Improving Sugar Beet (*Beta vulgaris* L.) Productivity by Inoculation with *Gluconacetobacter spp.*

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Abstract

Improving Sugar Beet (*Beta vulgaris* L.) Productivity Following Inocuation with *Gluconacetobacter spp.*

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Gluconacetobacter diazotrophicus is nitrogen fixing endophyte that associates with sugar cane. Fourteen strains of *Gluconacetobacter spp.*, including *Gluconacetobacter diazotrophicus*, were tested for capability of fixing nitrogen in sugar beet at 1 mM and 10 mM NO_3^-. Inoculation at different growth stages with different bacteria concentrations was assessed in sugar beets provided 5 mM NO_3^- to evaluate its impact on infection level and capacity of nitrogen fixation. A 16S rRNA based PCR technique was applied for identification of isolated strains. GUS labeled strains were also used to investigate the possible sites that the bacteria infect the host plant. Results showed that certain strains improved plant growth significantly. A greater biomass increase and higher nitrogen fixation was found at lower nitrogen treatments. Utilization of GUS-labelled strains of *Gluconacetobacter diazotrophicus* indicated that root tips, root hairs and the intersection of lateral roots were possible sites of infection by the bacterium.

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bp	base pair
cm	centimeter
CFU	colony forming unit
d/n	day: night
DW	dry weight
g	gram
GUS	beta-glucuronidase
M	mol per litre
mg	milligram
mM	millimol per litre
NDFA	nitrogen derived from atmosphere
PCR	polymerase chain reaction
PGPR	plant growth promotion rhizobacteria
ppm	parts per million
RMP	rounds per minute
S.E	standard error
µl	microlitre
µm	micrometer
v/v	volume: volume

1. GENERAL INTRODUCTION

The earth's climate is changing continuously, but unlike earlier dramatic changes that occurred in planet's history, this time human influence is adding to this change. Increased using of fossil fuels by humans is enhancing the greenhouse effect, generating unpredictable climates along with warmer temperatures overall. Climate change has become a part of scientific agenda since the 1970; however, it only attracted significant international concern starting in the 1990s. Now, global climate change is not only the scientific and social issue but more importantly it is also economical and political issue worldwide (Dawson and Spannagle, 2009).

Approximately 50 different gases are released by human, contributing to the greenhouse effect. However, many of those gases are only in small quantities that do not significantly contribute to global climate change. The significant contributors include: carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Carbon dioxide is the most important greenhouse gas, contributing 77% of 2004 emission, followed by methane, 14%; nitrous oxide, 8%, and the synthetic gases, 1% (IPCC, 2007; WRI, 2005). Consumption of fossil fuel is responsible for the most significant source of CO_2 emissions, accounting for more than 70% global emission (WRI, 2005). CO_2 emissions from fossil fuel consumption have dramatically increased up to 145% followed economic growth during 1970-2004 (IPCC, 2007; IDA, 2007). The bulk of 8% N_2O emission derives from fertilized agricultural soil due to increased food demand for rising of world population (IDA, 2007).

Carbon dioxide is an essential substrate of photosynthesis in plants and other photoautotrophs, as well as an important greenhouse gas which absorbs and re-emits infrared radiation. However, release of CO_2 from burning fossil fuels greatly contributes to increasing of atmospheric CO_2 concentration, which negatively affects earth's surface temperature causing more warmer and unpredictable climate. In 2010, the atmospheric CO_2 concentration reached to 390 ppm (parts per million) from 280 ppm of pre-industrial times (Pieter, 2009) and present level is higher than at any time during last 800 thousand years (Jonathan, 2006). Global temperatures have increased by 0.76 °C since the industrial revolution and it has been speculated that there will be 0.5-1.0 °C increase in global temperature in next 50 years, even if emission of greenhouse gases is inhibited completely. GHGs emitted until today guarantees further increase of 1.5-2.0 °C compared to pre-industrial times (Dawson and Spannagle, 2009). It is hypothesized that humans will adapt this magnitude of global temperature change regardless of direct adverse effects. However, studies indicate that exceeding 2 °C of temperature increase most likely results in significant adverse impacts on natural ecosystems and biodiversity (IPCC, 2002; Lanchbery, 2006). Base on the Intergovernmental Panel on Climate Change (IPCC) research date, climate change may accounts for the single largest factor for biodiversity loss in 20th century. Coupled with the fact that current species extinction is above natural extinction rate due to human damages, it is estimated that more than half of the species on earth may no longer exist by 2100 (Meyer, 2006). Further, warmer climate melts ice sheets, causing rising of sea levels and shifts in climate zones. Thus, greenhouse effect not only influences the biodiversity of natural ecosystems but also will have huge impact on agriculture production due to changing of annual and seasonal precipitation patterns,

and increasing frequency of extreme climates. Reduction of agriculture production is a serious issue considering 3 billion population growth is expecting in next 40 years (Cohen, 2005). Thus, global crop production has to increase to meet the demand of 9 billion people by 2050. Even though the changes in climate conditions might increase the crop yields in some regions, such as northern part of Canada, changes in temperature and water availability will negatively influence crop production of most regions, especially in tropical regions.

From an energy point of view, fossil fuels are non-renewable sources and account for more than 80% of world energy consumption (IEA, 2006). The world oil production reached its peak in 2000, followed by coal and natural gas (US Department of Energy, 2004). About 90% of discovered oil is already in production, leaving very little growth for future field discovered (Koppelaar, 2006). However, demand and consumption will increase at 1-2 % per year up to 2030 (IEA, 2006). In the last 30 years, more fossil fuels have been consumed compared to last two preceding centuries and it has been estimated that the consumption will double by 2050 (WRI, 2005). Fossil fuels are vital part of global economy and any changes that might occur in this energy sector will directly have impact not only on global economy but most countries' political stability as well. Thus, depleting the oil, coal and natural gas reservoirs will impact every country on the planet. On the other hand, the elevating atmospheric CO_2 level from fossil fuel consumption urgently demandes a switch to more greener and renewable energy sources.

A possible solution for mitigating greenhouse gas emissions derived from fossil fuel consumption is finding and obtaining primary energy from renewable sources and carbon storage. Bioefuel as a renewable energy sources, are promising due to the fact that it is derived from recent living organisms or metabolic waste and can be efficiently stored, unlike other renewable sources; therefore, biofuels is not only a greener energy source but also a sustainable source. Biofuel can be in liquid, solid and gas forms. Solid forms of biofuel (biomass), such as wood, manure and crop residues, are the major energy source of 2.5 million people in developing world (Dawson and Spannagle, 2009). Biogas, derived from anaerobic digestion of city and animal wastes to produce methane (CH_4), is gas form of biofuel. All commercial biofuels comes in liquid form as either bioethanol or biodiesel and bioethanol blends up to 10% gasoline can be used in regular cars without any modification of engines. Unlike bioethanol, biodiesel can directly replace the fossil fuel derived diesel. Bioethanol, a substitute for gasoline, is produced from sugar and starch rich crops, while biodiesel is generated from plant and animal oils. Historically, both liquid forms of biofuels have been used as fuel over a hundred years (Dawson and Spannagle, 2009). The transport sector currently produces 14% of greenhouse gas emissions using conventional fuels and the demand for the fuel is estimated to grow as much as 50-60% by 2030 (Ibid). Biofuels are already proven to be an important part of greenhouse gas mitigation. The International Energy Agency estimates that biofeul production most likely increase up to 6-8% per year until 2030, indicating 4-6 fold increase in production. Thus, biofuels will fuel 4-7% of transportation by 2030 (IEA. 2006a). Some studies even indicate that biofeul could possibly provide 10 % of transportation fuel by 2030 (WBCSD, 2007).

Bioethanol production has rapidly increased during the 1990s and the production double in the decade, reaching 20 billion litres in 2000. Corn-based ethanol production in US accounted for 40% of world bioethanol production in 2000, while Brazil remained the largest producer of biofuel production in the world. More than 40% of its transportation runs with bioethanol generated from sugar cane (WBCSD, 2007; Isherwood, 2006). In 2006, the bioethanol production reached 52 billion litres, doubling the production in short six years (Ibid).

Brazilian sugar cane-based ethanol production at 0.2-0.5 $US/litre is much more efficient and cheaper than corn-based production at 0.6-0.8 $US/litre (WBCSD. 2007; Crutzen *et al.* 2007). One of the major reasons for lower cost of production is that Brazilian sugar cane requires less amounts of nitrogen fertilization due to a nitrogen fixing bacteria, *Gluconacetobacter diazotrophicus*, which is specific to sugar cane and can contribute up to 150 kg of N ha^{-1} per year (Boddy *et al.* 1991, 1995; Cavalcante *et al.* 1988; James, 2000; Sevilla *et al.* 2000). One unique characteristic of this bacterium is that it thrives in medium as high as 10% of sugar concentration (Cavalcante & Dobereiner, 1988). Given the fact that sugar cane is a tropical and sub-tropical plant, it cannot be grown in Canada. However, considering sugar beet is suited for temperate climates and the sugar content is 25 % higher than that in sugar cane (World Bank, 1998; Weeden, 2000), it is hypothesized that introduction of this endophytic nitrogen fixing bacterium, *Gluconacetobacter diazotrophicus*, into sugar beet might also benefit the new host plant

with fixed nitrogen through nitrogen fixation. The potential nitrogen fixation in sugar beet will reduce the high costs on fertilization, making it more desirable plant for ethanol production at a lower cost in Canada.

A series of studies have been conducted to introduce the nitrogen fixing endophytic diazotrophs species, *Gluconacetobacter diazotrophicus* spp. *and Gluconacetobacter johannae* spp., into sugar beet. Forteen strains of *Gluconacetobacter* spp. were tested to find the most effective nitrogen fixing stains. The screening experiment was conducted both in 1 mM and 10 Mm nitrogen concentrations to assess the possible impacts of different nitrogen concentrations on nitrogen fixation. Sugar beet seeds were also inoculated at different growth stages to evaluate if inoculation time affects nitrogen fixation. Sugar beet seeds were also inoculated with different bacteria concentrations at seed and seedling stages to evaluate if inoculant number is an additional factor that influences nitrogen fixation in the plant. In all experiments, the ^{15}N dilution technique was used to quantify nitrogen fixation by the endophytic diazotrophs. Bacteria were also isolated from inoculated plants and the 16S rRNA molecular technique was applied to confirm the isolates. Finally, using GUS (beta-glucuronidase) labelled strains of *Gluconacetobacter diazotrophicus*, infection sites of sugar beet by bacteria were visualized with aid of microscope.

2. 0 LITERATURE REVIEW

2.1 Biofuel

Biofuels are a wide range of fuels which are mainly derived from plant-based sources, or biomass. Generally, these fuels can be in a solid, liquid or gaseous state (Demirbas, 2009). Bioethanol and biodiesel are the mostly widely used biofuels and are more similar to conventional petroleum fuel and in liquid form. Ethanol is mainly generated from either fermentation of sugar from sugarcane, sugar beet and sorghum; or saccharification of starch from corn, wheat and manioc. Second generation bioethanol comes from hydrolysis of cellulosic biomass, such as trees and grasses but it is still in developmental stages (Zuurbier and Vooren, 2008). Bioethanol can be directly used as fuel for vehicles but normally it is used as blending agent with gasoline to boost octane and reduce carbon emissions. Biodiesel is mainly produced from vegetable oils, animal fats or recycled grease. Major sources of vegetable oils are rapeseed, soybean, canola, corn and palm oil. Algae is considered an alternative future feedstock for biodiesel and represents the third generation of biofuels, producing 200 times more yield (per acre) than vegetable oils. Difficulties lie in creating cost-effective cultivation system (Sheehan, 1998).

Biofuels have been considered automobile fuel since the emergence of auto industry one century ago. However, production of biofuel is hampered by abundant and cheap petroleum (Zuurbier and Vooren, 2008). Nevertheless, interests on biofeuls like ethanol and biosiesel increased dramatically in last few years (EIA, 2007). Growing emissions of

pollutants and high oil prices are major reasons for this increase. Additionally, scarcity of fossil fuel sources, and reducing greenhouse gas emissions urge immediate transition to more sustainable and renewable alternative energy source, while increasing demanding on energy caused by increasing of world population and development of third world countries making this change imperative (Sensoz *et al.* 2000). Transportation consumes 30% of the world's energy, of which, 99% comes from traditional fossil fuel (EIA, 2007). Thus, transportation sector expects this transition more than any other sector.

2.1.1 Biomass

Biomass is organic feedstock derived from plants through photosynthesis, including woody plants, agricultural residues, organic waste by-products, energy crops, municipal green waste and forestry (Schuck, 2006). Biomass is the oldest form of renewable energy and is the most used renewable energy source in the today's world, reaching 13.8% in 2000 compared to 6.7 % in 1990 of global total annual energy (Klass, 2004). In general, biomass contributes around 10% of total energy supplies in industrialized countries compared to up to 30% of developing counties (IEA, 2007). Canada as world's top exporter of wood products, over 60% of renewable energy comes from biomass- mostly from wood residues and Canada's grain ethanol production from biomass reached more than 1 billion tonnes in 2008 (IEA, 2006).

World renewable energy represnts 18% of global energy supply, of which, over 55 %

comes from traditional biomass- wood products (Demirbas, 2001). However, as demand for energy is increasing exponentially in response to continuous growth of world population and scarcity of non-renewable energy sources, it is necessary to exploit new and more abundant energy sources via developing newer technologies, such as cellulosic ethanol production (Champagne, 2008) and wood feedstocks are more abundant than starch, sugar or oil (Rajagopal and Zilberman, 2007). Switchgrass and *Miscanthus* are major perennial grasses undergoing trials to test the breakdown of cellulose in cell walls of plants to convert into ethanol, holding enormous potential for future ethanol production (Lynd, 1996). Even though this technology is still developing, it will make feasible the utilization of the nongrain parts of crops, such as corn stover, rice hust, sorghum stalk, bagasse of sugarcane and any wooden materials for ethanol production, increasing sources of biomass (Wyman, 1999; Lynd, 1996).

Biomass as the fourth largest energy source after coal, oil and natural gas, and is a major source of energy for 50% of world's population (Karekezi and Kithyoma, 2006); its development and production depends on land availability. Currently, 0.19% of world land is using for energy crops, represents only 0.5-1.7% of agricultural land (Ladanai and Vinterback, 2009). Therefore, future energy from biomass greatly depends on extension of land availability and agriculture is the largest potential contributor of future food and world biomass for renewable energy supply.

2.1.2 Oil Seed biofuel crops

Biodiesel, in contrast with ethanol, is generated from oilseed crops and animal fats (Demirbas, 2001; Sheehan, 2000). Among 350 oil-bearing crops, only soybean, palm, sunflower, cottonseed, rapeseed, and peanut oils are considered potential biodiesel source (Goering *et al.* 1982). Using vegetable oils as energy source has started a hundred years ago when diesel engine was invented and diesel engines run can run on various vegetable oils back then (Bartholomew, 1981; Nitscheke and Wilson, 1965). Now biodiesel can be blended with fossil fuel up to 50% (IEA, 2007b).

Vegetable oil is attractive for biodiesel production from environmental point of view and it is renewable. Currently, the major disadvantages of these crops are low yield and high inputs, and that they may raise a variety of concerns such as increased food prices. However, increasing prices of fossil fuel has renewed interest in vegetable oils (Demirbas, 2008). Oil seed and nut kernels contain 20-60% of oil (Demirbas, 2008). USA is the world largest soybean producer and soybean is becoming primary biodiesel source along with rapeseed, while palm oil is produced mainly in Malaysia and Indonesia. Whereas, the rapeseed is the most commonly produced in Europe (Demirbas, 2008), Germany producing 50% of global biodiesel in 2005 (Martinot, 2005). Increased demand for agriculture to produce biodiesel, biofuel overall, may increase the declined income of farms; therefore reduces subsidies for farmers (Hazell and Pachauri, 2006).

