybridization with a DIG-labelled 4.3-kb *Hind*III fragment containing *nif*HDK genes from *G. diazotrophicus* UAP 5560 carried on a plasmid pNHAd4.

7.2.4 Polymerase chain reaction of 16S – 23S rRNA intergenic region

PCR amplification of the intergenic region of the 16S–23S rRNA of the isolates obtained was carried out to try and detect differences between the various isolates. The forward primer used in this study was an 18-mer 5'-GTAACAAGGTAICCG-3' while the reverse primer was a 15-mer 5'-GCCIAGGCATCCACC-3'. The PCR products were run on a gel and photographed.

7.3 Results

Table 7.1. Origins of isolates of *G. diazotrophicus* obtained from coffee, tea and banana plants

Crop	Region	Soil pH	Number/g fresh root[a]	Cultivar/ clone	Age of plant
Coffee	Kiganjo	4.02	2.1×10^6	SL 28	25 years
	Chomo	3.81	1.7×10^5	Ruiru 11	5 years
Tea	Kambaa	3.55	3.2×10^4	303/259	10 years
	Chomo	4.33	1.4×10^6	54/40	1 year
Banana	Maragwa	5.02	1.0×10^5	Kampala	10 months

[a]Colony forming units; mean of three replicates obtained after 5 days of incubation.

Isolates of endophytic *G. diazotrophicus* were obtained from coffee, tea and banana samples as shown (Table 7.1), and from the rhizosphere soil of some of the roots from which the endophytes were recovered (data not shown). Not all the plants yielded isolates. Within the roots, the bacterium was found in numbers ranging from 10^4 to 10^6 per gram fresh weight (Table 7.1). The isolation frequency of *G. diazotrophicus* was about 25% of the samples examined (data not shown). All the isolates had the same RFLP profile (Fig 7.1).

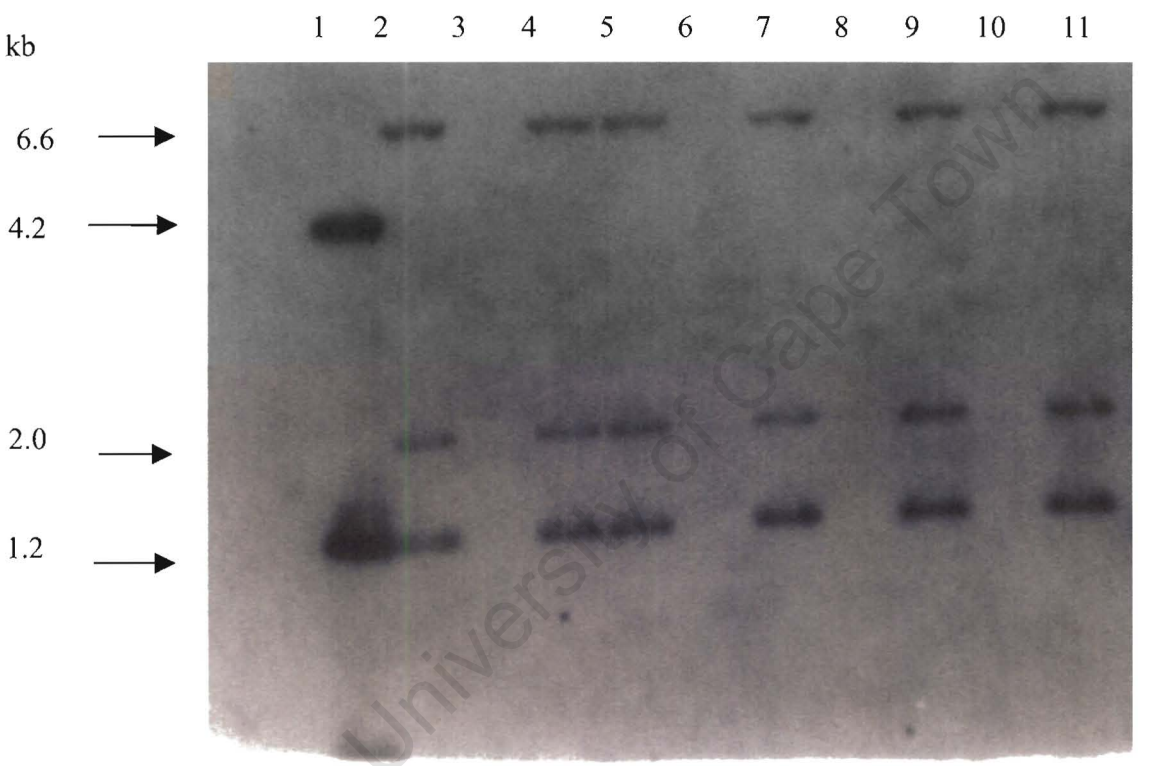


Fig.7.1. Autoradiogram of a Southern blot of total *Eco*R1-digested DNA hybridized with a DIG-labeled *nif*HDK probe of *G. diazotrophicus* UAP–5560. Lanes: 1, a 4.3-kb fragment of the *nif*HDK gene; 2, PAL5; 4, coffee isolate (root tissue); 5, banana isolate (rhizosphere); 7, tea isolate (root tissue); 8, tea isolate (rhizosphere); 9, coffee isolate (root tissue); 11, banana isolate (root tissue).

