DEVELOPMENT OF FREE-LIVING DIAZOTROPHIC (FLD) INOCULANTS AND THEIR EFFECTS ON CROP GROWTH

By

MEDHIN HADISH KIFLE

B.Sc. Biology, University of Asmara, Eritrea

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ABSTRACT

In this study several free-living diazotrophs (FLD) were isolated and screened for their nitrogen fixing ability on a range of crops grown in greenhouse, hydroponics and field trials.

Rhizosphere isolates of free-living diazotrophs (FLD) may be effective biofertilizer inoculants, and may improve plant health where crops are grown with little or no fertilizer, as is the case in the Developing World. FLD isolates from rhizospheric soils in KwaZulu-Natal were assessed by growing them on N-free media, which is a key isolation method. They were then evaluated for their nitrogenase activity by quantifying ethylene production from acetylene by gas chromatography (GC). The free living isolates that produced greater quantities of ethylene were detected by an acetylene reduction assay (ARA). These were further assessed for colony formation on N-free media with different carbon sources, and at a range of temperatures (20, 25 and 30^0C) and pH values (6.0, 7.0 and 8.0). Isolates G3 and L1 were identified using DNA sequencing by Inqaba Biotechnical Industries (Pty) Ltd as *Burkholderia ambifaria* Coenye et al, and *Bacillus cereus* Frankland, respectively. These isolates grew significantly better on an ethanol medium, at temperatures of 20, 25 and 30^0C and pHs of 6.0, 7.0 and 8.0. Isolates B3 (*Burkholderia* sp.) and D6 (*Bacillus cereus* Frankland) also grew well on an ethanol medium, but only at 20^0C and at a pH of 6.0 and 7.0, respectively, while Isolate E9 (*Burkholderia cepacia* Frankland) grew well on an ethanol medium only at 30^0C, and pH 6.0 and 7.0. Temperature and pH strongly influence FLD growth on N-free media using different carbon sources.

Further trials were conducted to screen the best isolates under greenhouse condition, using both seed treatments and drenching application techniques onto several crops. The drenching application resulted in an increase in the growth and N-total of all the evaluated crops, relative to an unfertilized control. Growth and N-total of maize and sorghum increased with seed treatments, but did not increase the growth of lettuce and zucchini.

Drenching of FLD isolates at 10^6cfu ml^{-1}, applied on weekly basis, resulted in an increase in the growth of lettuce. Increased doses and frequency of application of the FLD bacteria resulted in a decrease in lettuce growth. This led to the conclusion that application of FLD

bacteria at high doses and short intervals may create a situation where the applied FLD bacteria and the resident rhizosphere microbes compete for root exudates. High doses at low frequencies and low doses at high frequencies may be more effective on lettuce.

Inoculation of Isolate L1 (*B. cereus*) at 10^6cfu ml^{-1} or in combination with Eco-T® (*Trichoderma harzianum* Rifai), significantly increased growth of lettuce. This result may have been due to nitrogen fixation, or to secretion of growth promoting substances by both the FLD and *T. harzianum,* and to biocontrol effects of Eco-T®. Application of Isolate L1 (*B. cereus*) at 10^6cfu ml^{-1} with or without Eco-T® was an effective tool for enhancing plant growth and nitrogen fixation.

An FLD, Isolate L1 (*B. cereus*), was applied to lettuce plants together with a complete hydroponics fertilizer at 25% strength (Ocean Agriculture 3:1:3 (38) Complete), with the N level at 25mg l^{-1}. These plants grew significantly better than the control plants grown on 25% of normal NPK fertilization, or with an inoculation of L1 alone. This indicates that it may be possible to integrate FLD applications with the application of low levels of commercial fertilizers, which is what resource poor farmers can afford.

DECLARATION

I, Medhin Hadish Kifle, declare that

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Signed_____

Medhin Hadish Kifle

Signed_____

Prof. M.D. Laing (Supervisor)

DEDICATION

To the Hadish Kifle family and my Husband for the support and
Spiritual encouragement during my studies

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FOREWORD

The research presented in this thesis was conducted at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research presented is the result of two years of laboratory and field studies.

The main objectives of the research in this thesis were to produce solutions to the problem created by the high cost of mineral fertilizers, particularly nitrogenous fertilizer, by isolating free-living diazotrophs and screening them for their nitrogenase activity on different crops, to find the best possible biofertilizer inoculants.

The research is covered in eight chapters.

Chapter One is a review of the literature on the free-living diazotrophs (FLD), their growth condition and nitrogenase activity, with particular examples of *Azotobacter* and *Azospirillum*, and their effects on crop production when they are used as biofertilizers.

Chapter Two covers the isolation and *in vitro* screening of different diazotrophic isolates for their nitrogenase activity.

Chapter Three reports on greenhouse trials conducted on FLD isolates, selected from *in vitro* screening tests, for their effects on the growth of several crops (maize, sorghum, wheat, lettuce, zucchini and petunia) using seed treatments or drench applications.

Chapter Four covers an evaluation of the impact of FLD applications on lettuce growth, and the determination of their optimum dose and frequency of application.

Chapter Five reports on an evaluation of the combination effect of an FLD, Isolate L1 (*Bacillus cereus*), combined with *Trichoderma harzianum* Rifai (Eco-T®) applied to lettuce crops.

Chapter Six covers an evaluation of the combined effect of an FLD, Isolate L1 (*B. cereus*), with NPK fertilization on lettuce growth in hydroponics.

Chapter Seven reflects on an evaluation of the effect of biofertilizer on growth, yield and N-level of maize in a field trial.

Chapter Eight reviews the results and conclusions of the experimental chapters, then makes recommendations for future research on the growth condition, application techniques, integration of FLDs with other microorganisms, and interaction of FLDs with fertilization.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Sustainable agriculture has an imperative to minimize the use of expensive chemical fertilizers because the use of nitrogenous fertilizers is becoming expensive in terms of financial constraints, utilization of fossil energy, and pollution of water supplies (Sprent 1979; Gordon and Jacobson, 1983). However, nitrogenous fertilizers play a crucial role in agriculture, and are a major determinant of crop yields. To obtain the best yields and high-quality food, this fertilizer must be made available to plants in adequate quantities. A lack of N results in poor plant nutrition and a corresponding reduction in yield. Over-fertilization with N also results in poor quality products, soil acidity, pollution of the environment and unnecessary expenses. A big problem globally is that the majority of the world's farmers, especially in Africa and Asia, cannot afford to put on nitrogenous fertilizers. These issues have led to a search for alternative sources of N, in particular, investigations into the use of nitrogen fixing microorganisms that increase soil fertility and crop yields from a number of agricultural crops (Wu *et al.,* 2005). If the use of these microbes was adopted widely, then this could lead to a reduction in the excessive use of organic N and inorganic fertilizers (Garg *et al.,* 2001) and to increased yields by resource-poor farmers.

Nitrogen is an essential plant nutrient that is commonly deficient in most soils, contributing to reduced agricultural yields throughout the world. Eighty percent of the atmosphere is molecular nitrogen but it is biologically unavailable except for fixation by prokaryotic organisms containing the enzyme nitrogenase (Sprent, 1979).

Biological nitrogen-fixation (BNF) processes change inert nitrogen to useful ammonia (NH_3) that is used to manufacture amino acids, proteins, nucleic acids, and other nitrogen-containing components necessary for life. BNF is usually associated with symbiotic *Rhizobium*-legume systems. Biological nitrogen-fixation has been demonstrated in free-living microorganisms: anaerobic fixation by *Clostridium pasteurianum* Winogradsky and aerobic fixation by *Azotobacter chroococcum* Beijerinck (Beijerinck, 1901). Since then varieties of

other prokaryotic organisms have been added to the list of free-living N fixers. Vadakattu and Paterson (2006) explained that there is a wealth of free-living bacteria in soils that are capable of fixing significant quantity of atmospheric nitrogen in the absence of legumes, using crop residues and root exudates as their energy sources. The free-living heterotrophic, aerobic bacteria fix nitrogen non-symbiotically or loosely in association with grasses (Krishnamoorthy and Rema, 2004).

The aim of this study was to find nitrogen-fixation conditions for free-living diazotrophs (FLD) in chemically defined, nitrogen free media. Parameters such as pH and temperature were to be tested during the optimization studies. Furthermore, FLD isolates were to be inoculated either as seed treatment or by drenching into the rhizosphere of several crops (maize, wheat, lettuce, sorghum, zucchini and marigold/zinnia) containing nitrogen free growth media under optimized conditions in order to verify the growth promotion capacity and nitrogen-fixation of selected isolates on different crops, plus the levels of plant N and dry/wet biomass were to be measured.

1.2 Biofertilizers

Biofertilizers, more commonly known as microbial inoculants, are artificially multiplied cultures of certain soil organisms that can improve soil fertility and crop productivity (Roger and Watanabe, 1986). The main sources of bio-fertilizers are bacteria, cyanobacteria (Whitton and Potts, 2000; Irisarri *et al*., 2001; Mishra and Pabbi, 2004) and fungi that may be a partial or complete substitute for chemical fertilization (Bashan, 1998). Microbial inoculants are cost effective, environmentally friendly and also preserve soil health by enhancing and maintaining its fertility (Sprent, 1979; Abdul_Rahim, K., 2002).

Biofertilizers are classified into nitrogenous biofertilizers (*Rhizobium, Azospirillum* and *Azotobacteria*) (Krishnomoorthy and Rema, 2004), phosphorus solubilizers (*Phosphobacter* spp.) (Gaind and Gaur, 1991; Wu *et al.,* 2005), phosphorus mobilizers (mycorrhizae), sulphur solubilizers (*Thiobacillus thioxident* Waksman and Joffe), iron solubilizers (*Thiobacillus ferroxidents* Temple and Colmer) and vesicular arbuscular mycorrhizas (VAMs) (Krishnamoorthy and Rema, 2004).

Many bacteria have the ability to promote plant growth and thus can be utilized as bio-fertilizers that help expand the root system and improve seed germination (Bacilio *et al.,* 2003). *Azotobacter* is one of the plant growth promoting rhizobacteria (PGPR) that can be beneficial to plants by colonizing their root zone (Ahmad *et al.,* 2006). *Azospirillum* or other diazotrophs also enhance plant growth or nitrogen content that improve nutrient assimilation and alter root size (Kapulnik *et al.,* 1981a). In respect of their mode of action on plant, *Azospirillum* is a genus of fixing N bacteria which can be used as biofertilizers. However, Okon and Labandera-Gonzalez (1994) argued that the term 'biofertilizer' is not appropriate for *Azospirillum* because it does not replace nitrogen fertilizers but can supplement them.

1.3 Free-living nitrogen fixing bacteria

Microorganisms that fix nitrogen are called diazotrophs. The microbes that fix nitrogen independent of other organisms are called free-living bacteria. These are a potentially important group for nitrogen-fixation. A *Clostridium* sp. was the first gram positive, strictly anaerobic bacterium that was shown to be capable of nitrogen-fixation (Dixon and Wheeler, 1986). Members of the genus *Klebsiella* are facultative anaerobes with the flexibility to grow in both aerobic and anaerobic environments. However, they fix nitrogen anaerobically because they have no means of protecting nitrogenase from oxygen (Dixon and Wheeler, 1986).

Free-living, aerobic bacteria such as *Azotobacter chroococcum* Beijerinck and *Beijerinckia fluminensis* Döbereiner and Ruschel are capable of fixing atmospheric nitrogen non-symbiotically. Some bacteria, such as *Azospirillum* spp. and *A. paspali* Döbereiner, also fix nitrogen non-symbiotically. Studies have shown that aerobic, nitrogen-fixing species such as *A. brasilense* Corrig. Tarrand et al. and *A. lipoferum* (Beijerinck) Tarrand et al. are plant growth stimulating rhizobacteria that are associated with numerous grasses and cereals (Steenhoudt and Vanderleyden, 2000). The free-living *Azospirillum* spp. are better known as representatives of the best characterized genera of plant growth-promoting rhizobacteria (Steenhoudt and Vanderleyden, 2000) and are also considered to have a potential as nitrogen fixers in cereals.

Table 1.1 Examples of free-living nitrogen-fixing organisms (Dixon and Wheeler, 1986)

Archaebacteria	*Methanosarcina, Methanococcus*
Anaerobes	*Clostidium, Desulphovibrio, Desulfotomaculum*
Facultative Anaerobes	*Klebsiella, Erwinia, Enterobacter, Bacillus*
Microaerobes	*Azospirillum, Aquaspirillum*
Aerobes	*Azotobacter, Beijerinckia, Derxia, Bacillus*
Photosynthetic Bacteria	*Rhodopseudomonas, Rhodospirillum, Chromatium*
Cyanobacteria	*Anabaena, Calothrix, Nostoc*

1.3.1 The Genus Azotobacter

The genus *Azotobacter* is a group of nitrogen-fixing bacteria. Unlike rhizobia, they do not form root nodules or associate with leguminous crops. Beijerinck (1901) found the genus *Azotobacter* in neutral to alkaline soils. It comprises of large, free-living, obligatory aerobic rods that are capable of fixing nitrogen non-symbiotically. *Azotobacter* cells are rather large for bacteria, many isolates being almost the size of yeast cells, with a diameter of 2-4µm or more. Some strains are motile with peritrichous flagella (Thompson and Skerman, 1979; Bergersen, 1980).

1.3.2 The Genus *Azospirillum*

Like *Azotobacter, Azospirillum* species do not form root nodules or associate with leguminous crops. This genus is able to live on its own in soil, or in close associations with plants in the rhizosphere. *Azospirillum* was isolated from nitrogen-poor sandy soil in the Netherlands by Beijerinck in 1925 and was originally named *Spirillum lipoferum*. corrig. Tarrand et al. *Azospirillum* spp. are described as gram negative, rod-shaped, 1mm in diameter, and are very motile. Cells are about 1.0µm x 3.5mm in size with a single flagellum, when grown in massively parallel signature sequence (MPSS) broth. The genus produces lateral flagella when grown on MPSS agar at 30^0C. They form wrinkled, dark pink colonies when grown on MPSS agar. A formation of a white veil or bacterial band is visible when

inoculated onto NFb medium (N stands for new and Fb for Fábio Pedrosa) (Baldani et al., 1983; Bashan *et al.,* 1993) and Dobereiner's liquid medium (Döbereiner and Day, 1976).

1.4 Isolation of nitrogen-fixing bacteria

1.4.1 The Genus Azotobacter

Azotobacter is widely distributed in the rhizosphere, and is readily isolated from tropical and temperate regions (Tchan, 1984). Several methods have been developed for isolating nitrogen-fixing bacteria from the rhizosphere and roots of grass hosts. Beijerinck (1901) was able to isolate *Azotobacter* from soil using a liquid enrichment medium containing 0.02% K_2HPO_4 and 2% mannitol in tap water. Jensen (1965), cited by Thompson and Skerman (1979), stated that this method, sometimes with minor modifications, is still used extensively today. Additionally, Ahmad *et al*. (2006) were able to isolate *Azotobacter* from soil using nitrogen-free Jensen's medium, with sucrose as a carbon source instead of mannitol.

Brown *et al.* (1962) were able to isolate *Azotobacter* species by streaking of serial soil dilutions onto plates containing Brown N-free medium and successfully isolating species of *Azotobacter* that produced slimy, glistening, smooth, whitish, weakly convex, 2-10mm diameter colonies. Slight differences in size and sliminess of colonies were observed between different species.

For selective isolation of soil microorganisms, Aquilanti *et al.* (2004) employed strategies used by Pochon (1954) who used Winogradsky's enrichment solution for 7-14d, followed by streaking onto brown agar (Augier, 1956). He found that growth of most *Azotobacter* spp. was accompanied by the production of diffusible pigments.

Becking (1992) employed a soil paste-plate technique for isolation of the genus *Azotobacter.* Slimy and glistening colonies appeared upon the smoothed soil paste surface. On the other hand, Pochon (1954) isolated *A. chroococcum* through the direct sowing of single soil grains onto a mannitol nitrogen-free medium. The utilization of this combined method led to the

isolation of 35 *Azotobacter*-like cultures and also proved to be the best strategy in terms of reliability and selectivity (Aquilanti *et al.,* 2004).

1.4.2 The Genus *Azospirillum*

The medium, NFb, is a commonly used medium for the isolation of *Azospirillum* (Rodríguez Cáceres, 1982). Modification with Congo-red-NFb is semi-selective, which permits the recognition of *Azospirillum* colonies on plates and facilitates the isolation of pure cultures since *Azospirillum* absorbs Congo-red and the colonies appear dark red or scarlet with typical colony characteristics (Bashan *et al*., 1993). *Azospirillum* can also be isolated using a semi-solid malate enrichment medium (Rodríguez Cáceres, 1982). Mandimba *et al* (1986) was able to isolate *Enterobacter* and *Azospirillum* strains from the rhizosphere of maize and rice using the same medium.

1.5 Growth media

Both *Azotobacter* and *Azospirillum* can grow on nitrogen deficient media (Tejera *et al.,* 2005). These species are able to grow on a wide variety of carbohydrates, alcohols and organic acids but grow best on those that are nitrogen deficient (Thompson and Skerman, 1979; Ravikumar *et al.,* 2004). *A. lipoferum* grows vigorously on malate, succinate, lactate, or pyruvate but moderately on galactose or acetate and poorly on glucose or citrate (Okon *et al.,* 1976). Glucose supports little or no growth. Different strains of *Azospirillum* differ greatly in their utilization of amino acids for growth and their effects on nitrogen-fixation. For example, *A. brasiliense* grows poorly or not at all on media with glutamate, aspartate, serine, or histidine as the sole nitrogen and carbon source (Hartmann and Burris, 1987).

1.6 Factors affecting growth

In soil ecosystems, the survival and growth of free-living nitrogen-fixing populations are affected by many physical and chemical conditions, including the concentration and nature of organic components excreted by plant roots, as well as by the nature of interactions with other microbial populations (Bastolla *et al.,* 2005).

1.6.1 Temperature

In relation to temperature, *Azotobacter* is a typical mesophilic organism. As a result, temperature is one of the major factors determining the immediate activity of nitrogen-fixing populations present at a given time, both with regard to general metabolism, growth and nitrogen-fixation. Most studies reveal that the optimum temperature for *Azotobacter* growth is 25-30^{0}C. Vegetative *Azotobacter* cells die at high temperatures (45-48^{0}C). On the other hand, the minimum temperature for growth of *Azotobacter* lies a little above 0ºC (Mishustin and Shilnikova, 1969; Didonet and Magalhaes 1997). Maximal growth of *Azospirillum* occurs at 30^{0}C, and no growth takes place at 37^{0}C (Eckert *et al.,* 2001).

1.6.2 pH

Beijerinck (1901) explained that *Azotobacter* prefers neutral soils with a pH between 7.0-7.5, although it is able to develop on media with a pH range of 4.5-5.5. Eckert *et al.* (2001) noted that under fairly acid conditions, *Azotobacter* may be capable of surviving for short periods and for more than 2 years if pH is 6.0 or above. These authors also showed that *A. lipoferum* requires a similar pH as other *Azotobacter* species.

1.6.3 Inorganic salts

Beside the carbon source, nitrogen-fixing bacteria need several salts to grow successfully in nitrogen-free media. Sabra *et al*. (1999) stated that growth of *Azotobacter* is largely dependent on the presence of phosphorus and potassium compounds in the medium because the absence or deficiency of phosphorus in the medium slows development of the culture (Sabra *et al.*, 1999). Iron and molybdenum are co-factors of the nitrogenase enzyme, responsible for nitrogen-fixation and are essential for growth (Brock *et al.,* 1994; Cornish and Page, 1998; Cornish and Page, 2000). Growth of some *A. brasilense* and *A. lipoferum* strains

is severely inhibited by iron limitation and by competition with foreign microbial iron chelators (Hartmann, 1988). Addition of $FeSO_4$ decreased the viable cell count of a mutant strain of *Azotobacter vinelandii* Lipman (Edwards *et al.,* 2000); while in another study it increased growth (Vermani *et al.,* 1997). Calcium (Ca) and magnesium (Mg) also play an important role in the metabolism of nitrogen-fixing bacteria. A deficiency of Ca in the medium leads to prolongation of the lag phase, but its action is not regarded as specific for nitrogen-fixation. However, the concentration of Ca salts must not exceed a certain optimum (Mishustin and Shilnikova, 1969) because higher levels of Ca are inhibitory. Magnesium ions are essential for transformation and growth (Page and Sadoff, 1976). The action of copper on some free-living diazotrophs is toxic, even in very low concentrations (Becking, 1992).

1.6.4 Oxygen

Aerobic free-living diazotrophs requires oxygen for propagation (Mishustin and Shilnikova, 1969). When *Azotobacter* grows in a N-free culture, increasing dissolved oxygen tension improves cell concentration (Pena *et al.,* 2000). Initiation of growth is prevented by vigorous aeration but proceeds normally with gentle aeration (Dalton and Postgate, 1968). Parker (1954) also reported that there was an inhibition of respiration and growth at higher oxygen concentrations.

1.7 Cyst formation

Extensive capsules or slime layers are produced by free-living nitrogen fixing bacteria on carbohydrate containing media. *Azotobacter* spp. have several features that allow them to survive in the harsh environment of the soil (Socolofsky and Wyss, 1961). One feature is the formation of cysts (Thompson and Skerman, 1979). Cysts are resting cells that are surrounded by a protective coat, and they are resistant to various chemical and physical agents (Nobutake *et al.*, 2006). *Azotobacter* cysts are more resistant to various harmful agents than vegetative cells, including resistance to gamma radiation, sonic treatment, and desiccation (Socolofsky and Wyss, 1962). *Azotobacter* cysts can sometimes resist a temperature of 50^0C (Schreven, 1962). Addition of 0.3% n-butanol as a carbon source to the solid Burke's N-free medium may lead formation of cysts by *Azotobacter* spp. (Socolofsky and Wyss, 1961; Postgate, 1982). However, utilization of sucrose and glucose as the carbon sources can permanently suppress cyst formation (Socolofsky and Wyss, 1961). *Azospirillum*

spp. also overcome unfavorable conditions by cyst formation (Bashan, 1999). Bleakley *et al.* (1988) found that *A. lipoferum* can grow and produce cysts on nitrogen-free beta-hydroxybutyrate agar similar to the encystment of *Azotobacter*. Cells accumulate poly-beta-hydroxybutyrate and grow in chains or filaments that eventually lose motility and form capsules. However, the encapsulated cells are not resistant to desiccation.

1.8 Shelf life

Azotobacter is capable of surviving for short periods in fairly acid conditions but at a pH of 6.0 or above can survive for approximately two years. According to Aquilanti *et al*. (2004), *Azotobacter* spp. can also survive at -80^0C on tryptic soy broth (TSB) oxide with 50% (w/v) glycerol for long periods and at 4^0C on tryptic soy agar (TSA) plates for short periods. Thompson and Skerman (1979) explained that the cells from mature cultures survive desiccation and storage over silica gel for maximum periods of 6 to >24 months. *Azotobacter* cultures are also better maintained on sealed slants (Antheunisse, 1973). Aquilanti *et al*. (2004) used cheap and easily available waste materials as carriers for biological inoculants (*A. chroococcum*, *A. brasilense* and *Bacillus circulans* Jordan), but found that when fly ash was used as the carrier the diazotrophs had a long shelf life.

1.9 Factors affecting nitrogen-fixation

Some bacteria can convert nitrogen gas into ammonia by the process termed nitrogen-fixation. These bacteria are either free-living or form symbiotic associations with plants or other organisms. Conversion of molecular nitrogen to ammonium is performed through the action of a nitrogenase complex, the synthesis and activity of which is strongly repressed by both ammonium nitrogen and molecular oxygen (Steenhoudt and Vanderleyden, 2000). While fixing nitrogen with the aid of their enzyme complex nitrogenase, diazotrophs are affected by several factors.

1.9.1 Temperature

Nitrogenase is active over a fairly narrow temperature range. At the lower limits of 5-10ºC, nitrogenase activity is low, whereas at the upper limits, 37-40ºC, nitrogenase activity falls off rapidly because of the sensitivity of the enzyme to heat (Sylvia *et al*., 1999). Darbyshire

(1972) found that the most nitrogen was fixed by pure *Azotobacter* cultures at 28°C. Additionally, *Azospirillum* has been identified mostly in tropical soils but is unable to fix nitrogen at temperatures above 40^0C. Different species of *Azospirillum* behave differently in terms of nitrogenase activity, nitrogenase syntheses and growth above 42^0C (Aggarwal and Chaudhary, 1995).

1.9.2 pH

Both the growth of *Azotobacter* and nitrogen-fixation are weakened in acidic environments (Mishustin and Shilnikova, 1969). *Azospirillum* does well in soils with high levels of organic matter and high moisture content, and requires a pH level of above 6.0.

1.9.3 Oxygen

Nitrogen-fixation is inhibited by oxygen since dinitrogenase reductase is rapidly and irreversibly inactivated by oxygen. In aerobic bacteria, nitrogen-fixation occurs in the presence of O_2 in whole cells. However, in enzyme preparations, such organisms protect nitrogenase from oxygen inactivation by removal of oxygen during respiration, production of oxygen-retarding slime layers, or by compartmentalization in special cells called heterocysts (Brock *et al.,* 1994). High oxygen solution rates inhibit nitrogenase in whole bacteria (Yates, 1970), indicating that nitrogen-fixation by *A. chroococcum* is decreased with increasing agitation at Continuous growth conditions. *Azospirillum* is a microaerobic organism that requires a low level of oxygen for the expression of nitrogenase activity (Tarrand *et al*., 1978) and nitrogen-fixation occurs in microaerobic nitrogen-limited conditions (Eckert *et al*., 2001). The nitrogenase activity of the microaerophilic bacteria *A. brasilense* and *A. lipoferum* was completely inhibited by approximately 0.02atm of oxygen in equilibrium with the solution (Hartmann and Burris 1987; Mugnai *et al*., 1994).

1.9.4 Inorganic Salts

Some nutrients present in the growth medium also take part in nitrogen-fixation. The fixation of nitrogen by *Azotobacter* starts when the concentration of a phosphate reaches 0.04mg ml^{-1}. If the concentration of phosphate in solution is about 8mg ml^{-1}, then the assimilation of

nitrogen stops altogether and nitrogenase activity is detected only when the solubilization of phosphate increases (Becking, 1992). The nitrogenase complex needs magnesium ions to be active, so magnesium requirement for nitrogen-fixation is considerable (Sylvia *et al.,* 1999). Molybdenum is essential for most strains of *Azotobacter*. This need is shown both during fixation of molecular nitrogen and during development of nitrates (Mishustin and Shilnikova, 1969). This is a consequence of the fact that molybdenum is needed for the expression of the nitrogenase enzyme complex. It is known that vanadium stimulates nitrogen-fixation in a number of organisms, including various species of *Azotobacter*, some Cyanobacteria and phototrophic bacteria and *Clostridium pasteurianum* Winogradsky (Brock *et al.*, 1994). Vanadium causes the organism to express vanadium-based nitrogenase in a molybdenum deficient medium. In *Azotobacter*, ammonium salts (or nitrates) depress hydrogenase activity, but not completely (Postgate, 1982). *A. vinelandii* requires a high complement of iron and an efficient iron acquisition system to support nitrogen-fixation (Fekete *et al,*. 1983). The addition of ammonium, glutamine, oxygen, nitrate or nitrite, together with either iron limitation or a shift of temperature, can cause different levels of inhibition of nitrogenase activity (Mugnai *et al.,* 1994). Addition of ammonium chloride inhibited nitrogenase activity of *A. brasilense* and *A. lipoferum* whereas *A. amazonense* Magalhaes et al. showed only partial inhibition (Hartmann, 1988).

1.9.5 Nitrogen

In many cases when nitrogen-fixation is evaluated as nitrogenase activity, it is depressed in the presence of combined nitrogen in the medium (Laane *et al*., 1980). Small doses of nitrogen containing-minerals (especially ammonium salts) stimulate nitrogen assimilation, but larger doses restrain this process (Mishustin and Shilnikova, 1969).

1.10 Detection of nitrogen-fixation

There are several ways to detect nitrogen-fixation:

1.10.1 The stable isotope (N^{15}) method

The most definitive measurements of biological nitrogen-fixation make use of the stable, heavy isotope, ^{15}N, and require access to a mass spectrometer (Sprent, 1979). In this method,

incorporation of $^{15}N_2$ (labeled dinitrogen) into plant or microbial cells is measured. Samples are exposed to an atmosphere of about 10% $^{15}N_2$, usually in a balance of argon or helium, to eliminate competition from $^{14}N_2$. Following incubation, the samples are digested and the ^{15}N content of the materials is determined using a mass spectrometer. Detection of ^{15}N in tissues or cells provides definitive proof of nitrogen-fixation and allows very accurate quantification of the amount of nitrogen-fixation (Sylvia *et al*., 1999). This method is accurate but is time consuming and expensive, both in terms of equipment needed and production of the $^{15}N_2$ isotope (Burns & Hardy, 1975).