Biodiesel from animal fat, $ 0.4-0.5/ litre of gasoline equivalent (lge), is relatively cheaper than vegetable oil, $0.6-0.8/lde (IEA, 2007b). Currently, biodiesel production is more expensive compared to petrodiesel, and biodiesel production is heavily depend on feedstock and land availability more than bioethanol production, nor it can displace current fossil fuel use (Bala, 2005). As US an example, If all the oilseed production, animal fats and recycled grease were used for biodiesel production, only 4 billion gallons of diesel could be produced, less than current production of corn-based ethanol (Schnepf, 2007). Therefore, the research on converting non-food plant materials, including agricultural biomass waste such as corn stover and cereal straws, and energy crops into synthetic biodiesel is underway, which surely brings the potential to produce a great amount of biodiesel from cellulosic materials avoiding of agricultural supply and price concerns (CRS, 2007).

2.1.3 Sugar crops

Sugar and starch rich crops like sugarcane and corn are major sources to produce world's ethanol today. Additional crops include cereals, sorghum, potatoes and sugar beet. There are three main means to produce ethanol from biomass: fermentation of sugar from sugarcane, sugar beet and sorghum; saccharification of starch from corn, wheat and manoic; and hydrolysis of cellulosic materials (Goldemberg, 2007). Currently, corn in US and sugarcane in Brazil produce 90 % of world ethanol (Goldemberg, 2007). Even though 98% of US ethanol comes from corn, accounting for 20% of corn produced annually, however it can only represents about 3.6% of annual gasoline demand and cost

for corn based ethanol is twice compared to sugarcane-ethanol production (Worldwatch Institue, 2006). Under the most optimistic condition, tripling the corn-based ethanol production to over 60% of corn annual production can only replace 7.2% of US energy supply (CRS, 2007).

Further, the notion that ethanol from corn reduces green house gas emissions is only establishes when no fossil fuel and fertilizers are added. Additionally, for sugarcane-ethanol, the net energy gain is 8.5: 1 and most biodiesel, the net gain is 2.5 : 1 while corn-based ethanol cannot be compared to those numbers even when it bears the highest yield possible (Gail, 2007). Being a food crop is another major drawback of corn, indicating it might have to compete with agricultural food land and can have negative effects on food prices. Therefore, the interest in converting municipal wastes and animal manures, as well as cellulose biomass, into sugars by hydrolysis is also developing along (Champgne, 2007).

Sugarcane based ethanol is considered green and is produced at competitive price with gasoline. In addition, sugarcane waste (bagasse) can be used as energy source to ethanol plant, reducing energy costs (CRS, 2007). Brazilian sugarcane, a tropical plant, has already producing 16 billion litres per year, dominating the world bio-ethanol production (Goldemberg, 2007). Brazilian ethanol is entirely depending on sugarcane and reached peak in the 1980s, then declined due to lower fossil fuel prices. However, sugarcane-ethanol production increased dramatically beginning of this century due to higher oil prices and lower production costs (Goldemberg, 2007).

Sugarcane needs much less fossil fuel input,10-12%, compared to corn and reduces 90% of CO_2 compared 15-25% of reduction in corn, whereas sugar beet needs relative high amounts of inputs and reduces 50-60% emission compared to gasoline. Corn based ethanol is quite energy intensive process and energy input is as high as 60-80%. In terms of cost, sugarcane is more viable and comparable to fossil fuels, costing $0.3/lge (litre of gasoline equivalent) compared to $0.3-0.4/lge of gasoline, while corn (US) and sugar beet (EU) cost $0.6-0.8/lge with potential reduction to $0.4-0.6lge, where sugar beet energy output is higher than corn (IEA, 2007b).

2.1.3. 1 Sugar beet

Sugar beet (*Beta vulgaris* L.) is a biennial temperate climate root crop, herbaceous dicotyledon, and belongs to the family *Chenopodiaceae.* During its first year of growth, it accumulates sugar and stores them in its large tuber-like root to over winter. During the next year, it produces flowers and seeds; therefore, it is normally sown in spring and harvested at the end of the first year unless being grown for seed. Sugar beet only has 250 years of history and percentage of sucrose in beet was as low as 7.5% during the 1800s. Then it was raised to 17 % (fresh weight) using progeny test methods by Luois Vilmorin in the late 19th century and sugar content can vary between 15-25 % depending on growth conditions (Singh *et al.* 1985; Martin *et al.* 2006). For sugar processing sugar beet, it contains 17% sugar and 75% water (World Bank, 1998). Sugar beet is on average grown 17.8 million acres, 6 million ha (2000-2003) and production is about 266 million tons or 18 tons/acre (Martin *et al.* 2006). Before the twentieth century, sugarcane accounted for

95% of world sugar production, however, by late twentieth century sugar beet accounted for 40% of world sugar consumption (Atanassov, 1986).

Sugar beet germinates at about 16 ^{0}C but germination is more rapid at 28 ^{0}C. The optimum temperate for plant growth is 24 ^{0}C, however, 17-20 ^{0}C is most desirable for root growth. Temperature directly influences sugar content of beet; the highest sugar content occurs temperature between 19 -22 ^{O}C and temperatures at 30 ^{O}C or above negatively effects sugar accumulation (Terry, 1970; Brandes *et al.* 1941). However, cool nights does not do harm to the beet. Application of access nitrogen or application in the late season causes decrease of sugar content and increases impurities in the beet (Mortvedt *et al.* 1999). Normally sugar beet requires about 8 kg of nitrogen for per ton of roots and applying before seedling or mid-season is desirable for higher content of sugar and for higher yield (Martin *et al.* 2006).

Sugar cane as world's main source for sugar production, it is not grown in Canada due to climate is too cold for tropical plant. While, the nation's the largest portion, 90%, of sugar depends on importing raw sugar from sugar cane, and the rest depends on sugar beet grown in Canada (Agriculture and Agri-Food Canada, 2009). In 2006, sugar beet is grown almost 20,000 hectares mostly in Alberta and Ontario, producing 1.2 MT of sugar beets, which is 35% higher compared to 2001 statistics (Statistics Canada, 2008). Alberta accounts for 81% of Canada's sugar beet area and sugar content can reach 19% by weight compared to 10% of sugar cane. However, an average yield of sugar beet is 50 T/ha,

producing 6-7 tons of sugar on average. While 1 hectare of sugar cane producing about 100 tonnes of biomass, equals to 5-10 tonnes of raw sugar (Statistics Canada. 2008).

1.2 Nitrogen fixation

The growths of plants require the availability of mineral nutrients and nitrogen is the most limiting among all the mineral nutrients. Nitrogen is a necessary building block for nucleic acid and proteins. The earth's atmosphere contains nearly 80% of nitrogen in gas form (Dixon & Wheeler, 1986). However, a vast majority of organisms cannot access this abundant supply of nitrogen source due to the strong triple bond between the two nitrogen atoms, making the molecule non-reactive, until it is fixed (combined) in the form of ammonium (NH_4^+) or nitrate (NO_3^+). Nitrogen is fixed industrially by Haber-Bosh process, converting combined nitrogen and natural gas into ammonia ($N_2 + 3H_2 = 2NH_3$), a high energy consuming process. In nature, lightening also fixes nitrogen at certain level, ionizing the molecules in the atmosphere and enables them to combine and form nitrogen oxide, which dissolves in rain and forms nitrite (NO^2-) and nitrate. However, atmospheric nitrogen fixation only accounts for less than 10% of totally annual yield of fixed nitrogen (Kar *et al.* 2002). By far biological nitrogen fixation by soil microorganisms has the most significant role in agriculture after industrial fertilizers (Dong *et al.* 1995). Nitrogen fixing bacteria combines nitrogen with hydrogen to create compounds that are easily usable by plants; therefore, recovering the fertility of soil via biological nitrogen fixation has profound ecological effects and has a great agronomic

importance. Some of these bacteria are found in soil while some others live in association with roots of plants. In exchange for fixed nitrogen, the plant provides the bacteria with organic acids as energy source for growth. Thus, both partners benefit from this relationship and it is where they gained the name symbiotic nitrogen fixation.

2.2.1 Legumes

Legumes, pod-bearing plants, belong to the family of Fabaceae (Leguminosae), which is the third largest family of flowering plants (Hirsch *et al.* 2001). Increasing productivity of non-legume crops by crop rotations with legumes was described by Romans over 2000 years ago. However, the production of beneficial effect of legumes was not explained until 1800 (William, 2008). Now, it is widely accepted that the positive effect of legumes in increasing crop productivity attributes to those nitrogen fixing soil bacteria called rhizobia, belonging to the genus *Rhizobium* (William, 2008).

Rhizobium resides within root nodules of legumes. Association of legumes with *Rhizobium spp.* has an extreme importance in sustainable agriculture. Ten percent of legume N is left in the root after harvesting and N directly deposited into soil during root development have an utmost importance in maintaining soil fertility (Kipe-Nolt and Giller, 1993; Giller, 2001). The importance of legumes is not only because of legumes conserve nitrogen fertilizers, but also seeds and plants contain high percentage of protein; thus, legumes are nutritionally important for both humans and animals (Vance *et al.*

2000). Legumes take 11% (approximately 275 million hectares) of world's agriculturally arable land and provide more than 33% of mankind's nutritional N requirement (Food and Agricultural Org. of the United Nations, 2003; Vance *et al.* 2000).

2.2.2 Legume-*Rhizobium* symbiosis

The agricultural importance of the symbiotic relationship of legume-rhizobia is well established, in which a legume supplies rhizobium with carbohydrates as energy source and rhizobium supplies the host with fixed nitrogen in the form of ammonia. Despite the fact that Leguminosae is a massively large family, including about 750 genera and 20,000 species, not all genera or species form root nodules, a tumor-like root structure, associated with rhizobium (Dixon and Wheeler, 1986). Thus, legume-rhizobium symbiosis is highly species specific.

The uniqueness of this symbiosis lies in the fact that this association maximizes the transfer of fixed nitrogen to the plant from rhizobia residing harbor (Karl and William, 2002). In legume symbiosis, root nodules not only protect the bacteria by providing them a shelter but also providing with a low O_2 content, which has a vital significance because nitrogenease, the enzyme that catalyzes biological nitrogen fixation, is denatured by O_2. However, low concentration of nitrogen must be available for bacteria to survive and generate energy to drive nitrogen fixation (Karl and William, 2002).

Effective infection of legumes by rhizobia and establishment of legumes-rhizobia symbiosis involves recognition of signal molecules between the host and the bacteria in the soil (Hirsch *et al.* 2003; Zhang *et al.* 2002). Rhibozia living in soil are able to sense flavonoids, plant secondary metabolites, secreted by host roots and these flavonoid molecules trigger production of a set of nodulation molecules known as Nod factors by the bacteria (Zhang and Smith, 1995; 1996). Then, recognition of Nod factors by the host plant roots leads to a number of biochemical and morphological chances, such as cell division, root hair curling and elongation (Dixon and Wheeler, 1986). Root hair growth is re-directed to encircle the rhizobia multiple times (Sprent and Sprent, 1990).

Further, not only can this symbiosis relieve the requirements for nitrogen fertilizers during the growth of leguminous crops, it can also result in yields in nitrogen-deficient soils where most cereals and non-leguminous crops would barely survive. Additionally, inoculation of legume crops is not only an inexpensive means of N fertilization, compared to chemical fertilization, at a rate of $ 2-3 per acre but it does not pollute the water and soil (Vessey, 2004). Thus, Applying additional nitrogen fertilizer, however, suppresses N_2 fixing symbiosis due to the reason that plants have enough nitrogen in the soil and do not have to spend energy to form nodules to obtain more nitrogen by feeding the rhizobium. Globally, about 70-80% of total 17.2 x 107 tons of fixed nitrogen is reduced by legume-rhizobium symbiosis (Burris, 1980; Ishizuka, 1992).

2.3 Free-living nitrogen fixing bacteria and plant growth promoting rhizobacteria

Following increasing discovery of various groups of nitrogen-fixing microorganisms, the concept that plants stimulate proliferation of microorganism in root zone was firmly established by 19th century. The roots influence surrounding soil environment biologically by excreting a significant amount of organic carbon into it. Thus, the term rhizosphere, a dynamic environment and hosts wide range of microorganisms, was coined as the soil surrounding the roots under plant influence (Starkey, 1985; Rovira, 1991). The loss of organic maters into soil during root growth actively boosts microbial population around the root, stimulating microbial activity (Whipps, 1990; Morgan and Whipps, 2001). Among a dozen genera of isolated nitrogen fixing bacteria by 1969, *Azotobacter*, *Clostridum* and *Klebsiella* were categorized as bacteria that fix nitrogen in a non-symbiotic way, free-living state (Stelwart, 1969). Free-living nitrogen fixers (diazotrophs) include saprophytes living on plant residues, rhizosphere bacteria which has close associate with plant roots and bacteria that live within plants (endophytes). Unlike symbiotic nitrogen fixation, those free-living bacteria are not intimately related to legume plants and always actively chase the energy sources in soil to find required energy for nitrogen fixation (Vadakattu and Paterson, 2006).

The significance of free-living nitrogen fixing bacteria on nitrogen enrichment was a bit of controversial topic in the beginning. Some reported that the nitrogen contribution of

free-living bacteria was negligible (Mishuastin and Shilnikova, 1969), while it is becoming clear that non-symbiotic free-living plant-beneficial microorganisms has a great potential (Dobereiner, 1968; Vadakattu and Paterson, 2006) and they have different mechanisms to promote plant growth, such as morphological and physiological changes of the roots and production of plant growth hormones (Dobbelaere *et al.* 2001). In some cases, free-living microorganisms indirectly promote plant growth by suppressing the plant diseases (Moeene-Loccoz and Defago, 2004; Bashan and de-Bashan, 2002) or parasitic plants (Bouillant *et al.* 1997; Miche *et al.* 2000). However, in most cases, the beneficial effects of free-living microorganisms stay undiscovered. In addition, those undefined beneficial effects combine with other multiple effects related to variability in plant genotypes, soil composition, microclimatic conditions and difference in soil biota (Moenne-Loccoz *et al.* 2006). Further, the quantity of fixed nitrogen by free-living diazotrophs in natural condition is insignificant compared to legume-rhizobia symbiose based nitrogen fixation, unless organic substrates are added (Delwiche and Wijler, 1956). However, vastly unknown contribution of free-living plant-plant beneficial microorganisms to plant growth and health is not negligible.

Many rhizosphere microorganisms benefit from root exudates and have positive impacts on plant growth and health either symbiotically or associatively. Those associative microorganisms are called plant growth-promoting rhizobacteria (PGPR). PGPR enhance plant growth with various mechanisms both directly and indirectly. Some direct influences are enhancing seed germination, development and modification of root morphology, solubilisation of mineral nutrition, decreasing pollutant toxicity (heavy

metals), promotion of mycorrhizal functioning, utilization and phytohormones, such as auxins and cytokines (Jacoud *et al.* 1998, 1999; Dobbelaere *et al.* 2001; Glick *et al.* 1999). Indirect effects involve suppression of phytophathogenic bacteria and phytoparasitic nematodes (Cronin *et al.* 1997; Walsh *et al,* 2001; Burdman *et al.* 2002).

2.3 Nitrogen Fixing Endophytes

Endophytic bacteria refers to those that reside within the plant without causing visible disease symptoms (Schulz and Boyle. 2006); equally only those bacteria that can be isolated from surface-sterilized plant tissues and extracts from within plant tissues (Hallmann et al., 1997). Since the mid 20th century, numerous endophytic studies have been reported on various plant tissue parts, such as seeds (Mundt and Hinkel, 1976), tubers (Trevet and Hollis, 1948), roots (Philipson and Blair, 1957), stems and leaves (Henning and Villforth, 1940), and fruits (Sharrock *et al.* 1991).