A single PCR band was obtained with all the samples tested (Fig. 7.2).

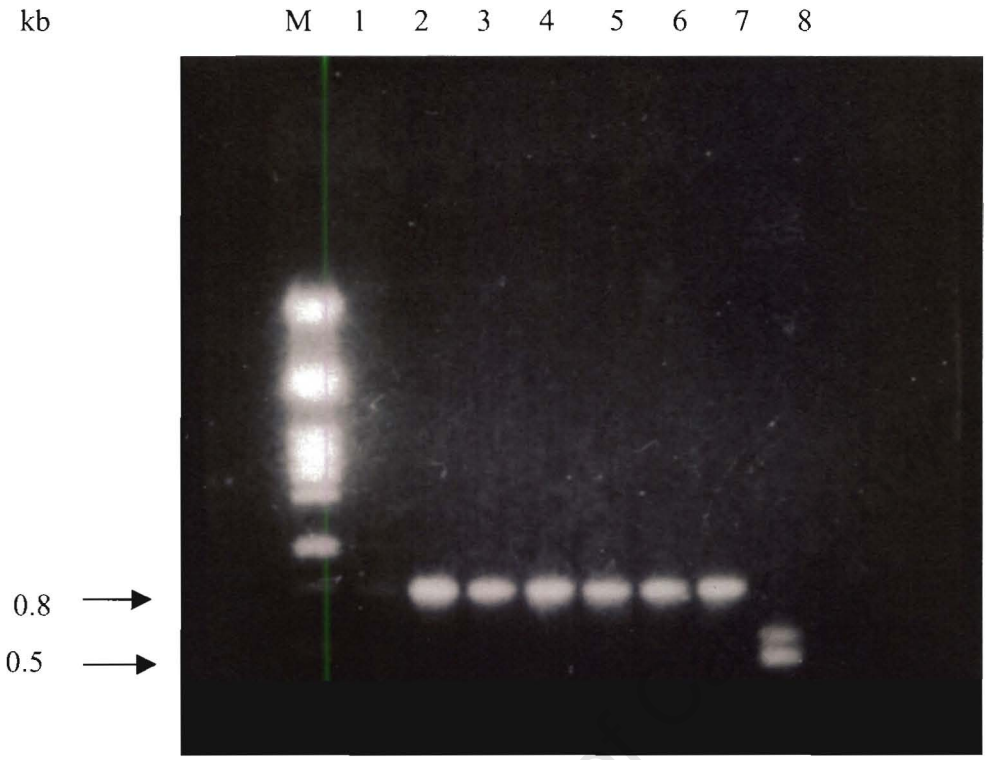


Fig. 7.2. PCR products following amplification of the 16S–23S rRNA intergenic region of genomic DNA from isolates of *G. diazotrophicus*. Lanes: M, Molecular weight markers; 1, PAL5; 2, banana isolate (rhizosphere), 3, banana isolate (root tissue); 4, coffee isolate (rhizosphere); 5, coffee isolate (root tissue); 6, tea isolate (rhizosphere); 7, tea isolate (root tissue), 8, *E.coli*.

7.4 Discussion

This diazotroph has been reported to occur within sugarcane tissues by several authors (Cavalcantes and Dobereiner, 1988; Gillis *et al.*, 1989; Caballero-Mellado and Martinez-Romero, 1994; James *et al.*, 2001). The numbers reported range from 10^3 to 10^7 colony forming units per g fresh weight (Reis *et al.*, 1994; Muthukumarasamy *et al.*, 1999), while it was found at between 10^4 to 10^6 colony forming units per g gresh weight in this

study (Table 7.1). In a previous study where *G. diazotrophicus* was isolated from coffee tissues, no numbers were determined (Jimenez-Salgado *et al.*, 1997). The isolation frequency of 25% in this study was also within the range of the data reported in the previous study in which the isolation rates ranged from 15% to 40% from the rhizosphere and inside the roots of coffee plants growing in acidic soil (Jimenez-Salgado *et al.*, 1997).

In this study, all the isolates were found to have the same RFLP (Fig. 7.1) whereas in the study on isolates from coffee plants in Mexico, four different banding patterns were observed (Jimenez-Salgado *et al.*, 1997). However, the isolates in that study were obtained from plants growing in diverse geographical regions up to 750 km apart whereas in this study, samples were obtained from a 60 km radius. It is therefore possible that the limited area and ecological diversity from which the samples in this study were obtained reduced the possibility of obtaining isolates with greater diversity. However, others have reported limited genetic diversity when studying the *nif* structural gene organization on the chromosome of endophytic *G. diazotrophicus* isolates from sugarcane. A shared *nif* structural gene organization on the chromosome was reported (Caballero-Mellado and Martinez-Romero, 1994).