1.10.2 The nitrogen difference method

The nitrogen difference method is adequate for active nitrogen fixers, but it will not detect increases of less than about 1% in the plant N, even when uniform samples can be taken (Burris and Wilson, 1972). Increases in plant N can be detected with the use of the Kjeldahl method, in which all forms of nitrogen in the sample are converted to ammonia by the digestion application followed by distillation of the ammonia to detect calorimetrically or by titration (Bergersen, 1980).

1.10.3 Acetylene reduction assays (ARA)

The ability of the nitrogenase complex to reduce acetylene (C_2H_2) to ethylene (C_2H_4) forms the basis of the acetylene reduction assay. In this method, the microbial system to be measured is exposed to an atmosphere containing 10% acetylene and incubated under appropriate conditions. Samples of the gas phase are periodically removed and injected into a gas chromatograph for quantification of ethylene production from acetylene (Dixon and Wheeler 1986; Sylvia *et al.,* 1999). This method is far simpler and faster than other methods (Brock *et al.*, 1994). "The theoretical relationship between acetylene reduction and nitrogen-fixation follows from 2 electrons being needed to reduce C_2H_2 to C_2H_4 and 6 electrons to reduce nitrogen to $2NH_3$, that is $3C_2H_2$ equivalent to 1 nitrogen" (Sprent, 1979).

Nitrogenase will reduce a number of substrates, including reducing acetylene to ethylene. This is of particular interest because it provides the basis for the estimation of nitrogenase activity by gas chromatography (Table 1.2).

Table 1.2 Substrates reduced by nitrogenase (Dixon and Wheeler, 1986).

Name	Formula	Major Products
Protons	H^+	H_2
Nitrogen	$N\equiv N$	$NH_3 + H_2$
Nitrous oxide	$N\equiv N^+—O^-$	$N_2 + H_2O$
Azide	$[N\equiv N^+ —N]^-$	$N_2 + NH_3 + N_2H_4$
Acetylene	$CH\equiv CH$	$CH_2 = CH_2$
Allene	$(CH_2=C=CH_2)$	$CH_3 - CH = CH_2$
Cynide	$[C\equiv N]^-$	$CH_4 + NH_3$
Alkyl cyanides	$R-C\equiv N$	$RCH_3 + NH_3$
Alkyl isocyanides	$R-N+ = C^-$	$RNH_2 + CH_4$
*Methyl acetylene is a probable intermediate		

1.11 Biofertilizer inoculants and nitrogen-fixation

For many years, *Rhizobium* inoculants have been produced around the world as a biofertilizer. Today nonsymbiotic, associative rhizosphere FLD bacteria are starting to be used on a large scale. The major breakthrough in the use of FLD for plant inoculation occurred in the late 1970s when *Azospirillum* was found to enhance non-legume plant growth (Dobereiner and Day, 1976) by directly affecting plant metabolism (Bashan *et al*., 1993). *Azotobacter* spp. and *Azospirillum spp.* secrete growth promoting hormones such as auxins, gibberellins and cytokinin into culture media (Azcón and Barea, 1975). Seed treatments with suspensions of these bio-inoculants have increased seed germination and growth of plants (Albrecht *et al.,* 1981). The benefits of biofertilizers using associative FLD bacteria can improve yields of host plants by 10-20%, replace 30-50% of the total amount of nitrogen required from chemical fertilizers, as well as enhancing shoot growth and root development (Baldani *et al.,* 1983). *Azospirillum* inoculants can increase plant dry weight and nitrogen assimilation by 25% as they are able to scavenge for nutrients, alter root permeability and fix nitrogen (Pacovsky *et al.,* 1985). In Israel, beneficial effects on the growth and yield of many crops have been obtained from inoculation with *Azospirillum* inoculants (Kapulnik *et al*., 1983).

1.12 Scope and potential application of FLD inoculants

Bacteria are continuously studied, investigated and exploited for their possible role in plant growth and health. Some of the investigations are in early or laboratory stages; others are undergoing greenhouse and field trials.

The simplest inoculation method used is by application of bacteria in a liquid suspension, either directly to the soil, or as a seed treatment. This technique has been used in numerous greenhouse and field experiments (Albrecht *et al.,* 1981; Millet and Feldman, 1984; Fallik *et al.,* 1988) but may be inadequate because *Azospirillum* survives poorly in soil in the absence of a carrier. When *Azotobacter* inoculants are applied to cereal seeds, they need adhesives such as guar gum as a sticker in order to retain a maximum number of *Azotobacter* cells on the seeds (Mor *et al.,* 1995).

1.13 FLD inoculants and their effects on various aspects of crop growth

1.13.1 Inoculants of *Azotobacter* spp.

Several studies have shown that *A. chroococcum* has beneficial effects on plant yields and improves the growth of plants by various mechanisms under certain environmental conditions. Recently *Azotobacter* strains have been usefully employed both in aquaculture and agricultural systems (Garg *et al.*, 2001) because it has the ability to fix nitrogen and solubilize phosphates (Pandey *et al.,* 1998; Kumar *et al.*, 2001), secrete stimulating hormones like gibberellins, auxins and cytokinins (Azcón and Barea, 1975), excrete ammonia (Narula *et al.,* 1981), produce antifungal substances (Lakshminarayana *et al.,* 1992) and produce siderophores (Suneja *et al.,* 1994).

Azcón and Barea (1975) found that strains of *Azotobacter* secreted growth promoting hormones into their culture media. Pre-treatment of seeds with a suspension of *Azotobacter spp.* resulted in improved seed germination and plant growth. Moreover, seed inoculation with *Azotobacter* spp. without fertilization increased grain and stover yield (Meshram and Shende, 1982).

Narula *et al.* (2005) found that *Azotobacter* applied to wheat and cotton increased yield, dry weight and survival rate of *Azotobacter* and also fixed 25-30kg ha^{-1} of nitrogen in the wheat field. Additionally, the nitrogen concentration in wheat grains and roots may be increased due to *Azotobacter* bio-inoculants (Wadad and Vlassak, 1988; Kader *et al.,* 2002). Similarly, inoculation of *A. chroococcum* onto *Brassica napus* cv. ISN-129 produced an increase in seed yield and total dry matter, especially when no external nitrogen was applied (Singh and Bhargava, 1994). In another trial, mungbean seeds were also inoculated with *A. chroococum*, resulting in increased nodulation, nitrogen-fixation and seed yield (Yadav & Vashishat, 1991). According to Oblisami *et al*. (1979), the effect of *Azotobacter* inoculants alone resulted in better growth of root systems when compared to that due to growth regulators (gibberellins (GAs) and indole-3-acetic acid (IAA)) applied alone. In other study, under field condition, *A. chroococcum* was sprayed on two varieties of paddy rice and a variety of wheat. Considerable increases in yield was attained from both the crops (Iswaran *et al.*, 1978). A foliar spray of *A. chroococcum* significantly increased grain and straw yield of a rice crop when applied on the 15^{th}, 30^{th}, and 45^{th} day after transplanting (Kannaiyan *et al.*, 1980).

1.13.2 Inoculants of *Azospirillum* spp.

Many *Azospirillum* strains produce plant hormones in liquid culture. The major hormone produced is indole-3-acetic acid (IAA) (Rao, 1983; Barbieri *et al*., 1986) and several gibberellins (Bottini *et al.,* 1989). As a result of plant hormone production, *Azospirillum* is also recognized as a growth promoting microorganism.

Reports show that *Azospirillum* inoculants can increase yield in field grown plants (Kapulnik *et al.,* 1981a; Rao*,* 1983; Watanabe and Lin, 1984). Even moderate yield increases attributed to inoculation with *Azospirillum* are considered commercially valuable in modern agriculture. Inoculation of *A. lipoferum* on rice seedlings (*Oryza sativa* L. var. OR42) enhanced phosphate uptake of plants, and increased fresh and dry weights of shoots (Murty and Ladha, 1988). Inoculation of *A. brasilense* Strains Cd and Sp-246 to wild cactus seeds improved seed germination and seedling growth parameters (Puente & Bashan, 1993). Selected isolates of *A. brasilense* has the potential to enhance the development and growth of several members of the Gramineae family and to increase total shoot and root weights, total-N content, plant height and leaf length (Kapulnik *et al.*, 1981b). Boddey *et al.* (1986) also found that

inoculation of wheat with *A. brasilense* (isolated from surface sterilized wheat roots) and the *A. brasilense* type strain (Sp7) increased wheat grain yield and nitrogen content. Increased nitrogen content was not due to nitrogen-fixation but due to nitrate reductase activity of the bacteria in the roots. After inoculation with *A. brasilense*-Cd ATCC 29729, *Setaria italica* L. showed increased nitrogen content and shoot dry weight.

1.14 Application in combination with other bio-inoculants, chemical fertilizers and hormones

Zahir and Arshad (1996) found that the application of *Azotobacter* in combination with L-tryptophan (an auxin precursor) significantly increased the grain yield (18.4%), fresh biomass (16.7%), 1000-grain weight (14.5%), and plant N uptake (40%) in maize. Wheat inoculated with *Azotobacter* spp. in combination with *Azospirillum* spp. also had increased yields and uptake of nitrogen (Rai and Gaur, 1988). According to Meshram and Shende (1982), a combination of nitrogen fertilization plus an *Azotobacter* inoculant increased yield, and it was economically most efficient at lower doses of N fertilization. Jatasara *et al.* (2000) found that application of nitrogen fertilizer levels of 60kg N ha^{-1} in combination with *Azotobacter* produced higher grain, and straw yield and nitrogen utilization efficiency when growing two varieties of oats. *Azotobacter* either alone, or in combination with ammonium, also had beneficial effects on the yield of wheat, which amounted to saving about 20% of normal application of ammonium (Kader *et al.,* 2002).

Table 1.3 Free-living diazotrophic bacteria for which evidence exists that their stimulation of plant growth is related to their ability to fix nitrogen

Free-living diazotrophs	Relationship to host	Host crop	N-fixed	References
Azotobacter spp	Rhizospheric	Wheat	30%	Kloepper *et al*. (1989)
Azospirillum spp.	Rhizospheric	Maize	58%	Garcia de Salamone and Döbereiner (1996)
Azoarcus spp	Endophytic	Kallar grass	26%	Hurek *et al*. (2002)
Acetobacter diazotrophicus	Endophytic	Sugarcane	60-80%	Boddey *et al*. (1995)
Bacillus spp.	Endophytic	Wheat	43%	Kloepper *et al*. (1989)
Burkholderia vietnamensis	Endophytic	Rice	42%	Govindarajan *et al*. (2007)

1.15 References

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CHAPTER TWO

ISOLATION AND PRELIMINARILY SCREENING OF FREE-LIVING DIAZOTROPHS (FLD) FOR NITROGENASE ACTIVITY

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Science and Agribusiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

Bacteria with the ability to grow on nitrogen-free media, with nitrogenase activity under aerobic conditions, were isolated from soils collected from four different agricultural locations in KwaZulu-Natal (South Africa). Isolates grew as circular, large and small colonies; the cells were Gram-negative rods.

Thirty one diazotrophic isolates that grew well on nitrogen-free media were isolated from rhizosphere soils. All the isolates were screened for nitrogenase activity. The nitrogen-fixing activities were calculated from their C_2H_4 reducing activities. All of the bacterial strains studied had nitrogenase activity, as measured by the acetylene reduction assay method. The activities varied from 0.54 - 150nmo of C_2H_4 h^{-1}, indicating that the levels of N-fixation by the FLD isolates varied from negligible to highly significant ($P<0.001$).

Three different media using mannitol, glucose and ethanol as carbon sources were used to evaluate growth activity of the selected isolates (B3, D6, E9, G3 and L1) that generated greater than 50nmo of C_2H_4 h^{-1}. Variations were observed in the maximal growth rates of nitrogen-fixing isolates with respect to substrates, temperatures and pH levels.

2.1 Introduction

An inexpensive, environmentally-friendly alternative to expensive chemical nitrogen fertilization is biological nitrogen fertilizer (BNF). Research has shown that BNF is the most efficient way to supply the large amounts of nitrogen needed by plants to produce high-yielding crops (Ladha *et al*., 1986).

Biological nitrogen fixation is carried out by symbiotic bacteria, FLD and blue green algae. Nitrogen fixing bacteria (diazotrophs) convert atmospheric nitrogen gas (N_2) to ammonia (NH_3). Nitrogenase is the enzyme that is responsible for nitrogen fixation. Nitrogen-fixation by free-living and associative bacteria commonly occurs in forest soils (Dawson, 1983; Limmer and Drake, 1996). Free-living diazotrophs detected in association with plant roots include *Acetobacter diazotrophicus* Gillis et al*, Herbaspirillum seropedicae* Baldani *et al, Azoarcus spp. and Azotobacter* spp. (Steenhoudt and Vanderleyden, 2000). Kennedy *et al.* (1997) reviewed the use of diazotrophs on cereal crops to achieve considerable levels of nitrogen fixation. The symbiotic association between legumes and the gram negative bacteria collectively called rhizobia is an agriculturally important association. *Rhizobium* spp. usually infect leguminous trees, and shrubs, causing root nodulation. The nitrogen fixing symbiosis between *Rhizobium* spp. and legumes is used to great advantage in agriculture. In contrast, *Frankia* is an actinomycete, which is known for actinorhizal symbioses with non-legumes.

The desire for an inexpensive source of nitrogen for cereals and pasture grasses has focused attention on non-symbiotic nitrogen fixation. Day *et al*. (1975) at Rothamsted (UK) suggested that considerable amounts of nitrogen are routinely fixed by non-symbiotic bacteria in fields of cereal crops.

Members of the Azotobacteraceae family have the ability to fix atmospheric nitrogen non-symbiotically. At present, strains belonging to the species *Azotobacter vinelandii* Lipman and *Azotobacter chroococcum* Beijerinck are being employed as soil inoculants in rainy areas, and warm and alkaline soils (Pandey *et al.,* 1998). They occur in forest litter and in plant rhizospheres (Granhall and Lindberg, 1978). The genus *Azospirillum* is a group of non-symbiotic free-living diazotrophs that lives in association with the roots of members of the grass family, supplying nitrogen and promoting the yield of crops (Ladha *et al.,* 1997).

The family Azotobacteraceae is a group of aerobic nitrogen fixers that is commonly isolated and researched FLDs.

The objective of this research was to isolate a spectrum of FLD bacteria from a mixture of crop rhizospheres, to evaluate their growth in culture, and their nitrogenase activity on nitrogen free media.

2.2 Materials and methods

2.2.1 Origin of soil samples

Nitrogen-fixing bacteria were isolated from rhizosphere soils of three crops (maize, millet and beans) that were grown at the university farm, Ukulinga, the government research station at Cedara and in greenhouses of the University of KwaZulu-Natal, Pietermaritzburg, South Africa.

2.2.2 Isolation, culture and media

FLDs were isolated from rhizosphere soil by placing 10g of soil into one of three media, using mannitol, ethanol and glucose as carbon sources in a liquid culture, incubated at room temperature for seven days in the dark. Serial dilutions were then preformed and plated onto the same solid media, as described by Aquilanti *et al*. (2004). FLDs were recognized by the appearance of slimy, glistening colonies. All isolates were purified by streaking on to tryptic soy agar (TSA) plates.

2.2.3 Screening of isolates

Isolates were gram stained, then screened for growth and colony morphology on N-free media, as used by Turner and Gibson (1980) and Thompson and Skerman (1979), at pH 6.0, 7.0 and 8.0, containing mannitol, ethanol or glucose as their sole carbon sources. Colony morphology was recorded after 7 days incubation at temperatures of 20, 25 and 30^0C. The colonies were quantified as Colony Forming Units (CFU) per ml.

2.2.4 Nitrogenase activity

The nitrogen-fixing isolates were grown under nitrogen-fixing conditions (Thompson and Skerman, 1979; Bergersen, 1980). The isolates were then evaluated for their ability to fix nitrogen on nitrogen-free medium by the acetylene reduction assay. One colony of each isolate was placed onto N-free Burke's medium (with mannitol as the carbon source) and agitated for 48h at 150rpm. Ten milliliters of each isolate culture was placed in a 29ml bottle and closed with a red rubber septum (SIGMA-ALDRICH, William Freeman and Co., Ltd.). Two ml of acetylene gas was injected by a gas-tight syringe through the rubber stopper into the bottle which was then incubated for 2h at room temperature. An uninoculated bottle containing free-nitrogen medium was injected with 2ml acetylene to serve as a control. Ethylene concentration was analyzed by injecting a 0.5ml gas sample into a gas-chromatograph equipped with a flame ionization detector. The temperature of the detector, injection port and oven were 150, 100 and 50^0C respectively.
Nitrogenase activity was expressed as nmo of ethylene formed per culture per hour, and was calculated according to a modified Turner and Gibson (1980) equation:

$A = (C_2H_4 \times (PV/RT))/2$, where:
A = nitrogenase activity (nM C_2H_4 per culture per hour],
C_2H_4 = ethylene peak area,
P = atmospheric pressure in atmospheres (1.0),
V = volume of acetylene injected into the culture tube (liters),
R = gas constant 82.054ml-atm/mol ^{0}K,
T = temperature in Kelvins (273^0C).

2.2.5 Statistical analysis

A general linear model (GLM) was used to run an ANOVA on the C_2H_4 production and the interaction of FLD isolates x media x temperature x pH. If the ANOVA was significant, ($P<0.05$), then the means were compared using the least significant difference (LSD) test.

2.3 Results

2.3.1 Isolation

Isolation of FLD bacteria was originally conducted using nitrogen free media, as described by Thompson and Skerman (1979), Bergensen (1980) and Turner and Gibson (1980). Isolates that were able to growth on these media were selected. Numerous gelatinous, slimy white, large and small colonies were found to consist of small and large sized rods.

2.3.2 Growth of FLD in liquid enrichment cultures

In order to isolate FLD bacteria, nitrogen-free media was used with ethanol, mannitol or glucose as the carbon source. After seven days of stationary incubation at room temperature in the dark, the color of the cultures with mannitol or glucose as the carbon sources changed to milky-white and there was slight growth on the walls of liquid. Bubbles were formed in the medium with ethanol as a carbon sources (Fig. 1.1).

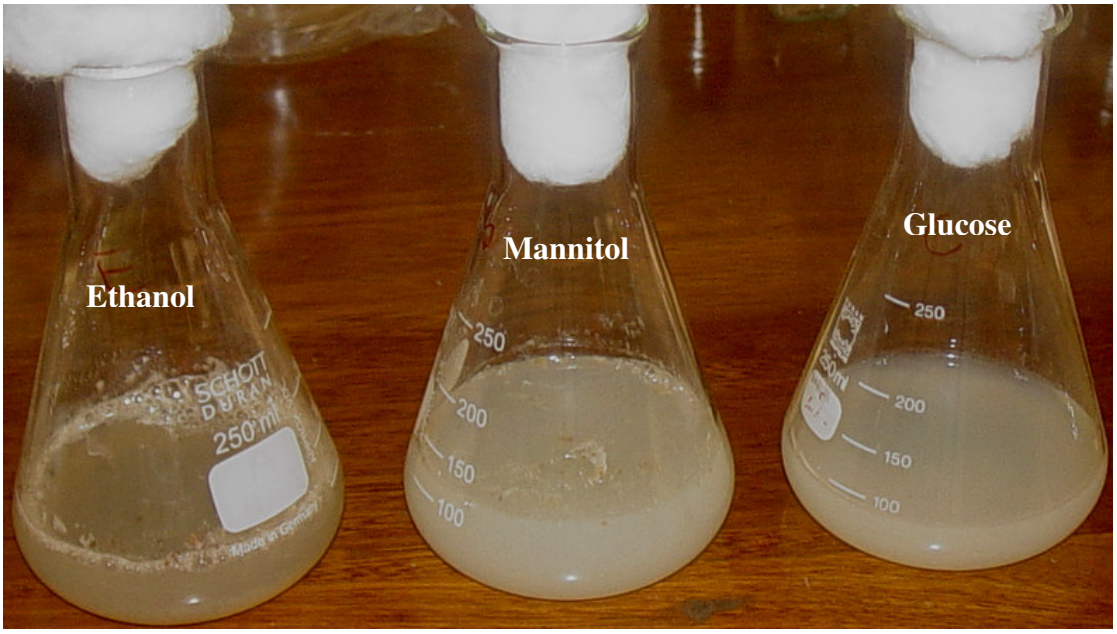

Fig 1.1 Growth of free-living diazotrophs on different liquid media

2.3.3 In vitro Screening of FLD Isolates on N-free media

From the enrichment cultures, FLDs were then purified onto similar solid media and were tested for colony formation. Isolates formed large, white, translucent, circular, smooth, milky and slightly raised colonies on mannitol agar, whereas on glucose agar the colonies were relatively smaller, transparent moist, soft and with an irregular edge (Fig. 1.2). On ethanol agar plates, the colonies were small, circular, and slimy and white (Fig. 1.2).

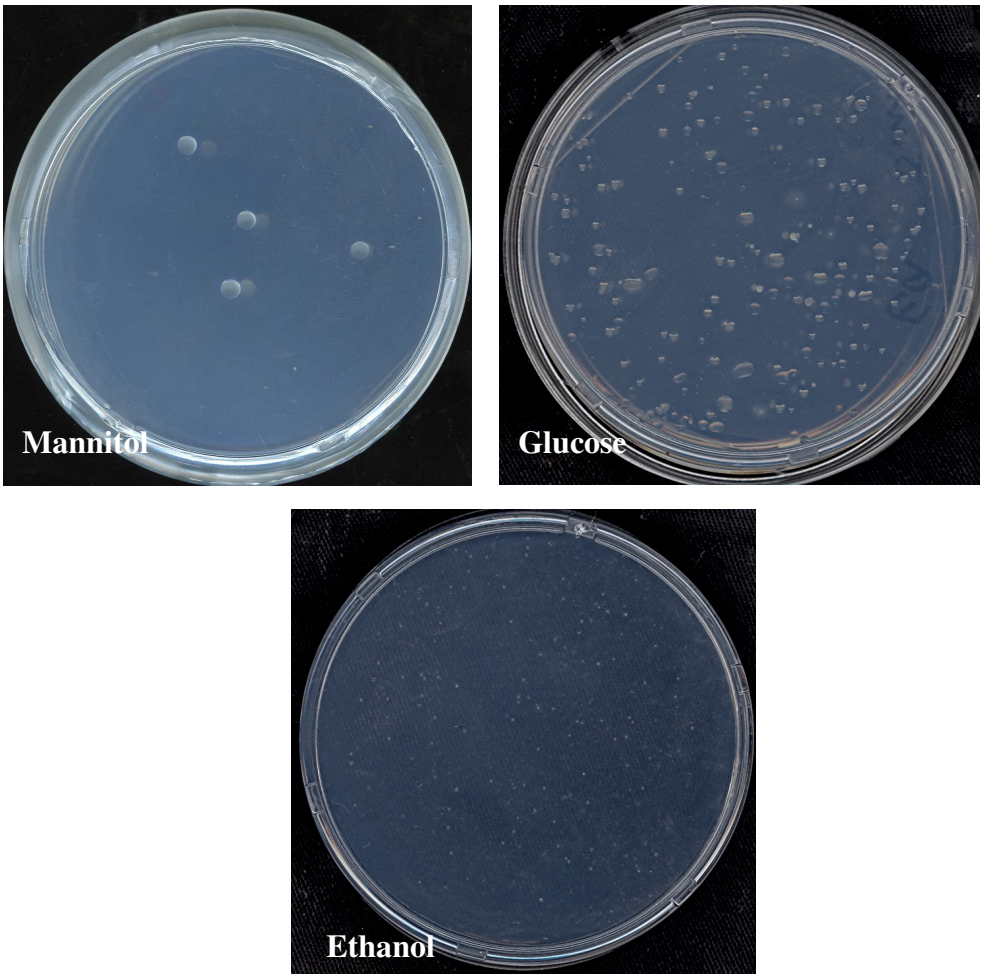

Fig 1.2 Colonial morphology of FLD isolates grown on different nitrogen-free media after 48h incubation at 28^0C.

2.3.4 Microscopic observation on the morphological characteristics of FLD isolates

Microscopically cells of Isolate B3 on TSA were short, almost spherical, and motile; occurring singly, in pairs or in chains, and was Gram negative. Cells of Isolate E9 were large cocci and motile. Isolate L1 cells occurred singly or in pairs, and were Gram-positive. Cells of Isolate D6 were large, motile rods, and cells of Isolate G3 were also rods, motile and Gram-positive (Fig. 1.3, 1.4, 1.5).

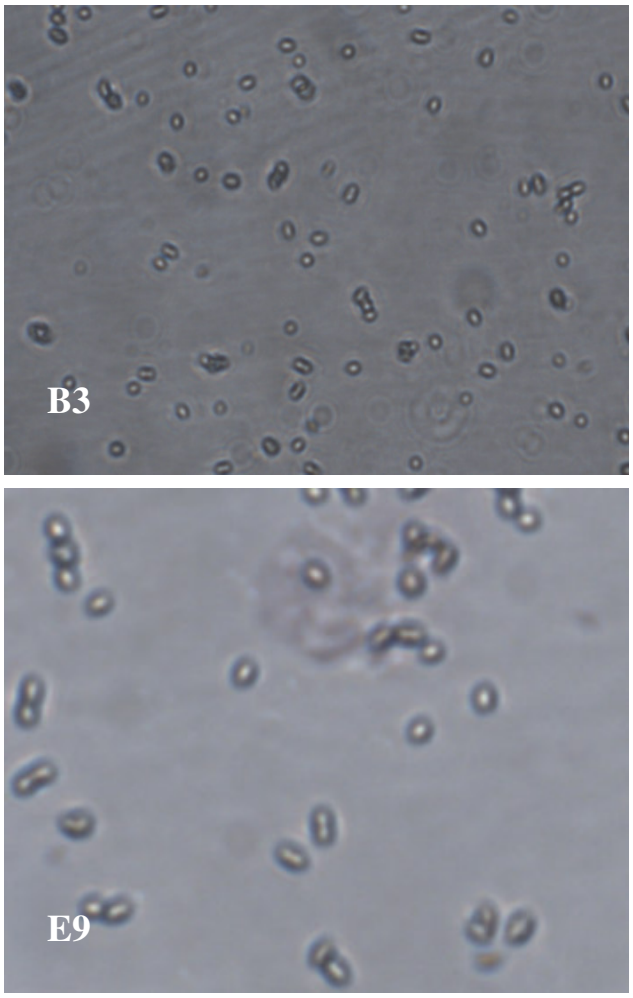

Fig 1.3 Photomicrographs of selected diazotrophic bacteria. Cells of diazotrophic isolates (B3 and E9) grown on TSA medium. All plates were inoculated from a single colony and incubated at 28^0C for 48h (x400 magnification).

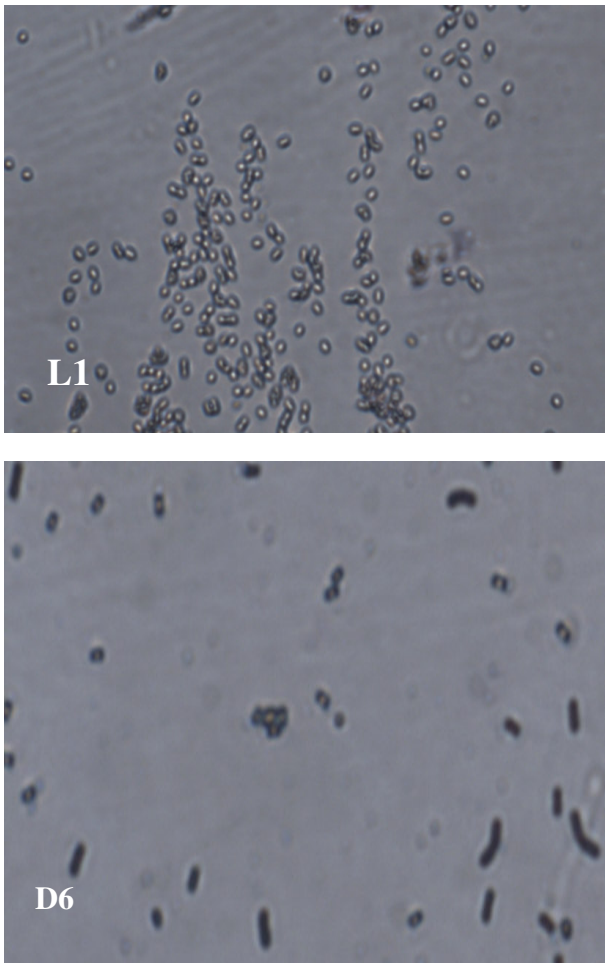

Fig 1.4 Photomicrographs of selected diazotrophic bacteria. Cells of diazotrophic Isolates L1 and D6 grown on TSA medium. All plates were inoculated with single colony and incubated at 28^0C for 48h (x400 magnification)

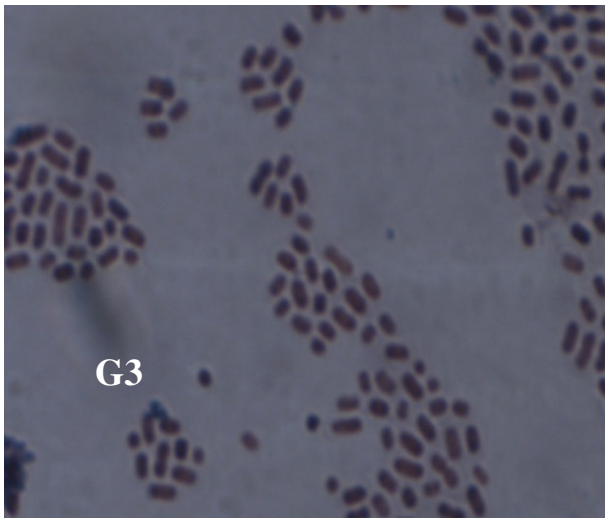

Fig 1.5 Photomicrographs of selected diazotrophic bacteria. Cells of Isolate G3 grown on TSA medium. All plates were inoculated with single colony and incubated at 28^0C for 48h and (x400 magnification).