At early stages, endophytes were believed to be weak pathogens, however, discoveries in recent years confirmed several beneficial effects on host plants, such as promotion of plant growth, increased resistance to plant pathogens and parasites (Hallmann *et al.* 1997). Promotion of plant growth can be achieved by several mechanisms, such as production of plant hormones, synthesis of siderophores, nitrogen fixation, solubilisation of minerals (e.g. phosphorous) or through enzyme activitys, e.g. suppression of ethylene by 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Whipps, 2001; Hurek and

Reinhold-Hurek, 2003; Cherin and Chet, 2002). In recent years, the most interest has focused on the nitrogen fixation mechanim, especially endophytic diazotrophs (Dobereuner and Pedrosa, 1987; Estrada *et al.* 2002). Generally, endophytes do not induce a hypersensitive response in the plant. Most endophytic bacteria colonize intercellular spaces (Hinton and Bacon, 1995), although it has been shown that they also colonize intracellularly, e.g. Azoarcus spp. (Hurek *et al.* 1994).

Endophytic bacteria mostly originate from seeds (Adams and Kloepper, 1996), vegetative planting material (Dong *et al.* 1994), rhizosphere soil (Mahaffee and Kloepper, 1997) and phyllaffee (Beattie and Lindow, 1995). Endophytic colonization starts with colonizing root surface, then entry of bacteria through germinated breaches and move into starchy endosperm, further translocating to radical and coleoptile. Gradually, endophytes spreading to all parts of the plant (Hinton and Bacon, 1995). In terms of population density, it is highly variable depending on plant species and other factors. However, roots have with average densities of 10^5 CFU/g compared to stem and leaves, 10^4 CFU/g and 10^3 CFU/g, indicating population number decreases acropetally (Hallman and Berg, 2006). Plant organs, such as flowers, fruits and seeds have even lower colonization numbers and, most cases, numbers are below detecting thresholds.

2.3.2 Association of endophytes with sugar cane

Four million vehicles in Brazil run on ethanol produced from sugar cane grown on 8% (four million hectares) of nation's cropped area, providing more than 10 billion litres of fuel-alcohol annually (Boddey *et al.* 1991). Sugar cane contains 13.3% saccharose (about 10-15% sucrose) and 14% fiber (World Bank, 1998). Sugar cane is a tropical perennial C4 grass (family Poaceae) and produces massive amounts of biomass which, however, demands significant input of nitrogen, more than 250 kg /Ha. (Fuenctes-Ramirez *et al.* 1999). Nevertheless, applications of N fertilizer to sugar cane by Brazilian farmers are not adequate enough to cover the theoretical loss of nitrogen when plants are harvested (Boddy *et al.* 1995; Ruschel, 1981). In many parts of country, sugar cane has been continuously planting more than 100 years without nitrogenous fertilizer, and yet none of cane yield or soil N reserves fall with the time (Dong *et al.* 1994; Cavalcante and Dobereiner, 1988). In Brazil, the rates of nitrogen fertilisation are normally as low as 60 kg per hectare and the effect of adding additional the nitrogen fertilizer is negligible (Reis, V.Lee *et al.*, 2007; Ruschel *et al.*, 1984). These results lead scientists to suspect that sugar cane might have been benefiting from BNF (Neyra and Dobereiner, 1977; Ruschel, 1981).

Based on the ^{15}N isotope dilution and N-balance studies, it was confirmed that some Brazil sugar cane varieties are particularly effective in obtaining N up to 60 to 80%, equivalent to 200 kg N ha-1 year, of plant N which supposedly comes from sugarcane associated biological nitrogen fixation (BNF) (Boddey *et al.* 1991; Urquiage *et al.* 1992). However, both N-fixation techniques are not direct means to link BNF with endopytic

diazotrophic in sugar cane (James *et al.* 1997). Even though many genera of nitrogen fixing bacteria, such as *Bacillus* (Seldin *et a*l. 1984), *Azotobacter* (Singh *et al.* 1981), *Derxia* , *Enterobacter* , *Hlesiella* , *Beijerinckia* (Dobereiner, 1961) and *Azospirillum* (Dobereiner, 1988), have been isolated from the rhizosphere of sugarcane (Seldin *et al.* 1984; Singh *et al.* 1981; Dobereiner, 1961, 1988; Reis *et al.* 2007), none have enough numbers to be responsible for the high rates of N fixation, indicating rhizosphere free-living bacteria are not major contributors to sugar associated symbiosis. Then interest was caste on endophytes, bacteria that live inside the host plant without causing any disease symptoms, as a prime contributor of fixed N to sugarcane. *Acetobacter diazotrophicus* (*Gluconacetobacter diazotrophicus*) and *Herbaspirillum* are most recognized endophytes for their substantial numbers, up to 10^7/g fresh weight, isolated from within surface sterilized roots, stems and leaves (Cavalcane *et al.* 1988; Yamada *et al.* 1997; Boddey *et al.* 1995, 2000; Reis *et al.* 1994; Dobereiner *et al.* 1995). Large numbers of bacteria are of critical importance to correlate the BNF to specific N_2-fixing bacteria. In soybean, for an example, a large nodule should contain 10^9 bacteria (Baldani *et al.* 1986). Theoretically, sugar cane plants should have similar bacteria numbers to account for 80% nitrogen fixation (Urquiaga *et al.* 1992; James and Olivares, 1997a). The lower number of bacteria in sugarcane, compared to soybean, can be ascribe to the fact that sugarcane has lower %N compared to soybean so demands for N is not as high as soybean (James and Olivares, 1997a).

Endophytes do not survive for long time in the soil without having a host plant (Baldani *et al.*, 1992; Reis *et al.*, 1994; Olivares *et al.*, 1996). Interestingly, it is also

reported that *Gluconacetobacter diazotrophicus (G. diazotrophicus)* is present in low concentrations in the rhizosphere of sugarcane and is explained most probably because soil close to the host plant is enriched in sucrose (Li and MacRae 1991). It is also worth mentioning that increased soil moisture extends survival of *G. diazotrophicus* (Oliveira *et al.* 2004). Since endophytes live within the plant tissue, they supposedly fix nitrogen more efficiently than associative diazotrophs that live in the rhizosphere, such as *Azosphirillum* and *Azotobacter* (Patriquin *et al.* 1983; Triplett, 1996). Further, endophytes do not have to compete with other rhizosphere microbes for nutrient resources and have easy access to carbon substrates supplied by plant (Sprent and James, 1995; Hallmann *et al.* 1997). Moreover, endophytic diazotrophs live and grow in a relatively low pO_2 environment within dense plant tissues, which protects the nitrogenase from excess oxygen and is necessary for expression of nitrogenase (Patriquin *et al.* 1983; Gallon, 1992; Baldani *et al.* 1997).

2.3.2 *Gluconacetobacter diazotrophicus*

G. diazotrophicus is gram-negative, obligatory aerobic, rod-shaped and acid producing endophyte originally isolated roots and stems of sugarcane (Dong *et al.* 1994; Cavalcante and Dobereiner, 1988). G. *diazotrophicus,* belongs to alpha subgroup of the *Proteobacteria* (Gillis *et al.* 1989), grows best in sucrose-rich medium, can grow in up to 30% sucrose, fixes N_2 on N-free semi-solid (Cavalcante and Dobereiner, 1988) and solid LGIP medium supplemented with 10% sucrose (Pan and Vessey, 2001; Dong *et al.* 1995).

Nitrogenase activity by the bacteria is inhibited by excessive flux of O_2 (Gallon, 1992; Hunt *et al.* 1993; Layzell *et al.* 1990). Even though *G. diazotrophicus* showed capability of growing extreme conditions such as high sugar concentration and relatively low pH (Stephan *et al.* 1991; Chanway, 1998; James *et al.* 1998), this bacterium does not survive well in soils, particularly in natural soils (Baldani *et al.* 1997).

Even though endophytic diazotrophs are suspected as the main contributors of fixed N_2 to sugarcane growth, up until now it was not sure which specific bacteria was responsible for the majority of N--fixation. However, *Gluconacetobacter diazotrophicus,* previously known as *Acetobacter diazotrophicus* (Gillis *et al.* 1989), has long been the main interest for such plant-associated BNF and proposed as major contributor and has been estimated to fix up to 150 kg of N ha^{-} per year in sugarcane (Boddy *et al.* 1991. 1995; Cavalcante *et al.* 1988; James, 2000; Sevilla *et al.* 2000). The better colonization of sugarcane by *Gluconacetobacter diazotrophicus* might be related to properties of the bacteria, such as the optimal growth of sugar level is 10%, which resembles sugar content of sugar cane, and it reduces acetylene at pH levels 3 (Cavalcante and Dobereiner, 1988).

The bacterium also been isolated from different hosts such as coffee (Jimenez *et al.* 1997; Madhaiyan *et al.* 2004), potato (Paula *et al.* 1992), sorghum (Isopi *et al.* 1995), pineapple (Tapia *et al.* 2000), carrot, radish (Madhaiyan *et al.* 2004) and mealy bugs (Ashbolt *et al.* 1990; Caballero *et al.* 1995). More recently, isolation of *G. diazotrophicus* from economical important rise crop is reported (Muthukumarasamy R. *et al.* 2005). Further, inoculation of *G. diazotrophicus* not only promotes the plant growth in sugar rich

crop of sugar cane but also enhanced biomass of none sugar rich crops, such as rice plant (Riggs *et al.* 2001; Saravanan *et al.* 2007a). However, based on its growth conditions, theoretically *G. diazotrophicus* grows better in sucrose-rich crops. It is reported that bacterium does not have nitrate reductase and nitrogen fixation is not inhibited by as high as 80 mM of nitrate (Boddy *et al.* 1991; Gillis *et al.* 1989; Li *et al.* 1991; Stephan *et al.* 1991) nor is nitrogenase greatly inhibited by ammonium (Fu *et al.* 1988) , however, more recent works indicate that high N-fertilization levels in sugar cane field limits population of *G. diazotrophicus* (Fuentes-Ramirez *et al.* 1993, 1999; Muthukumarasamy, 1999; Reis Junior *et al.* 2000). In addition, population of *G. diazotrophicus* is susceptible to different cultivars of plant and is directly influenced by plant genotype (da Silva *et al.* 1995). Further, varieties of G. *diazotrophicus* isolated from Brazilian sugarcane is more diverse than the ones isolated in Mexico and is most probably because of different nitrogen-fertilization rates in two countries, indicating high N-fertilization also negatively effects population diversity of the bacterium (Caballero-Mellado *et al.* 1995). However, it is suggested that population dynamics of *G. diazotrophicus* influenced by changes in plant physiology induced by nitrogen, which subsequently affects plant association with the endophyte (Fuentes-Ramirez *et al.* 1998). As for the symbiosis of *G. diazotrophicus* with sugarcane, high doses of nitrate reduces the concentrations of sucrose in leaves, resulting decrease the sugar and sucrose in stem (da Silveira *et al.* 1991).

Nevertheless, more recent studies suggest that the beneficial growth effect may have come from plant hormonal effects on plant morphology and enhanced nutrient uptake rather than BNF, such as generation of indole-acetic acid (IAA) and gibberellins (Bastian

et al. 1998; Sevilla *et al.* 1998; Bastian *et al.* 2000; Fuentes- Ramirez *et al.* 1993). Solubilization of plant macro and micronutrient like P and Zn also are possible plant-growth-promoting traits (Saravanan *et al.* 2007a; Mowade and Bhatta-charyya, 2000), allowing both microorganisms (Rodriguez and Fraga, 1999) and plants (Kuklinsky-Sobral *et al.* 2004) to have access those unavailable nutrients. *G. diazotrophicus* is also considered as a biocontrol agent for is characteristics of defending the plant from pathogens and nematodes (Muthukumarasamy *et al.* 2000; Blanco *et al.* 2005; Saravanan *et al.* 2007b).

2.3.3 Co-innoculation

It has been demonstrated that co-inoculation of diazotrophs resulted in greater increase of biomass in sugarcane compared to individual inoculation, up to 29.2%, proving inoculation with mixture of diazotroph species is a best means to improve crop productivity through more effective nitrogen fixation process (Oliveira *et al.* 2002). In the latest study, several endophytic diazotrophs were being tested, including *G. diazotroph*, to evaluate the role of mixture inoculations. It was suggested that consortium of endophytic diazotrophs maximizes N_2 fixation and contribution of BNF in response to combined inoculation is affected by strain mixture of inoculum and plant genotype; soil and nitrogen fertiliziation are also considered additional influencing factors (de Oliveira *et al.* 2006). As for sugar beet, studies report that dual inoculation of nitrogen fixing and

phosphate solubilizing *Bacillus* strains have positive responses on yields of sugar beet compared to individual ones (Cakmakci *et al.* 1999, 2001, 2006; Sahin *et al.* 2004).

2.3.4 Association with vesicular-arbascular mycorrhiza (VAM)

More than 95% of plant taxa form associations with certain soil fungi that aid in taking up phosphate from soil. These fungi that form symbiotic association with roots of plants are called mycorrhizal fungi and the association itself is referred to as mycorrhiza. Mycorrhizas are devided into three morphological different groups, depending on the penetration of the root cells or not: (arbvscular) endomycrorrhizas, ectomycohhizas and ectenomycorrhizas (Gianinazzi *et al.* 2006). The endomycohrriza characterized by inter-and-intracellular funga growth in root cortex, forming specific fungal structures, such as vesicles and arbuscles. This growth trait gives the arbascular endomycorrhiza the alternative name, vesicular arbuscular mycohrriza (VAM) (Quilambo, 2003). VAM is the most widespread plant root symbiosis, forming association with more than 80% of plant families (Smith and Read, 1997). In addition, mycorrhizal plants have higher tolerance to heavy metals, root pathogens, drought, high temperatures, lower soil pH and to transplantation shock (Bagyaraj, 1997).

VAM is one of the most known factors in improving growth and nitrogen content in legumes (Barea and Azcon-Aguilar, 1983). Various bacteria have occurred within and on the surface of vesicular-arbuscular mycorrhizal fungus (VAM) (Mosse, 1962; Varma *et al.*

1981) and improvement of nitrogen fixation via associations of N fixing bacteria with spores of VAM is confirmed (Tilak *et al.* 1989), including *G. diazotrophicus* (Paula *et al.* 1991). In many higher plants *Gluconacetobacter* and AM fungi form intercellular association (Barea *et al.* 1987), in which fungi increases both nitrogen and phosphate uptake; therefore it is speculated that enhanced plant growth is generated by providing significant amounts of N through P-medidated mechanism (Cooper and Tinker, 1978). Synergistic effects of *G. diazotrophicus* on AM fungi colonization of sucrose-rich plants, such as sugarcane, sweet potato and sweet sorghum have been recorded (Paula *et al.* 1991; Isopi *et al.* 1995; Reis *et al.* 1999). In additional, the entry of *G. diazotrophicus* and other diazotrophs into sugarcane and sweet sorghum roots is facilitated by mycorrhization, indicating the symbionts may also interact each other (Paula *et al.* 1992; Vessey, 2003). Further studies indicate that co-inoculation of VAM with three species, N-fixing, P and K solubilizing, of bacteria had significant positive effects on plant growth and biomass of maize compared to controls, resulting higher rate of root infection in the presence of bacterial inoculation (Wu *et al.* 2005). More interestingly, VAM also generates hydrolytic enzymes and it might possibly work together with cell degrading enzymes of *G. diazotrophicus*, which might be beneficial for one or both symbiosis (Adriana-Anaya *et al.* 2006).

2.3.5 Infection of the host plant

It is necessary to elucidate the sites that bacteria enter the plant in understanding biological importance of where bacteria invade the plant. James *et al.* (1994) suggested that lateral root junctions and loose cell of root cap are the possible sites for infection. The very same result is confirmed by using immunogold labeling with antibodies specific to the bacteria (Reis J *et al.* 1995). Later studies reported that root hair and root tips are also possible infection sites for *G. diazotrophicus* (Bellone *et al.* 1997). In most recent plant-associated microorganisms study, using green florescent protein gene, *gfp*, marked *G. diazotrophicus* strain, it was further confirmed that lateral root emergence, the junctions between root cap and root axis in close to apex and root hairs in rice plants are possible sites that bacteria infect the plant (Rouwus *et al.* 2010). The exact same result is further confirmed using histochemical localization of *gus*A gene expression in sorghum and rice (Luna *et al.* 2010).