Some of the isolates obtained using LGI media had characteristics indistinguishable from that of *G. diazotrophicus* PAL5 (i.e. formation of a yellow surface pellicle on semi-solid media and similar colony morphology on solid media) but their *nif*HDK genes were not detected by hybridization to those of *G. diazotrophicus* UAP-5560. It is possible that these are other diazotrophs whose structural nitrogenase genes have a different organization from those of *G. diazotrophicus*. In the study by Jimenez-Salgado *et al.* (1997), such isolates were reported, which did not hybridize to the *nif*HDK genes under stringent conditions. However, these authors reported that the colony morphologies of those other isolates were very different from that of *G. diazotrophicus*, although they also formed yellow pellicles on semi-solid medium.

PCR of the 16S–23S rRNA intergenic region of strains obtained in this study was carried out in an attempt to detect any differences between the strains which may not have been

apparent in the analysis of the RFLP of the *nif*HDK genes. In prokaryotes, the spacer regions between 16S and 23S rRNA genes exhibit a high degree of sequence and length variation between different genera and species. Even within a single genome, there are frequently multiple rRNA genetic loci with spacer regions showing a significant degree of variation. PCR amplification of these intergenic regions has been used to detect differences not only between different species of bacteria, but also between different strains (Jensen *et al.*, 1993). In this study, a single PCR product was obtained with all the samples tested (Fig. 7.2), once more indicating no genetic diversity.

CHAPTER EIGHT

GENERAL DISCUSSION

This study has clearly demonstrated that rhizobia can and do infect non-legumes, especially landraces of African sorghum and millet (Chapter 2). This adds to the body of knowledge already available which show that rhizobia are able to enter the roots of cereal crops and colonize internal tissues. Such cereal crops could therefore serve as a reservoir for rhizobia and help in their survival from one cropping season to the other, especially when dealing with intercropping and rotational systems. The fact that rhizobia located inside sorghum roots could induce nodule formation in soybean plants supports the notion of cereals as alternative hosts for rhizobial survival. The fact that all the strains tested could produce detectable amounts of IAA shows that cereal crops can benefit from this plant growth hormone. Further interest was the observation that nutrent concentration in organs of sorghum plants were altered by rhizobial inoculation. This could have implications for mineral acquisition in the field, especially under low nutrient conditions which are common in Africa. What remains unknown though is the mechanism by which nutrient uptake was enhanced.

Because lumichrome is known to stimulate alfalfa plant growth (Phillips et al., 1999) this rhizobial metabolite was studied and shown to be widely secreted by rhizobia (Chapter 6) from four genera, namely *Rhizobium, Bradyrhizobium, Mesorhizobium* and *Sinorhizobium.* Functionally, this molecule was shown to have a marked effect on the growth and development of various crop species under glasshouse conditions (Chapter 3). This implies that rhizobia do not just only fix N in homologous legume hosts, but that they also produce signals that influence plant growth. This means that in rotations and mixed cropping systems, both legumes and non-legumes could potentially enhance their growth via the activity of this metabolite released by soil rhizobial. It is also possible that some of the rhizobial growth effects previously attributed to plant hormones such as IAA and cytokinnins from rhizobia, could actually be due to lumichrome. Although the mechanisms by which lumichrome induces growth promotion remain unknown, the data show that root respiration was altered in some crops species. For instance, it increased root respiration in maize and CO_2 so produced can promote growth of rhizobia and AM fungi in the soil with potential for improved N and P in symbiotic legumes.

Lumichrome effects on field-grown cowpea plants (Chapter 4) was manifested in decreased stomatal conductance and transpiration rate, and this could have a positive effect in the water relations of plants growing in dry arid areas of Africa where water use efficiency is important for increased crop yields. Whether these observed effects of lumichrome are developmentally driven, remains to be known.

The mineral concentration of tissues of field-grown plants was also influenced by lumichrome via an unknown mechanism. There was an improved uptake of some mineral nutrients by some plants with lumichrome supply. It is therefore possible that the observed differences in mineral concentration when sorghum was inoculated with rhizobia may be due to the effect of lumichrome produced by rhizobial strains.

Lastly, a preliminary study of the presence of *Gluconoacetobacter diazotrophicus* in coffee, tea and banana from Kenya (Chapter 7) showed its presence in these important Kenya crops. Further studies to characterize these isolates are needed as well as studies on the interaction of this diazotroph with these crops to determine if the bacterium influences their growth in any way, and if so by what mechanisms.

Taken together, the data from this study show that diazotrophs have multiple effects on plant development than previously thought.

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