2.3.5 Nitrogenase activity of FLD isolates

FLDs isolated from nitrogen-free media were tested for their nitrogenase activity using a method described by Turner and Gibson (1980).

The FLDs produced ethylene within the range of 0.058 – 152.45nmo of C_2H_4 per hour (Table 2.1). Of the 32 isolates, 18 produced less than 20nmo of C_2H_4 per hour and only five had greater than 50nmo of C_2H_4 per hour (Table 2.1).The first 12 isolates produced very small amount of ethylene with no significant differences (within the range 0.24 – 3.45nmo of C_2H_4 per hour). Isolates Br2 and F7 produced 18.67 and 20.12nmo of C_2H_4, respectively. Isolate F2 produced significantly more C_2H_4 (26.67 nmo of C_2H_4 h^{-1}). Greater than 50nmo of C_2H_4 $hour^{-1}$ were recorded from Isolates E9, B3, G3, L1 and D6 (Table 2.1 and Appendix 2.1, Fig. 2.1).

Table 2.1 Preliminary screening of FLD isolates for their nitrogenase activity (nmo C_2H_4 h^{-1}) measured by the Acetylene Reduction Assay (ARA) after two hours incubation

FLD Isolates	ARA nmo C_2H_4 h^{-1}		FLD Isolates	ARA nmo C_2H_4 h^{-1}	
Control	0.24	a [1]	MA2	4.37	Cd
O25	0.58	a	L6	4.98	D
K	1.16	abc	Br2	18.67	E
K3	1.20	abc	F7	20.12	E
A8	1.74	abcd	F2	26.67	F
B1	1.83	abcd	L2.0	30.49	G
SB3	1.83	abcd	F5.0	32.52	G
G3.2	1.85	abcd	L4	32.62	G
MA14	2.20	abcd	F5	38.22	H
E1	2.47	abcd	F5.5	38.55	H
G2.0	2.61	abcd	L2	44.52	I
K1	2.67	abcd	E9	50.94	J
H01	3.10	abcd	B3	81.05	K
O13	3.45	abcd	G3	89.40	L
M6	3.73	bcd	L1	150.47	M
K6	4.17	cd	D6	152.45	M
P	<0.001				
DMRT	5.2				
s.e.d.	1.735				
c.v.%	8.0				

1. Means followed by the same letter are not significantly different, using Duncan's New Multiple Range Test at alpha <0.05

2.3.6 Growth of FLD on different media, temperature and pH

Isolation of free-living nitrogen fixing bacteria was originally conducted using mannitol, glucose and ethanol as the carbon sources, as described by Bergensen (1980) and Thompson and Skerman, (1979).

The putative nitrogen-fixing bacteria isolates with high C_2H_4 levels, measured using ARA, were retained for further screening as nitrogen fixing bacteria. Some of the other bacterial isolates were stored at -80^{0}C on 65% v/v glycerol in Tryptone Soy Broth (TSB) but the least active isolates were discarded.

Isolates that generated more than 50 nmo of C_2H_4 hr^{-1} (Table 2.1) were used to determine growth activity on different media, held at different temperatures and with different pH values.

Isolate E9 grew well on the ethanol medium at 25^0C and at pH 8.0 and also grew well on the ethanol and glucose media at 30^0C and pH 6.0 and 7.0 (Table 2.2). Isolates L1 and G3 grew well on the ethanol medium at all ranges of temperature and pH values (Table 2.2).This isolate also grew well on Jensen's Medium at 20^0C pH 7.0 and at 25^0C and all pH values, and slightly less vigorous growth on Burke's Medium at 20^0C and pH 7.0 and 8.0. On the other hand, Isolate E9 grew poorly on the mannitol medium at all evaluated temperatures and pH.

Isolate B3 grew well on Burke's and Jensen's Media at all temperatures and pH 6.0 and 7.0 (Table 2.2).The best growth was shown by Isolate G3 and L1 on ethanol medium at all ranges of temperature and pH (Table 2.2). Isolate B3 grew significantly less on ethanol medium except at 20^0C pH of 7.0 (Table 2.2).

Table 2.2 Colony Forming Units (CFU) of FLD bacterial isolates grown at different temperatures and pH values on various N-free solid media after a seven day incubation period

Bacteria	Media	T (C^0)	pH	Growth (CFU) x10^4	
E9	Burke's (mannitol)	20	6	71.33	lmnopqrstuvwxyzabcdefghijklmno
			7	37.67	Abcdef
			8	57.33	Cdefghijklmnopqrstuvwx
		25	6	71.33	lmnopqrstuvwxyzabcdefghijklmno
			7	69.33	jklmnopqrstuvwxyzabcdefghijklm
			8	59.67	Efghijklmnopqrstuvwxyza
		30	6	43.00	Cdefghij
			7	63.67	Fghijklmnopqrstuvwxyzabcdef
			8	53.00	Cdefghijklmnopqrstu
	Ethanol	20	6	39.33	Bcdefg
			7	32.67	Abcd
			8	31.67	Abc
		25	6	70.33	klmnopqrstuvwxyzabcdefghijklmn
			7	83.33	Xyzabcdefghijklmnop
			8	90.33	Fghijklmnop
		30	6	89.33	Efghijklmnop
			7	77.00	Stuvwxyzabcdefghijklmnop
			8	71.00	lmnopqrstuvwxyzabcdefghijklmno
	Jensen's (glucose)	20	6	66.33	hijklmnopqrstuvwxyzabcdefghi
			7	57.33	Cdefghijklmnopqrstuvwx
			8	48.33	Cdefghijklmno
		25	6	47.00	Cdefghijklmn
			7	68.00	hijklmnopqrstuvwxyzabcdefghijk
			8	84.33	Yzabcdefghijklmnop
		30	6	83.67	Xyzabcdefghijklmnop
			7	45.00	Cdefghijkl
			8	46.33	Cdefghijklm
D6	Burke's (mannitol)	20	6	53.00	Cdefghijklmnopqrstu
			7	76.67	Rstuvwxyzabcdefghijklmnop
			8	79.67	Uvwxyzabcdefghijklmnop
		25	6	38.00	Abcdef
			7	48.67	Cdefghijklmnop
			8	76.00	Qrstuvwxyzabcdefghijklmnop
		30	6	67.00	hijklmnopqrstuvwxyzabcdefghij
			7	66.00	hijklmnopqrstuvwxyzabcdefghi
			8	53.00	Cdefghijklmnopqrstu
	Ethanol	20	6	60.00	Efghijklmnopqrstuvwxyza
			7	50.00	Cdefghijklmnopqr
			8	51.00	Cdefghijklmnopqrs
		25	6	31.67	Abc
			7	49.67	Cdefghijklmnopq
			8	44.00	Cdefghijk
		30	6	48.67	Cdefghijklmnop
			7	56.33	Cdefghijklmnopqrstuvw
			8	76.67	Rstuvwxyzabcdefghijklmnop
	Jensen's (glucose)	20	6	59.33	Efghijklmnopqrstuvwxyz
			7	62.67	Fghijklmnopqrstuvwxyzabcde
			8	45.67	Cdefghijklm
		25	6	68.33	ijklmnopqrstuvwxyzabcdefghijkl
			7	41.33	Cdefgh
			8	51.67	Cdefghijklmnopqrst
		30	6	68.67	ijklmnopqrstuvwxyzabcdefghijkl
			7	90.33	Fghijklmnop
			8	85.67	Zabcdefghijklmnop

Table 2.2 continued

			6	65.67	Ghijklmnopqrstuvwxyzabcdefghi
		20	7	67.00	Hijklmnopqrstuvwxyzabcdefghij
			8	82.33	Wxyzabcdefghijklmnop
			6	82.33	Wxyzabcdefghijklmnop
	Burke's (mannitol)	25	7	53.67	Cdefghijklmnopqrstuv
			8	58.33	Defghijklmnopqrstuvwxy
			6	82.67	Wxyzabcdefghijklmnop
		30	7	69.33	Jklmnopqrstuvwxyzabcdefghijklm
			8	69.33	Jklmnopqrstuvwxyzabcdefghijklm
			6	64.00	Fghijklmnopqrstuvwxyzabcdefg
		20	7	57.33	Cdefghijklmnopqrstuvwx
			8	56.00	Cdefghijklmnopqrstuvw
			6	66.67	Hijklmnopqrstuvwxyzabcdefghi
B3	**Ethanol**	25	7	74.67	Opqrstuvwxyzabcdefghijklmnop
			8	72.33	Mnopqrstuvwxyzabcdefghijklmnop
			6	74.67	Opqrstuvwxyzabcdefghijklmnop
		30	7	85.67	Zabcdefghijklmnop
			8	47.67	Cdefghijklmn
			6	57.67	Cdefghijklmnopqrstuvwxy
		20	7	68.00	Hijklmnopqrstuvwxyzabcdefghijk
			8	71.00	Lmnopqrstuvwxyzabcdefghijklmno
			6	14.33	A
	Jensen's (glucose)	25	7	17.00	Ab
			8	42.33	Cdefghi
			6	61.33	Fghijklmnopqrstuvwxyzabc
		30	7	72.33	Mnopqrstuvwxyzabcdefghijklmnop
			8	91.67	Hijklmnop
			6	94.00	Klmnop
		20	7	90.67	Ghijklmnop
			8	84.33	Yzabcdefghijklmnop
			6	87.33	Bcdefghijklmnop
	Burke's (mannitol)	25	7	88.67	Defghijklmnop
			8	58.00	Defghijklmnopqrstuvwxy
			6	60.67	Fghijklmnopqrstuvwxyzab
		30	7	90.33	Fghijklmnop
			8	97.00	Nop
			6	58.33	Defghijklmnopqrstuvwxy
		20	7	82.67	Wxyzabcdefghijklmnop
			8	69.33	jklmnopqrstuvwxyzabcdefghijklm
			6	69.33	jklmnopqrstuvwxyzabcdefghijklm
L1	**Ethanol**	25	7	57.67	Cdefghijklmnopqrstuvwxy
			8	68.00	hijklmnopqrstuvwxyzabcdefghijk
			6	71.00	lmnopqrstuvwxyzabcdefghijklmno
		30	7	58.00	Defghijklmnopqrstuvwxy
			8	60.67	Fghijklmnopqrstuvwxyzab
		20	6	58.33	Defghijklmnopqrstuvwxy
			7	82.67	Wxyzabcdefghijklmnop
			8	69.33	jklmnopqrstuvwxyzabcdefghijklm
		25	6	69.33	jklmnopqrstuvwxyzabcdefghijklm
	Jensen's (glucose)		7	57.67	Cdefghijklmnopqrstuvwxy
			8	68.00	hijklmnopqrstuvwxyzabcdefghijk
		30	6	71.00	lmnopqrstuvwxyzabcdefghijklmno
			7	58.00	Defghijklmnopqrstuvwxy
			8	60.67	Fghijklmnopqrstuvwxyzab

Table 2.2 continued

			6	87.67	Cdefghijklmnop
		20	7	82.00	Wxyzabcdefghijklmnop
			8	75.67	Qrstuvwxyzabcdefghijklmnop
			6	65.33	Ghijklmnopqrstuvwxyzabcdefgh
	Burke's (mannitol)	25	7	66.00	Hijklmnopqrstuvwxyzabcdefghi
			8	82.33	Wxyzabcdefghijklmnop
			6	42.33	Cdefghi
		30	7	34.00	Abcde
			8	42.33	Cdefghi
			6	80.00	Vwxyzabcdefghijklmnop
		20	7	94.00	Klmnop
			8	96.67	Nop
			6	97.67	Op
G3	**Ethanol**	25	7	95.00	Lmnop
			8	86.33	Abcdefghijklmnop
			6	92.33	Ijklmnop
		30	7	98.33	P
			8	94.00	Klmnop
			6	78.33	Tuvwxyzabcdefghijklmnop
		20	7	70.67	Klmnopqrstuvwxyzabcdefghijklmn
			8	88.00	Cdefghijklmnop
			6	91.67	Hijklmnop
	Jensen's (glucose)	25	7	81.67	Wxyzabcdefghijklmnop
			8	62.33	Fghijklmnopqrstuvwxyzabcd
			6	59.33	Efghijklmnopqrstuvwxyz
		30	7	63.00	Fghijklmnopqrstuvwxyzabcde
			8	86.33	Abcdefghijklmnop

P	$<0.001^{***}$
DMRT	162.1
s.e.d	10.4
Cv%	18.7

Effect	**F - Value**	**P – Value**	**s.e.d**
Bacteria	40.98	$<0.001^{***}$	1.997
Media	1.3	0.274^{ns}	1.547
Temperature	11.21	$<0.001^{***}$	1.547
pH	0.03	0.975^{ns}	1.547
Bacteria. Media	16.01	$<0.001^{***}$	3.458
Bacteria. Temperature	12.2	$<0.001^{***}$	3.458
Media. Temperature	5.68	$<0.001^{***}$	2.679
Bacteria. pH	1.88	0.064^{ns}	3.458
Media. pH	4.99	$<0.001^{***}$	2.679
Temperature. pH	0.7	0.596^{ns}	2.679
Bacteria. Media. Temperature	6.4	$<0.001^{***}$	5.99
Bacteria. Media. pH	2.08	0.009^{**}	5.99
Bacteria.Temperature.pH	4.14	$<0.001^{***}$	5.99
Media.Temperature.pH	3.19	0.002^{**}	4.64
Bacteria. Media. Temperature. pH	3.69	$<0.001^{***}$	10.374

Description: *** highly significant, ^{ns} not significant, T = Temperatures (20, 25 and 30^0C), Media: N_2-free media (mannitol, ethanol and glucose as carbon sources); pH (6.0, 7.0 and 8.0); CFU= Colony forming units. Means followed by the same letter are not significantly different using Duncan's New Multiple Range Test at alpha 0.05.

2.4 Discussion

Thirty five strains of FLDs were initially isolated from rhizospheric soils on nitrogen free media containing ethanol, mannitol or glucose as the carbon source, according to the method of Thompson and Skerman (1979). Pochon (1954) isolated 45 different strains of FLD bacteria (*Azotobacter* sp.) on N-free medium with mannitol as the carbon source. Similarly, Jensen (1965) isolated *Azotobacter* spp. using the same carbon source. Ahmed *et al*. (2006) also isolated *Azotobacter* spp. using the same medium with sucrose as the carbon source. These isolates were tested for nitrogenase activity using GC analysis, as described by Turner and Gibson (1980). It appears that many FLD strains are present in soils globally. Five of the local isolates (B3, E9, L1, D6 and G3) produced substantial levels of ethylene. If an appropriate isolation protocol is followed, then it is relatively easy to discover strains of FLD bacteria in local soil samples.

The five isolates producing the most ethylene did not appear to be related to each other, based on their colony growth patterns on different media. Furthermore, cells of these isolates were both Gram positive and Gram negative. There were also significant differences between the growth of the isolates at different temperature, pH and media with each of the carbon sources. This is in agreement with findings of Jensen (1965) and Eckert *et al.* (2001). These isolates were further screened in the greenhouse, and were identified using DNA sequencing, as discussed subsequently.

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Appendix 2.1

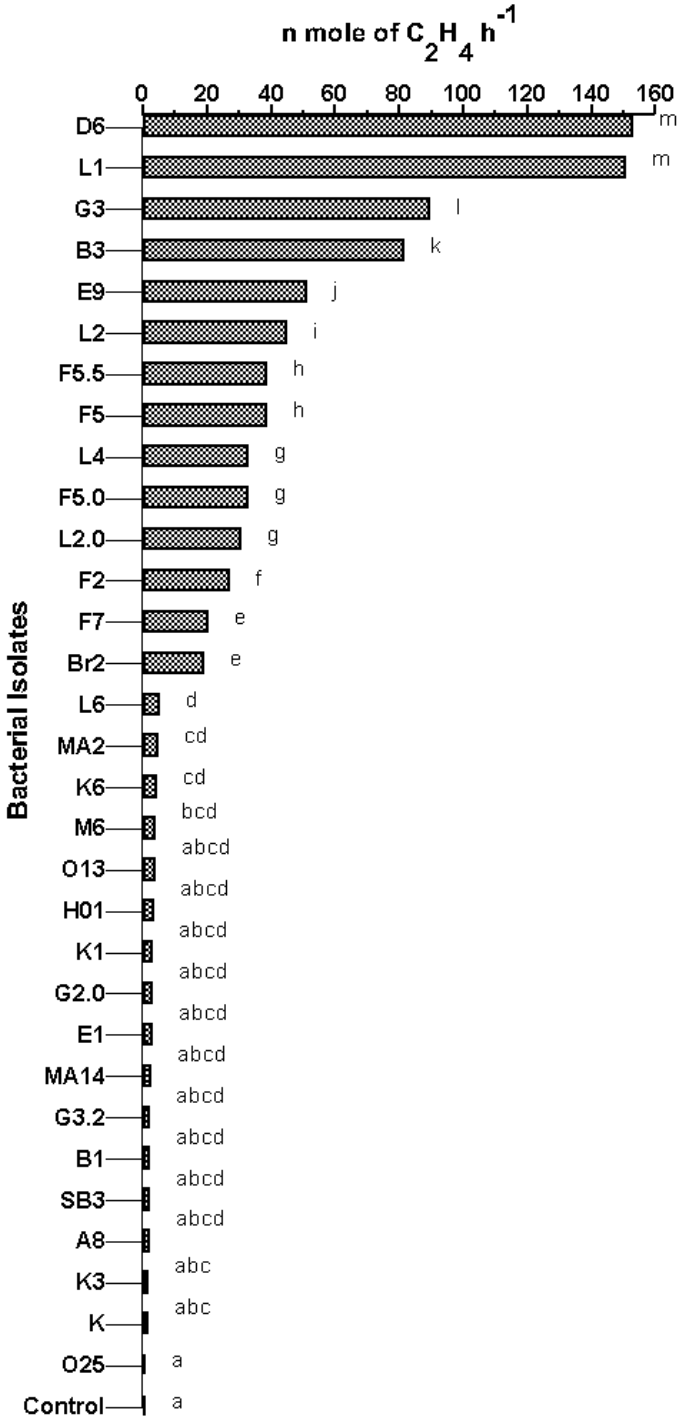

Fig 1.6 Potential for nitrogen fixation by bacterial isolates grown on a nitrogen free medium (with mannitol as the carbon source), evaluated from their nitrogenase activity (nmo C_2H_4 h^{-1})

CHAPTER THREE

SCREENING OF FREE-LIVING DIAZOTROPHS (FLD) AS BIOFERTILIZERS UNDER GREENHOUSE CONDITIONS

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Science and Agribisiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

Free-living nitrogen fixing (diazotrophic) (FLD) bacterial isolates that had showed the ability to fix nitrogen *in vitro* were screened as biofertilizers under greenhouse conditions on several crops. These FLDs were applied as a seed treatment and as a drench, except for petunia that was only treated by drenching. Increased growth, yield and nitrogen contents resulting from inoculation of plants with the FLD isolates were attributed to nitrogen fixation by the inoculants. The effects arising from FLD inoculations were different for different crops and bacterial isolates. Application by drenching of all FLD isolates significantly increased wet and dry biomass, yield and plant N of maize, wheat and sorghum ($P<0.001$). They also significantly increased lettuce, zucchini and petunia biomass, root weight and plant N, when compared to the untreated and unfertilized control. However, Isolate G3 (*Burkholderai ambifaria* Coenye et al.) caused no significant increase in plant N when applied by drench onto zucchini and petunia seedlings.

Seed treatments with the FLD isolates caused a highly significant increase in wet and dry biomass, yield and plant N of maize, sorghum and wheat and lettuce crops. However, plant N of lettuce seedling inoculated by seed treatment was not significantly different from the untreated control. There were significant differences in the efficacy of different FLD inoculants ($P<0.001$) that was reflected in differences in wet and dry biomass weight of zucchini when applied by seed treatment. However, there was no significantly difference in plant N.

3.1 Introduction

Microorganisms are important in agriculture in order to promote the recycling of plant nutrients and to reduce the need for chemical fertilizers. Nitrogen-fixing bacteria are important for plant nutrition by increasing N uptake by the plants, and play a significant role in the bio-fertilization of crops. Biological N fixation provides a major source of nitrogen for plants, which plays a key role in environmental friendly agricultural practices.

Members of the Azotobacteraceae family have the ability to fix atmospheric nitrogen non-symbiotically. At present, strains belonging to the species *Azotobacter vinelandii* Lipman and *Azotobacter chroococcum* Beijerinck are employed as soil inoculants in rainy regions and in warm and alkaline soils (Pandey *et al*., 1998). *Burkholderia* species are known to exhibit activities involved in nitrogen-fixing, bioremediation, plant growth promotion, or biological control *in vitro* (Caballero-Mellado *et al*., 2007). Plant–associated *Bacillus* spp., sometimes referred to as yield-increasing bacteria (YIB), have also been used to increase yields of many crop plants (Shen, 1997).

Members of the genus *Azotobacter* are obligate aerobic, heterotrophic, free-living diazotorophs (FLD), with the capacity to produce plant growth stimulants (Thompson and Skerman, 1979). The members of the genus have been developed as a crop inoculants for cereals (Wani *et al*.,1988), oil seeds, cotton and vegetables because of their ability to produce plant growth regulators (Dobbelaere *et al*., 2003), siderophores and to excrete ammonia (Garg *et al*., 1997).

Brown *et al*. (1964) found that tomato growth increased when the crop was inoculated with *Azotobacter.* Similar results were obtained by Rovira (1965) with wheat following inoculation with *A. chroococcum*, *Clostridium pasteurianum* Winogradsky and *Bacillus polymyxa* (Prazmowski) Mace. Yields of rice in field trials increased with applications of *Azotobacter spp*. (Yanni and El-Fattah, 1999). Narula *et al*. (2005) noted that yields increased when cotton plants were inoculated with *Azotobacter* spp.

According to Yanni and El-Fattah (1999), nitrogen accumulation by rice plants increased when inoculated with *Azotobacter spp.* Similarly, inoculation of seeds with *A. chroococcum* increased the weight and N content of panicles of *Setaria italica* (L.) (Yahalom *et al*., 1984*)*. Inoculation with *Azospirillum brasilense* Corrig. Tarrand et al. significantly increased cotton

plant height and dry weight and increased N uptake by cotton plant up to 0.91mg per plant (Fayez and Daw, 1987).

Inoculation with *A. brasilense* increased wheat grain yield by up to 30% and other yield components significantly under field conditions (Dobbelaere *et al*., 2002; Rao and Charyulu, 2005), but only at lower rates of fertilizer-N (50–60kg N per ha). Sixty to 80% of sugarcane nitrogen content is derived from BNF and it believed that *Acetobacter diazotrophicus* Gillis et al. is responsible for much of this BNF (Boddey and Döbereiner, 1995).
The objective of this greenhouse study was to evaluate the effects of five FLD isolates for their nitrogen fixing potential on several crops. The five FLD isolates were: B3 (*Burkholderia sp*.), D6 (*Bacillus cereus*), E9 (*Burkholderia cepacia*), G3 (*B. ambifaria*) and L1 (*B. cereus*).

3.2 Materials and methods

3.2.1 Source of free-living diazotrophs

FLD isolates were isolated from soils where vegetables, maize, millet and beans had previously been cultivated, and were cultured on N-free media, according to Bergensen (1980) and Thompson and Skerman (1979). Details of these media are in Appendix A. One gram of soil was added to 10ml distilled water and serial dilutions were made (10^4). Aliquots 0.1ml from each dilution were plated in triplicate, on to nitrogen-free agar plates (with mannitol, glucose or ethanol as carbon sources), using the spread plate method, and then incubated at 28 ± 2^0C for 5 days. Five isolates were selected for further experimenting based on their levels of nitrogenase activity. These isolates were identified by Inqaba Biotechnical Industries (Pty) Ltd[1]., based on BLAST analysis of their genomes.

3.2.2 Preparation of cell suspensions

Free-living diazotrophic isolates were cultured separately in 250ml conical flasks containing 100ml of sterilized tryptone soy broth (Merck) medium. Each flask was inoculated with a

[1] Inqaba Biotechnology Industries (Pty) Ltd., P.O. Box 14356, Hattfield 0028, Pretoria, South Africa.

loop full of an isolate previously cultured on tryptone soy agar (TSA) (Merck) (28^0C, 48h). Three replicates were made for each isolate. The flasks were incubated at 30^0C for 72h in a water bath shaker at 150rpm (GFL® 1083, Labortechnik). Cell suspensions were centrifuged at 10,000 x g for 20min (Beckman J2 HS centrifuge). Cell pellets were then re-suspended and washed twice with sterile distilled water. Final cell pellets were diluted with sterile distilled water (approximately 500ml). Cell numbers were adjusted to approximately 10^8cfu ml^{-1} for each of the free-living diazotrophic isolates as determined by dilution plating and use of a counting chamber. This procedure was repeated each time fresh cell suspensions were needed.

3.2.3 Crops evaluated

Seeds of yellow grain maize (*Zea mays* L Colorado; Sahara Type; Lot.No. YR001YO); sorghum (*Sorghum bicolor* (L) Moench); wheat; lettuce (Great Lakes) were obtained from McDonalds Seed Company (Pty) Ltd[2]. Seeds of zucchini (*Cucurbita pepo* L, F1-Hybrid Squash Partenon), were obtained from Starke Ayres Seed Company[3]. Petunia seedlings were obtained from Sunshine Seedlings[4].

3.2.4 Application methods

3.2.4.1 Seed treatment

The prepared cell suspension (10^8cells ml^{-1}) of each free-living diazotrophic isolate was added separately to each of the five conical flasks and labeled. Two grams of a sticker, gum Arabica[5]; were dissolved in 100ml of tap water, stirred and allowed to stand for 1h. This allowed the gum to dissolve and to form a homogeneous suspension. The suspension was divided into five 250ml beakers, each containing 20ml aliquots of the sticker. Twenty milliliters of the FLD isolate's cell suspensions were added into each flask that already contained the 20ml aliquots of sticker. This resulted in a total volume of 40ml of bacterial-sticker suspension in each of the five beakers.

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[2] McDonalds[2] Seed Company (Pty) Ltd., Pietermaritzburg, Republic of South Africa
[3] Starke Ayres Seed Company, P.O.Box 304, Eppindust 7475, South Africa
[4] Sunshine Seedling Service, Pietermaritzburg, Republic of South Africa
[5] gum Arabica, SIGMA

An appropriate number of maize seeds was placed separately into each of the five bacterial suspensions and stirred. The seeds were left for two hours to allow adhesion of bacteria to the seed coat. The treated seed were placed on paper towels and air-dried overnight. The treated seeds were planted into pots filled with composted pine bark. There were three pots for each replicate and three replicates for each treatment giving 3x3x5 total of 45 pots. The pots were watered every day with a fertilized solution (Table 3.1) and of 0.02g l^{-1} of balanced micronutrients (Microplex, Ocean Agriculture Muldersdrift, South Africa)[6]. All pots were left in a greenhouse tunnel (20-30^{0}C) till harvest.

Table 3.1 Fertilizer used with out Nitrogen

Source	**K**	**P**	**Ca**	**Mg**
KH_2PO_4 (220mg l^{-1})	63 mg l^{-1}	50 mg l^{-1}	-	-
KCl (324mg l^{-1})	170 mg l^{-1}	-	-	-
KSO_4 (149mg l^{-1})	67 mg l^{-1}	-	-	-
$CaCl_2$.6(H2O) (1095mg l^{-1})	-	-	200 mg l^{-1}	-
$MgSO_4.7H_2O$ (203mg l^{-1})	-	-	-	20 mg l^{-1}
Total required	300 mg l^{-1}	50 mg l^{-1}	200 mg l^{-1}	20 mg l^{-1}

The plants were harvested at their base level and weighed their wet weight. Subsequently wet biomass was dried at 70^{0}C for 48h and dry weight of seedlings per plot (pot) was measured. Dried biomass was taken for plant-N analysis. Only above-ground stems and leaves were weighed. Lettuce and petunia root dry weight were measured, together with zucchini fresh fruit yield. Each experiment was repeated at least twice.

3.2.4.2 Drenching

For drenching, the cell suspension of the FLD isolates was counted using a haemocytometer and adjusted to 10^8cells ml^{-1}. Then each seedling in a pot was inoculated with 10ml of FLD cell suspension. Each treatment had three replicates and each replicate had three pots that were inoculated (3x3= 9 pots).