2.3.6 Localization of bacteria

It is of significant importance to examine where bacteria locate themselves and fix nitrogen. Even though endophytes generally colonize the intercellular spaces and vascular tissues, the exact location of major endophyte's of sugarcane, *Gluconacetobacter diazotrophicus,* is a quite controversial. James et al (1994, 1998) reported that they found the bacteria in the cortex of plant roots and in stem xylem vessels. They also proposed that root xylem might be potential infection route for stem and leaf xylem infection.

Moreover, it has been shown that *G. diazotrophicus* can grow and fix nitrogen on 1% concentration of sucrose (Boddy *et al.* 1991), supporting that *G. diazotrophicus* is not required to live only in sucrose-rich environment (Hawker, 1965; Dong *et al.* 1994, 1997) but also can grow and fix nitrogen in xylem where it contains 0-9% sucrose (Welbaum *et al.* 1992) and has lower pO_2 which allows for nitrogenase expression (Gallon, 1992). However, Dong *et al.* (1994) argued that *G. diazotrophicus* is confined only to the intercellular apoplast (spaces) of sugar cane where plentiful nutrients and pressure (Welbaum *et al.* 1990). They concluded that it is most unlikely to colonize the xylem vessel not only because of host defence reaction (Kao J *et al.* 1980; Olivares FL *et al.* 1997) but due to discontinuity of xylem vessel, blocking transportation of *G. diazotrophicus* within xylem (Dong *et al.* 1997). Nevertheless, Fuentes-Rammirez *et al.* (1999) using GUS-labeled strain and SEM (scanning electron microscopy) asserted that *G.diazotrophicus* colonizes the stem xylem and intercellular spaces of sugar cane. Further, neither James *et al.* (1994), nor Fuentes-Rammirez *et al.* (1999) have found the host defence response of sugar cane toward *G. diazotrophicus.* It is well established that some cell wall degrading enzymes assist the penetration of rhizobia into plant root (Mateos *et al.* 1992, 2001; Jimenez-Zurdo *et al.* 1996) and it is also speculated that some plant morphological changes might occur, forming continuous vessels by hydrolyzing the cell walls, induced by *G. diazotrophicus* (Esau K. 1997; Adrinao- Anaya *et al.* 2006).

2.4 Conclusion

It has been shown that endophytic diazotroph, *G. diazotrophicus,* is not only able to infect sugar cane but also infects sucrose-rich crops, such as sweet potato and sweet sorghum when the bacterium is co-inoculated with mycorrhizal fungus (Paula *et al.* 1991, 1992). Also, the latest study showed that isolates of *G. diazotrophicus* from sugarcane, sweet potato, pineapple and wild cane showed appreciable amount of nitrogenase activity (Prabudoss and Stella, 2009). Plus, there is one report that *G. diazotrophicus* has been isolated from sugar beet (Madhaiyan *et al.* 2004). All of those studies give an insight that inoculation of sugar beet with nitrogen fixing endophyte, *G. diazotrophicus spp.,* could most probably promote plant growth and increase yield through nitrogen fixation at lower cost compared to chemical fertilizers, making sugar beet a potential alternative to produce ethanol in Canada. Additionally, using nitrogen fixing endophytic bacteria as biofertilizer reduces the costs on expensive chemical fertilizers while decreases the carbon dioxide production and eliminates the environmental pollution, such as leaching of nitrates into groundwater, making the sugar beet is more viable plant to grow at cheaper price in more greener way. Further, sugar concentration of sugar beet is 25% higher than sugar cane (Singh et al. 1985; Martin *et al.* 2006; World Bank, 1998; Weeden, 2000) and yields can be 10 times greater than corn and wheat (Vessey, personal contact).

Although sugar beet production cannot be compared to imported raw sugar cane in Canada, increased energy price and demand for ethanol production are positive signs that there is a future for sugar beet production in this country.

3.0 Objectives

The fourteen strains of *Gluconacetobacter spp.* were screened for their capability of infecting sugar beet along with nitrogen fixiation. A commonly used strain, PAL5T, was selected for the rest of the studies. Based on some studies that are already published, inoculation at different growth stages and bacterial concentration of inoculum has significant importance in plant response to inoculation (Bashan, 1986). The inoculant strain was also isolated from plant tissue and was confirmed by a molecular PCR technique. In addition, the possible entry sites for bacteria into host plant were investigated by using GUS gene labelled strains. Following were specific research topics:

- Testing 14 strains of *Gluconacetobacter* spp. for capacity of improving biomass of sugar beet.
- Investigation of effect of different nitrogen concentrations on nitrogen fixation in sugar beet.
- Assessment of the optimum inoculation stage.
- Evaluation of the least inoculant number.
- Molecular confirmation of bacteria isolates by PCR.
- Determination the possible sites of bacteria entry the host plant with GUS strains.

4. Materials and Methods

Fourteen strains of *Gluconacetobacter* spp. (Table. 1) were selected out of 30 strains for inoculation of sugar beet plants based on higher activity of acetylene reduction both in semisolid LGI-P medium and semisolid LGI-P mixed with ground beet tissue medium (Houman Fei, Saint Mary's university).

4.1 Experiment I : Screening of *Gluconacetobacter* spp.

In collaboration work with Dr. Houman Fei, 14 strains of *Gluconacetobacter* spp., including 11 strains of *G. diazotrophicus* and 3 strains of *G. johannae* (Table. 1), were screened for their nitrogen fixation capacity in sand soil pots in a greenhouse.

4.1.1 Plant growth and soil

Seeds of , *Beta vulgaris* L. variety-Beta 5833R, from Betaseed Inc. (ON, Canada) were germinated in Petri dishes lined with one layer of filter paper wetted with distilled water at room temperature in dark. When seedlings reached about one inch in length, they were transferred into 3 L pots containing 3 kg of silica sand (one plant/pot). Plants were grown in the greenhouse of Saint Mary's University, Halifax, NS.

4.1.2 Greenhouse conditions

The plants were grown in greenhouse during winter with a controlled temperature of 20-27°C (n/d) and with a photoperiod of 16/8 h (d/n). Supplemental light was supplied by High Pressure Sodium lamps at 350-370 m^{-2} s^{-1}. Plants were irrigated every day with the same volume of water (50 ml/pot) and the volume was increased to 100 ml/pot, 200 ml/pot and 300 ml/pot over four months of growth period. Throughout the experiment, pots were rearranged to ensure exposure to light intensities.

Plants were watered twice a week with a modified Knop's solution (Mohr and Schopfer, 1861) containing 1 mM NO_3^- and 10 mM NO_3^-, respectively. Knop's solution contains: 0.656 g/L $Ca(NO_3)_2$, 0.202 g/L KNO_3, 0.250 g/L KH_2PO_4, 0.120 g/L $MgSO_4$, 2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2 \cdot 4H_2O$, 0.22 mg/L $ZnSO_4 \cdot 7H_2O$, 0.08 mg/L $CuSO_4 \cdot 5H_2O$, 0.02 mg/L $H_2MoO_4 \cdot H_2O$, 6.95 mg/L $FeSO_4 \cdot H_2O$; pH was adjusted to 6.0 with 1% acetic acid solution. Each strain had five replicates both in 1 mM and 10 mM experiments. Treatment solution was enriched with 1% of $^{15}N\ O_3^-$, while ^{15}N was not added to control solutions.

4.1.3 Harvest

The plants were harvested after 12 weeks of growth. The shoots, beets (lower hypocotyls or upper root) and fibrous roots were collected separately. Around 2 g of

fresh beet tissue was sampled and kept at -80°C for detecting the presence of inoculated bacteria in the interior plant using polymerase chain reaction assay (PCR). All other tissues were dried at 80°C for 3 days to measure dry biomass. Dry biomass of roots, shoots and beets were compared, respectively.

4.1.4 Culture of bacteria

All 14 strains of *Gluconacetobacter* spp. were cultured in modified liquid LGI-P medium (Cavalcante and Dobereiner, 1988; Pan and Vessey, 2001) at 30°C until the broth OD value reached to 0.6, λ 600 nm. Each sugar beet seedling was inoculated with 5 ml of bacteria broth (OD=0.6, λ 600 nm) after the emergence of the first true leaves.

The modified LGIP liquid medium contains (quantities per litre): 0.2 g K_2HP_4; 0.6 g KH_2PO_4; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 0.002 g $Na_2MoO_4.2H_2O$; 0.01 g $FeCl_2.6H_20$; 0.5% bromothymol blue solution in 0.2 M KOH; Biotin 0.0001g; Pyridoxal HCl 0.0002 g; $(NH_4)_2SO_4$ 1.32 g; crystallized cane sugar 100g; final pH 5.5 adjusted with 1% acetic acid solution. Additional 15g L^{-1} of agar is used in solid medium and semisolid medium for isolation contained 2 g L^{-1} agar. The medium is autoclaved for 15 min after adding all chemicals and pH is adjusted around 5.7 before autoclaving.

4.1.5 Quantification of nitrogen fixation by ^{15}N isotope dilution

Several methods have been developed for quantification of plant-associated heterotrophic nitrogen fixation both in grasses and cereals, including ^{15}N isotope dilution (McAuliffe *et al.* 1958), ^{15}N natural aboundance (Shearer and Kohl, 1986), and acetylene reduction (Hardy *et al.* 1968). Each method has its unique a set of calculations which are subject to certain inaccuracy. However, stable isotope techniques (^{15}N) have been generally considered the most popular due to the higher accuracy for quantitative measurements of symbiotic N fixation (Danso, 1995). The ^{15}N –dilution technique was used in this thesis for its higher reliable quantification assumption.

The principle of ^{15}N isotope dilution technique (MacAuliffe *et al.* 1958) relies on the fact that use of ^{15}N-labeled fertilizer, which is diluted in plants that assimilate ^{14}N from atmosphere in symbiosis with microorganisms. Thus, ^{15}N isotope dilution technique indirectly evaluates nitrogen fixation by estimating the dilution of ^{15}N-labeled fertilizer by $^{14}N_2$ derived from the atmosphere. The amount of dilution is proportional to the amount of fixed nitrogen. The fixed N in the plants or ^{15}N can be measured by mass spectrometer based on isotope ratio (Mariotto, 1983; Knowles and Blackburn 1993; Unkovich *et al.* 2001). The estimation is more accurate and straightforward in sand cultures because of known amounts of enriched ^{15}N in fertilizer, whereas in soil the $^{14}N_2$ released from soil organic materials is also absorbed by plants, causing errors in estimation (Boddy and Victoria, 1986).

Based on the following formula (Busse, 2000), if the ^{15}N content in certain parts of the inoculated plant tissue is lower than that found in control plants, this indicates the nitrogen in inoculated plant was derived from the air by biological nitrogen fixation via the aid of endophytic diazotroph.

1) The percentage of N derived from atmosphere (Ndfa) for individual plant was calculated by folloing for equation:

   $AT\%\ Ndfa = [(AT\%_{Ref} - AT\%_{Treat}) / AT\%_{Ref}] \times 100\%$

   Where AT refers to atom per centage of ^{15}N. Ref is reference plant (control) and Treat is treatment plants. Reference plants were not labled with ^{15}N isotope.

2) Total nitrogen fixed (mg $plant^{-1}$) was calculated by the following equation:

   $Totoal\ N\ fixed = AT\%\ Ndfa \times Biomass\ (mg\ plant^{-1}) \times N\%$

3) Nitrogen derived from atmosphere (Ndfa):

   $Ndfa\% = (Fixed\ Ndfa / Total\ N) \times 100\%$

Table 1. 14 strains of *Gluconacetobacter spp.*, used for sugar beet inoculation.

Species	Strain	Source	Institution	Genetically engineered	Country of origin	Other designations /culture listings	Reference
G. diazotrophicus	SRT4 LsdA'	Lazaro Hernandez	CGEB [1]	Under expression of levan sucrase A	Cuba		Hernandez et al., 2000. Arch Microbiology, 172:120-124
G. diazotrophicus	SRT4 LsdB+ +	Lazaro Hernandez	CGEB [1]	Over expression of levanase B	Cuba		
G. diazotrophicus	CFNE 550	Jesus Caballero-Mellado	UNAM [2]		Mexico		Caballero-Mellado et al. 1995. Appl. Environ. Microbiol. 61:3008-3013.
G. diazotrophicus	PSP22		EMBRAPA [3]		Brazil		Caballero-Mellado et al. 1995. Appl. Environ. Microbiol. 61:3008-3013.
G. johannae	UAP-CF51	Jesus Caballero-Mellado	UNAM [2]		Mexico		Jimenez-Salgado et al. 1997. Appl. Environ. Microbiol. 63:3676-3683.
G. johannae	CFN-CF52	Jesus Caballero-Mellado	UNAM [2]		Mexico		Jimenez-Salgado et al. 1997. Appl. Environ. Microbiol. 63:3676-3683.
G. johannae	CFN-CF76	Jesus Caballero-Mellado	UNAM [2]		Mexico		Luis E. Fuentes-Ramirez, et al. 2001. Intl. J. Syst. Evol. Micorbiol. 51:1305-1314.

G. diazotrophicus	SRT4	Lazaro Hernandez	CGEB[1]		Cuba		Coego A. et al. 1992. Rev Lat-amer Microbiol 34:189–195
G. diazotrophicus	PAL5 T		EMBRAPA[3]		Brazil	PAI 5; PAL 5; ATCC 49037; CCUG 37298; CIP 103539; DSM 5601; LMG 7603; NCCB 89154	Caballero-Mellado et al. 1994. Appl. Environ. Microbiol. 60:1532–1537.
G. diazotrophicus	PAL5 T LsdA-	Lazaro Hernandez	CGEB[1]	Underexpression of levan sucrase A	Cuba		
G. diazotrophicus	PAL5 T LsdB ++	Lazaro Hernandez	CGEB[1]	Overexpression of levanase B	Cuba		
G. diazotrophicus	PAL5	Lazaro Hernandez	CGEB[1]		Cuba	PAI 5; PAL 5; ATCC 49037; CCUG 37298; CIP 103539; DSM 5601; LMG 7603; NCCB 89154	Z. DONG, et al. 1995. Appl. Enviro. Microbiol. 61:1843-1846.
G. diazotrophicus	PAL3		UNAM[2]		Brazil	Pal 3; PAL 3; LMG 8066	Caballero-Mellado et al. 1994. Appl. Environ. Microbiol. 60:1532–1537.

G. diazotrophicus	UAP AC7	Jesus Caballero-Mellado	UNAM[2]		Mexi-co		Tapia-Hernández A, et al. 2000. Microb Ecol 39:49–55.
G. diazotrophicus	1772	Jesus Caballero-Mellado	UNAM[2]		Austra -lia		Caballero-Mellado et al. 1995. Appl. Environ. Microbiol. 61:3008-3013.
G. diazotrophicus	T2	Lazaro Hernandez	CGEB[1]		Cuba		F.G. Loiret, et al. 2004. Journal of Applied Microbiology 2004, 97, 504–511

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Strains containing the term LsdA- have been genetically engineered for the underexpression of levan sucrose A (IsdA) gene.

Strains containing the term Lsd++ have been genetically engineered for the overexpression of levanase B (IsdB) gene.

4.2. Experiment II: Assessment of best inoculation stage experiment

To evaluate if inoculation at different growth stages of sugar beet has impacts on plant biomass (Bashan, 1986), inoculation at four different growth stages (seed, seedling, seven leaves and 14 leaves) was carried out. The strain PAL5T was selected for inoculation due to the positive results it gave from screening experiment.

4.2.1 Culture of bacteria

Strain PAL5T was cultured in modified liquid LGI-P medium (Cavalcante and Dobereiner, 1988; Pan and Vessey, 2001) at 30°C until the broth OD value reached 0.7, λ 600 nm. Bacteria growth curve was obtained by serial dilution at different OD values.

4.2.2 Plant growth conditions

Sugar beet seed was supplied from a seed distributer as the fruiting structure of sugar beet, the achene. Each achene contains a single sugar beet seed. Hensceforth, when the term sugar beet "seed" is used in this thesis, what is actually been referred to is the seed within the sugar beet achene.

A commercial seed coating was removed by shaking the seeds in the 50 ml flasks with distilled water for five minutes. Then the seeds were washed thoroughly several

times with distilled water. Seeds were planted to the pots, each pot containing three seeds.

Sugar beets were grown during summer with a minimum temperature regime of 18/22 °C (n/d) in a greenhouse. Plants were watered every day with the same volume of water (50 ml/pot) or with Knop's solution and volume was increased to 100 ml/pot, 200 ml/pot and 300 ml/pot in over four months of growth. Treatments were watered twice a week with nutrition solution (Knop's) enriched with 1% of ^{15}N and un-enriched nutrition solution was used for control plants. The nitrogen concentration of nutrition solution was 5 mM. The nutrition solution had been applied 10 days after plantation (one seedling/pot).

4.2.3 Inoculation

The soil surface area immediately above where the sugar beets were planted was inoculated by application of 5 ml of broth, OD = 0.7, λ 600 nm, at different growth stages (ie., seed, seedling, seven leaves and 14 leaves). Control plants were un-inoculated.

4.2.4 Harvest

Plants were harvested after for four months of growth, from the beginning of June to beginning of October in 2008. The shoots, beets (hypocotyls, the upper roots) and roots (fibrous roots) were collected separately and dried at 80°C for 3 days.

4.3. Experiment III: Titre experiment

To determine whether inoculation with different bacteria concentrations results in differences in biomass, sugar beets were inoculated with three different concentrations of bacteria in the inoculant. Strain PAL5T of G. *diazotrophicus* was used in this greenhouse experiment. Additionally, to confirm the results of experiment II, sugar beets were inoculated at seed and seedling growth stages.

4.3.1 Culture of bacteria

The strain PAL5T was cultured in modified liquid LGI-P liquid medium at 30°C until the broth OD value reached to 0.5, λ 600 nm. Bacteria number was confirmed by dilution plating at OD of 0.5, λ 600 nm; each dilution had four replicates. The same dilution plating was repeated four times and the mean number from those four individual experiments has been used for titre experiments.

4.3.2 Plant growth conditions

Purchased sugar beet seeds had a seed coating which enhances seed germination and protection from pathogenic bacteria.. However, coatings were removed by rinsing with high pressure tap water for three to five minutes in order to minimize the interference of

seed coating with the inoculation. Then the seeds were washed thoroughly several times with distilled water.

Sugar beet seeds were grown in sand-pots, each pot containing 3 kg of sand and three beet seeds, with a minimum temperature regime of 18/22 °C (n/d) at Saint Mary's University's greenhouse from May to September in 2009. Each plant was watered with same volume of water, 50 ml/ pot, every day in the first month of growth and the water volume had been gradually increased to 100 ml pot^{-1}, 200ml pot^{-1} and 300 ml pot^{-1} in four month of growth perioed in response to plant growth. Treatments were watered twice a week with Knop's nutrition solution enriched with 1% of $^{15}NO^{3-}$ and un-enriched nutrition solution was applied to control plants. The nutrient solution was applied 10 days after plantation, after seedlings were thinned down to one plant in each pot. Nitrogen concentration of nutrition solution had been kept at 5 mM in entire experiment.

4.3.3 Inoculation at seed and seedling stages

Sugar beets were inoculated at seed and seedling stages with three different bacteria concentrations (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1}, 10^6 CFU ml^{-1}). However, at seed inoculation stage, the seeds were dipped into different concentration of bacteria broth for half an hour, and then the rhizosphere soil was inoculated with 5 ml/pot of broth for the second time after plantation. Thus, seeds were inoculated twice in different methods in the seed titre experiment. The second seed experiment was carried out in exact two weeks after the first seed experiment was set up. In second seed titre experiment, the

rhizosphere soil around the seeds was inoculated with 5 ml/pot of bacteria broth and seeds were inoculated only once.

4.3.3.1 Seed inoculation (inoculated twice)

After the commercial seed coatings were removed, the treatment seeds were dipped into three different inoculants (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}), respectively, for 30 min. Then seeds were transferred to pots, each pot containing three seeds. Then seeds were rhizosphere inoculated with 5 ml of additional broth at different concentrations, such as 10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}. Control seeds were dipped into pure LGI-P liquid medium, and then the rihzosphere soil of seeds were inoculated with additional 5 ml of pure LGI-P medium.

4.3.3.2 Seedling inoculation (inoculated once)

After the first pair of true leaves opened (15 days after planting), 5 ml of PAL5T broth at the concentrations of 10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}were applied to the sand around each stem, respectively. Control plants were inoculated with 5 ml of pure LGI-P medium. Ten days after plantation, the nutrition solution applied to treatment plants twice a week. Inoculant numbers were confirmed by serial dilution at OD of 0.5, λ 600nm.

4.3.3.3 Seed inoculation (inoculated once)

Two weeks after the seed and seedling titre experiment started, another seed inoculation experiment was set up. Seeds were planted into the pots (three-seed/pot), then the soil surface as of seeds was inoculated with 5 ml of broth at different bacteria concentrations (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}). Control was inoculated with pure LGI-P medium only. Bacteria number was confirmed by serial dilution at OD of 0.5, λ 600nm, repeated four times.

4.3.3.4 Harvest

Plants were harvested after 12 weeks of growth. Approximately, 7.0 g of fresh beet tissues from each treatment was aseptically isolated and kept at -80°C for PCR analysis. ^{15}N abundance was analyzed by mass spectral meter at Agriculture and Agri-Food Canada Research Centre at Lethbridge (AB, Canada).

4.4 Experiment IV: Assessment of sites of *Gluconacetobacter* infection by use of GUS labelled strains (GUS assay)

To visualize the possible infection sites of sugar beet plants by *G. diazotrophicus* strains, a reporter gene assay was applied. Among six reporter genes that have been reported in studies of gene expression in higher plants, *lacZ* gene from *Escherichia coli*, encodes β-galactosidase, is the most frequently used reporter gene. However, high levels of endogenous β-galactosidase activity in plants make it difficult or impossible to detect chimeric β-galactosidase by enzymatic methods (Jefferson, 1986). Nevertheless, a gene fusion marker that uses *uidA*, or *gus*A, as reporter gene that encodes the enzyme β-glucuronidase (GUS), was used in this thesis (Jefferson, 1986).

The major advantage of GUS-reporter system is that the hydrolysation of the substrate X-gluc (5-bromo-4-chloro-3-indoly glucuronide) results in production of an insoluble blue colour in those cells displaying GUS activity (Jefferson, 1987). Since plant cells do not contain any GUS activity, the production of a blue color when stained with X-gluc indicates the activity of the promoter that drives the transcription of the *gus*A-chimeric gene in that GUS labeled strains of *G. diazotrophicus* (Jefferson, 1987).

Sugar beet plants were grown in sand soil in greenhouse and harvested after 12 weeks of growth. All dry samples were ground and sieved thorugh 60 mesh screen. ^{15}N

abundance was analyzed by mass spectral analysis at Stable Isotope Facility of University of Alberta (Alberta, Canada).

4.4.1 Culture of GUS strains

Two GUS labelled strains of *Gluconacetobacter diazotrophicus* (kindly provided by Dr. Jesus Caballero- Mellado) were used for this experiment; UAP-5541/PRGS561, which constitutively expresses GUS, and UAP-5541/PRGH562 with a *nif*H:: *gus*A transcriptional expression of GUS (Fuentes-Ramirez et al. 1999). Both strains were cultured in LGI-P liquid medium, containing 45 mg/L streptomycine (was added after autoclaving), at 30 ^{0}C until OD value reached to 0.5, λ 600 nm.

4.4.2 Confirmation of GUS strains

The GUS gene marked *G. diazotrophicus* strains were constructed (Fuentes-Ramirez *et al.* 1999) to have intrinsic resistance towards streptomycin. Thus, GUS strains, UAP-5541/PRGS561 and 5541/PRGH562, were pre-screened in LGI-P liquid medium containing streptomycin at 45mg L^{-1} (Fuentes-Ramirez *et al.* 1999; Cocking *et al.* 2006). Then plated on LGIP solid medium, containing 50mg L^{-1} X-Gluc, with and without bromthymol blue, to confirm the inserted plasmids were not lost. The formation of dark blue colonies indicated *gus*A gene expression in bromthymol blue containing medium. Control plates either did not contain X-Gluc.

4.4.3 Sterilization of sugar beet seeds and germination

Commercial seed coatings were flushed off seed with high pressure tap water, then quickly rinsed with 70% ethanol up to 10 seconds; followed by sterilization with 3% commercial bleach, containing 0.05% Twin 20 for three minutes and washed seven times with sterilized distilled water. Then seeds were aseptically transferred to Petri dishes with filter paper in the bottom. Each Petri dish contained 20-25 seeds and an adequate amount of sterilized water was added for germination. The Petri dishes were then sealed with parafilm and germinated in growth chamber in the dark at 18/22 °C (n/d) for up to 5-6 days.

4.4.4 Inoculation with GUS strains

Germinated beet seeds were transplanted into 50 ml glass beakers, containing either MS medium or 1% agar medium under an airflow and at one plant per container. Then each seedling was inoculated with 0.1 ml of the GUS-labelled strains from culture broths with OD value of 0.4, λ 600nm. Finally, beakers were sealed with parafilm and place in growth chamber for further growth. Photoperiod was 16/8 h (d/n) at 18-22 °C (n/d).

In another experiment, germinated seeds were transplanted into the small 300 ml plastic pots containing sterilized sands. Plants were watered with sterilized distilled water throughout experiment. Each pot containing one seedling was inoculated with 5 ml broth

of either with UAP-5541/PRGS561 strain or with 5541/PRGH562 strain with OD value of 0.4, λ 600nm. Control plants were inoculated with PAL5T strain as positive control. Additionally, another set of control plants were inoculated with sterilized water. Plants from each treatment were grouped together and placed in bigger transparent plastic containers separately, preventing the seedlings from dehydration and cross-contamination. Then plants were incubated at growth chamber for four to six days, respectively. Photoperiod is 16/8 h (d/n), 22/18 °C (d/n). Each treatment had six replicates each time and the same experiment was repeated for five times to further confirm the results.

4. 4.5 GUS staining

Plants were harvested four and seven days after inoculation and washed carefully to separate sands from roots. Then the seedlings were transferred into 5 ml clear autoclaved vials. Seedlings were fixed, washed and stained based on the procedures on kit (β-glcuronidase (GUS) reporter gene staining kit, Sigma).

Staining solution was added after washed with washing solution and vials were degassed for 20 minutes in vacuum desiccator. Then vials were covered with aluminum foil and placed in incubator at 37°C in the dark for 12 hours. At this point, blue colour develops over time; however, sometimes had to incubate up to 24 hours or more to allow the blue colour appear. Solutions were prepared fresh each time. After the blue coulour

developed, the green chlorophyll was removed by desiccating the samples with ethyl alcohol series, such as 25%, 35%, 50%, 70% and 90% for half hour. Samples can be stored at 4 °C for long periods now, or examined under the microscope.

4. 4.6 Microscopic observation

Samples were observed under dissecting microscope (OLYMPUS-SZ61, Japan) and pictures were taken with camera (INFINITY-lite, Japan) using INFINITY CAPTURE software.

4.5 Experiment V: Detection of isolated bacteria strain with 16S rRNA based PCR

To further confirm that it was the inoculated bacteria contributing to the increased sugar beet biomass, inoculated bacteria were isolated aseptically from frozen plant tissues. The identity of isolated bacteria as *G. diazotrophicus* was confirmed using polymerase chain reaction (PCR) assay.

4.5.1 Isolation of bacteria

Frozen plant tissues were thawed and sterilized with 0.1% $MgCl_2$ for five minutes. Then tissues were rinsed with sterilized distilled water for five times or more. The surface layer of beet was removed by a scalpel, and this piece was homogenized with sterilized plastic-tipped drilled bit in 0.5 ml of 10% sucrose. The homogenized beet tissue was passed through a 5 μm of syringe filter and was inoculated into tubes containing 5 ml semisolid LGI-P medium (Vladimir *et al.* 1998). Inoculated tubes were incubated at 30 °C for 10-15 days until yellowish colour was visible. Then bacteria were plated on Petri dishes to further purify.

In addition, the homogenized beet tissue in 0.5 ml of 10% sterilized sucrose solution was filtered through 5 um of filtration, and then centrifuged for 10 minutes at 13,000 rpm. After the supernatant was discarded, 0.5 ml of 10% autoclaved sucrose was added and vortexed for immediate PCR analysis.

4.5.2 Amplication of 16S rRNA genes

In this PCR experiment, species specific primers were used to identify the bacterium that was isolated from sugar beet. PCR for detection of *Gluconacetobacter* spp. was performed by genetic method based on 16S rRNA gene sequence with the species-specific primers AC (5′- CTGTTTCCCGCAAGGGAC- 3′) and DI (5' - GCGCCCCATTGCTGGGTT- 3') due to 16S rRNA is highly conserved in different species of bacteria (Kirchhof *et al.* 1998). The primer pair AC-DI targets the 445 amplicon (Sievers et al. 1998). Primer pair for all 16S rDNA is RB (5'-AGA GTT TGA TYM TGG CTC AG-3') and RM (5'-GGA CTA CCA GGG TAT CTA ATC C-3'), which is homologous to regions conserved in all 16 S rRNAs, was used in the same PCR reaction to prove the PCR approach. RB and RM universal primers target a fragment of 800 bp (Mahhaiyan *et la.* 2004).

4.5.3 PCR Condition

Amplification was carried out in 50- µl reactions. Suspension of the bacteria colonies in 1 ml of sterilized water or filtered sugar beet extraction was centrifuged at 13000 rpm for 10 min. 2 µl of supernatant was used for PCR. Then following components, which were kept on ice, were added in order: 1uM of each primer (AC, DI,RB and RM), 10 ul buffer, 1.25 mM dNTP and 1.5 mM $MgCl_2$. After 10 minutes of denaturation at 95 °C, 2 U of Taq-Polymrase was added, followed by 35 cycles: 95°C for

1 min, 52°C for 2min, 72°C for 2 min, and final cycle at 72°C for 10 min. The amplification products were anaylysed by electrophoresis in 1% agarose gels. Gel was run for 30 minutes at 100 volt. Ethidium bromide was pre-added to agrose gel.

After separation of PCR products with 1% agarose gel, it was viewed and photographed using Algha imager TM1200 documentation and analysis system.

4.6 Statistics

Data was tested for normal distribution. Normally distributed data was analyzed by ANOVA with JPM (7) software. However, data that was not normally distributed analyzed by MANOVA using SYSTAT (12) software due to the robustness of MANOVA to the deviation from normality. Then data was further tested by ANOVA via SYSTAT 12 software. In all analysis, the means were compared in a pair-wise fashion to the un-inoculated control to maximize the potential of indicating if any individual strain stimulated plant biomass and contributed fixed N to the host plants.