[6] Microplex, Ocean Agriculture (Pty) Ltd. P.O.Box 741, Muldersdrift, 1747, South Africa

Each of the five broth cultures was separately dispensed, and 4ml aliquots were directly drenched onto the base of the plants. Each treatment had three replicates and each replicates had three pots that were inoculated per drench volume, and per FLD isolates. This procedure was repeated each week for four weeks.

3.2.4.3 Controls

Two controls were set up in this study. In the first, untreated seeds were planted in nine pots filled with composted pine bark. These were labeled as "control-none" and received water only. In the second, the other nine pots served as a positive control and were supplemented weekly with fertilizer (N: P: K; 3:1:3(38) complete)[7] and was labeled as "NPK".

Treatments:

1. Isolates E9 (*Burkholderia cepacia* Frankland) spore suspension at 10^8cells ml^{-1}
2. Isolates D6 (*Bacillus cereus* Frankland) spore suspension at 10^8cells ml^{-1}
3. Isolates G3 (*Burkholderia ambifaria* Coenye et al.) spore suspension at 10^8cells ml^{-1}
4. Isolates B3 (*Burkholderia* sp.) spore suspension at 10^8cells ml^{-1}
5. Isolates L1 (*Bacillus cereus* Frankland) spore suspension at 10^8cells ml^{-1}
6. Control-none (uninoculated and unfertilized)
7. Control- NPK (fertilized with NPK and uninoculated)

Seedlings from each pot were harvested at maturity at their base and placed in a brown paper bag. The plant material was subsequently dried in an oven at 55^0C. Once dried, the content of each bag was weighed and the mean weight per plant was calculated. The above procedure was conducted on maize, sorghum, wheat, lettuce, zucchini and petunia.

3.2.5 Nitrogen analysis

The micro-Kjeldahl technique of Willis *et al*. (1996) was followed. See Appendix 2 for details.

3.2.6 Statistical analysis

[7] Ocean Agriculture (Pty) Ltd. P.O.Box 741, Muldersdrift, 1747, South Africa

A general linear model (GLM) was used to run an ANOVA on the wet biomass and dry weight of seedlings, root weight yield and plant N. If the F test was significant at $P < 0.05$, then the means were compared using a least significant difference (LSD) test.

3.3 Results

3.3.1 FLD isolates applied as drenches to maize seedlings

FLD isolates drenched to maize significantly increased in wet and dry weight of maize seedlings relative to the untreated and unfertilized control (P<0.001) (Table 3.2 and Table 3.13). Isolate L1 increased significantly higher dry weight than Isolate E9, B3, D6 and G3 (P<0.001) (Table 3.2 and Table 3.13).

Treatments with Isolates D6 (*B. cereus*), E9 (*B. cepacia*), B3 (*Bukholderia sp*.) and L1 (*B. cereus*) significantly increased the yield of maize relative to the Isolate G3 and the untreated control (P<0.001) (Table 3.2 and Table 3.13).

All the FLD isolates tested significantly increased plant N in maize by 11-36.7%, relative to the untreated control (P<0.001) (Table 3.2 and Table 3.13) (see also Appendix 3.1 for Fig. 3.1).

Table 3.2 Effects of FLD isolates (applied as drench), on growth, yield and plant N of maize

	Drenched							
	Maize							
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Yield (g)**		**Plant N (mg g^{-1} DW)**	
Control-none	751	a	169	a	63	a	19.2	a
E9 (*B. cepacia*)	1067	b	268	b	223	bc	24.2	c
B3 (*Burkholderia* sp.)	1166	bc	237	b	206	b	21.3	b
G3 (*B. ambifaria*)	1270	bc	280	b	151	a	24.9	cd
D6 (*B. cereus*)	1311	bc	282	b	249	bc	25.7	cd
L1 (*B. cereus*)	1413	c	373	c	355	c	26.2	d
NPK fertilizer	1796	d	455	d	522	d	35.7	e
P	<.001		<.001		<.001		<.001	
l.s.d.	272.8		104.8		132.6		1.79	
s.e.d.	129.9		49.9		63.1		0.852	
Cv%	14.7		23.9		35.3		4.9	

Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.2 FLD isolates applied as seed treatments to maize crop

The same FLD isolates, applied as seed treatments, increased plant N of the maize crop significantly (P<0.001), by 12-31% relative to untreated control (Table 3.3 and Table 3.13) and see Appendix 3.2 for Fig. 3.2. Significant differences (P<0.001) in the wet weight, dry weight and yield were observed when FLD isolates were applied as seed treatments. Isolate L1 (*B. cereus*) caused significant increases in wet and dry weight of maize plants when compared to the untreated and the other free-living diazotrophic isolates (P<0.001) (Table 3.3) and see Appendix 3.4 for (Fig. 3.4). Yield was significantly increased by all isolates except Isolate G3 (P<0.001). Isolate L1 (*B. cereus*) caused the largest yield increase, but this was still less than the fertilized treatment.

Table 3.3 Effects of FLD isolates (applied as seed treatments), on growth, yield and plant N of maize

Seed Treated								
	Maize							
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Yield (g)**		**Plant N (mg g^{-1} DW)**	
None	168	a	56.0	a	70.2	a	19.5	a
D6 *(B. cereus)*	338	b	112.7	b	126.2	b	25.7	cd
G3 *(B. ambifaria)*	355	b	118.2	b	112.1	ab	25.3	cd
E9 *(B. cepacia)*	371	bc	123.8	bc	135.8	b	23.8	bc
B3 *(Burkholderia sp.)*	393	bc	131.1	bc	146.4	b	21.8	b
L1 (*B. cereus*)	502	c	167.5	c	224.2	c	25.2	d
NPK	807	d	269.0	d	295.3	d	36.2	e
P	<.001		<.001		<.001		<.001	
l.s.d.	133.3		44.42		53.9		2.063	
s.e.d.	63.4		21.14		25.65		0.982	
Cv%	21.4		21.4		22.9		5.6	

Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.3 FLD applied as drenches to sorghum seedlings

All of the isolates inoculated onto young emerging sorghum seedlings as a cell suspension resulted in a significant increase in plant N (P<0.001), increasing it by 25-69% when compared with the control (Table 3.4 and Table 3.13) and see for Fig. 3.3 in Appendix 3.4 There was also a significant difference between treatments with different FLD isolates.

Additionally, the application of FLD isolates applied as a drench, irrespective of NPK fertilization, satisfied the need for nitrogen to the level of 43-51% of N requirements.

Sorghum seedlings treated with a cell suspension of FLD isolates showed increases in wet and dry biomass compared to the untreated control (P<0.001). However, there were also significant differences in the wet and dry weight between the treatments (Isolates B3, D6, G3 and L1) relative to Isolate E9 and the untreated control (P<0.001). At harvest, significant difference in yield resulted from treatments by the FLD isolates (P<0.001) (Table 3.4).

Table 3.4 Effects of FLD isolates (applied as seed drench), on growth, yield and plant N of sorghum

Drenched

Sorghum

Treatments	Wet weight (g)		Dry weight (g)		Yield (g)		Plant N (mg g^{-1} DW)	
Control	90.5	a	22.63	a	5.95	a	13.75	a
E9 *(B. cepacia)*	135.4	b	33.85	b	9.11	a	18.75	bc
D6 *(B. cereus)*	176.0	c	44.00	c	10.62	b	22.00	d
G3 *(B.ambifaria)*	189.1	c	47.27	c	10.57	b	20.50	cd
B3 *(Burkholderia sp.)*	193.8	c	48.44	c	11.57	b	17.25	b
L1 (*B. cereus)*	198.5	c	49.62	c	12.86	b	20.75	cd
NPK	341.7	d	85.41	d	31.82	c	40.00	e
P	<.001		<.001		<.001		<.001	
s.e.d.	13.05		3.263		1.797		1.148	
l.s.d.	27.42		6.854		3.775		2.411	
Cv%	9.8		9.8		19.2		7.9	

Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.4 FLD isolates applied as seed treatments to sorghum

However, all of the bacterial treatments applied as seed treatments resulted in a significant increase in plant N, 32%-83% when compared to the untreated control (P<0.001) (Table 3.5) and see Fig.3.4.in appendix 3.4. There was no significant difference in N increases among the different FLD isolates.

Plants grown from sorghum seeds treated with FLD isolates developed significantly greater wet and dry weight than the untreated control (P<0.001) (Table 3.13). Again, there were no significant differences in the wet and dry weight as a result of treatments with different FLD isolates.

At harvest, significant differences in yield resulted from FLD treatments (P<0.001) (Table 3.5). Yields of sorghum plants treated with Isolate L1 were greater than the yield of plants treated with Isolate G3 (*B. ambifaria*), D6 (*B. cereus*), or the untreated control (P<0.001) (See also Fig. 3.4 in Appendix 3.4). Seed treatment of sorghum seeds with five FLD isolates resulted in positive effects on growth, yield and plant N of sorghum plants. Relative to the untreated and unfertilized control, the plants treated with FLD were bigger and had greater wet and dry weight, yield and plant N due to the seed treatments (P<0.001).

Table 3.5 Effects of FLD isolates (applied as seed treatments), on growth, yield and plant N of sorghum

Seed Treated								
	Sorghum							
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Yield (g)**		**Plant N (mg g^{-1} DW)**	
Control	40.3	a	19.2	a	2.7	a	9.2	a
G3 (*B.ambifaria*)	73.2	b	30.6	b	5.3	b	16.1	b
L1 (*B. cereus*)	73.8	b	29.4	b	6.3	c	16.8	b
D6 *(B. cereus*)	73.9	b	31.9	b	5.3	b	13.8	b
B3 (*Burkholderia* sp.)	79.9	b	36.3	b	5.4	bc	15.6	b
E9 (*B.cepacia*)	81.8	b	36.1	b	5.5	bc	12.1	b
NPK	121.6	c	61.3	d	15.1	d	34.3	c
P	<.001		<.001		<.001		<.001	
s.e.d.	6.64		3.97		0.475		2.342	
l.s.d.	13.95		8.34		0.997		4.921	
Cv%	12.1		16.1		10.3		20.3	

Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.5 FLD isolates applied as drenches to wheat plants

Application of bacterial cell suspensions into emerging seedlings of wheat weekly for one month had a positive effect on plant N levels, increasing plant N by 31-51% relative to the untreated control. However there was no significant difference between treatments with five different FLD isolates (Table 3.6 and Table 3.13). Drenching of all FLD isolates onto wheat seedlings was increased seedling wet and dry weights, and yield relative to the untreated control (P<0.001) (See Appendix 3.5 for Fig. 3.5).

Table 3.6 Effects of FLD isolates (applied as drench), on growth, yield and plant N of wheat

Drenched

	Wheat							
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Yield (g)**		**Plant N (mg g^{-1} DW)**	
Control-none	7.7	a	1.9	a	1.2	a	8.7	A
G3 (*B. ambifaria*)	14.3	b	3.5	b	2.4	b	11.5	B
E9 (*Burkholderia cepacia*)	14.3	b	3.5	b	2.4	b	11.2	Ab
D6 *(B. cereus*)	14.4	b	3.6	b	2.6	b	12.5	B
B3 (*Burkholderia* sp.)	15.2	b	3.8	b	2.5	b	13.2	B
L1 (*B. cereus*)	15.8	b	3.9	b	2.6	b	13.5	B
NPK fertilizer	36.0	c	9.0	c	6.0	c	23.7	C
P	<.001		0.001		<.001		<.001	
s.e.d.	1.01		0.2524		0.2493		1.239	
l.s.d.	2.121		0.5302		0.5238		2.604	
Cv%	8.5		8.5		12.3		13	

Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.6 FLD isolates applied as seed treatments to wheat plants

Isolates of FLD bacteria, applied as seed treatments to wheat seeds, were tested for their potential to fix nitrogen and increase plant growth. Three of five isolates caused no significant difference to the wet and dry weights of wheat seedlings relative to untreated and unfertilized control. Five of the isolates caused significant increases in grain yield (P<0.001) (Table 3.7 and see Fig. 3.6 in Appendix 3.6). Isolate E9 (*B. cepacia*) was significantly more effective than three other FLD isolates (L1, D6 and G3) and the untreated control in terms of their wet weight and grain yields. Isolates of free-living nitrogen fixing isolates applied as seed treatments to wheat seeds increased plant N by 58-78.5% compared to untreated control (P<0.001) (Table 3.13).

Table 3.7 Effects of FLD isolates, applied as seed treatments, on growth, yield and plant N of wheat

Seed treated								
	Wheat							
Treatments	Wet weight (g)		Dry weight (g)		Yield (g)		Plant N (mg g^{-1} DW)	
Control	1.7	a	0.8	a	0.8	a	2.8	a
G3 (*B.ambifaria*)	2.5	ab	1.2	ab	1.4	b	4.4	b
E9 (*B. cepacia*)	3.9	c	1.8	c	2.3	c	4.6	b
D6 (*B. cereus*)	2.7	b	1.4	bc	1.6	b	4.8	b
B3 (*Burkholderia* sp.)	3.0	bc	1.5	bc	1.7	b	5.0	b
L1 (*B. cereus*)	2.5	ab	1.3	ab	1.5	b	4.6	b
NPK	7.4	d	3.7	d	4.4	d	14.8	c
P	<.001		<.001		<.001		<.001	
l.s.d.	1.042		0.5471		0.6093		1.501	
s.e.d.	0.496		0.2604		0.29		0.714	
Cv%	20.5		21.5		20.6		17.3	

Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.7 FLD isolates applied as drenches to lettuce seedlings

In this experiment all five free-living diazotrophic isolates caused significantly increases in wet, dry weight, root dry weight and plant N related to the untreated control. Isolate L1 was significantly more effective at increasing plant N relative to the untreated control and the rest of the FLD isolates (P<0.001) (Table 3.8 and Table 3.13).

FLD Isolates B3 (*Burkholderia sp.*), E9 (*B. cepacia*), D6 (*B. cereus*) and L1 (*B. cereus*) caused highly significant increases in lettuce wet weight, relative to the untreated control (P<0.001) (Table 3.8) (See also Fig. 3.7 in Appendix 3.7). Moreover, application of Isolates G3 (*B. ambifaria*) resulted in a greater wet weight than Isolates E9 (*B. cepacia*), B3 (*Burkholderia sp.*) and D6 (*B. cereus*) and the untreated control (P<0.001). Inoculation of bacterial isolates by drenching resulted in highly significant increases in dry weight relative to the untreated control (P<0.001). Root development was also improved by inoculation of FLD applied by drenching to lettuce seedlings. Five of the isolates were effective and caused significant increases in root dry weight compared to the untreated control (P<0.001).

Table 3.8 Effects of FLD isolates, applied as drench, on growth, yield and plant N of lettuce

Drenched								
Lettuce Seedling								
Treatments	**Wet Weight (g)**		**Dry Weight (g)**		**Root Dry Weight (g)**		**Plant N (mg g^{-1} DW)**	
Control	3.48	a [1]	1.39	a	0.69	a	3.58	a
D6 (*B. cereus*)	6.94	b	2.82	b	1.43	b	7.27	b
B3 (*Burkholderia* sp.)	8.87	bc	3.46	bc	1.72	bc	7.04	b
E9 (*B. cepacia*)	9.64	c	4.07	c	2.01	bc	6.47	b
L1 (*Bacillus cereus*)	10.41	cd	4.44	c	2.22	c	8.37	c
G3 (*B.ambifaria*)	12.31	d	4.46	c	2.24	c	6.07	b
NPK-fertilizer	24.6	e	9.51	d	5.54	d	16.96	d
P	<.001		<.001		<.001		<.001	
l.s.d.	2.28		1.01		0.71		1.26	
s.e.d.	1.08		0.48		0.34		0.60	
C.v%	14.1		15.80		21.0		10.60	

[1] Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.8 FLD isolates applied as seed treatments to lettuce crops

Inoculation of the FLD isolates by seed treatment, particularly Isolates G3 (*B. ambifaria*), B3 (*Burkholderia* sp.) and D6 (*B. cereus*), were not effective at increasing wet weight relative to the untreated control (Table 3.9 and see Fig 3.8 in Appendix 3.8). On the other hand, Isolates E9 (*B. cepacia*), D6 (*B. cereus*) and L1 (*B. cereus*) increased wet weight relative to other isolates (G3 (*B. ambifaria*) and B3 (*Burkholderia* sp.)) and the untreated control (Table 3.9 and Table 3.13). A significant increase resulted from the application of the five FLD isolates on dry weight, compared to the untreated control. Isolates E9 (*B. cepacia*) and L1 (*B. cereus*) caused increased root dry weights compared to the control. Plant N of treated and untreated lettuce seedlings were not significantly different.

Table 3.9 Effects of FLD isolates, applied as seed treatments, on growth, yield and plant N of lettuce

Seed treated								
	Lettuce							
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Root dry weight (g)**		**Plant N (mg g^{-1} DW)**	
Control	1.13	a [1]	0.36	a	0.17	a	2.45	a
G3 (*B.ambifaria*)	1.44	a	0.63	ab	0.27	ab	3.39	a
B3 (*Burkholderia* sp.)	1.70	ab	0.81	b	0.30	ab	3.68	a
D6 (*B. cereus*)	1.77	abc	0.72	b	0.29	ab	3.96	a
E9 (*B. cepacia*)	2.25	bc	0.70	b	0.43	b	3.77	a
L1 (*Bacillus cereus*)	2.49	c	0.86	b	0.39	b	3.59	a
NPK	6.34	d	3.05	c	2.34	c	14.34	b
P	<.001		<.001		<.001		<.001	
l.s.d.	0.78		0.34		0.21		1.60	
s.e.d.	0.37		0.16		0.10		0.74	
C.v%	21.30		22.50		23.30		20.90	

[1] Means with the same letter in the same column are not significantly different at P<0.05, based on Fisher's LSD test

3.3.9 FLD applied as drenches to zucchini plants

Wet weight, dry weight and fruit yield of zucchini increased when FLD isolates were applied by drenching to zucchini seedlings. However, there was no significant increase in plant N as a result of application of the five bacterial isolates; relative to the untreated control (Table 3.10 and see Fig. 3.9 in Appendix 3.9).

Table 3.10 Effects of FLD isolates, applied as drench, on growth, yield and plant N of zucchini

Drenched

	Zucchini seedlings							
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Fruit (g plant^{-1})**		**Plant N (mg g^{-1} DW)**	
Control	19.2	a	6.40	a	50.5	a	4.64	a
B3 (*Burkholderia* sp.)	45.6	b	15.21	b	129.3	b	5.42	a
L1 (*B. cereus*)	45.8	b	14.43	b	130.1	b	5.88	a
G3 (*B. ambifaria*)	45.9	b	15.29	b	148.4	b	5.75	a
E9 (*B. cepacia*)	49.9	b	14.14	b	126.6	b	5.31	a
D6 (*B. cereus*)	52.0	b	14.84	b	147.1	b	5.31	a
NPK-fertilizer	143.1	c	47.71	c	282.7	c	26.73	b
P	<.001		<.001		<.001		<.001	
l.s.d.	16.26		4.452		27.06		1.405	
s.e.d.	7.74		2.119		12.88		0.669	
C.v%	19.1		16.4		12.6		11.2	

[1] Means with the same letter in the same experiment are not significantly different at P<0.05, based on Fisher's LSD test.

3.3.10 FLD isolates applied as seed treatments to zucchini plants

Significant increases in wet weight, dry weight, fruit yield and plant N were observed when FLD isolates were applied as seed treatments to zucchini seeds (P<0.001) (Table 11 and see Fig. 3.10 in Appendix 3.10). Additionally, Isolates L1 and E9 caused a greater increase in plant N relative to the other isolates and the untreated control.

Table 3.11 Effects of FLD isolates, applied as seed treatments, on growth, yield and plant N of zucchini biomass, fruit yield and plant N

Seed Treated

Zucchini

Treatments	Wet weight (g)		Dry weight (g)		Fruit (g plant $^{-1}$)		Plant N (mg g^{-1} DW)	
Control-none	11.4 [1.1]	a	3.8 [0.7]	a	10.7 [1.0]	a	2.4 [0.5]	a
G3 (*B. ambifaria*)	14.6 [1.2]	b	4.8 [0.8]	b	17.4 [1.3]	b	2.6 [0.6]	b
B3 (*Burkholderia* sp.)	17.3 [1.3]	c	5.7 [0.8]	b	18.4 [1.3]	b	3.2 [0.6]	b
D6 (*B. cereus*)	19.4 [1.3])	c	6.4 [0.9]	c	21.4 [1.3]	b	3.4 [0.6]	b
L1 (*B. cereus*)	21.7 [1.4]	d	7.2 [0.9]	c	21.4 [1.4]	b	4.5 [0.7]	c
E9 (*B.cepacia*)	22.8 [1.4]	d	7.6 [0.9]	c	21.5 [1.4]	b	3.6 [0.7]	c
NPK- fertilizer	58.0 [1.8]	e	19.4 [1.3]	d	126.8 [2.1]	c	25.8 [1.4]	d
P	(<.001)		(<.001)		(<.001)		(<.001)	
l.s.d	(0.07)		(0.07)		(0.12)		(0.05)	
s.e.d	(0.03)		(0.03)		(0.06)		(0.03)	
C.v%	(3.7)		(5.0)		(5.9)		(4.9)	

[1] Means with the same letter in the same column are not significantly different at P<0.05

Values in parenthesis represent transformed means using Log base 10 transformations

3.3.11 FLD isolates applied as drenches to petunia seedlings

Unlike the above crops, the FLD isolates were applied by drenching only. Biomass was increased due to drenching with the bacteria except for root dry weight of Isolate E9 (*B.cepacia*). All isolates except Isolate G3 (*B. ambifaria*), increased plant N by 24-40% when compared with the untreated and unfertilized control (Table 3.12 and see Fig. 3.11 in Appendix 3.11).

Table 3.12 Effects of FLD isolates, applied as drenches, on growth, yield and plant N of petunia

Drenched

Petunia seedlings

Treatments	Wet weight (g)		Dry weight (g)		Root dry weight (g)		Plant N (mg g^{-1} DW)	
Control	3.6 (0.63)	a	0.4 (0.13)	a	0.07 (0.03)	a	2.2 (0.49)	a
E9 (*B.cepacia*)	3.9 (0.66)	b	0.6 (0.19)	b	0.10 (0.04)	a	2.7 (0.57)	c
G3 (*B. ambifaria*)	5.7 (0.81)	c	0.9 (0.27)	c	0.15 (0.06)	b	2.2 (0.50)	a
D6 (*B. cereus*)	7.1 (0.89)	d	1.1 (0.32)	d	0.20 (0.08)	d	3.1 (0.61)	e
B3 (*Burkholderia sp.*)	7.4 (0.93)	e	1.0 (0.30)	cd	0.21 (0.08)	d	2.5 (0.55)	b
L1 (*B. cereus*)	7.5 (0.89)	e	1.0 (0.30)	cd	0.15 (0.06)	c	2.9 (0.59)	d
NPK-fertilizer	38.9 (1.60)	f	6.6 (0.87)	e	1.39 (0.38)	e	6.0 (0.84)	f
P	(<0.001)		(<0.001)		(<0.001)		(<0.001)	
l.s.d.	(0.20)		(0.13)		(0.03)		(0.13)	
s.e.d.	(0.10)		(0.06)		(0.01)		(0.06)	
C.v%	(15.10)		(25.20)		(20.10)		(14.40)	

[1] Means with the same letter in the same experiment are not significantly different at P<0.05, based on Fisher's LSD test

Values in parenthesis represent transformed means using Log base 10 transformations

Table 3.13 Summary of the effects of FLD isolates applied by drench or seed treatments on several crops

Evaluated Crops	Application methods	Wet weight	Dry weight	Plant -N	Yield	Root Dry weight
Maize	Drenched	+++	+++	+++	+++	
	Seed treated	++	++	++	++	
Sorghum	Drenched	+++	+++	+++	+++	
	Seed treated	++	++	++	++	
Wheat	Drenched	+++	+++	+	++	
	Seed treated	+	+	+	++	
Lettuce	Drenched	+++	+++	+++		+++
	Seed treated	+	+	NS		++
Zucchini	Drenched	+++	+++	NS	+++	
	Seed treated	++	++	NS	++	
Petunia	Drenched	+	++	NS		++

Description: $^{+++}$ highly significant, $^{++}$ highly significant, $^{+}$ significant, ^{NS} not significant

3.4 Discussion

Nitrogen fixation by free-living microorganisms associated with plants has been of interest for many years. Application of a range of diazotrophic rhizobacteria increased vegetative growth and grain yield of crop plants such as rice, wheat, maize, sugarcane and cotton (Kennedy *et al*., 2004).

The FLD isolates were applied by drench or seed treatment and, in many cases, significantly increased the growth, yield of maize, sorghum and wheat crops in greenhouse trials. The control plants showed very poor growth, which may be attributed to nutrient deficiency compared to plants treated with FLD isolates. Isolate L1 (*B. cereus*) was consistently superior to the other four isolates in promoting growth and plant N, both as adrench or a seed treatment applied to maize, sorghum or wheat.

Application of FLD isolates to maize by drench significantly increased the yield (43%-68%), wet weight (59%-78%), dry weight (59%-82%), and plant N (60%-73.4%). In lettuce, the wet weight increased 28%-50%, dry weight by 30-47%, root dry weight by 26%-40% and the plant N by 36%-49%. Similarly, the wet weight of petunia increased by 10%-19.2%, the dry weight by 9.1%-15%, the root dry weight by 7.2%-15% and plant N by 37%-51.6%.

These results compare well with those of Boddey *et al*. (1995), who showed that inoculation of *Acetobacter diazotrophicus* increased plant N by 60-80% in sugarcane, and Zahir and Arshad (1996) who found that inoculation of *Azotobacter* spp. increased the yield of potato. FLD isolates have also been reported to increase the wet weight (40-58%), dry weight (39.6-58%), yield (28-40%), and the plant N (47-55%) of sorghum, and to increase the wet weight (60%-67%), dry weight (50%-58%), yield (35%-41%), and plant N (35%-49%) of wheat when applied by drench. In contrast, Kader *et al*. (2002) and Narula *et al*. (2005) found that wheat accumulated less plant N (about 20%) when inoculated with *Azotobacter* spp.

Seed treatment with FLD isolates increased the biomass (42%-62%), yield (38%-76%) and plant N (60-70%) in maize. It also increased the biomass (67%), yield (35%-42%), and plant N (35%-49%) in sorghum.

Inoculation of FLD isolates applied by drench increased the plant N (36%-49%) in lettuce and (42%-52%) in petunia. However, there was no significant increase in plant N in zucchini. These results are in agreement with Kloepper *et al*. (1989) who inoculated *Azotobacter* spp onto wheat, and Kennedy *et al*. (2004) who showed that inoculation of diazotrophic plant growth-promoting rhizobacteria could result in increased biomass, as well as crop yield of maize, rice, wheat, sugarcane and cotton. Stimulation of different crops by rhizobacterial inoculation has been demonstrated by other research in both laboratory and field trials. For example, it was reported that wheat yield increased up to 30% when treated with *Azotobacter*

inoculants and up to 43% with *Bacillus* inoculants (Kloepper *et al*., 1989). Biological nitrogen fixation (BNF), associated with cereals and grasses, contributes to the growth and nutrition of several crops. Inoculation of wheat seedlings with different wild type strains of *A. brasilense* resulted in an increase in the number and length of lateral roots (Barbieri *et al*., 1988). The results of our studies on the effects of FLD on growth and nitrogen fixation are in agreement with the results of Boddey and Döbereiner (1988), who reported a significant increases in growth and nitrogen levels in forage grasses and sugarcane when inoculated with *Azospirillum* spp.

The application by drenching of the FLD isolates caused a growth promoting effect on lettuce and petunia seedlings and improved plant N per plant, in a greenhouse study. Though there was no significant increase in plant N, FLD isolates applied by drenching improved growth and fruit yield of zucchini plants as well. This could be because of the production of plant growth hormones and other biologically active substances by the FLD isolates (Rózycki *et al*., 1999).

The results showed that FLD isolates have the potential to fix nitrogen and to promote growth of several crops. Either drench or seed treatment application of FLD to cereals (maize, sorghum or wheat) contributed to an increases in growth and plant N. This may be an indicator of that these FLD isolates live in association with cereal roots that could enhance the performance of individual plants.

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Appendix 3.1

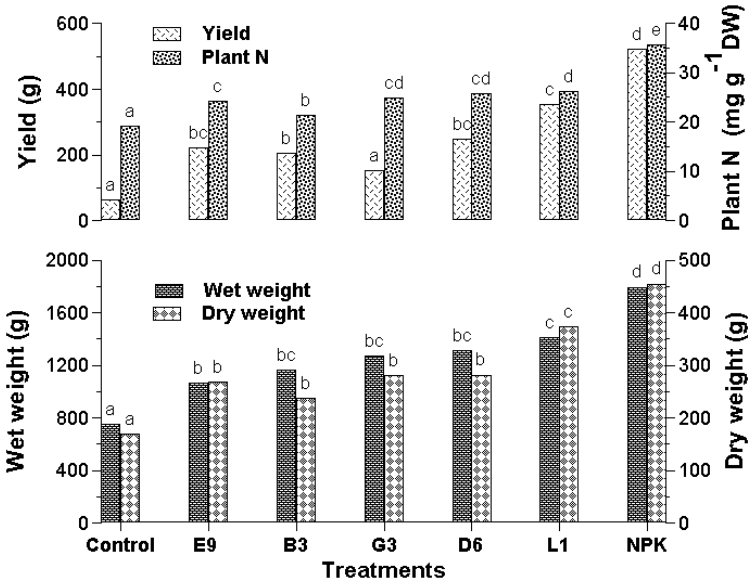

Fig.3.1 A histogram of the effects of FLD isolates, applied by drenching, on growth, yield and plant N of maize.