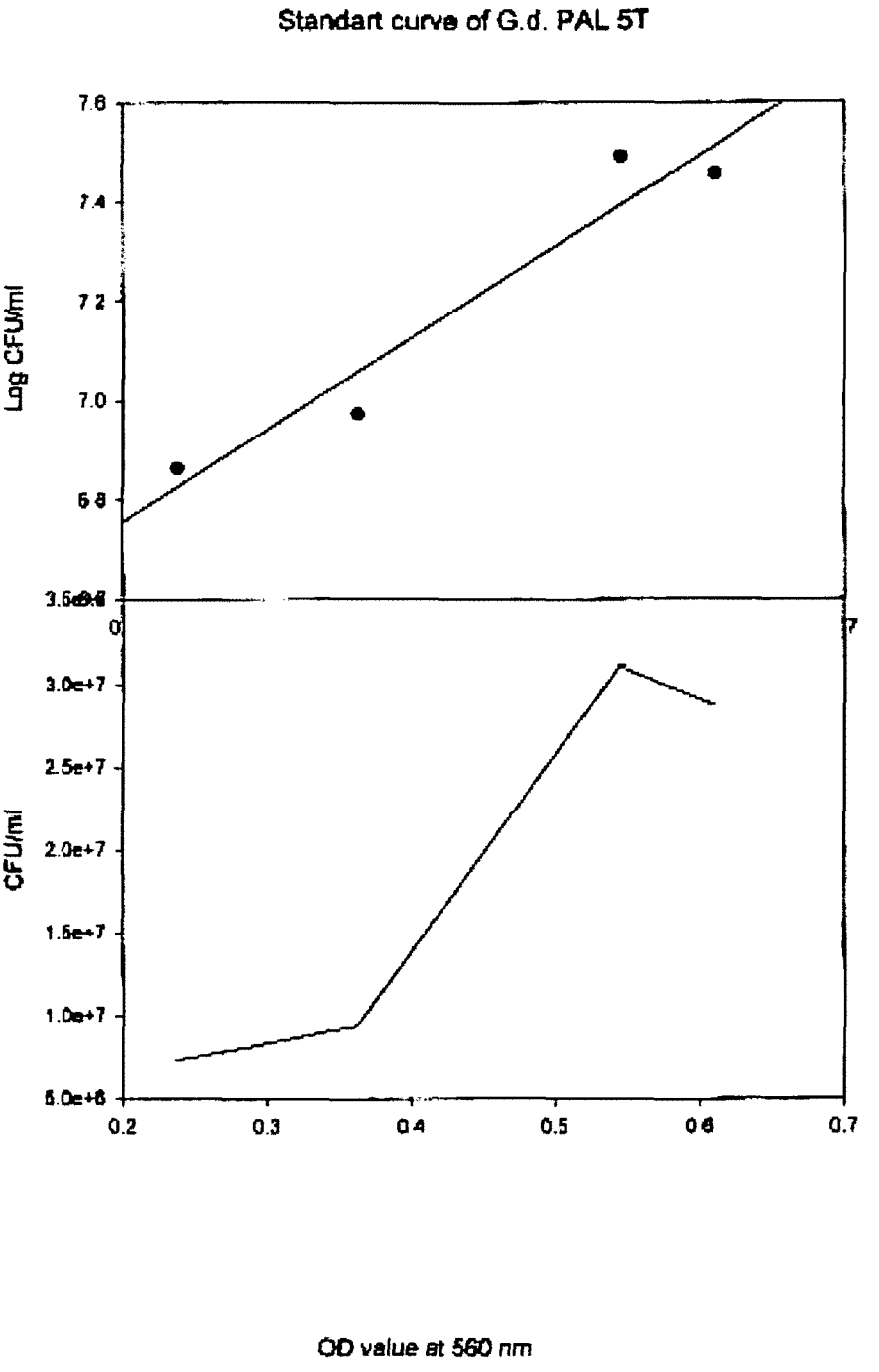
In the titre experiments, samples were further analysed by MANOVA with SYSTAT software due to its capability of processing un-parametric samples. As a result, the MANOVA test also showed that there was significant different between treatment and control, $P < 0.05$. Then samples further were analysed by Fisher's least-significant-difference test. The results supported the MANOVA test outcome, $P < 0.05$, proving results of original analysis were correct.

5.0 Results

5.1 Bacteria growth curve and enumeration of inoculums

The growth curve of PAL5T strain in liquid L-GIP medium varied regardless of repetition of the same experiment for many times. Thus, all growth curves were combined to have one representative curve (Figure. 1). Plate counting was also conducted at a fixed OD value, OD=0.5, 600nm. In four individual serial plate counting experiments, the result was almost the same each time. Thus, the mean number from those four different experiments was used for diluting the inoculant (10^8/ml). Additionally, right after inoculating the sugar beet seeds and seedlings, the original inoculant used for inoculation was plated for further confirmation of previous results. Once again, all results were matched (10^8/ml).

Figure. 1 **Growth Curve of the strain PAL5T.**



5.2 Experiment (I): Screening of 14 strains of *Gluconacetobacter* spp.

Fourteen strains of *Gluconacetobacter spp.*, including three *Gluconacetobacter johannae* strains, were screened for their capability of stimulating fixing nitrogen in different nitrate concentrations. The dry biomass of beets, shoots and roots of treatments were compared to control plants in each of 1 mM and 10 mM NO^{3-} concentration experiments.

5.2.1 Biomass of sugar beet grown on 1 mM NO_3^-.

Six of fourteen strains of *Gluconacetobacter spp.* significantly increased biomass of the beets compared to the control, indicating inoculation by some strains did have positive impact on plant growth (Table. 2). The strain of *G. d.* PAL5T LsdB++ had the highest improvement for beet growth, reaching a significant increase of 110.6% (Table. 2).

Table. 2 **Beet biomass of sugar beets treated with 1 mM NO_3^-.**

Each value is a mean of 5 replicates. Significance was compared only between control and each treatment. Treatments listed with different levels of significance (a or b) are statistically different from each other with α =0.05, where (a) is not significantly different and (b) is significantly different from control. S.E = standard error.

Gluconacetobacter	**Beets**		**Signif.**	**% increase**
spp.	**DW (mg)**	**S.E.**	**α =0.05**	
Control(uninoculated)	1826.4	306.0	a	0.0
G. d. SRT4 LsdA$^-$	2266.2	300.1	a	24.1
G. d. SRT4 LsdB^{++}	3129.6	481.2	b	71.4
G. d. CFNE 550	2504.6	377.9	a	37.1
G. d. PSP 22	2295.8	77.1	a	25.7
G. j. UAP-Cf 51	3279.0	417.5	b	79.5
G. j. CFN-Cf 52	2183.4	267.5	a	19.5
G. j. CFN-cf 76	2731.6	501.7	a	49.6
G. d. SRT4	3247.2	363.7	b	77.8
G. d. PAL5T	2731.8	322.2	a	49.6
G. d. PAL5T LsdA$^-$	3093.4	398.6	b	69.4
G. d. PAL5T LsdB^{++}	3847.0	545.9	b	110.6
G. d. UAP AC7	2500.0	328.3	a	36.9
G. d. 1772	3087.8	227.1	b	69.1
G. d. T2	1754.6	231.7	a	-3.9

In the shoots, the dry biomass increase ranged from 3.3% to 57% as compared to un-inoculated control plants (Table. 3). Even though the percentage of increase in shoots was much lower than the beets, 12 out of 14 strains significantly increased shoot biomass.

Table 3. **Shoot biomass of sugar beets treated with 1 mM NO_3^-**

Each value is a mean of 5 replicates. Significance was compared only between control and each treatment. Treatments listed with different levels of significance (a or b) are statistically different from each other with α =0.05, where (a) is not significantly different and (b) is significantly different from control. S.E = standard error.

Gluconacetobacter	**Shoots**		**Signif.**	**% increase**
spp.	**DW (mg)**	**S.E.**	**α =0.05**	
Control(uninoculated)	1800.4	181.8	a	0.0
G. d. SRT4 LsdA$^-$	2134.8	89.2	a	18.6
G. d. SRT4 LsdB^{++}	2441.2	104.8	b	35.6
G. d. CFNE 550	2378.0	174.9	b	32.1
G. d. PSP 22	2532.8	151.6	b	40.7
G. j. UAP-Cf 51	2439.6	223.0	b	35.5
G. j. CFN-Cf 52	2568.8	117.3	b	42.7
G. j. CFN-cf 76	2488.4	121.4	b	38.2
G. d. SRT4	2381.4	184.3	b	32.3
G. d. PAL5T	2677.4	129.5	b	48.7
G. d. PAL5T LsdA$^-$	2415.0	175.3	b	34.1
G. d. PAL5T LsdB^{++}	2589.2	170.9	b	43.8
G. d. UAP AC7	2827.4	282.8	b	57.0
G. d. 1772	2604.6	93.8	b	44.7
G. d. T2	1859.8	150.5	a	3.3

A similar improvement of biomass accumulation occurred in roots inoculated with most of strains. The ranged of increase was from 16.1% to 59.3%; number of strains that had significant increase reached to 7 (Table. 4).

Table 4. **Fibrous root biomass of sugar beets treated with 1 mM NO_3^-.**

Each value is a mean of 5 replicates. Significance was compared only between control and each treatment. Treatments listed with different levels of significance (a or b) are statistically different from each other with α =0.05, where (a) is not significantly different and (b) is significantly different from control. S.E = standard error.

Gluconacetobacter	**Fibrous Roots**		**Signif.**	**% increase**
spp.	**DW (mg)**	**S.E.**	**α =0.05**	
Control(uninoculated)	571.4	81.3	a	0.0
G. d. SRT4 LsdA$^-$	663.2	71.1	a	16.1
G. d. SRT4 LsdB^{++}	703.8	95.4	a	23.2
G. d. CFNE 550	827.8	72.6	b	44.9
G. d. PSP 22	792.8	81.2	b	38.7
G. j. UAP-Cf 51	691.8	46.4	a	21.1
G. j. CFN-Cf 52	777.4	120.5	a	36.1
G. j. CFN-cf 76	912.0	45.5	b	59.6
G. d. SRT4	727.0	118.6	a	27.2
G. d. PAL5T	910.2	108.3	b	59.3
G. d. PAL5T LsdA$^-$	787.8	62.2	b	37.9
G. d. PAL5T LsdB^{++}	878.2	35.3	b	53.7
G. d. UAP AC7	765.4	90.5	a	34.0
G. d. 1772	856.8	44.7	b	49.9
G. d. T2	685.0	120.7	a	19.9

5.2.2 Biomass of sugar beet biomass grown on 10 mM NO_3^-

The dry biomass of inoculated sugar beets grown on 10 mM NO_3^- was compared with control plants. Beets, shoots and roots were compared separately.

In beets, 10 of 14 strains increased the dry biomass, proving that inoculation had direct growth impact on plant growth. The highest percentage of increase in dry beets was 67.3%.

Table. 5 **Beet biomass of sugar beets treated with 10 mM NO_3^-.**
Each value is a mean of 5 replicates. Significance was compared only between control and each treatment. Treatments listed with different levels of significance (a or b) are statistically different from each other ($\alpha = 0.05$), where (a) is not significantly different and (b) is significantly different from control. S.E= standard error.

Gluconacetobacter **spp.**	**Beets**		**Signif.**	**% increase**
	DW (mg)	**S.E.**	**$\alpha = 0.05$**	
Control(uninoculated)	15943.2	2338.1	a	0
G. d. SRT4 LsdA$^-$	21461.2	1583.9	b	34.6
G. d. SRT4 LsdB^{++}	23352.6	280.4	b	46.5
G. d. CFNE 550	20880.4	930.3	a	31
G. d. PSP 22	22376.8	1445	b	40.4
G. j. UAP-Cf 51	21183.6	3500.1	a	32.9
G. j. CFN-Cf 52	21952.2	2438	b	37.7
G. j. CFN-cf 76	26035.4	1391.2	b	63.3
G. d. SRT4	26671.4	1969.1	b	67.3
G. d. PAL5T	22711.4	1879.3	b	42.5
G. d. PAL5T LsdA$^-$	19984	1789.2	a	25.3
G. d. PAL5T LsdB^{++}	22736.4	3825.9	b	42.6
G. d. UAP AC7	24248.6	2652.4	b	52.1
G. d. 1772	21317	1916.7	a	33.7
G. d. T2	22099.6	1959.4	b	38.6

In the shoots, the increased dry biomass of inoculated sugar beets was not significant except for the treatment that was inoculated with strain *G. d.* PAL5T, resulting 27.2 % dry biomass increase. Two strains, *G. j.* UAP-Cf 51 and *G. d.* PAL5T LsdB^{++}, had negative growth effects on shoot. Interestingly, this negative growth effect of both stains had been not found on beets (Table. 5), where strain *G. d.* PAL5T LsdB^{++} significantly increased beet dry biomass.

Table. 6 **Shoot biomass of sugar beets treated with 10 mM NO_3^-.**
Each value is a mean of 5 replicates. Significance was compared only between control and each treatment. Treatments listed with different levels of significance (a or b) are statistically different from each other with α =0.05, where (a) is not significantly different and (b) is significantly different from control. S.E= standard error.

Gluconacetobacter	**Shoots**		**Signif.**	**% increase**
spp.	**DW (mg)**	**S.E.**	**α =0.05**	
Control(uninoculated)	13532.4	959.2	a	0
G. d. SRT4 LsdA$^-$	13760.6	731.3	a	1.7
G. d. SRT4 LsdB^{++}	13895.4	978	a	2.7
G. d. CFNE 550	13635.4	1480.1	a	0.8
G. d. PSP 22	14991.4	873.3	a	10.8
G. j. UAP-Cf 51	12534.4	1810.9	a	-7.4
G. j. CFN-Cf 52	14373.6	1204.8	a	6.2
G. j. CFN-cf 76	14441	866.3	a	6.7
G. d. SRT4	14032.3	1033.1	a	3.7
G. d. PAL5T	17215.4	1543.8	b	27.2
G. d. PAL5T LsdA$^-$	14731.4	766.8	a	8.9
G. d. PAL5T LsdB^{++}	13353.4	672.6	a	-1.3
G. d. UAP AC7	14725	1736.2	a	8.8
G. d. 1772	13641.4	374.7	a	0.8
G. d. T2	12728	956.4	a	-5.9

Four out of 14 strains significantly increased the fibrous root biomass. The increase ranged from 17.8% to 75.5% (Table. 7).

Table 7. **Fibrous root biomass of sugar beets treated with 10 mM NO_3^-**
Each value is a mean of 5 replicates. Significance was compared only between control and each treatment. Treatments listed with different levels of significance (a or b) are statistically different from each other with α =0.05, where (a) is not significantly different and (b) is both statistically different from control (α =0.05). S.E= standard error

Gluconacetobacter	**Roots**		**Signif.**	**% increase**
spp.	**DW (mg)**	**S.E.**	**α=0.05**	
Control(uninoculated)	3359.2	336.8	a	0
G. d. SRT4 LsdA$^-$	5439.6	1241.3	b	61.9
G. d. SRT4 LsdB^{++}	3958.8	515.3	a	17.8
G. d. CFNE 550	4448.2	344.1	a	32.4
G. d. PSP 22	4457.4	593.6	a	32.7
G. j. UAP-Cf 51	4164	616.9	a	24
G. j. CFN-Cf 52	4065.8	233.5	a	21
G. j. CFN-cf 76	4432	652.1	a	31.9
G. d. SRT4	4724.4	345	a	40.6
G. d. PAL5T	5487.8	534	b	63.4
G. d. PAL5T LsdA$^-$	5130	599.6	b	52.7
G. d. PAL5T LsdB^{++}	5896.4	802.3	b	5.5
G. d. UAP AC7	4643.2	666.8	a	38.2
G. d. 1772	4493.2	233.2	a	33.8
G. d. T2	4670.8	709.8	a	39

Most of strains did not show a significant effect on shoot biomass accumulation at the 10 mM NO_3^- treatment; only the strain *G. d.* PAL5T had significant increase on beet shoots (Table. 6). Even though the magnititude of inoculation impact on fibrous roots at 10 mM NO_3^- treatment was higher, fewer strains had significant biomass increase on fibrous roots (Table. 4 and Table. 7).

The highest percentage of beet and shoot biomass increase occurred at the 1 mM treatment NO_3^- , 110.6 % and 57%, respectively (Table .2 and Table. 3). However, the comparison of the increments between 1 mM NO_3^- and 10 mM NO_3^- treatments showed that the overall improvement of beet growth was much lower in the 1 mM NO_3^- treatment than on 10 mM NO_3^- (Table. 8).

Table. 8 **Comparison of tissues biomass from 1 mM NO_3^- and 10 mM NO_3^- treatments**

Biomass from 1 mM NO_3^- and 10 mM NO_3^- treatments were compared for each tissue. Data were analysied by t-test using SYSTAT 12 software (α=0.05). S.E=standard error.