Appendix 3.2

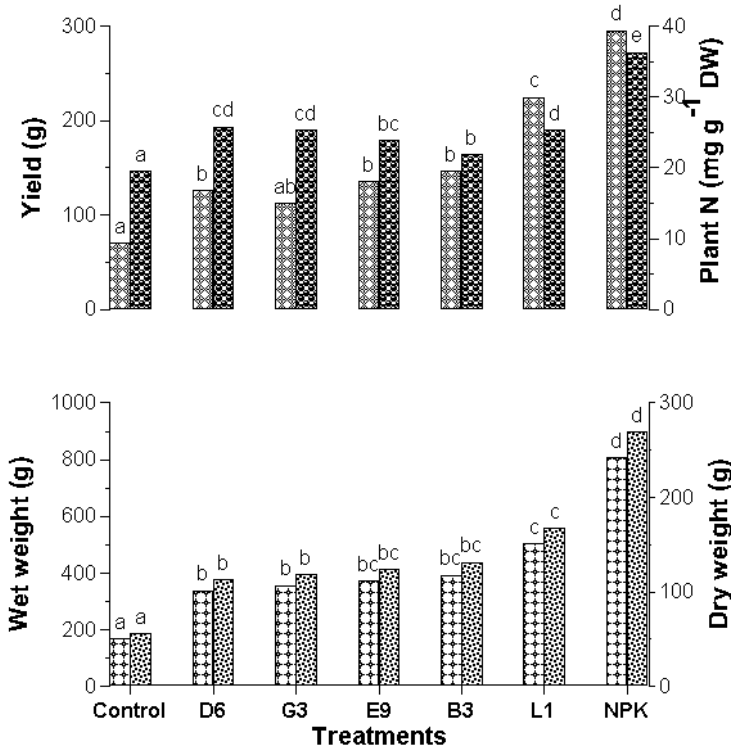

Fig. 3.2 A histogram of the Effects of FLD isolates, applied by seed treatment, on growth, yield and total N of maize

Appendix 3.3

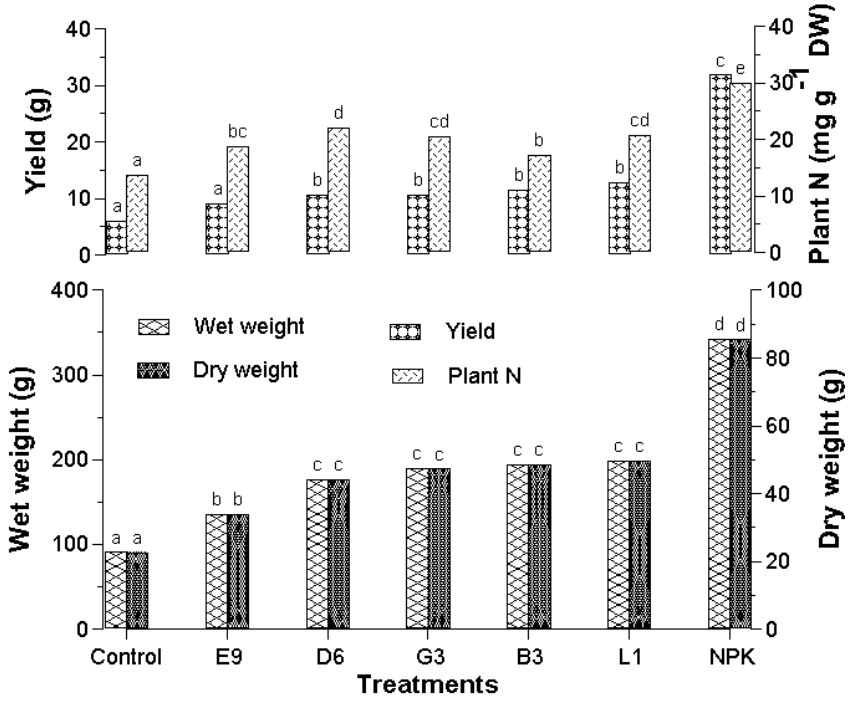

Fig. 3.3 A histogram of the effects of FLD isolates, applied by drench, on growth, yield and plant N of sorghum

Appendix 3.4

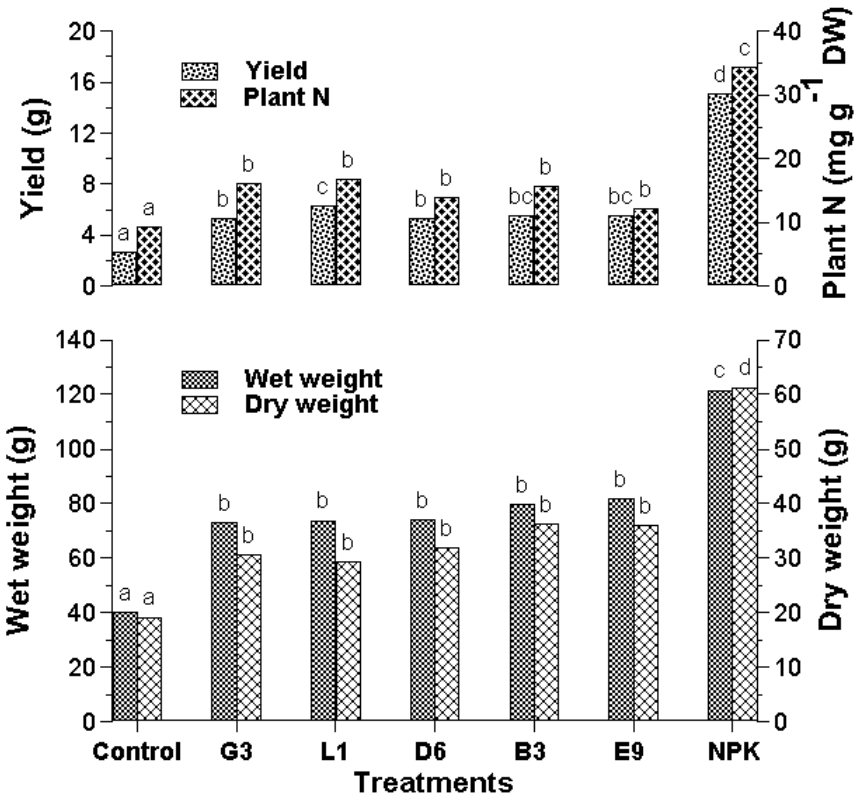

Fig.3.4 A histogram of the effects of FLD isolates, applied by seed treatments, on growth, yield and plant N of sorghum

Appendix 3.5

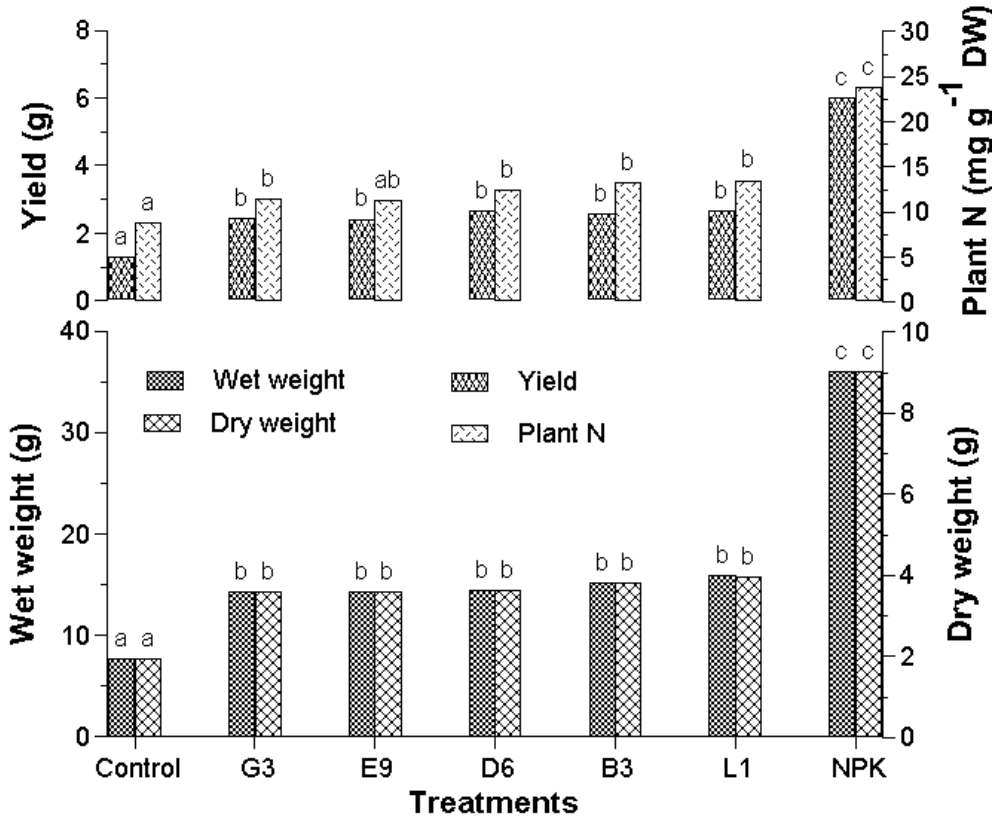

Fig. 3.5 A histogram of the effects of FLD isolates, applied by drench, on growth, yield and plant N of wheat plants

Appendix 3.6

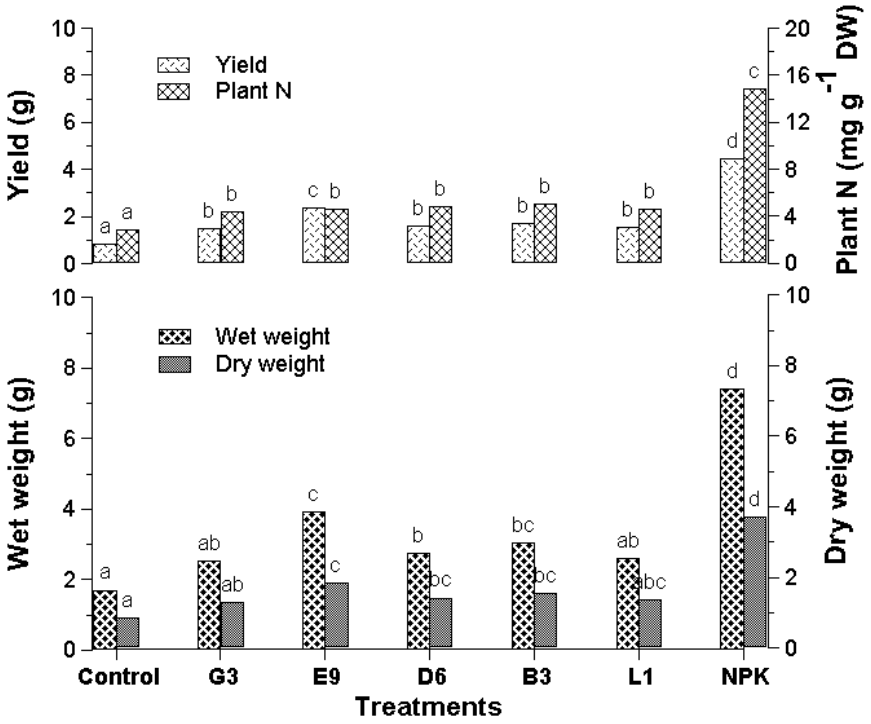

Fig. 3.6 A histogram of the Effects of FLD isolates, applied by seed treatments, on growth, grain yield and plant N of wheat

Appendix 3.7

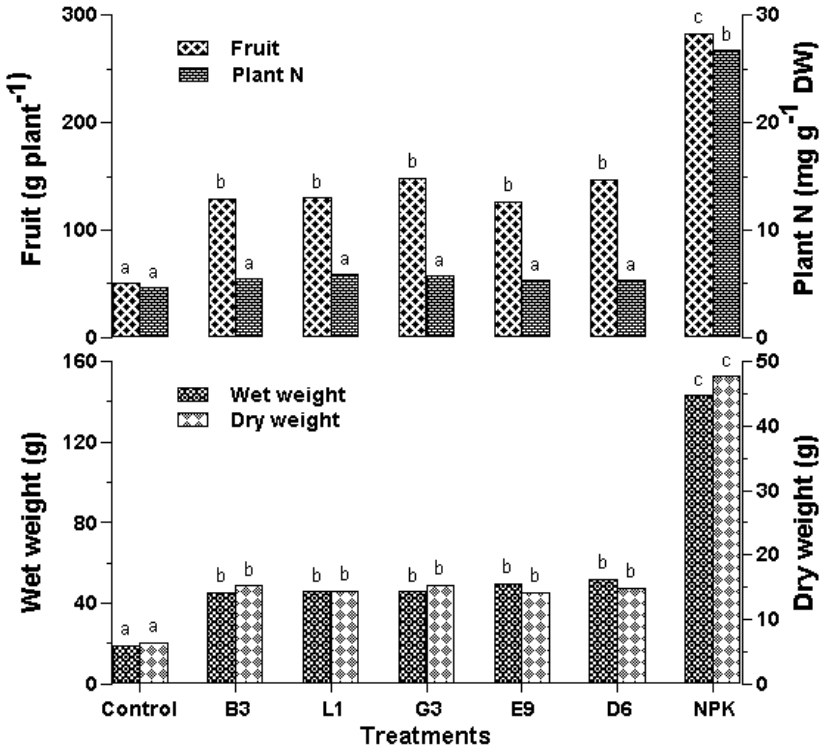

Fig.3.7 A histogram of the effects of FLD isolates, applied by drenching, on growth, yield and plant N of lettuce

Appendix 3.8

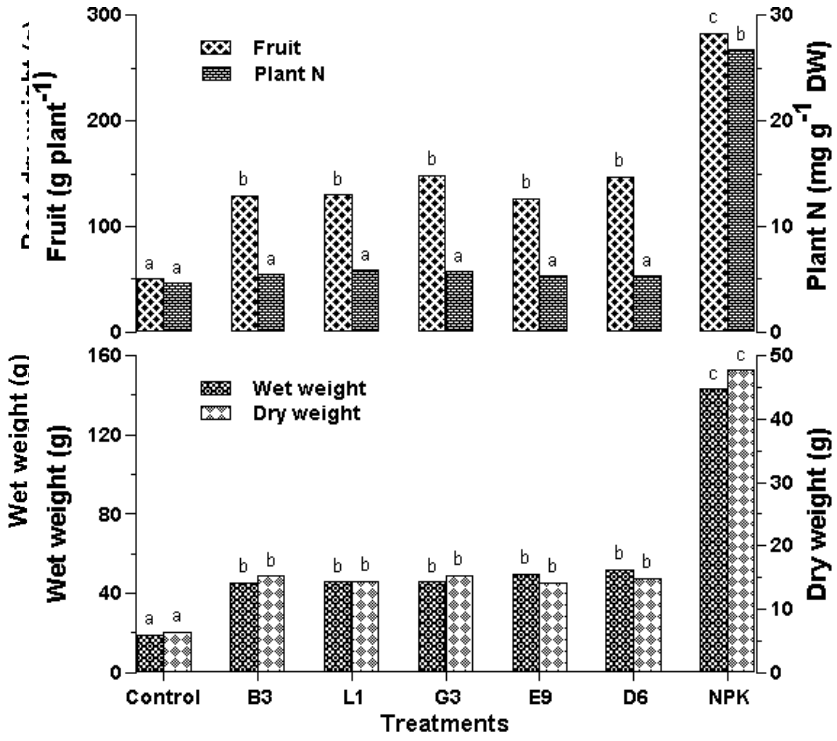

Fig. 3.8 A histogram of the effects of FLD isolates, applied by seed treatments, on growth, yield and plant N of lettuce

Appendix 3.9

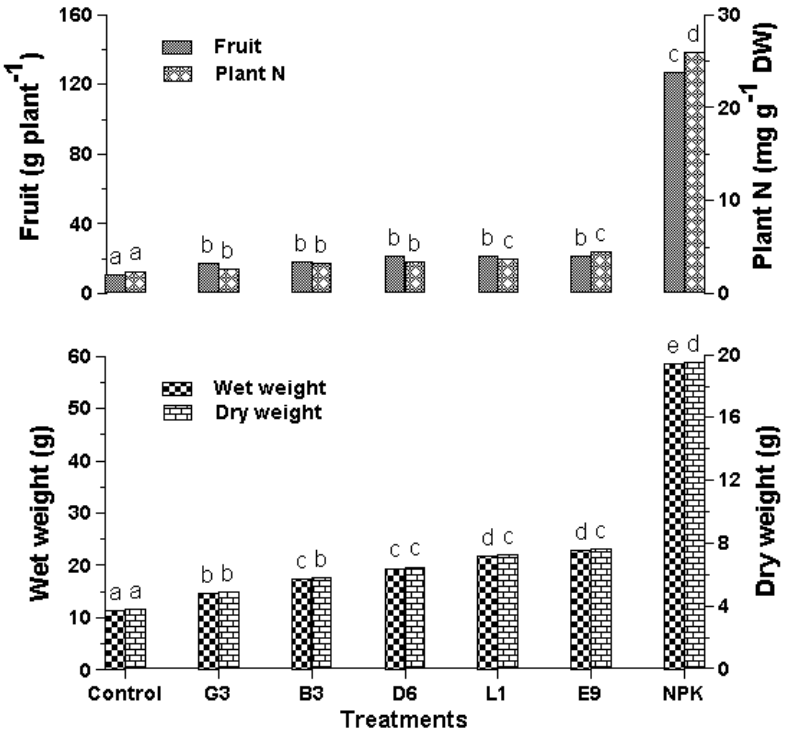

Fig. 3.9 A histogram of the effects of FLD isolates, applied by drenching, on growth, yield and plant N of zucchini

Appendix 3.10

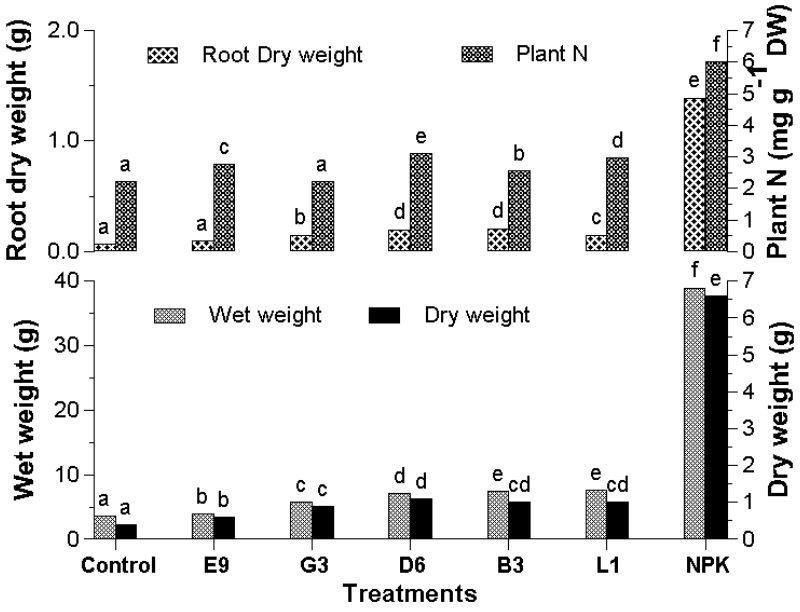

Fig. 3.10 A histogram of the effects of FLD isolates, applied by drench, on growth, yield and plant N of zucchini

Appendix 3.11

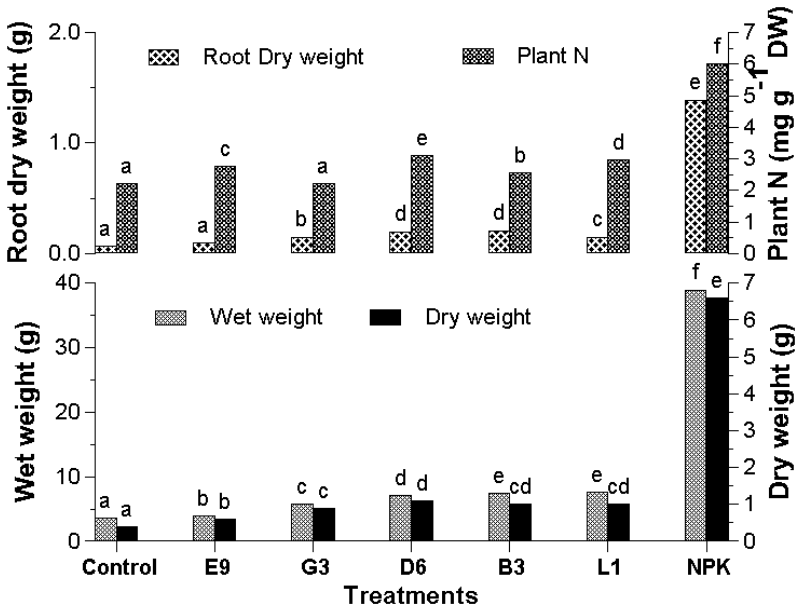

Fig. 3.11 A histogram of the effects of FLD isolates, applied by drenching, on growth, and plant N of petunia seedlings

CHAPTER FOUR

DETERMINATION OF OPTIMUM DOSE AND FREQUENCEY OF APPLICATION OF FREE-LIVING DIAZOTROPHS (FLD) ON LETTUCE

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Science and Agribisiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

Free-living diazotrophic isolates L1 (*Bacillus cereus* Frankland) and Br2(*Bacillus subtilis* (Ehrenberg) Cohn) were evaluated in greenhouse trials for their optimum dose and frequency of application on a single lettuce variety (Great Lakes) seedlings in a composted pine bark medium. Bacterial isolates were grown in Burke's broth and were applied as a drench at four different doses (10^5, 10^6, 10^7 and 10^8 colony forming units (cfu) ml^{-1}) and at different frequency of application (1, 7, 14 and 21 days). Two months later wet weight, dry weight and plant N levels were measured.

Lettuce growth and plant N level responded positively to both bacterial isolates inoculated at 10^6cfu ml^{-1} weekly and every two weeks. Isolate Br2 inoculated at 10^6cfu ml^{-1} weekly fixed 32.4% plant N when applied weekly and 26.7% when applied every two weeks. Isolate L1, at the same dose, applied weekly or every two weeks, fixed 27.7% and 29.1% of plant N requirement, respectively. The lettuce seedlings responded less well to a higher dose at 10^8cfu ml^{-1} of either isolates applied weekly and every two weeks. The response to dosage depended on frequency of application. Doses of 10^6 cfu ml^{-1} of both isolates applied weekly or every two weeks had the best effect on lettuce seedling growth and plant N.

4.1 Introduction

Nitrogen is often a limiting nutrient for crop production. However, the cost of nitrogen fertilizers has increased drastically, in line with oil prices. Extending biological nitrogen fixing ability to non-legumes by using free-living diazotrophs would be a useful technology for increasing crop production (Kennedy and Tchan, 1992).

A number of microorganisms are known to have beneficial effects on plant growth. Plant growth promoting rhizobacteria (PGPR) are commonly used as inoculants for improving the growth and yield of agricultural crops (Khalid *et al*., 2004). Among these are plant growth promoting rhizobacteria that form a symbiotic relationship with plants such as nitrogen fixing *Rhizobium* sp., as well as free-living diazotrophs (FLD) associated with the roots of grasses (Mishustin, 1970; Kloepper *et al*., 1980; Glick, 1995).

In a greenhouse study on sugar beet, three different *Bacillus* isolates fixed nitrogen and increased growth (Çakmakçi *et al*., 2006). Similarly, inoculation with a strain of *Bacillus* sp. also increased growth of roots and shoot parts of rice plants (Beneduzi *et al*., 2008). In another study, Hafeez *et al*. (2006) noted that selected *Bacillus* sp. used as bio-inoculants onto wheat resulted in increases in plant biomass, root length, and plant N and phosphorous content.

Narula *et al*. (2005) found that inoculation with *Azotobacter* sp. increased wheat and cotton yield, dry weight, and plant N. Similarly, nitrogen concentration in wheat grain and root tissue may increase due to *Azotobacter* bioinoculants (Kader *et al*., 2002). In another study, inoculation of *Azotobacter chroococcum* Beijerinck onto *Brassica napus* cv. $ISN^{-1}29$ produced an increase in seed yield and total dry matter when no external nitrogen was applied (Prabhjeet and Bhargava, 1994).

Other FLD detected in association with plant roots and found to fix nitrogen include: *Acetobacter diazotrophicus* Gillis et al., *Herbaspirillum seropedica* Baldani et al. (James and Olivares, 1998), *Azoarcus* sp. (Hurek *et al*., 2002) and *Azospirillum* sp. (Steenhoudt and Vanderleyden, 2000).

Inoculation of *Azospirillum* onto cereals and non-cereal species typically results in increases in plant dry weight and in the amount of nitrogen in shoots (Baldini and Döbereiner, 1980; Albrecht *et al*., 1981; Bashan, 1986; Kapulnik *et al*., 1987). Similarly, significant increases in growth and dry matter were obtained by Venkateswarlu and Rao (1983) for pearl millet following inoculation with *Azospirillum brasilense* Corrig. Tarrand et al.

Recent studies have shown that several other bacterial species such as *H. seropedicae*, and *Burkholderia* spp. increased fresh weight and plant N of rice through biological nitrogen fixation (Baldani *et al*., 1997; Baldani *et al*., 2002; Rodrigues *et al*., 2008).

The aim of this study was to evaluate effectiveness of two FLD isolates on lettuce growth under greenhouse conditions; and to evaluate the effects of different doses and frequencies of application, on lettuce biomass and plant N levels in the lettuce plants.

4.2 Materials and methods

4.2.1 Preparation of inoculants

From frozen stocks, FLD bacterial strains L1 and Br2, were grown in Burke's medium (Bergensen, 1980). Isolate L1 was previously identified as *B. cereus* and demonstrated good multi-crop effects on plant growth and nitrogen-fixation activity. Isolate Br2 (*Bacillus subtilis* (Ehrenberg) Cohn) was supplied by Dr Brendon Neumann[8]. The bacterial inocula were produced by growing the bacteria in 250ml of Burke's broth, on a rotary shaker (150rpm for 72h at 28^0C). In the log phase of growth, bacterial suspensions were centrifuged (10,000 x g for 10 min at 4^0C) and washed three times in a phosphate buffer. Final cell pellets were diluted with sterile distilled water and cell numbers were adjusted to 10^5, 10^6, 10^7 and 10^8cfu ml^{-1} for each of the two bacterial isolates, as determined by dilution plating and use of a counting

[8] *Dr. Brendon Neumann, PHP (Pty) Ltd., Nottingham Rd., South Africa*

chamber. This procedure was repeated each time fresh cell suspensions were needed. Five milliliters of each bacterial suspension was applied to each seedling.

4.2.2 Microbial inoculation

The prepared cell suspensions (10^5, 10^6, 10^7 and 10^8cfu ml^{-1} cells of both FLD isolates were added separately to each of the eight conical labeled flasks. Two grams of a sticker, gum Arabic[9], were dissolved in 100ml of tap water, stirred and allowed to stand for 1h. This allowed the substance to dissolve and form a homogeneous suspension. The suspension was further divided into eight 50ml beakers, each containing 10ml aliquots of the sticker. To each of the beakers, 10ml of the bacterial cultures was added separately, and stirred. This resulted in a volume of 20ml of sticker-bacterial suspension in each of the eight beakers of a 1:1 suspension.

Seed coating took place in a plastic bag. The bag was filled with 200g seeds. The bacterial-sticker suspension was added at a rate of 0.1ml g^{-1} of seeds. The use of a sticker was to increase the amount of inoculant that would adhere to seeds in order to increase the number of bacteria stuck onto each seed. The bag was closed in such a way to trap air as much as possible. The bag was shaken for two minutes, until all the seed were uniformly wetted with the sticker suspension. The bag was opened and the seed spread onto paper towels and air-dried overnight. The coated seeds of lettuce (cultivar Great Lakes) were planted into 32 pots filled with composted pine bark. The pots were watered with tap water contained soluble fertilizer applied at rate of 0.224g ℓ^{-1} KH_2PO_4, 0.149 gℓ^{-1} K_2SO_4, 0.324 gℓ^{-1} KCl, 0.203g ℓ^{-1} $MgSO_4$, 1g ℓ^{-1} $CaCO_3.12H_2O$ to give 300mg ℓ^{-1} and 50mg ℓ^{-1} and left in a greenhouse (20-25^{0}C) till harvest.

The seedlings were subsequently drenched with the FLD isolates after seedling emergence, at different doses and frequencies. Seedlings were dosed with 10^8, 10^7, 10^6, 10^5cfu $ml^{-1}$of bacteria at rate of 1ml liquid culture $plant^{-1}$. Doses were applied every 7, 14, and 21 days.

[9] gum Arabic sticker, from *Acacia* sp., SIGMA

Frequency	Days of **inoculations** (drenching repeat)				
1 (seed treated at planting)	1				
7 (drench every week)	1	7	14	21	28
14 (drench every two week)	1	14	28		
21 (drench after three weeks)	1	21			

The experiments used a complete randomized block design with three replicates, for the three factors tested: inoculation with the FLD isolates Strain L1 (*B. cereus*) or Strain Br2 (*B. subtilis*); doses of 10^5, 10^6, 10^7 and 10^8 cfu ml^{-1}; and frequencies of 1, 7, 14 and 21 days. : 1 = seed treated prior to plant, 7 - every week, 14 = every two weeks and 21 = every three weeks, after emergence of seedlings, respectively. The treatments were applied for one month in order to establish the bacteria. Two months later wet and dry biomass weight and plant N were measured.

4.3 Controls

Two controls were set up in this study. Untreated seeds were planted in nine pots filled with composted pine bark, and received water only. The other nine pots served as a positive control and were supplemented weekly with a balance NPK fertilizer called 3:1:3 (38) Complete[10].

4.4 Nitrogen total analysis

The analytic technique of Willis *et al*. (1996) was used. See Appendix B for details.

4.5 Statistical analysis

The GenStat 9th edition was used for analysis of variance. When the *F*-test was significant, the treatment means were compared using the Least Significant Difference (LSD) test.

[10] Ocean Agriculture (Pty) Ltd. P.O. Box 741, Muldersdrift, 1747, South Africa

4.6 Results

4.6.1 Effects of two FLD bacteria on wet and dry biomass and nitrogen accumulation of lettuce

The response of lettuce seedlings to treatments with Isolate Br2 (*B. subtilis*) at a dose of 10^5cfu ml^{-1} applied once as a seed treatment were not significant for growth compared to control-none. The single inoculation (seed treated) was not effective (Table 4.1) (See Appendix 4.1 for Fig.4.1) However, when Isolate Br2 at doses of 10^6, 10^7, 10^8cfu ml^{-1} applied at all frequencies significantly increased in plant N ($P<0.001$).

Isolate L1 (*B. cereus*) at doses of 10^5, 10^6, 10^7 cfu ml^{-1} applied at all frequencies were significantly able to increase plant N of lettuce seedlings relative to the untreated and unfertilized control ($P<0.001$). Moreover, at doses of 10^8 applied at all frequencies except at 21d did not significantly increase in biomass when compared to the control-None (Table 4.1) (See Appendix 4.1 for Fig. 4.1). Isolate L1 (*B. cereus*) at a dose of 10^8cfu ml^{-1}, applied weekly did not significantly increase in plant-N relative to the Control-None ($P<0.001$) (Table 4.1) (See Appendix 4.1 for Fig.4.1). Application of Isolate L1 (*B. cereus*) at a dose of 10^5cfu ml^{-1}, applied once at the time of planting and 21d later, supplied 18.3% and 13.6% of plant N requirement respectively (see Appendix 4.1 for Fig.4.1). Moreover, 18.6%, 27.7%, 18.7% and 9.6% of plant N demand was provided by applying Isolate L1 at doses of 10^5, 10^6, 10^7 and 10^8cfu ml^{-1} at a frequency of once a week (See Appendix 4.1 for Fig.4.1).

When FLD Isolate L1 (*B. cereus*) was applied to lettuce seedlings at 10^5cfu ml^{-1} at all frequencies of application, 13.9% - 18.9% of plant N were generated by the bacteria. When Isolate L1 was applied at 10^6cfu ml^{-1} weekly or every two weeks, it provided 27.7% – 29.1% of nitrogen demand. At doses of 10^7 and 10^8cfu ml^{-1}, applied at 21d, produced 19.1% and 19.9% of plant N, respectively. Less nitrogen was produced when Isolate L1 was applied at doses of 10^7 and 10^8cfu ml^{-1} weekly and every two weeks (9.6% and 10.3%; 15.0% and 18% of nitrogen demand, respectively) (Table 4.1). Significant difference was observed in frequency, concentration and the interaction of frequency x concentration applications ($P<0.001$) (Table 4.2).

Table 4.1 Response of lettuce (wet and dry weight and plant N) after two months in a greenhouse to varied dosages and frequencies of application of two FLD isolates

Bacteria	Doses (cfu ml^{-1})	Frequency (weeks)	Wet biomass weight (g)	Dry biomass weight(g)	Total N (mg g^{-1})
Br2	10^5	1	2.35 ab	0.91 ab	7.87 cde
		7	7.42 defg	3.69 cde	8.30 cde
		14	6.69 cdefg	3.04 abcde	8.63 de
		21	3.85 abcd	1.45 abc	8.53 cde
	10^6	1	4.15 abcde	1.43 abc	9.37 de
		7	9.69 g	4.81 e	9.53 e
		14	8.23 efg	3.93 de	9.20 de
		21	7.35 cdefg	3.21 bcde	9.27 de
	10^7	1	5.67 abcdefg	2.52 abcde	9.10 de
		7	7.57 defg	3.79 cde	8.97 de
		14	7.37 cdefg	3.19 bcde	8.13 cde
		21	6.32 bcdefg	2.52 abcde	8.53 cde
	10^8	1	7.37 cdefg	3.61 cde	7.23 cde
		7	4.14 abcde	2.05 abcd	8.50 cde
		14	6.17 bcdefg	2.77 abcde	7.20 cde
		21	6.13 bcdefg	2.54 abcde	8.10 cde
L1	10^5	1	5.73 bcdefg	2.39 abcd	6.90 bcde
		7	6.49 cdefg	2.80 abcde	7.23 cde
		14	5.26 abcdef	2.41 abcd	6.83 bcde
		21	4.64 abcdef	1.97 abcd	6.83 bcde
	10^6	1	6.49 cdefg	3.02 abcde	8.17 cde
		7	8.53 fg	4.38 de	9.23 de
		14	8.69 fg	4.24 de	8.37 cde
		21	5.70 abcdefg	2.39 abcd	8.20 cde
	10^7	1	4.96 abcdef	2.01 abcd	8.47 cde
		7	6.17 bcdefg	2.68 abcde	6.63 bcd
		14	5.95 bcdefg	2.52 abcde	8.33 cde
		21	6.04 bcdefg	2.90 abcde	7.07 bcde
	10^8	1	5.04 abcdef	2.16 abcd	8.80 de
		7	3.63 abcd	1.47 abc	4.57 ab
		14	3.25 abc	1.18 ab	8.33 cde
		21	5.85 bcdefg	2.56 abcde	5.87 bc
Control			1.69 a	0.71 a	3.37 a
NPK			35.48 h	18.39 f	28.37 f
P			<0.001	<0.001	<0.001
s.e.d			0.109	0.1114	0.1314
DMSE			4.2	1.43	1.85
C.V%			16.10	24.60	21.30

Means followed by the same letter are not significantly different using Duncan's New Multiple Range Test at alpha 0.05.

Continued Table 4.1

Effect	Wet weight			Dry weight			Plant N		
	P	s.e.d	l.s.d	P	s.e.d	l.s.d	P	s.e.d	l.s.d
Bacteria	(0.36)^{NS}	(0.03)	(0.06)	(0.30)^{NS}	(0.03)	(0.06)	(0.88)^{NS}	(0.01)	(0.02)
Frequencies	(0.12)^{NS}	(0.04)	(0.08)	(0.04)**	(0.04)	(0.08)	(0.04)**	(0.02)	(0.03)
Concentrations	(0.004)**	(0.04)	(0.08)	(0.01)**	(0.04)	(0.08)	(0.01)**	(0.02)	(0.03)
Bacteria .Frequencies	(0.16)^{NS}	(0.06)	(0.11)	(0.22)^{NS}	(0.06)	(0.11)	(0.66)^{NS}	(0.02)	(0.05)
Bacteria .Concentrations	(0.45)^{NS}	(0.06)	(0.11)	(0.42)^{NS}	(0.06)	(0.11)	(0.99)^{NS}	(0.02)	(0.05)
Frequencies.Concentrations	(0.01)**	(0.08)	(0.16)	(0.01)**	(0.08)	(0.16)	(0.002)**	(0.03)	(0.07)
Bacteria .Frequencies.Concentrations	(0.52)^{NS}	(0.11)	(0.22)	(0.54)^{NS}	(0.11)	(0.23)	(0.10)^{NS}	(0.05)	(0.09)
CV%		(16.7)			(26.0)			(4.6)	

Description: ** highly significant, ^{ns} not significant

4.7 Discussion

At harvest after two months, there were significant differences in wet and dry weight of lettuce plants ($P<0.001$), as a result of inoculation with Isolates L1 and Br2 at various doses and frequencies.

At 10^5, 10^6, 10^7cfu ml^{-1} weekly application of either isolates was the most successful frequency of application. Similar result obtained by Okan and Labandera-Gonzalez (1994) who determined that the optimum concentration of an *Azosprillum* isolate was 10^7cfu ml^{-1} for inoculation onto a range of different host plants. However, at 10^8cfu ml^{-1}, a single seed treatment or a seed treatment and a single drench at 21d was more effective than every 7 or 14d applications. According to Bai *et al*. (2002), co-inoculation of plant growth promoting rhizobacteria (PGPR) strains increased nodule number, plant dry weight and fixed nitrogen at optimal dose (10^8cells per seedling). Okon and Itzigsohn (1995) also suggested that a bacterial concentration of 10^9-10^{10}cells g^{-1} or ml^{-1} was the optimum concentration of FLD bacteria when applied as a seed treatment, especially for crops with small seeds.

The high dose and high frequencies were less effective than at low doses and low frequency. This may be probably due to an increase in substrate requirements of the bacterial populations. It seems clear that there is an optimum dose x frequency for FLD.
Indeed, our study showed the application of FLD isolates at 10^5 cfu ml^{-1} doses at 1d (seed treated) and 21d application did not result in a significant increase in biomass compared to the untreated control. At a dose of 10^{-8}cfu ml^{-1} applied at a range of frequencies, there were significant increases in biomass, compared to the untreated control. These results provide evidence that the doses and frequency of application may affect the establishment of inoculum in the rhizosphere of plants. The most interesting result from a commercial perspective is that the dose of 10^8cfu ml^{-1} applied by seed treatment was effective, because this would be relatively cheap to manufacture, and and as a treatment, it is easy and relatively cheap to apply, especially for field crops when drenching is not a realistic option, other than at planting.

Overall, the results demonstrated the beneficial effect of FLD isolates, increasing biomass and plant N content of lettuce compared to the control (un-fertilized and un-inoculated).

However, biomass was still much less than the NPK treatment and it is unlikely that these FLD treatments will replace nitrogen fertilization in the immediate future in commercial agriculture.

In conclusion, this study showed that inoculation of lettuce with FLD Isolates L1 (*B. cereus*) and Br2 (*B. subtilis*) increased yield and nitrogen content in lettuce. Dose and frequency of application interacted significantly.

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Appendix 4.1

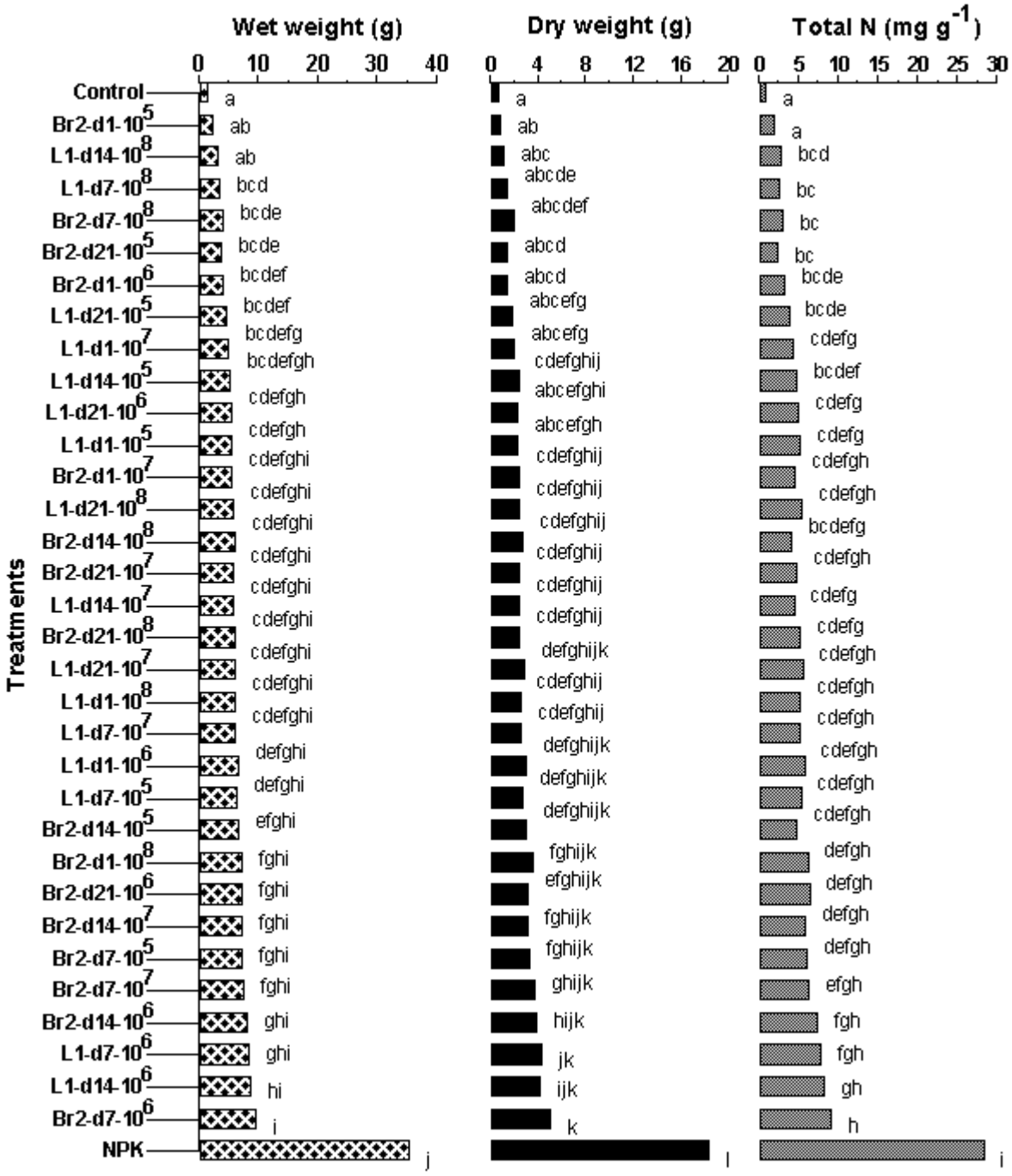

Fig. 4.1 Response of lettuce seedlings to treatment with Isolates L1 and Br2, applied at different doses and frequencies

CHAPTER FIVE

EFFECTS OF INOCULATION WITH FREE-LIVING DIAZOTROPHS (FLD) AND *Trichoderma harzianum* (Eco-T®) ON LETTUCE GROWTH

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Science and Agribusiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

Mixed inoculations of Isolate L1 (*Bacillus cereus* Frankland) at 10^6 and Eco-T® (*Trichoderma harzianum* Rifai) at 10^8cfu ml $^{-1}$ resulted in significant increases in growth and nitrogen content of lettuce. The pair of microbes was tested for their compatibility *in vitro* on PDA agar and there was no apparent antagonistic interaction. Weekly applications of Isolate L1 (*B. cereus*) at 10^6cfu ml^{-1}, applied alone, or in combination with *T. harzianum* (Eco-T®), resulted in significant increases in wet weight, dry weight and nitrogen content ($P<0.001$). Similarly, a single inoculation of Isolate L1 at 10^8cfu ml^{-1}, a single inoculation of Eco-T® and the combined inoculation of Eco-T® and Isolate L1 at 10^8cfu ml^{-1} increased yields, and the effect of combining microbes was additive. Inoculations of Isolate L1 (*B. cereus*) at 10^8 and 10^6cfu ml^{-1} with *T. harzianum* (Eco-T®) satisfied the need for nitrogen for lettuce growth to the level of 21% and 42.7%, respectively. Application of Isolate L1 (*B. cereus*.) alone at doses of 10^8 and 10^6cfu ml^{-1} satisfied the needs of lettuce for nitrogen to the level of 22% and 44.9%, respectively. Combinations of Isolate L1 (*B. cereus*) at 10^6 and 10^8cfu ml$^{-1,}$ with *T. harzianum* (Eco-T®) were either additive or synergistic for a number of growth parameters measured.

5.1 Introduction

Application of microbial inoculants has the potential to maintain soil fertility and promote plant growth (Bashan, 1998; Kennedy *et al*., 2004). Plants depend on nitrogen from the soil for their health and growth, and cannot acquire it from the gaseous nitrogen in the atmosphere. The primary way in which nitrogen becomes available to them is through nitrogen-fixing organisms that convert atmospheric nitrogen into nitrates or nitrites as part of their metabolism, and the resulting products are released into environment.

Non-symbiotic (free-living, associative or endophytic) nitrogen fixing genera such as *Azospirillum, Azotobacter, Acetobacter, Alcaligenes, Arthrobacter, Acinetobacter, Bacillus, Burkholderia* and *Azoarcus* may promote plant growth directly by fixation of atmospheric nitrogen, solubilization of phosphate, siderophore production or production of plant growth hormones (Okon and Kapulnik, 1986; Kloepper *et al*., 1989; James and Olivares, 1998; Steenhoudt and Vanderleyden, 2000).

Free-living diazotrophs (FLD) associated with plants have been shown to improve the productivity of non-leguminous crops (Boddey *et al*., 1995; Döbereiner *et al*., 1995; Boddey *et al*., 1998). The genus *Azospirillum* was discovered in nitrogen-poor soils of Netherlands by Beijerinck (1925) (*in* Holguin *et al*. (1999)). When inoculated onto cereals such as wheat (Kapulnik *et al*., 1983), sorghum (Alagawadi and Gaur, 1992; Sarig *et al*., 1988), millet (Rao *et al*., 1985) and maize (Garcia De Salomone and Döbereiner, 1996) and noncereal species, increased plant growth and levels of nitrogen in shoots and grains occurred (Baldini and Dobereiner, 1980; Kapulnik *et al*., 1981; Bashan, 1986; Rao *et al*., 1987; Barbieri *et al*., 1988; Das and Saha, 2003).

Trichoderma is a genus of asexually reproducing fungi that are frequently isolated soil fungi. Certain isolates of *Trichoderma* species can provide plant growth promotion in the absence of any major pathogens (Inbar *et al*., 1994). For example: *Trichoderma harzianum* 1295–27 has been shown to solubilize phosphate and micronutrients that are then made available to enhance plant growth (Altomare *et al*., 1999). However, Whipps (1997) noted that multiple microbial interactions involving bacteria and fungi in the rhizosphere, sometimes promote plant growth better than a single species of microorganism. With this idea in mind, Zaidi and Khan (2007) inoculated *Azospirilum* spp. in combination with phosphate-solubilizing

microorganisms and a arbuscular mycorrhizae (AM) fungus and recorded increases in growth, grain yield, and N content of wheat plants.

According to Rovira (1965) (*in* Ki and Milic (2001)), wheat increased its growth when inoculated with *Azotobacter chroococcum* Beijerinck, *Clostridium pasteurianum* (sic) Winogradsky and *Bacillus polymyxa* (Prazmowski) Mace. Inoculation of nitrogen fixing isolate PNF11, along with other bacterial strains such as *Azotobacter* sp. (AZS_3), fluorescent *Pseudomonas* (Ps_5), and *Bacillus* sp. (Bc_1), improved plant growth and yield of *Vigna radiata* T44 (Ahmad *et al.*, 2006).

The main objective of this research was to evaluate the ability of FLD bacterial Isolate L1 (*B. cereus*), in combination with Eco-T® (*Trichoderma harzianum* Rifai) on the growth of lettuce cultivar Great Lakes.

5.2 Materials and methods

5.2.1 Production of the FLD isolate

An FLD Isolate L1 was previously isolated from soils where vegetables, maize, millet and beans had previously been cultivated. Isolate L1 was cultured in 250ml conical flasks containing 100ml of sterilized tryptone soy broth (Merck) medium incubated at 28^0C for 72h in a water bath shaker at 150rpm (GFL® 1083, Labortechnik). The cell suspension was then centrifuged at 9000 x g for 20min (Beckman J2 HS centrifuge). Cell pellets were then resuspended and washed twice with sterile distilled water. Final cell pellets were diluted with sterile distilled water. Cell number was adjusted to 10^6 and 10^8cfu ml^{-1}. This procedure was repeated each time a fresh cell suspension was needed.

5.2.2 Source of *Trichoderma*

A commercial strain of Eco-T® (*T. harzianum*)[11], was used for inoculation. It contained approximately 10^8 conidia g^{-1}.

[11] PHP Ltd, Nottingham Road, RSA

5.2.3 *In vitro* dual culture of Isolate L1 *(B. cereus*) and *T. harzianum*

A single streak of Isolate L1 was made bisecting a 90mm diameter petri dish containing potato dextrose agar (PDA) (Merck). A 4x4mm agar disk colonized with Eco-T® (*T. harzianum*) was placed at the edge of the plate on either side of the bacterial streak. The dual culture bioassay was replicated three times. Plates inoculated solely with Eco-T® served as a control. Plates were incubated at 28^0C and were checked after six days for indications of a microbial interaction.

5.2.4 Greenhouse trial

Seedling trials were carried out in pots kept under shade house conditions. Lettuce seeds were planted into pots filled with composted pine bark growing medium (CPB). Five seeds were planted per pot and were thinned to two plants per pot after germination. Trials consisted of eight treatments, including two control treatments. The pots were placed in an ice-cream container (used as a water reservoir) and watered every week. As a first control, seeds were planted in eight pots and watered from tap water and labeled as "Control-None". The other eight pots served as a positive control and were supplemented weekly with fertilizer called 3:1:3(38) (Complete)[12] at a rate of 1g ℓ^{-1} and was labeled as "NPK". The other treatments were watered with only micronutrients (Microplex)[13] at 0.02g ℓ^{-1}. Each treatment was replicated four times. Pots were arranged in a complete randomized block design.

5.2.5 Seedling drench

Prior to inoculating with the FLD organism, untreated lettuce seeds (cultivar Grate Lakes) were planted into pots filled with composted pine bark. There were two pots in each replicate and four replicates for each treatment (2 x 4 x 6) giving a total of 48 pots.

For drenching, 5ml quantities of a cell suspension (10^6 and 10^8cfu ml^{-1}) of Isolate L1 (*B. cereus*.) were inoculated onto seedlings right after emergence and weekly for one month. One day later 4ml of an Eco-T® suspension, containing approximately 10^8conidia ml^{-1}, was drenched into each pot.

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[12] Ocean Agriculture, Muldersdrift, Republic of South Africa
[13] Ocean Agriculture, Muldersdrift, Republic of South Africa

5.2.6 Seeds

Lettuce (cultivar Great Lakes) seeds were used in this study. Untreated lettuce seeds were obtained from Starke Ayres Seed Company (Pty) Ltd., Pietermaritzburg, Republic of South Africa.

5.2.7 Biomass measurements

Lettuce plants were harvested at soil level when two months old. After measuring the wet weight, the plants were placed in paper bags and dried at 70^0C for 48h. The total dry weight of plants per pot was determined. Only above-ground stems and leaves were weighed.

5.2.8 Nitrogen analysis

The analysis technique of Willis *et al*. (1996) was used. See Appendix B for details.

5.2.9 Statistical analysis

A general linear model (GLM) was used to run an ANOVA on the wet and dry weight of seedlings and plant N. If the F test was significant, ($P<0.05$), then the means were compared using the least significant difference (LSD) test.

In order to determine the effects of Eco-T® (*T. harzianum*) and Isolate L1 (*B. cereus*) treatments, the plant growth parameters were changed into percentage. The observed wet weight, dry weight and plant N were compared to the expected biomass and plant N under the assumptions of an independent effect. The expected Wet weight, Dry weight and plant N percentage (W_E) for Eco-T® was calculated as follows:

$WL1W_E = WL1 + W_E (1-WL1)$ (Finney, D.J. 1964).

Where WL1 at 10^6 or 10^8 and W_E are the observed growth parameters caused by Isolate L1 and Eco-T®alone, respectively.

Results from an X^2 test were compared to the X^2 table of values (df = 1, $P \leq 0.05$).

$X^2 = (O-E)/E$

Where,

O = the observed growth parameters for either of Eco-T® or Isolate L1 (at 10^6cfu ml^{-1} or 10^8cfu ml^{-1}) treatments

E = the expected growth parameters for either the Eco-T® or Isolate L1 (at 10^6cfu ml^{-1} or 10^8cfu ml^{-1}).

An additive interaction would be indicated if $X^2 < 3.84$. A synergistic interaction would be indicated if $X^2 > 3.84$.

If O>E, then the interaction would be considered synergistic.

If O<E, then the interaction would be considered antagonistic.

5.3 Results

5.3.1 *In vitro* interaction between Eco-T® (*T. harzianum*) and Isolate L1 (*B. cereus*)

Eco-T® (*T. harzianum*) and Isolate L1 (*B. cereus*) did not inhibit each other in any of combinations and interactions tested (Fig 5.1).

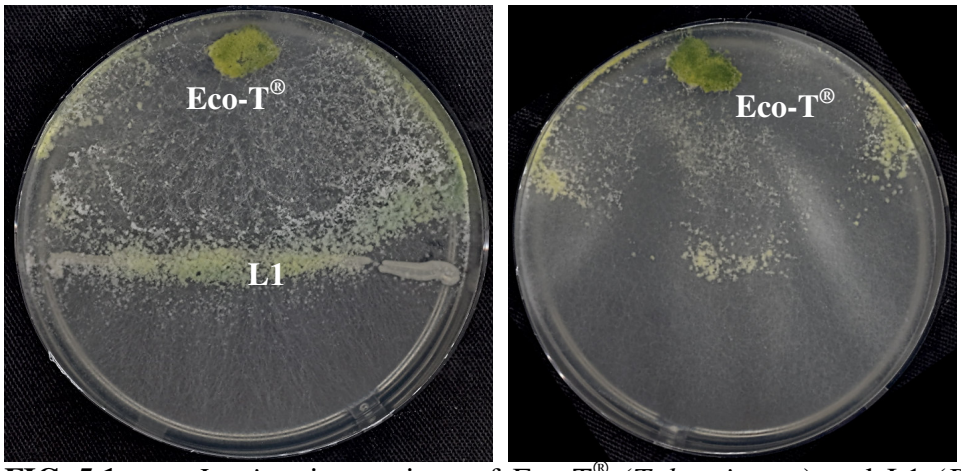

FIG. 5.1 *In vitro* interactions of Eco-T® (*T. harzianum*) and L1 (*B. cereus*). Eco-T® overgrew Isolate L1 after five days incubation at 28^0C on PDA agar plates. There was no inhibition zone.

5.3.2 Effect of single and dual inoculation of Eco-T® (*T. harzianum*) and Isolate L1 (*B. cereus*) on lettuce growth

In this experiment, as reflected in Table 5.1, a single inoculation of Isolate L1 applied alone at a concentration of 10^6cfu ml^{-1} resulted in a significant (P<0.001) increase in biomass and plant N relative to the untreated control, L1 at a concentration of 10^8cfu ml^{-1}, Eco-T® and

Eco-T® with L1 at 10^8cfu ml^{-1}. Isolate L1 at a concentration of 10^8cfu ml^{-1}alone and in combination with Eco-T® gave less wet and dry weight and plant N.

Inoculation of Isolate L1 only at a concentration of 10^6cfu ml^{-1} satisfied the need for nitrogen to the level of 44% of nitrogen requirements, and 42.7% when applied in combination with Eco-T®. Inoculation of Isolate L1 alone at a concentration of 10^8cfu ml^{-1} satisfied the need for nitrogen to the level of 23.5% of nitrogen requirements and 22.9% when inoculated in combination with Eco-T®.