	1 mM NO_3^-	S.E	10 mM NO_3^-	S.E	P- value (α =0.05)
Beet (mg)	**2760.86**	**101.63**	**22643.61**	**515.42**	**P < 0.001**
Shoot (mg)	**2452.74**	**43.18**	**14154.31**	**280.63**	**P < 0.001**
Root (mg)	**784.23**	**20.64**	**4716.19**	**150.87**	**P < 0.001**

5.2.3 Quantification of nitrogen fixation in sugar beet tissues

Quantification of nitrogen fixation in sugar beet tissues was calculated based on ^{15}N dilution technique. Nitrogen content of tissues from both the 1 mM NO_3^- and 10 mM NO_3^- treatments, as well as the control was tested to be able to compare the nitrogen fixation between treatments and control.

The improvement of biomass accumulation by inoculation with 14 strains of *Gluconacetobacter spp.* in all beet tissues were well matched with corresponding nitrogen fixation in both treatments on 1 mM N and 10 mM N. The fixed nitrogen in the plant derived from atmosphere was higher in treatment on 1 mM NO_3^- than 10 mM NO_3^- treatment (Table.11). The highest nitrogen fixation was found in the roots inoculated with the strain *G. d.* PAL5T LsdB++ in 1 mM NO_3^- treatment; the nitrogen derived from atmosphere reached to 24.4% (Table. 9). Interestingly enough, the fixed nitrogen in the sugar beet plants gradually increased in the order of shoots, beets and fibrous roots in both treatments and all inoculations (Table. 9 and Table. 10).

Table 9. **Nitrogen fixation in sugar beet treated with 1 mM NO_3^-** ($P<0.05$). S.E=standard error.

Gluconacetobacter **Spp.**	**Nitrogen Derived from Atmosphere (Ndfa) (%)**					
	Shoots	**S.E.**	**Beets**	**S.E.**	**Roots**	**S.E.**
Control(uninoculated)	0.0	0.0	0.0	0.0	0.0	0.0
G. d. SRT4 $LsdA^-$	9.5	2.7	10.8	2.4	15.3	3.8
G. d. SRT4 $LsdB^{++}$	11.7	3.1	12.4	2.5	16.9	3.3
G. d. CFNE 550	10.1	2.4	9.9	1.5	13.9	2.2
G. d. PSP 22	10.0	0.9	11.0	0.7	15.5	0.7
G. j. UAP-Cf 51	9.1	1.4	12.1	1.6	15.0	1.9
G. j. CFN-Cf 52	10.5	0.7	11.7	0.8	16.2	1.6
G. j. CFN-cf 76	12.8	1.2	13.6	1.4	18.1	1.7
G. d. SRT4	12.1	2.5	14.3	2.3	18.7	2.5
G. d. PAL5T	15.8	1.2	16.8	1.1	21.9	1.4
G. d. PAL5T $LsdA^-$	13.8	2.0	13.7	3.2	21.4	1.9
G. d. PAL5T $LsdB^{++}$	17.9	1.8	18.6	2.1	24.6	2.7
G. d. UAP AC7	16.0	2.6	17.4	2.6	21.7	1.9
G. d. 1772	15.6	1.0	17.2	0.4	22.6	1.2
G. d. T2	8.3	1.7	9.8	2.0	14.7	2.4

Table 10. **Nitrogen fixation in sugar beet treated with 10 mM NO_3^-** ($P<0.05$). S.E=standard error.

Gluconacetobacter	**Nitrogen Derived from Atmosphere (Ndfa) (%)**					
Spp.	**Shoots**	**S.E.**	**Beets**	**S.E.**	**Roots**	**S.E.**
Control(uninoculated)	0.0	0.0	0.0	0.0	0.0	0.0
G. d. SRT4 $LsdA^-$	0.0	0.6	1.6	0.8	3.4	1.2
G. d. SRT4 $LsdB^{++}$	0.0	0.6	1.0	0.7	3.2	1.4
G. d. CFNE 550	0.0	0.4	0.8	0.4	3.1	0.9
G. d. PSP 22	0.0	0.6	1.4	1.0	3.5	0.9
G. j. UAP-Cf 51	0.0	0.9	1.1	1.1	4.6	1.9
G. j. CFN-Cf 52	0.0	0.9	1.4	1.4	4.9	1.6
G. j. CFN-cf 76	0.2	0.9	2.3	0.8	3.1	1.4
G. d. SRT4	0.4	0.7	2.2	0.6	3.1	1.5
G. d. PAL5T	0.0	0.5	1.4	0.6	3.6	1.2
G. d. PAL5T $LsdA^-$	0.0	0.6	2.3	1.4	4.5	1.3
G. d. PAL5T $LsdB^{++}$	0.0	0.4	1.6	0.6	3.9	0.9
G. d. UAP AC7	0.5	0.4	2.7	0.8	5.0	0.7
G. d. 1772	0.0	0.7	1.5	0.6	3.2	1.0
G. d. T2	0.0	0.7	1.5	0.6	4.6	1.2

Red colour on Table. 9 and Table. 10 indicates the significantly different samples compared to control in dry biomass.

As it is shown on the table (Table. 11), the overcall nitrogen fixation in shoots, beets and fibrous roots of sugar beet at 1 mM nitrogen treatment is higher than those that at 10 mM treatment. This result statistically confirms that inoculated strains fix nitrogen more efficiently at lower nitrogen concentration (1 mM) compared to higher concentration (10 mM).

Table 11. **Comparison of nitrogen fixation in tissues from 1 mM NO_3^- to 10 mM NO_3^- treatments**

The %Ndfa from 1 mM and 10 mM treatment were compared for each tissue. Data were analysied by t-test using SYSTAT 12 software. S.E=standard error.

	1 mM (%Ndra)	S.E	10 mM (%Ndra)	S.E	P-value (α=0.05)
Shoot	12.382	0.543	1.825	1.449	P < 0.001
Beet	13.517	0.535	1.709	0.182	P < 0.002
Fibrous Root	18.320	0.627	3.850	0.283	P < 0.001

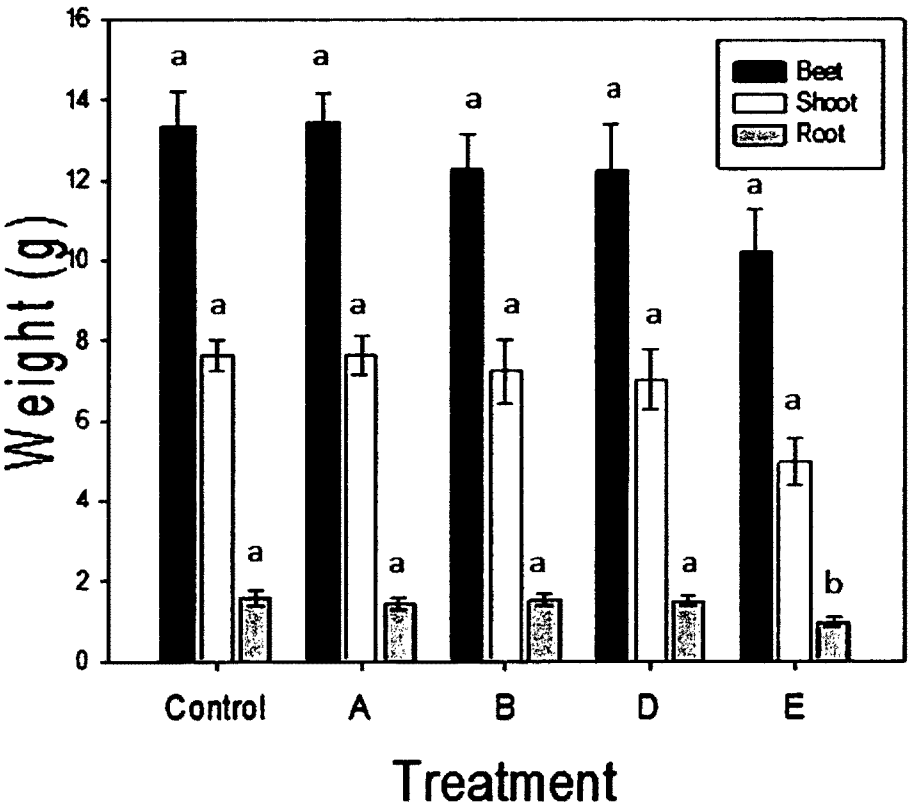
5.3 Experiment II: Assessment of best inoculation stage

To assess the effect of inoculation at different plant growth stages on accumulation of sugar beet biomass, beets were inoculated at four different stages (seed, seedling, seven leaves and fourteen leaves).

The dry biomass of beets, shoots and fibrous roots were compared to the control. There were no significant differences among four treatments in terms of beet dry weight (Figure. 2), indicating inoculation at different stages did not have significant effect on growth. Interestingly, there was also no significant difference in beet dry biomass of control and treatment plants (Figure. 2); showing inoculation had no effect on plant growth. Given that inoculation with strain PAL5T did not result in a statistically significant increase in beet dry biomass in the 1 mM NO_3^- treatment (Table. 2), but had a positive increase in dry beet biomass of the 10 mM NO_3^- treatment indicating an interaction between *G. diaztrophicus* strain and nitrogen fertility.

Interestingly, negative significant difference was found between shoots of fourteen-leave stage and control plants, indicating the inoculation reduced the shoot biomass at the fourteen-leaves stage (Figure. 2).

Figure. 2 **Dry biomass of beets, shoots and roots from different growth stages inoculation**. A, B, D and E referring to seed, seedling, seven leaves and fourteen leaves inoculation stages ($P<0.05$). Statisticaly significance within treatments was compared, where (a) is not significant different and (b) is significantly different. Control was un-inoculated. 8-9 replicates per treatment. Data were analysied by MANOVA using SYSTAT 12 software.



5.4 Experiment III: Titre experiment

To assess if the concentration of bacteria in the inoculant was one of the factors that influences biomass increase, sugar beets were inoculated with different concentrations of bacteria (titre). Additionally, to confirm the different inoculation stages do not have any impact on plant biomass increase, the seed and seedling inoculation was added to titre experiment.

5.4.1 Seed inoculation - titre

In this seed inoculation-titre experiment, seeds were dipped into different concentrations (10^2 CFU ml^{-1}, 10^4 CFU $ml^{-1}$and 10^6 CFU ml^{-1}) of PAL5T broth before planting. After planting the seeds, the soil above the seed was additionally inoculated with 5 ml/ pot of bacteria broth (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}).

There was significant differences in dry biomass of beets compared to control plants (Table. 12 and Figure. 3), showing inoculation did have positive effect on the plant growth. It is speculated that this biomass increase was mostly probably from biological nitrogen fixation. Interestingly, no significant differences were observed among treatments (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}) in terms of dry beet biomass (Table. 12), indicating that as low as 500 bacteria was enough to infect the plant to have

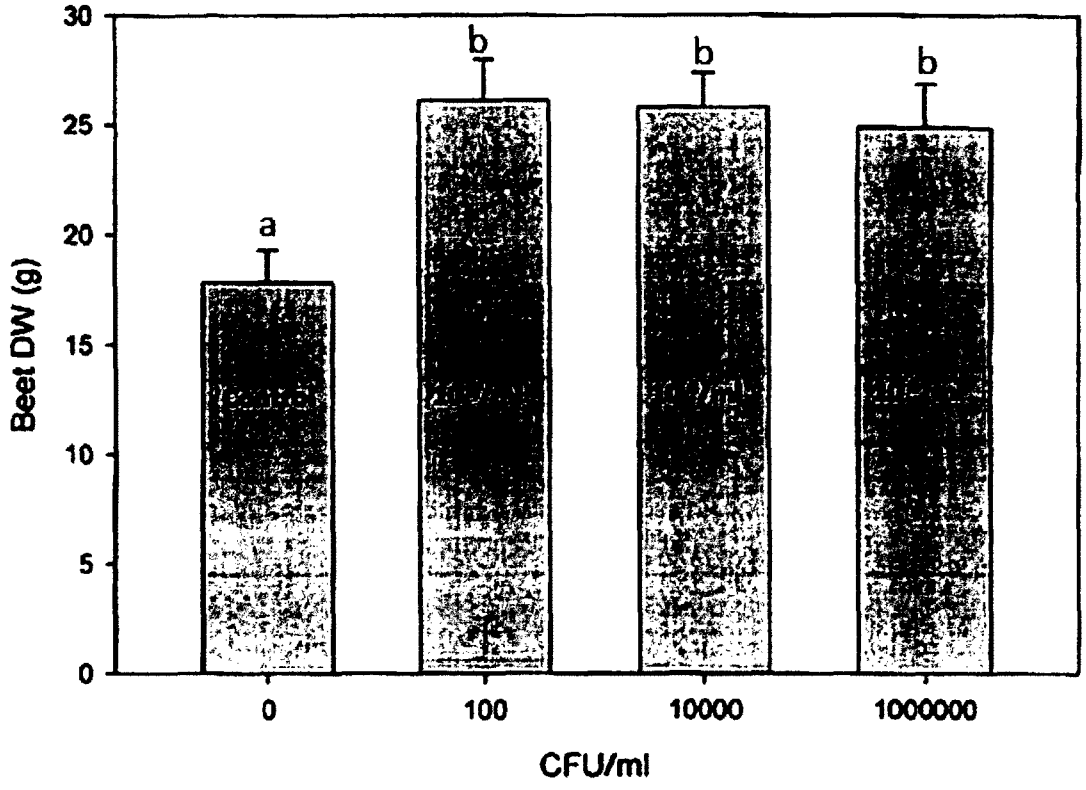
positive growth effect. The result also shows that growth effect caused by incoulant of 500 bacteria was the same with the effect of 5×10^3 and 5×10^4.

It is of biological importance that the lowest inoculant number resulted in the highest biomass increase (Figure. 3).

Table 12. **Biomass of beets in sugar beet inoculated at seed stage (inoculated twice).** The mean number represents of 8-13 replicates. Significance was compared only between control and each treatment ($P<0.05$). S.E=standard error.

Seed inoculation (twice) 5 ml broth ; 5 mM NO_3^-	**Beets**		**Signif.**
	DW (mg)	**S.E.**	**α=0.05**
Control (uninoculated)	**17.8**	**1.5**	**a**
10^2/ml	**26.1**	**1.9**	**b**
10^4/ml	**25.5**	**1.7**	**b**
10^6/ml	**24.9**	**2**	**b**

Figure.3 **Comparison of beet dry biomass of sugar beet**
Statistically significance of treatments was compared, where (a) is not significant different and (b) is significantly different. Control was un-inoculated. 8-9 replicates per treatment.The mean number represents of 8-13 replicates. Significance was compared only between control and each treatment ($P < 0.05$). Dry hypocotyl biomass was increased 46.5%, 44.8% and 39.5%, respectively.