Table 5.1 Effects on lettuce growth by inoculations of Isolate L1 (*B. cereus*) at various concentrations, with or without with Eco-T® (*T. harzianum*)

Treatments	Wet weight (g)			Dry weight (g)			Plant N (mg g^{-1})		
Control	5.60	(0.81)	a	0.71	(0.23)	a	1.03	(0.31)	a
Eco-T	12.3	(1.12)	b	1.78	(0.44)	bcd	2.39	(0.56)	b
$L1^{-1}0^8$	12.6	(1.12)	b	1.62	(0.40)	b	2.66	(0.55)	b
$L1^{-1}0^8$ + Eco-T	11.3	(1.09)	b	1.60	(0.41)	bc	2.59	(0.53)	b
$L1^{-1}0^6$	23.6	(1.38)	c	2.74	(0.57)	cd	4.44	(0.73)	c
$L1^{-1}0^6$ + Eco-T	22.9	(1.37)	c	3.02	(0.59)	d	4.30	(0.72)	c
NPK	53.6	(1.70)	e	6.00	(0.81)	e	8.76	(0.97)	d
Eco-T + NPK	95.3	(1.98)	f	10.67	(1.07)	f	9.42	(1.00)	d
P	<0.001			<0.001			<0.001		
s.e.d.	0.075			0.078			0.057		
l.s.d.	0.157			0.163			0.119		
C.V%	8.1			19.6			11.7		

Means with the same letter in the same column are not significantly different at P< 0.05, based on Fisher's LSD test

Values in parentheses represent transformed means using a log transformation

Table 5.2 Effects of combining FLD Isolate L1 at 10^6cfu ml^{-1} or 10^8cfu ml^{-1} and Eco-T® on growth of lettuce in hydroponics

Treatments	Growth parameters	Observed %	Expected%	X^2	Interaction
Eco-T +L1(8)	Dry Weight	50.3	51.7	0.037	Additive
	Wet Weight	42.7	41.5	0.037	Additive
	Plant N	50.7	50.3	0.003	Additive
Eco-T +L1(6)	Dry Weight	26.7	57.4	16.455	Synergistic
	Wet Weight	21.1	56.9	22.530	Synergistic
	Plant N	27.3	64.3	21.310	Synergistic
Eco-T + NPK	Dry Weight	177.8	100.0	60.580	Synergistic
	Wet Weight	178.8	100.0	62.093	Synergistic
	Plant N	171.8	100.0	51.558	Synergistic

Combination of Eco-T® and FLD Isolate L1 at 10^8cfu ml^{-1} resulted in significantly higher growth parameters than the Control-None. At this concentration, the interaction between Eco-T and Isolate L1 was additive, which indicates that the two agents were acting independently on the growth of lettuce (Table 5.2). However, at a concentration of 10^6cfu ml^{-1} application of Isolate L1, with or without Eco-T®, resulted in significantly higher wet, dry weight and plant N than the Control-None, or itself at 10^8cfu ml^{-1}.

5.4 Discussion

In this study, combining two biofertilizer organisms showed potential to reduce crop requirements for fertilizers. The use of two groups of organisms together has been proposed as one approach to improve plant growth (Barea *et al*., 1997). Benefits of using such combinations for plant growth promotion could result in increased crop mineral uptake and yields (Roesti *et al*., 2006; Rudresh *et al*., 2005). There is also potential for the exploitation of *Trichoderma sp*. for plant growth promotion and to provide biological control against sub-lethal pathogens (Vinale *et al*., 2008; Bailey *et al*., 1998). It is with this idea that the dual inoculation of FLD Isolate L1 (*B. cereus*) and Eco-T® (*T. harzianum*) was evaluated for the compatibility *in vitro* of the two organisms and their combined effects on the growth and nitrogen content of lettuce under greenhouse conditions.

Interactions between Eco-T® (*T. harzianum*) and FLD Isolate L1 (*B. cereus*) *in vitro* showed no indication of microbial inhibition, and no quantitative differences were recorded. This observation led to inoculate both microbes for plant growth improvement.

In the greenhouse trial, either solo or dual inoculations of FLD isolate L1 (*B. cereus*) with Eco-T® (*T. harzianum)* improved plant growth and nitrogen content of lettuce seedlings. Its interaction with Eco-T®was synergistic, which indicates that the two microorganisms enhanced the impact of the other for an enhanced effect on lettuce plant. The interaction between Eco-T® and NPK fertilizer were was also synergistic effects on lettuce growth, probably as a result of growth stimulation enhancing root structures, and/or the effect of biocontrol against sub-lethal pathogens.

The effects we observed on combined inoculations on lettuce growth are in agreement with the observations of Al-Nahidh and Gomah (1991) and Zaidi and Khan (2007). Both found that as a result of mixed inoculation with diazotrophic bacteria and VA-mycorrhiza increased growth and nutrient content of wheat. Dual inoculation with *Frankia* and mycorrhizal fungi also showed enhanced nitrogen-fixing ability and better growth of *Hippophae tibetana* Schlecht seedlings (Tian *et al*., 2002). The result is also consistent with the findings of Flouri *et al*. (1995) who studied dual inoculation of *Azospirillum* with a fungal biocontrol agent, *Phialophora radicola* Deacon, on wheat. Another successful study was on the combined inoculation of black mangrove seedlings with *Phyllobacterium sp*. and a fast-growing, phosphate-solubilizing bacterium *Bacillus licheniformis.* The seedlings developed more leaves than resulted from individual bacterium inoculation (Rojas *et al*., 2001).

Inoculation of the lettuce plants with FLD Isolate L1 at the lower dose (10^6cfu ml^{-1}), alone or in combination with Eco-T®, significantly increased lettuce biomass and nitrogen content compared to inoculations of Isolate L1 at 10^8cfu ml^{-1},with or without Eco - T ®. This reduced response to Isolate L1 applied at the higher dose (10^8cfu ml^{-1}) may have resulted from increased competitive interactions and population dynamics of microbial communities at the root-soil boundary. Growth and population dynamics, and carrying capacity of microbial communities in the rhizosphere is determined by the limited carbon resources released by plants as root exudates (Grayston *et al*., 1997). However, this contrasts with research done by (Rodriguez-Romero *et al*., 2005), where inoculation with arbuscular mycorrhizal fungus *Glomus manihotis* (Manihotina), followed by *Bacillus* spp. at 10^8cfu ml^{-1} 20 days later increased the fresh weight, aerial dry weight, and shoot length and leaf areas of banana plants more than a lower dose at 10^6cfu ml^{-1}.

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Appendix 5.1

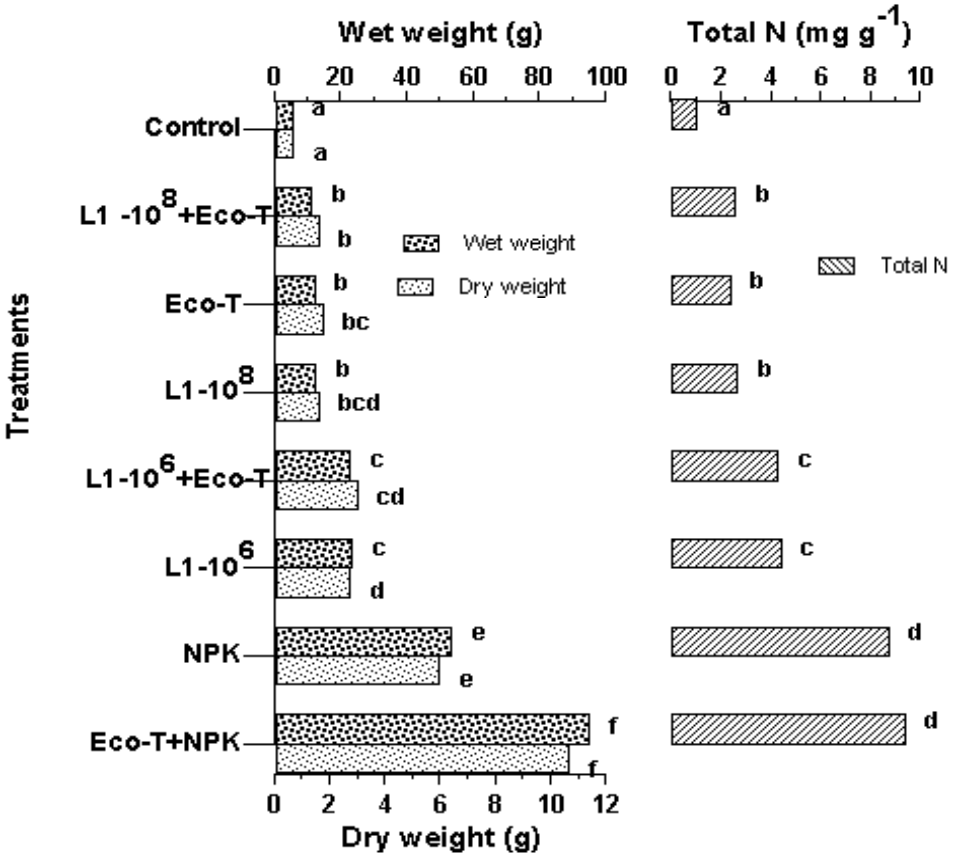

Fig. 5.2 Effects of inoculation of lettuce with Eco-T® (*T. harzianum*) and FLD bacterium Isolate L1 (*B. cereus*) at 10^6 or 10^8cfu ml^{-1}.

CHAPTER SIX

INTERACTION EFFECTS OF FREE-LIVING DIAZOTROPHS (FLD) COMBINED WITH VARIOUS NPK FERTILIZATION LEVELS ON LETUCE GROWTH IN HYDROPONICS

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Science and Agribusiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

A hydroponics experiment was conducted to study the potential of biofertilizers to be combined with reduced levels of commercial fertilizers for lettuce growth. Inoculation of a microbial inoculant Isolate L1 (*Bacillus cereus* Frankland*)* and minimal levels of mineral fertilizers improved the growth and plant N of a lettuce crop six weeks after transplanting the seedlings.

Application of a culture of FLD Isolate L1 (*B. cereus)* together with four different doses of nutrient solution (NPK) affected the growth and plant N of a lettuce crop. Inoculation with Isolate L1 (*B. cereus)* alone significantly increased wet weight, dry weight and plant N relative to an unfertilized control. A highly significant increase in wet weight was measured when Isolate L1 (*B. cereus*) was applied in combination with 25% or 50% NPK nutrient solution ($P<0.001$). It was concluded that application of appropriate microbial inoculants with reduced levels of mineral fertilizers in hydroponics had a positive effect on lettuce growth and provided adequate levels of plant N.

6.1Introduction

Today, most farmers in developing countries still practice subsistence agriculture. Declining soil fertility is a critical problem affecting their agricultural productivity. Over the past 50 years, modern agricultural systems doubled food production, mainly of cereals, by increasing inputs in fertilizers, pesticides and other production technologies (Matson *et al*., 1997; Tilman *et al*., 2001). However, because of the increasing cost of inorganic fertilizers, limited availability and uncertain accessibility (Bohlool *et al*., 1992; Odhiambo and Magandini, 2008), small scale farmers have not used them to maximize their agricultural production, because the majority of the world's farmers, especially in Africa and Asia, cannot afford to use nitrogenous fertilizers.

To solve this problem, attention is now focused on in developing the technology of biofertilizers. This is as an alternative approach to crop nutrition. Instead of using conventional fertilizers, beneficial micro-organisms are applied to the crops and they provide some or all of the macronutrients to crops. Biological nitrogen fixation (BNF) represents a renewable source of nitrogen for agriculture (Sabry *et al*., 1997). The *Rhizobium*–legume symbiosis represents the most established system of BNF and has showed great potential to improve the productivity and sustainability of agriculture (Peoples *et al*., 1995). *Rhizobium gallicum* is an efficient symbiont for bean cultivation (Mnasri *et al*., 2007). Nitrogen requirements can be met from symbiotic rhizobia that are able to nodulate legume roots (Aguilar *et al*., 2001). In addition, they provide a beneficial effect on the productivity of cereals and other crops grown in agricultural rotations with legumes by recycling of N through plant residues (George *et al*., 1992; Drevon *et al*., 2001).

Non-symbiotic associations between diazotrophic bacteria and non-leguminous plant roots are also an important source of BNF (Kennedy and Tchan, 1992). Strains of *Acetobacter* and *Herbaspirillum* can contribute significant quantities of nitrogen to sugarcane (Kennedy *et al*., 1997). Associative diazotrophic bacteria increased yield in a range of field crops (Kennedy *et al*., 2004). Free-living hetrotrophic diazotrophs (FLD) such as *Azotobacter sp*. may increase yields of rice by >20% (Yanni and El-Fattah, 1999). *Bacillus cereus* Frankland is also a non-symbiotic FLD bacterium that may provide crops with some of their nitrogen requirements (Seldin *et al*., 1983; Li and Zhang, 2000). Other *Bacillus* strains can also suppress plant pathogens and stimulate nutrient uptake by plants, either by promoting rhizobial symbiosis or

by directly fixing atmospheric nitrogen (Bloemberg and Lugtenberg, 2001; McSpadden Gardener, 2004).

Increases in oil prices have focused attention on the need to reduce costs of fertilization and to decrease pollution events in modern agriculture. For this reason, the use of biofertilizers is being studied widely in order to reduce applications of chemical fertilizers (Al-Nahidh and Gomah, 1991; Kader *et al*., 2002; Kadwe *et al*., 2004; Mekki and Ahmed, 2005; El Kramany *et al*., 2007; Hegazi *et al*., 2007; Mahfouz *et al*., 2007;).

Free-living diazotrophs (*Azotobacter chroococcum* Beijerinck*, Azospirillum liboferum* (Beijerinck) Tarrand et al*,* and *Bacillus megatherium,* applied with 50% of the recommended dosage of NPK fertilizer, increased growth parameters of oil content of fennel (*Foeniculum vulgare* Mill.) compared to NPK fertilizer treatments only (Mahfouz *et al*., 2007). According to Maheshwari *et al*. (1992), use of FLD (mainly *Azotobacter* spp.) alone increased palmarosa (*Cymbopogon martini* var. Motia) yield by 16% and when applied together with 80kg N fertilizer, yield increased by 29%.

The objective of this study was to determine the growth and plant N of lettuce plants as influenced by the application of a biofertilizer, Isolate L1, with different doses of inorganic N fertilizer in hydroponics. The ultimate goal was to see if it is possible to minimize inorganic fertilization N usage without incurring any yield loss.

6.2 Materials and methods

6.2.1 Bacterial culture

A culture of a non-symbiotic biological nitrogen fixing bacterium, previously isolated from the rhizosphere soil Isolate L1 (previously identified as *B. cereus,* using sequence analysis of the small ribosomal subunit, 16S r DNA, at Inqaba Biotech[14]) was used in this study to determine its influence on growth of lettuce. The bacterium was grown on TSB medium for 24h on a shaker at 150rpm and 30^0C.

[14] Inqaba Biotechnical Industries (Pty) Ltd. P.O.Box 14356 Hatfield 0028, Pretoria, South Africa

6.2.2 Hydroponics experiment

Lettuce seedlings were used for evaluating the effect of FLD on plant growth and N levels as a result of the inoculation of microbial inoculant Isolate L1 (*B. cereus*) and different doses of a mineral fertilizer in hydroponics. Seeds were planted in Speedlings® trays and left in a greenhouse until emergence. The roots of seedlings were washed clean with tap water, then transplanted into a hydroponics system. For the fertilization treatments 3:1:3(38) (Complete)[15] (NPK) was applied at five levels, i.e. zero, full (1g ℓ^{-1}), three-quarter (0.75g ℓ^{-1}), half (0.5g ℓ^{-1}) and one- quarter (0.25g ℓ^{-1}) dose rates, in the presence or absence of FLD inoculum. At the same time, 0.017g ℓ^{-1} of a mixed micronutrient product (Microplex)[16] was added to the solution. Nutrient solutions were renewed at one week intervals. Isolate L1 (*B. cereus*) was used as a bio-inoculant. During the period of treatment, a bacterial suspension (2ml) was added to a hydroponics solution in which the final population of the bacterium was approximately $1x10^6$cfu ml^{-1}. The bacterial suspension was renewed once every week. The different treatments were arranged in a complete randomized block design with three replicates for each treatment.

6.2.3 Plant harvest, growth parameters and N-analysis

Plants were harvested after six weeks and weighed, and then dried for nitrogen analysis. The nitrogen total was calculated by the following formula (Kalra, 1998):

g kg^{-1} N = [(µg ml^{-1} x color development reagent (ml))] / [g Sample(g)/50]

6.2.4 Nitrogen analysis

This was conducted as per the protocol of Willis *et al*. (1996). For details see Appendix 2.

6.2.5 Statistical analysis

GenStat 9th Edition was used for analysis of variance. When the *F*-test was significant, the treatment means were compared using the Least Significant Difference (LSD) test.

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[15] NPK: Ocean Agriculture 3:1:3(38) Complete, Muldersdnift, South Africa
[16] Microplex, Ocean Agriculture Muldersdnift, South Africa

6.3 Results

Integrated application of different rates of inorganic fertilizer (NPK) and the biofertilizer, Isolate L1 (*B. cereus*) increased the growth and plant N of lettuce under hydroponic conditions. A wet weight of 29.8g, dry weight of 8.0g and plant N of 6mg g^{-1} DW were obtained after the application of the FLD alone, an increase of 123% wet weight, 100% dry weight and 100% plant N, compared with untreated control.

In all parameters (dry weight, wet matter and plant N), the effect of FLD Isolate L1 (*B. cereus*) integrated with 75% and 100%NPK fertilizer was not significantly different from those of 75%NPK and 100% NPK fertilizer alone (Table 6.1 and Fig. 6.1).

Table 6.1 Effect of a biofertilizer (FLD Isolate L1) in combination with different doses of NPK fertilizer on lettuce growth and plants N

Bacteria	Wet weight (g)		Dry weight (g)		Plant N (g kg^{-1} DW)		
None	13.0	a	4	a	3	(0.61)	a
L1	29.8	b	8	b	6	(0.86)	b
25% NPK	38.1	c	11	bc	9	(0.96)	c
L1 +25% NPK	43.3	d	12	c	10	(1.03)	d
50% NPK	68.7	e	17	d	15	(1.19)	e
L1 +50% NPK	75.5	f	19	d	16	(1.22)	e
75% NPK	104.5	g	26	e	20	(1.32)	f
L1+75% NPK	108.5	g	27	e	22	(1.36)	f
100% NPK	142.4	h	36	f	28	(1.47)	g
L1 +100% NPK	141.3	h	35	f	28	(1.47)	g
P	<0.001		<0.001			(<0.001)	
s.e.d	1.6		3.79			(0.046)	
l.s.d	3.3		7.96			(0.096)	
C.V%	9.9		6.1			(4.9)	

Means with the same letter in the same column are not significantly different at $P < 0.05$. Values in Parenthesis represents transformed means using Log transformations

The interaction between FLD Isolate L1 with different strength of NPK fertilizer was additive and based on the two agents acting independently for lettuce growth (Table 6.2).

Table 6.2 Interaction effects of combining FLD Isolate L1 at 10^8cfu ml^{-1} with different levels of NPK fertilization on growth of lettuce

Treatments	Parameters	Observed%	Expected%	X^2	Interaction
L1 + 25%NPK	Wet weight (g)	30.4	42	3.20	Additive
	Dry weight (g)	33.3	47.7	3.30	Additive
	Plant N (mg g^{-1})	35.7	45.9	2.26	Additive
L1 + 50%NPK	Wet weight (g)	53.0	58.9	0.59	Additive
	Dry weight (g)	52.8	62.5	1.51	Additive
	Plant N (mg g^{-1})	57.1	63.1	0.56	Additive
L1 + 75%NPK	Wet weight (g)	76.2	81	0.29	Additive
	Dry weight (g)	75.0	78.1	0.12	Additive
	Plant N (mg g^{-1})	78.6	76.3	0.07	Additive
L1 + 100%NPK	Wet weight (g)	99.2	100	0.01	Additive
	Dry weight (g)	97.2	100	0.08	Additive
	Plant N (mg g^{-1})	100.0	100	0.00	Additive

6.4 Discussion

Recently the use of bio-fertilizers (FLD) in agriculture has received considerable attention because the production technology for biofertilizers is relatively simple and the cost is relatively low compared to chemical fertilizer plants. Bio-fertilizers improve plant growth; reduce the addition of more mineral nutrients to the soil, and are a renewable source of N.

The FLD Isolate L1 was applied to lettuce plants in hydroponics and these plants grew significantly better than the untreated control plants. Plant N was increased by 21.4%.

The response of lettuce growth and plant N to application of 25% of the recommended NPK level (25% NPK) integrated with Isolate L1 (*B. cereus*) was significantly better than that of the 25% NPK alone. The increase in plant N observed as a result of inoculation with the FLD alone was less than for the integrated application of the FLD with 25% NPK. Therefore combining NPK at 25% recommended dose combined with FLD Isolate L1 increased lettuce

wet and dry biomass by 30.4% and 33.3% respectively. Plant N was also increased by 34.7%. This is a good indication that it may be possible to integrate FLD applications with low levels of commercial fertilizers.

Application of FLD in combination with 50% NPK produced highly significantly increases in wet matter relative to application of 50% NPK alone. In other studies, when plant-growth promoting rhizobacteria (*Azotobacter* strains) were used as inoculants for biofertilization and phytostimulation of canola, some of the inoculants improved growth and yield parameters above the plants fertilized with 50% of the recommended dose of nitrogen (Zaied *et al*., 2007). According to Young *et al*. (2004), the combined effect of a biofertilizer with 50% chemical fertilizer increased lettuce yield by 25% compared to only chemical fertilizer. Similar results were achieved on rice yields by Jeyabal and Kuppuswamy (2001).

Application of 75% and 100% of dose of NPK fertilizer alone were not significantly different in growth and plant N compared to treatment with FLD and 75% and 100% doses of NPK fertilizer.

Combining FLD with reduced doses of NPK fertilizer may achieve optimum nutrient management for crop growth. There are many references in the literature that high levels of chemical fertilizer applications are not good for a soil in the long term (Byrnes, 1990). Therefore reducing the amount of NPK, by adding beneficial micro-organisms, has considerable potential to reduce the negative impacts of chemical fertilizer (Chen, 2006).

Small-scale farmers, also called resource-poor farmers, struggle to afford nitrogenous fertilizers. In many cases in Africa and Asia, their staple foods are grown without the benefit of any fertilizer, or with very limited fertilizer applications. In these cases, the low cost of FLD applications make this option an attractive one. Where a resource poor farmer does apply low levels of nitrogenous fertilizer, the FLD will still contribute more nitrogen and growth stimulating activities to the crop health. This approach may have its greatest benefit for resource-poor farmers.

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Appendix 6.1

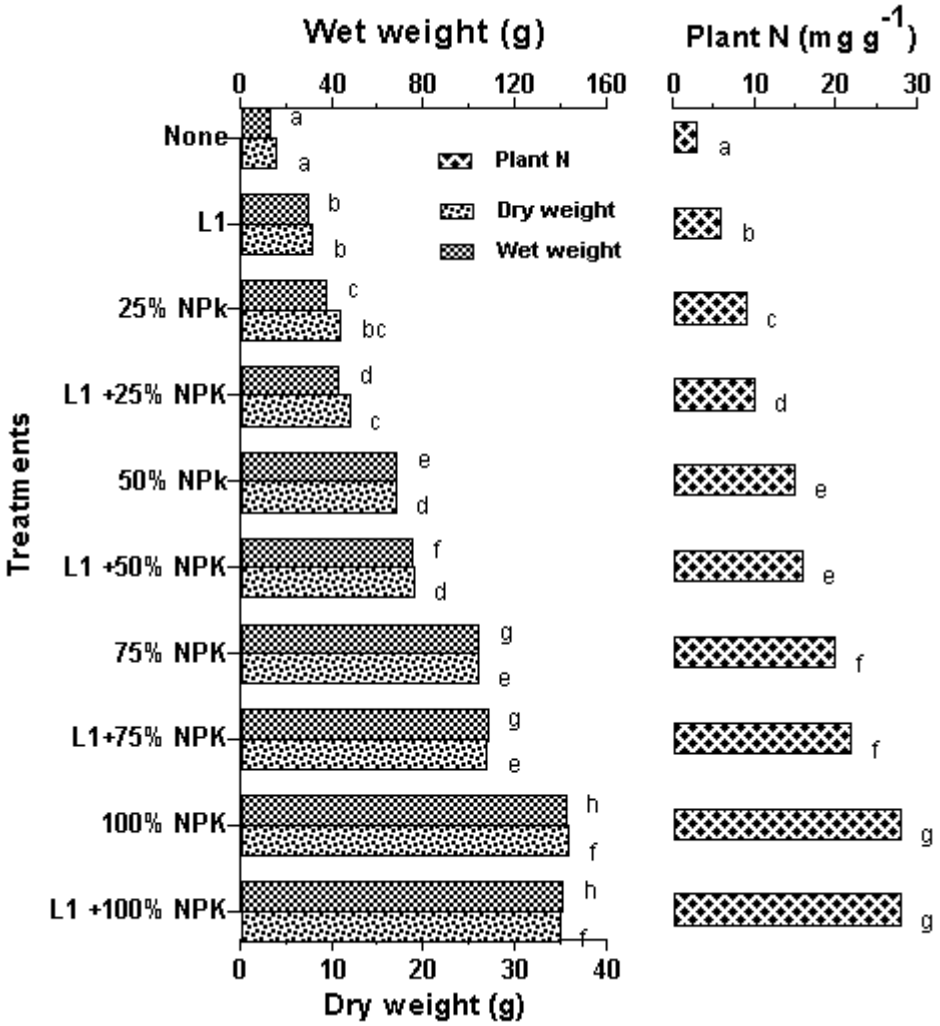

Fig 6.1 Effect of a biofertilizer (FLD), in combination with NPK fertilization, on lettuce growth and plant N in hydroponics

CHAPTER SEVEN

THE EFFECT OF BIOFERTILIZATION ON GROWTH, YIELD AND N-LEVEL OF MAIZE IN A FIELD TRIAL

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Science and Agribusiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

Three free-living diazotrophs (FLD), Isolates D6 (*Bacillus cereus* Frankland), L1 (*Bacillus cereus* Frankland) and Br2 (*Bacillus subtilis* (Ehrenberg) Cohn), were tested for their effects on plant growth, yield, N level and population dynamics over time in a field trial. The FLD bacterial communities of maize rhizosphere associated with field-grown maize roots were examined for quantitative changes in size, over time. Rhizospheric soil samples were taken 30, 60 and 90 days after planting (DAP). Individual bacterial colonies of FLD were sampled using mannitol N-free-agar to estimate the FLD number in the maize rhizosphere. Five treatments included a control (no fertilizer), chemical fertilizer (NPK), and three biofertilizers (D6, L1 and Br2). After inoculation of FLD isolates there was a decrease in number of FLD cells over time. Plants treated with Isolate D6, followed by L1 and Br2, maintained the highest number of FLD cells at 30 and 60 days after planting. The smallest FLD population was in the rhizosphere of plants treated with NPK fertilizer, and plants of the untreated control. At the time of harvesting all counts of Isolate D6 were higher than those of the other treatments. Dry and wet weight was also recorded for all treatments, and the FLD and NPK fertilizer treated plants were compared to the untreated control. Plants treated with NPK fertilizer did not have a significantly higher yield than plants treated with FLD isolates Br2 and D_6. Application of biofertilizers containing three different FLD Isolates significantly increased the growth of maize over time, as did NPK fertilizer treated plants. Inoculation of maize with different isolates of nitrogen fixing bacterial isolates in a field showed significant growth and yield responses.

7.1 Introduction

Cereals, mainly wheat, rice and maize, are the world's most important food sources. In many sub-Saharan African countries, small-scale farmers have depleted soil nutrients, much as 22kg of N ha^{-1}p.a. (Drechsel *et al*., 2001) because they are using the land without application of sufficient quantities of manure or fertilizer (Sanchez, 2002). To overcome the nutrient depletion, it is typical to use mineral fertilizers (Palm *et al*., 1997), but the increasing cost of fertilizers is a problem for both commercial and small-scale farmers (Johnson and Lay, 1974; Sanchez, 2002). An alternative approach is to use biofertilizer inoculations. There are many micro-organisms in the rhizosphere of plants that are able to exert beneficial effects on plant growth. Free-living rhizobacteria, such as *Azospirillum* spp. (Okon and Itzigsohn, 1995), *Bacillus* spp. (Seldin *et al*., 1984) and *Paenibacillus* spp. (Seldin *et al*., 1998) have been used as seed inoculants, to provide beneficial effects to host plants. According to Picard *et al*., (2008), cereals are able to maintain free-living diazotrophs (FLD) in their rhizosphere. Root exudation is a key driver of nitrogen fixation by diazotrophic bacteria residing in the soil and rhizosphere (Jones *et al*., 2003) because growth of the microbial population in a rhizosphere is controlled by the nutrients present in the exudates (Albrecht *et al*., 1981; Del Gallo and Fendrik, 1994; Angle *et al*., 1996; Grayston *et al*., 1997). Bacterial populations growing in a rhizosphere may also be influenced by the age of plant (Westover *et al*., 1997).

Experiments with the inoculation of free-living diazotrophic (FLD) bacterial populations associated with particular plants showed increases in plant productivity and significant increases in level of nitrogen in crops under some conditions (Evans and Barber, 1977; Gaskins *et al*., 1985; Schippers *et al*., 1987; Bashan and Levanony, 1990; Okon and Itzigsohn, 1995). However, not all attempts to increase nitrogen fixation activity have been successful. On the other hand, bacterial inoculation can be highly beneficial, even when nitrogen fixation is not affected (Gaskins *et al*., 1985; Elsas *et al*., 1986;).

The purpose of this field study was to study the effect of inoculation with FLD isolates onto a maize crop, compared with an uninoculated unfertilized control, and an uninoculated, fertilized control.

7.2 Materials and methods

7.2.1 Field experiment design

The field experiment conducted at Ukulinga Farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa. There were five treatments consisting of inoculation with three FLD isolates (D6, L1 and Br2), an uninoculated control and an uninoculated, fertilized control (NPK 3:1:3(38) Complete)[17] treatments. There were four replicates for each treatment in a complete randomized block design.

7.2.2 Inoculum preparation

Two FLD isolates, D6 and L1, were isolated previously, based on their nitrogen fixing ability in previous experiments. Isolate Br2 was provided by Dr Brendon Neumann[18]. Each FLD isolate was cultured separately in 250ml conical flasks containing 100ml of sterilized Tryptone soy broth (Merck) medium. Each flask was inoculated with a loop-full of FLD isolate cultured on tryptone soy agar (Merck) (28^0C, 48h). Three replicates were made for each isolate and incubated at 30^0C for 72h in a water bath shaker at 150rpm (GFL® 1083, Labortechnik). Cell suspensions were centrifuged at 10,000 x g for 20 min (Beckman J2 HS centrifuge). Cell pellets were then re-suspended and washed twice with sterile distilled water. Cell numbers were adjusted using a counting chamber (Tiefe 0.200mm Fuchs-Rosenthal, German) to approximately 10^8cells ml^{-1}.

7.2.3 Seed treatments

The prepared cell suspensions (10^8cells ml^{-1}) of each FLD bacterium were added separately to each of the three labeled conical flasks. Two grams of a sticker, gum arabica®[19], was dissolved in 100ml of tap water, stirred and allowed to stand for 1h. This was to allow the substance to dissolve and form a homogeneous suspension. Aliquots of the 20ml sticker were added into each beaker containing 20ml of bacterial cell suspension. This resulted in total

[17] Ocean Agriculture, P.O.Box 741, Mulders Drift 1747, Republic of South Africa
[18] Dr Brendon Neumann, PHP(Pty) Ltd., Nottingham Rd., South Africa
[19] gum arabic stickers, from Acacia tree, SIGMA

volume of 40ml of sticker-bacterial suspension in each of the three beakers. An appropriate number (100) of maize seeds was placed separately into each of the three bacterial-sticker suspensions and stirred. The seeds were left for two hours to allow bacterial adhesion to the seed coat. The treated seed were placed on paper towels and air-dried overnight and then planted.

7.2.4 Data collection

Plant samples were collected separately every month for the purpose of nitrogen estimation and FLD bacterial population estimation. Serial dilution was done onto mannitol N-free agar. Wet and dry weight was recorded. From each replicate, plant samples were dried and grounded in a Wiley mill. The plant N content in each plant sample was estimated by a microKjeldhal method (Willis *et al*., 1996) (See Appendix 2 for details).

7.2.5 Statistical analysis

Data was analyzed by analysis of variance using the statistical Analysis System Computer package (Version 9, GenStat®). If the F-test was significant, then a means separation was conducted using the LSD test.

7.3 Results

7.3.1 Estimation of FLD Populations

Numbers of FLD bacteria (as determined by cfu) in rhizosphere soils of maize plants were within the range of 4–1400 x $10^3 g^{-1}$ of soil and were in the following order: D6 > L1 > Br2 > NPK > Control. During the first month the FLD cfu counts in the rhizosphere of maize plants inoculated with Isolate D6 were significantly higher than those of other treatments (Table 7.1). In the second month, the number of FLD bacteria decreased in the rhizosphere of maize, to the range of $0.1^{-1}50$ x $10^3 g^{-1}$ of soil in the order: D6 > L1 > Br2 > NPK > Control. In the third month, the numbers of FLD bacteria were not significantly different between treatments, except for plants treated with Isolate D6 (Table 7.1) and see for (Fig. 7.2) in Appendix 7.2.

7.3.2 Growth and yield parameters

Maize plants treated with Isolate Br2 and D6 significantly increased in wet biomass and dry matter per plant at 90 days. The effect of NPK fertilizer application on plant growth was similar to growth of plants inoculated with FLD bacteria. Least growth parameters were recorded by the un-inoculated control plants (Table 7.1) and (see Appendix 7.3 for Fig. 7.3 and Fig. 3.4). The maximum yield was 301g $plant^{-1}$ for plants treated with NPK fertilizer, 285.9g $plant^{-1}$ treated with Isolate Br2, and 227.4g $plant^{-1}$ treated with Isolate D6. The plants treated with D6 had the highest FLD population throughout the growth period but it resulted in a lower yield than treatments with Br2 and NPK.

There was significant difference in plant N levels at 30 days as a result of inoculation with Isolates Br2 and D6 and L1 compared to uninoculated control. At 60 days, inoculation of plants with Isolate D6 resulted in significant lower plant N levels than plants inoculated with Br2 ($P<0.001$). At 90 days inoculation of plants with Isolate Br2 resulted in significant higher plant N levels than plants inoculated with Isolate L1 and D6 (Table 7.1) ($P<0.001$). Plants treated with NPK fertilizer reflected higher plant N levels than the other treatments at all plant growth stages. The level of plant N of maize plants was higher in the first month and then started to decrease towards harvest at 90 days (Table 7.1) and see (Fig. 7.1) in Appendix 7.1. Plant N levels in plants treated with NPK fertilizer were significantly higher than those treated with FLD and the untreated control plants (Table 7.1) and see Appendix 7.1 for (Fig. 7.1).

Table 7.1 Effect of FLD isolates on maize growth, yield and plant N levels in a field trial

30 days								
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Plant N (g kg^{-1} DW)**		**FLD (CFU x10^3)**	
None	27.7 (1.45)	a	3.4 (0.64)	a	21.6	a	4	a
L1	31.5 (1.45)	a	3.3 (0.61)	a	56.4	b	650	c
D6	41.1 (1.57)	ab	5.0 (0.74)	ab	50.8	b	1400	d
Br2	50.3 (1.68)	ab	6.0 (0.82)	b	61.2	b	291	b
NPK	60.6 (1.79)	b	6.4 (0.87)	b	94.8	c	13	a
P	0.031		0.031		<0.001		<.001	
s.e.d.	0.106		0.081		8.724		62.7	
l.s.d.	0.231		0.176		20.11		144.6	
cv%	9.4		15.5		18.7		16.3	

60 days								
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Plant N (g kg^{-1})**		**FLD (CFU x10^3)**	
None	129 (2.11)	a	16.08 (1.23)	a	10.24	a	0.1	a
L1	154 (2.15)	ab	19.30 (1.27)	ab	28.35	bc	25.0	b
D6	233 (2.32)	b	29.10 (1.43)	b	24.28	b	150.0	c
Br2	268 (2.39)	b	33.50 (1.50)	bc	32.04	c	15.0	b
NPK	331 (2.51)	b	41.30 (1.62)	c	47.56	d	0.1	a
P	0.003		0.003		<0.001		<.001	
s.e.d.	0.087		0.084		1.78		4.63	
l.s.d.	0.19		0.183		4.105		10.67	
cv%	5.4		8.4		7.7		14.9	

90 days										
Treatments	**Wet weight (g)**		**Dry weight (g)**		**Yield (g)**		**Plant N (g kg^{-1})**		**FLD (CFU x10^3)**	
None	278.0	a	34.8	a	183.5	a	3.40	a	0.01	a
L1	391.9	ab	49.0	ab	194.2	a	12.39	b	0.40	a
D6	496.3	bc	62.0	bc	227.4	ab	11.90	b	5.10	b
Br2	571.0	c	71.4	c	285.9	b	18.07	c	0.20	a
NPK	656.0	c	82.0	c	301.4	b	22.99	d	0.06	a
P	0.003		0.003		0.02		<0.001		<.001	
s.e.d.	78.25		9.78		35.71		1.274		0.2893	
l.s.d.	170.5		21.31		77.8		2.937		0.6672	
cv%	23.1		23.1		21.2		11.3		30.7	

Means with the same letter in the same column are not significantly different at $P < 0.05$, based on Fisher's LSD test. Values in parenthesis represents transformed means using log transformation

7.4 Discussion

The numbers of FLD cells were considerably higher in the rhizosphere of plants that were inoculated with Isolate D6, followed by Br2, L1 NPK and the untreated control. The results show that the cell counts of FLD bacteria in the rhizosphere of maize plants were higher in the first month of crop growth, and then started to decrease over time. This is in agreement with data obtained by other researchers, as number of bacteria (including diazotrophs) is influenced by the age of plants (Westover et al., 1997) because exudation rates are higher in seedlings than older plants (Del Gallo and Fendrik, 1994). In contrast, Mahaffee and Kloepper (1997) found that number of rhizospheric diazotrophs increased regardless of time.

Higher wet and dry weights were obtained as a result of FLD inoculations, compared to the untreated control. During the first month maize plants inoculated with Isolate L1, D6 and Br2 showed increased wet weights in the range of 13.9, 48.3 and 81.8% over the control. Inoculation of maize plants with FLD bacteria resulted in increases in wet and dry biomass and yield. This result is similar to those presented by Albrecht *et al*. (1981) and Baldini and Döbereiner (1980), when cereals and non-cereals were inoculated with *Azospirillum,* and Baldani *et al*. (1997) in a study on the effect of FLD on plant growth. In the present study, FLD inoculations improved maize growth significantly. Isolate Br2 was the most effective inoculant tested in the field experiment.

Yield was also increased by the FLD inoculum, and yield increase of field-grown maize ranged from 5.0 to 55.8% over the uninoculated control. Plants treated with NPK fertilizer did not yield significantly more than plants inoculated with Isolates Br2 and D6. This suggests that these bacteria had a direct effect on plant growth parameters, which, in turn, resulted in an increase in plant growth.

FLD inoculum increased plant N levels of maize. A similar result was found by Wu *et al*. (2005) on maize plant when inoculated with *pseudomonas* spp. In this study, inoculating with FLDs isolates reduced the quantity of nitrogen fertilizer needed by crops. The three FLD isolates were fairly efficient at nitrogen fixation, since the plant N levels of inoculated plants, measured at 30 days after planting (DAP), were more than 50% higher than that of the un-inoculated control. Though plants inoculated with Isolate D6 supported large FLD populations in their rhizosphere, inoculation with Isolate Br2 resulted in significantly higher

plant N levels. The decline in plant N over time (30, 60 and 90d) may be due to a relatively fixed level of N being diluted in increasing quantities of plant biomass.

This study has shown that N-fixing bacteria were able to increase wet and dry weight, and plant N of inoculated maize plants, and that these effects depend on the specific isolate of FLD bacterium inoculated.

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Appendix 7.1

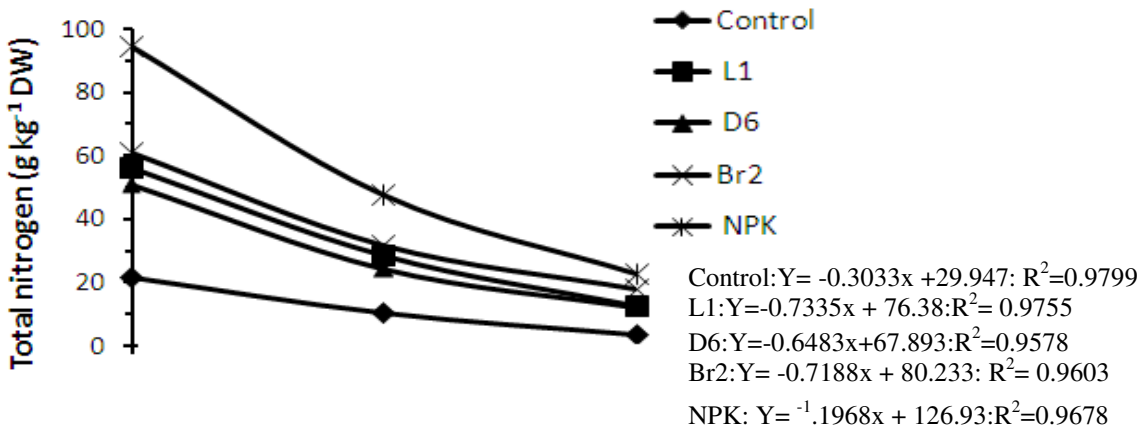


FIG. 7.1 Effect of FLD isolates on maize plant N in a three month field trial

Appendix 7.2

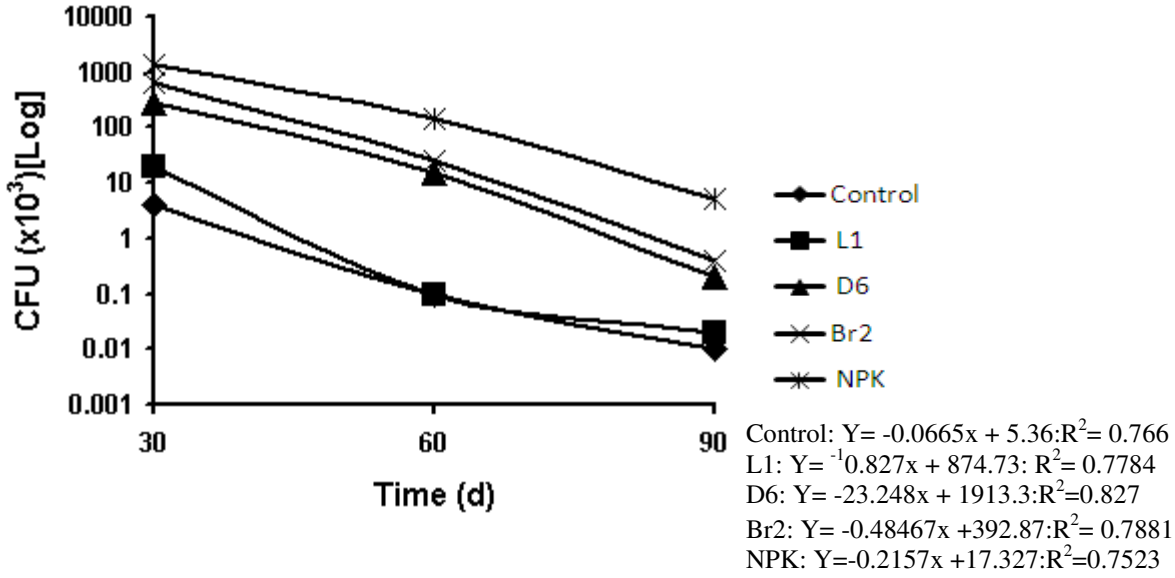
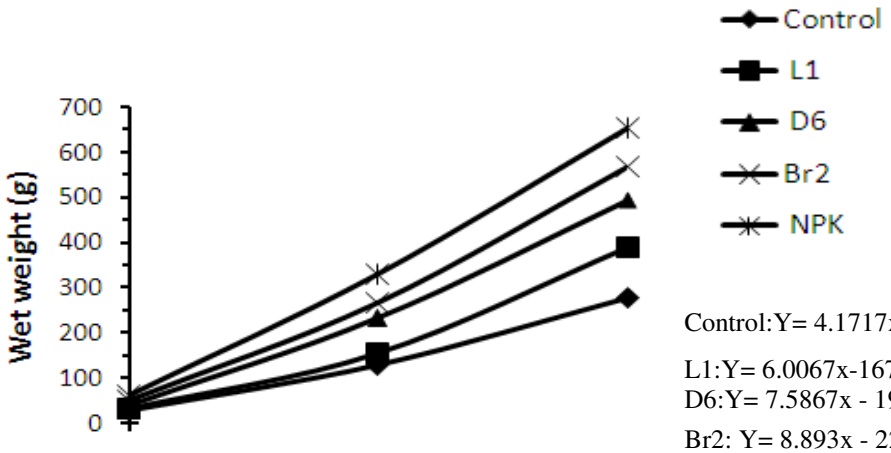


FIG. 7.2 Effect of the application of FLD isolates on the population of FLD bacteria isolated from the rhizosphere of maize plants in a three month field trial

Appendix 7.3



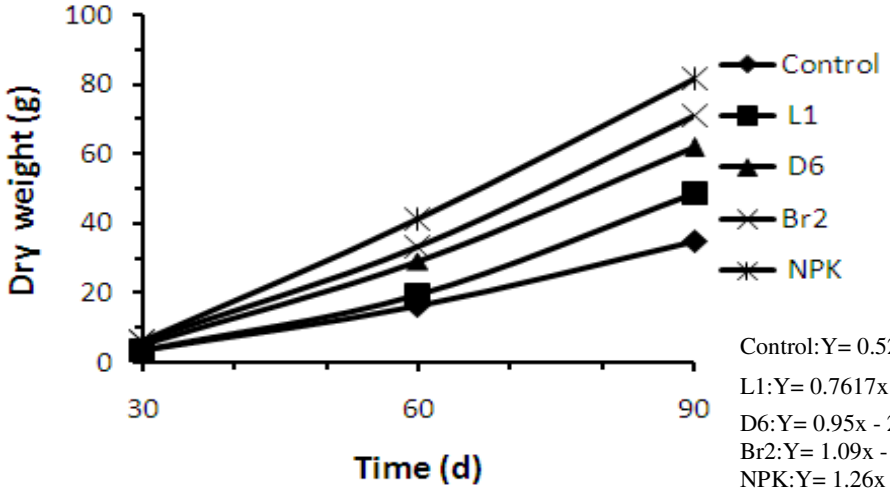
Control:Y= 4.1717x^{-1}05.4: R^2=0.9.88

L1:Y= 6.0067x-167.93: R^2= 0.967
D6:Y= 7.5867x - 198.4: R^2= 0.9919

Br2: Y= 8.893x - 224.27: R^2= 0.9911
NPK:Y= 9.9233x - 246.2: R^2 = 0.9972

FIG. 7.3 Effect of FLD isolates on maize wet weight in a three month field trial

Appendix 7.4



Control:Y= 0.5233x - 13.307:R^2= 0.9878

L1:Y= 0.7617x - 21.833:R^2= 0.9709

D6:Y= 0.95x - 24.967:R^2= 0.9921
Br2:Y= 1.09x - 28.433:R^2= 0.9916
NPK:Y= 1.26x - 32.367:R^2= 0.998

FIG. 7.4 Effect of FLD isolates on maize dry weight in a three month field trial

CHAPTER EIGHT

GENERAL OVERVIEW

8.1 Introduction

In many areas of the world there is increasing demand for a reduction in fertilizer inputs in agricultural crop production. And in other parts of the world, the poorest of farmers are seeking access to sources of nitrogen inputs for their crops that are more affordable than nitrogenous fertilizers. Eighty percent of our atmosphere is made up of nitrogen gas (N_2). This gas is of no use to most organisms and can only be beneficial to plant growth if it is first converted to ammonium and/or nitrate. This can be done through an industrial process (the Haber-Bosch reaction), used in the manufacture of nitrogenous fertilizers. Use of N-fertilizer input in developing countries is predicted to increase from the current 41 million tons of N per year to 77 million tons in 2010, which will cost billions of US dollars. It is desirable to reduce the expense and energy cost, pollution of groundwater, risk of chemical spills, and to decrease atmospheric nitrous oxide (an intoxicating "greenhouse" gas). To do this, the biological nitrogen fixation (BNF) process is important. One of the benefits of biological (microbial) nitrogen fixation is that it is largely regulated by the host plants, and nitrogen is only supplied on demand to host plants.

When managed properly, BNF can provide financial and environmental benefits by reducing external nitrogen inputs and improving the quality and quantity of internal plant resources. Efficient uses of microbial fertilizers have increased growth, yield and plant N in the case of rhizobia-legume associations, while reducing the impact of agricultural on the environment (Peoples *et al*., 1995). Biological nitrogen fixation is sustainable in that energy sources are abundant from host plant exudates (a renewable source other than from non-renewable fossil fuels). This results in a renewable source of nitrogen for crop production and agricultural sustainability (Peoples *et al*., 1995). BNF is also an efficient way for N_2 to be fixed by free-living diazotrophs (FLD) for the benefit of non-leguminous plants. Moreover, BNF integrating with reduced levels of organic and mineral N fertilizers can reduce application of mineral fertilizers containing N.

One of the major disadvantages of BNF is that the ability of a biological system to fix nitrogen is dependent on the presence of the enzyme nitrogenase. Furthermore, the nitrogen

fixing activity of a soil is strongly dependent on favorable moisture conditions, temperature, pH, oxygen, organic food sources, and the soil's physical and chemical properties. In an attempt to maximize crop production and nitrogen availability through BNF, it is thus important to determine optimum application rates for a range of crops under different growing conditions, and to determine compatibility of diazotrophs with other soil micro-organisms.

The findings presented in this thesis resulted from the evaluation of five FLD isolates for BNF activity and their effects on the growth of several crops. Experiments were conducted under greenhouse, hydroponics and field conditions with the use of seed treatments and drenching as the methods of applications. It was established that:

- Using an *in vitro* screening test, FLD isolates were evaluated for their nitrogenase activity through the Acetylene Reduction Assay (ARA). Of all the isolates, five produced above 50nmo of $C_2H_4\ h^{-1}$ and were kept for further screening.
- Most of the isolates grew on three different media with mannitol, glucose and ethanol as carbon sources, at a temperature range of 20-30^{0}C and a pH of 6.0 - 8.0. Best growth showed on the ethanol medium.
- Drenching application was superior to seed treatment for most crops tested under greenhouse conditions.
- Dual inoculation of FLD Isolate L1 and Eco-T® (*Trichoderma harzianum* Rifai) improved growth of lettuce. Isolate L1 was more effective at 10^6cfu ml^{-1} than it was at 10^8cfu ml^{-1}.
- Inoculation of FLD Isolate L1, combined with various doses of NPK fertilizer in a hydroponics system provided better nitrogen fixation at 25% NPK followed at 50% NPK. Nitrogen fertilizer was lowest when Isolate L1 was combined with 100%NPK fertilizer.
- In a field trial, Isolate Br2 increased yield of maize, equivalent to the inorganic fertilizer treatment (NPK).

8.2 Conditions that affect growth of FLD and nitrogen fixing activity

8.2.1 Current understanding

FLD growth and their nitrogen fixation activities are affected by media, temperature, pH and other conditions. FLD grow on N-free media held under optimum pH (Schubert *et al*., 1990) and temperature conditions. This is necessary because nitrogenase denatures at higher temperatures (Liengen, 1999).

8.2.2 Future research

Further trials need to be conducted under a wider range of pH and temperature conditions, to test the flexibility, efficacy and shelf life of FLD in a biofertilizer formulation. This will improve the chances of a versatile FLD being released as a commercial biofertilizer.

Large-scale screening of other nitrogen fixing bacteria, either endophytic, associative in rhizosphere of plants or free-living diazotrophs, needs to be under-taken in order to find other isolates that causes a differential growth response in plants via the transfer of fixed N products. Some of these isolates may be superior as commercial biofertilizers.

8.3 Application methods

8.3.1 Current understanding

With regard to the FLD seed treatment system, benefits were shown, such as increased growth, yield and plant N in maize, wheat and sorghum. But other crops such as lettuce and zucchini did not respond to FLD seed treatments. A drenching application of FLD inoculants was more effective and caused increased growth and plant N in all the evaluated crops (maize, wheat, sorghum, zucchini and petunia).

8.3.2 Integrated treatments

The drenching application technique that was studied in this thesis, combined with the right frequency and dose, could establish biofertilizers formulations that would be effective in liquid form.

Seed treatment inoculation techniques need to be studied in relation to deliver an optimum dose of biofertilizer to the field crops, in order to provide a cost effective delivery system for both large growers and small-scale farmers.

8.4 Integration of FLD with Eco-T® (*T. harzianum*)

8.4.1 Current understanding

In this study, Eco-T® and FLD Isolate L1 were tested for their *in vitro* compatibility on PDA. There was no inhibition zone between the two organisms, which showed promising potential for the combination to be used together for plant growth promoting applications. Inoculation of lettuce plants with Eco-T® and FLD Isolate L1, applied at 10^6cfu ml^{-1}, improved plant growth and plant N. Integration of FLDs with other microorganisms has to be tested for the optimum combination required for each organism to achieve a synergistic effect and to minimize competition effects. According to Jisha and Alagawadi (1996), the combination of phosphate solubilizing *B. polymyxa* and *T. harzianum* resulted in increased yield and nitrogen uptake in sorghum.

8.4.2 Future research

What remains to be resolved is whether this biofertilizer inoculant needs to be limited to a single strain of bacterium or not. If all of the biofertilizer needs can be resolved by a single strain of bacterium, then this would simplify the design and reduce the cost of inoculant products. However, it would be surprising if a single strain of bacterium could provide optimal activity. When applied to the target ecosystem, inoculum strains have to compete with all of the other microbes present in the soil. This competition could reduce the efficacy of the final product and therefore methods and strategies to improve biofertilizer performance should be studied.

8.5 Interaction of FLD with fertilization (NPK) in hydroponics

8.5.1 Current understanding

FLD Isolate L1 was applied to lettuce plans together with a complete hydropnic fertilizer at 25% strength, with the N level at 25mg ℓ^{-1}. These plants grew significantlly better than the control plants grown on 25% of normal NPK fertilization or Isolate L1 alone. Significant increases in wet and dry weight were obtained when Isolate L1 was inoculated, together with NPK at 25% strength (with the N level at 25mg ℓ^{-1}). This supports the findings of Zaied *et al*. (2007) and Young *et al*. (2004) who reported that inoculation of plant promoting rhizobacteria in combination with fertilizer at a 50% dose gave corresponding increases in crop growth parameters. According to Kennedy and Islam (2001), BNF-based systems are less competitive with mineral fertilizers but may provide an alternative source of nitrogen, to supplement chemical fertilizers, but not to replace them.

8.5.2 Future research

More complex experiments using different FLD isolates at different mineral fertilizer doses with different crops under different growth conditions, including field trials, could be undertaken to relate the dose effect of fertilization to FLD inoculum, and correlate this with plant growth and nitrogen fixation. Ultimately, the goal is to develop and market the integrated use of biofertilizers and therefore reduce the level of mineral fertilizer applications required, thereby reducing the cost and adverse effects on agricultural environments caused by artificial sources of N fertilizer.

8.6 References

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Appendix 1

N-free media:

1. ***Burke's Modified Medium*** (The concentration of the medium is expressed *as* g ℓ^{-1} in distilled water)

Mannitol	10.0
K_2HPO_4	0.64
KH_2PO_4	0.16
NaCl	0.2
$MgSO_4.7H_2O$	0.2
CaCl	0.1

Plus the following trace elements: mg ℓ^{-1}

$FeSO_4.7H_2O$	2.5
H_3BO_3	2.9
$CoSO_4.7H_2O$	1.2
$CuSO_4.5H_2O$	0.1
$MnCl_2.4H_2O$	0.09
$Na_2MoO_4.2H_2O$	2.5
$ZnSO_4.7H_2O$	2.1

Final pH was 7.4 ± 0.2

2. ***Jensen's Medium*** (The concentration of the medium is expressed as g ℓ^{-1} in distilled water*)*

Glucose	5
K_2HPO_4	0.8
$CaCl_2.2H_2O$	0.2
$FeSO_4.7H_2O$	0.04
$MgSO_4.7H_2O$	0.2
$Na_2MoO_4.2H_2O$	0.005

pH 7.0; K_2HPO_4 sterilized separately and added to the warm agar just before pouring plates.

3. Ethanol N-free medium

The same with Jensen's Medium, except that ethanol was used as the carbon source; 10ml of ethanol was added to 1 ℓ of water from a filter-sterilized solution.

Solid media were prepared from the above carbon sources by the addition of 2% agar.

Appendix 2

Nitrogen analysis

1. **Reagent preparation:**

(a) Salicylate

Using either no heat or very little heat, the following salts were dissolved: 32g of sodium salicylate (anhydrous), 40g of TSP (trisodium phosphate, sodium phosphate tribasic dodecahydrate, or $Na_3PO_4.12H_2O$, and 0.5g of sodium nitrosylpentacyanoferrate (III) (sodium nitroprusside) in 1ℓ of water.

(b) Hypochlorite (NaOCl)

100ml of commercially available bleach containing 3.5% sodium hypochlorite to water and dilute to 1 litre.

(c) Nitrogen standard

Stock solutions were prepared from ammonium chloride (1000mg of N ℓ^{-1}), diluted with water.

2. **Digestion procedure:**

At the end of each trial, total wet weight of leaves, stem and yield of each plant were recorded. The biomass was placed in a 56^0C oven to dry for 48h. The dry weight samples were weighed and ground in a Wiley mill. Samples were subjected to Kejldahl digestion (Willis et al., 1996) with minor modifications. 1g samples were weighed and placed into 200ml Kejldahl flasks. 10ml of concentrated sulphuric acid (98%v/v), 3.5g of K_2SO_4, and 35mg of selenium powder was added to the sample. The block heater was pre-heated to 100^0C and the temperature increased up to 425^0C for one hour or until the sample was clear. After 10-15 minutes, water was carefully added to make up a final volume of 50ml.

3. Colorimetric procedure:

1 ml of the digested sample were transferred to a sterile test tube and diluted with 10ml of sterile distilled water and 0.2ml of ammonia to bring the concentration within working range. 4ml of salicylate reagent followed by 1ml of hypochlorite reagent was added into the diluted sample. The sample was left to react for a minimum of 12 min. Absorbance at 685nm was then measured. A calibration curve was linear to 65mg ℓ^{-1}.

The nitrogen total was calculated by the following formula (Kalra, 1998):

$$g\ kg^{-1}\ N = (\mu g\ ml^{-1} \times \text{color development reagent (ml)}) / [g\ \text{Sample (g)}/100$$

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