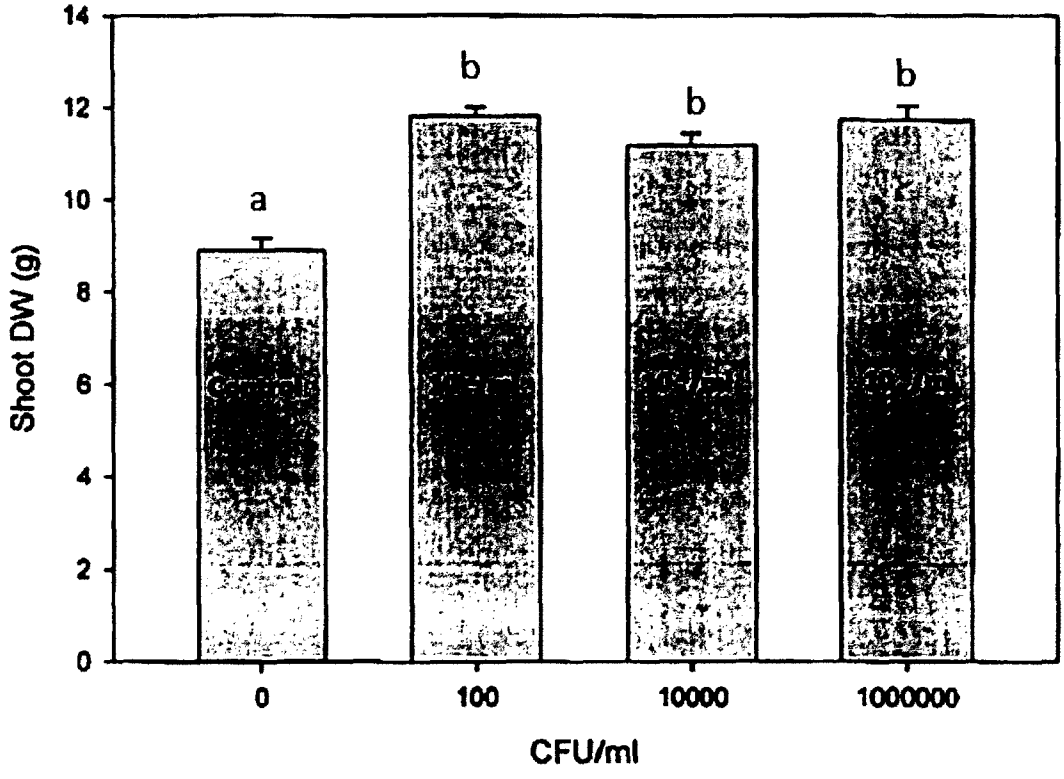


In shoots, significant difference was found between treatments and control (Figure. 4), proving seed inoculation also had positive growth effect on sugar beet shoots. The dry shoot increase ranged from 26.6% to 32.7%. The lowest inoculation number resulted in

the highest shoot increase, which is quite interesting considering the lowest inoculant number also gave the highest biomass increase in beets.

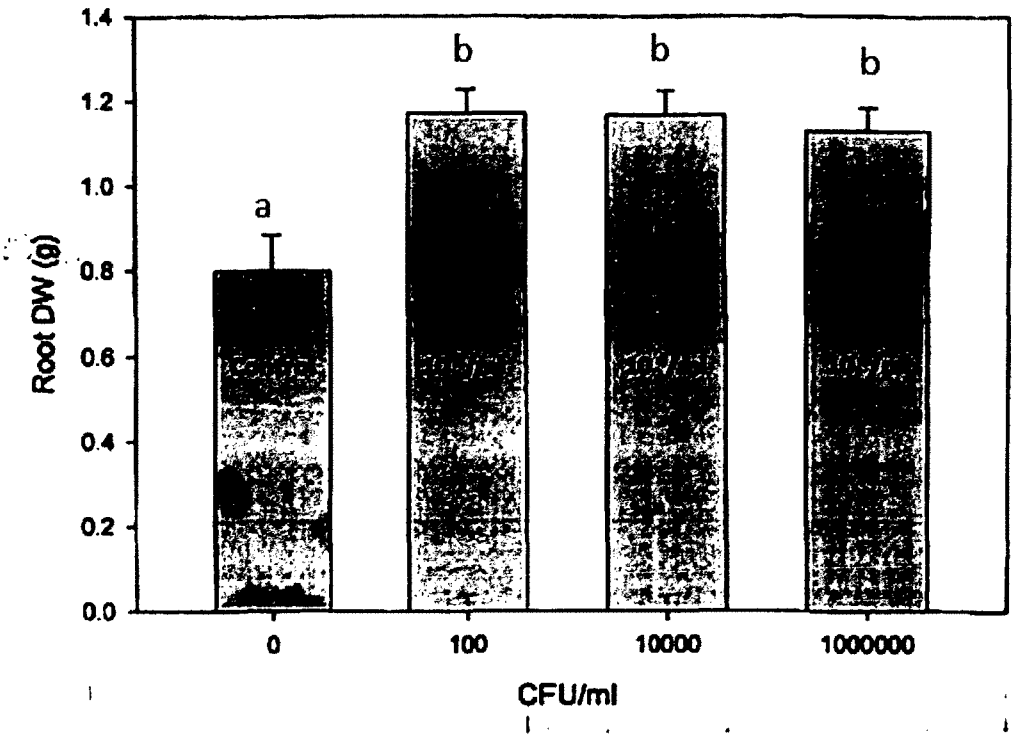
In shoots, inoculation increased the shoot biomass by 32.7% in 10^2 CFU/ml inoculant, 26.6% % in 10^4 CFU ml^{-1} inoculant and 31.8% in 10^6 CFU ml^{-1} inoculants, separately (Figure. 4).

Figure. 4 **Comparison of shoot dry biomass of sugar beets**
Statistically significance of treatments was compared, where (a) is not significant different and (b) is significantly different. Control (0) was un-inoculated ($P < 0.05$). Treatments were inoculated with 5 ml of broth at the concentrations of 10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}. Dry shoot biomass was increased 32.7%, 26.6% and 31.8%, respectively.



Similarly, the significant increase was also observed in fibrous roots and increase was 46.3%, 46.3% and 41.3% in three different inoculants in the order of 10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1} (Figure. 5). The highest inoculant number resulted in the lowest root biomass increase in comparison to other titre treatments, while the lowest inoculant number gave higher increase than the lowest inoculants.

Figure. 5 **Comparison of fibrous root dry biomass of sugar beets**
Statistically significance of treatments was compared, where (a) is not significant different and (b) is significantly different. Control (0) was un-inoculated. Treatments were inoculated with 5 ml of broth at the concentrations of 10^2 CFU /ml, 10^4 CFU /ml and 10^6 CFU /ml. Dry root biomass was increased 46.3%, 46.3% and 41.3%, respectively ($P < 0.05$).



Shoot and root samples were also re-analysed by MANOVA with SYSTAT 12 software. The results came out exactly same with previous analysis that there was significant difference between shoots and roots of treatment compared to control plants, $P < 0.05$.

In addition, beet, shoot and fibrous root samples were further analysed by Fisher's least-significant-difference test. The results were confirmed with MANOVA test by aid of SYSTAT 12 software, $P < 0.05$.

Interesting fact that germination rate of inoculated seeds was higher than un-inoculated seeds (roughly 30%, data not shown).

5.4.2 Seedling- titre inoculation

For seedling-titre experiment, seeds were germinated in pots and at the seedling stage, each plant was inoculated by applying 5 ml of broth on the soil surface around the stem with different bacteria concentrations (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}).

No significant beet biomass differences have been observed between treatments and control (Table. 13). Thus, samples were not analysed for ^{15}N content.

Table 13. **Biomass of beets in sugar beet inoculated at seedling stage**

Statistically significance of treatments was compared, where (a) is not significant different and (b) is significantly different. The mean number represents of 8 replicates. Significance was compared only between control and each treatment ($P<0.05$). S.E refers to standard error.

Seedling inoculation 5 ml broth ; 5 mM NO_3^-	**Beets**		**P-value**
	DW (mg)	**S.E.**	**$\alpha<0.05$**
Control (uninoculated)	**21.2**	**1.7**	**a**
10^2/ml	**23**	**1.8**	**a**
10^4/ml	**22.5**	**1.1**	**a**
10^6/ml	**18.2**	**1.1**	**a**

5.4.3 Seed inoculation-titre: inoculated once

In comparison to previous seed-titre experiment, in which seeds were inoculated twice in a different manner, seeds were only inoculated once right after planting with 5 ml of three different concentrations of bacteria broth (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}). Control was inoculated with pure L-GIP medium.

Interestingly enough, the experiment failed to give positive results in terms of significant dry plant biomass increase in beets (Table. 14). Therefore, quantification of nitrogen fixation test was not conduced considering not any significant biomass increase was observed.

Table 14. **Biomass of beets inoculated at seed stage (inoculated once)**
The mean number represents of 8 replicates. Significance was compared only between control and each treatment ($P<0.05$). (Experiment was set up 15 days after than previous two experiments started). S.E - standard error.

Seed inoculation (once) 5 ml broth ; 5 mM NO_3^-	**Beets**		**P-value**
	DW (mg)	**S.E.**	**α=0.05**
Control (un-inoculated)	**21.8**	**1.3**	**a**
10^2/ml	**22.8**	**1.3**	**a**
10^4/ml	**22.8**	**1.3**	**a**
10^6/ml	**22.6**	**1.4**	**a**

5.5 GUS assay (IV)

5.5.1 Confirmation of GUS expression in GUS-transformed strains

To confirm GUS expression in the GUS-transformed strains of *Gluconacetobacter diazotrophicus* (*G. diazotrophicus*), a series of individual experiments were conducted. GUS gene labeled *G. diazotrophicus* strains, UAP-5541/PRGS561 constitutively expressing GUS and 5541/PRGH562 with a *nif*H::gusA transcriptional fusion, were grown in liquid ATGUS medium [0.8% (w/v) agar, yeast extract (2.7g L^{-1}), glucose (2.7g L^{-1}), mannitol (1.8g L^{-1}), MES buffer (4.4 g L^{-1}), K_2HPO_4 (065g L^{-1}), pH 6.5, containing 45 mg/L streptomycin] to test growth condition (Edward, 2006).

Based on the fact that the optimal growth for *G. diazotrophicus* is 10% sucrose, we hypothesized that GUS strains might have faster in the original liquid LGI-P medium containing 10% sucrose (Gillis, 1989). The growth rate of GUS gene labelled *G. diazotrophicus* strains in liquid ATGUS medium (Cocking, 2006) was compared to liquid LGI-P medium based on OD values. Result indicated that the GUS strains grew faster in liquid LGI-P medium compared to ATGUS. Thus, liquid LGI-P medium is used for future culturing experiments.

GUS labeled *G. diazotrophicus* strains, UAP-5541/PRGS561 and 5541/PRGH562, were grown in LGI-P medium with and without streptomycin at 45mg/L. In the

streptomycin containing LGI-P medium, bacteria grew well which further indicated that bacteria retained resistance to the antibiotic.

Confirmation of GUS gene expression was tested on solid LGI-P medium containing X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt), 50 mg L^{-1}, and control medium did not contain X-Gluc. PAL5T was used as a second control to compare GUS gene labeled strains. X-Gluc was added in two forms, as a powder after autoclaving the medium and after dissolving in ethylenediaminetetraacetic acid (EDTA). The results showed that the colonies of GUS gene labeled strains plated on LGI-P medium containing X-Gluc turned to dark colour (Figure. 7 and Figure. 10), while colonies of medium did not contain X-Gluc remained as yellow (Figure. 8, Figure. 9 and Figure. 11). Further, the colonies of PAL5T did not show any colour changes in neither in X-Gluc containing medium nor non-containing one. All of those factors contributing the confirmation of GUS strains did not lose the GUS gene containing plasmids and were viable. In terms of the forms of adding the X-Gluc, the powder method resulted in darker colour compared to EDTA –dissolved X-Gluc. Nevertheless, the colony colour was supposed to be dark blue rather than dark based on previous research (Edward, 2006). Thus, another set of experiment was conducted to obtain the blue colour to further confirm the GUS expression. It was concluded that bromothymol blue was responsible for colour change from dark blue to blue (Figure. 6).

The experiment above was repeated adding one more factor, with and without a bromothymol blue. As expected, the colonies of medium did not containing

bromothymol blue gave a true blue colour compared to darker blue colour formed from bromothymol containing medium. Thus, the expression of GUS gene on L-GIP medium containing X-Gluc was finally confirmed and bacteria strains were viable.

Figure. 6 **Expression of *Gluconacetobacter diazotrophicus* 5541/PRGH562 strain on solid LGI-P medium (I).**
The medium did not contain bromothymol blue but was treated with X-Gluc. Blue colour showing presence of inserted PRGH562 plasmid in the bacteria.

Figure. 7 **Expression of *Gluconacetobacter diazotrophicus* 5541/PRGH562 strain on solid LGI-P medium (II).**
The medium did not contain bromothymol blue but was treated with X-Gluc.

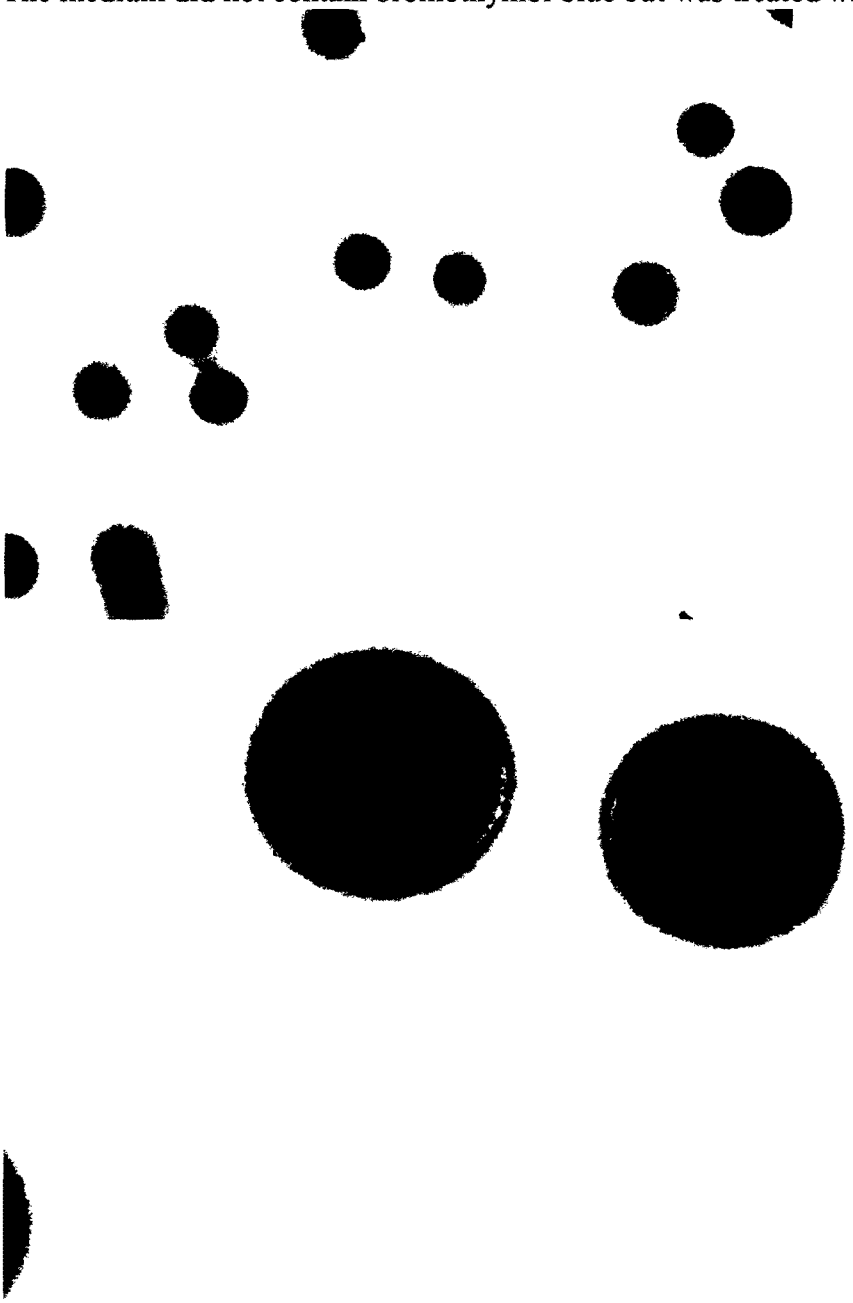


Figure. 8 **Colonies of *Gluconacetobacter diazotrophicus* 5541/PRGH562 strain on solid LGI-P medium (Control I).**
The medium contained bromothymol blue but was not treated with X-Gluc. Yellow is the original colour of bacteria colony on LGI-P medium, not developing any colour changes indicates GUS labelled strain, *Gluconacetobacter diazotrophicus* 5541/PRGH562, only expresses when the medium treated with X-Gluc.

Figure. 9 **Colonies of *Gluconacetobacter diazotrophicus* 5541/PRGH562 strain on solid LGI-P medium (control II).**
The medium did not contain bromothymol blue, nor was treated with X-Gluc.

Figure. 10 **Expression of *Gluconacetobacter diazotrophicus* 5541/PRGS561 strain on solid LGI-P medium (III).** The medium did not contain bromothymol blue but was treated with X-Gluc.

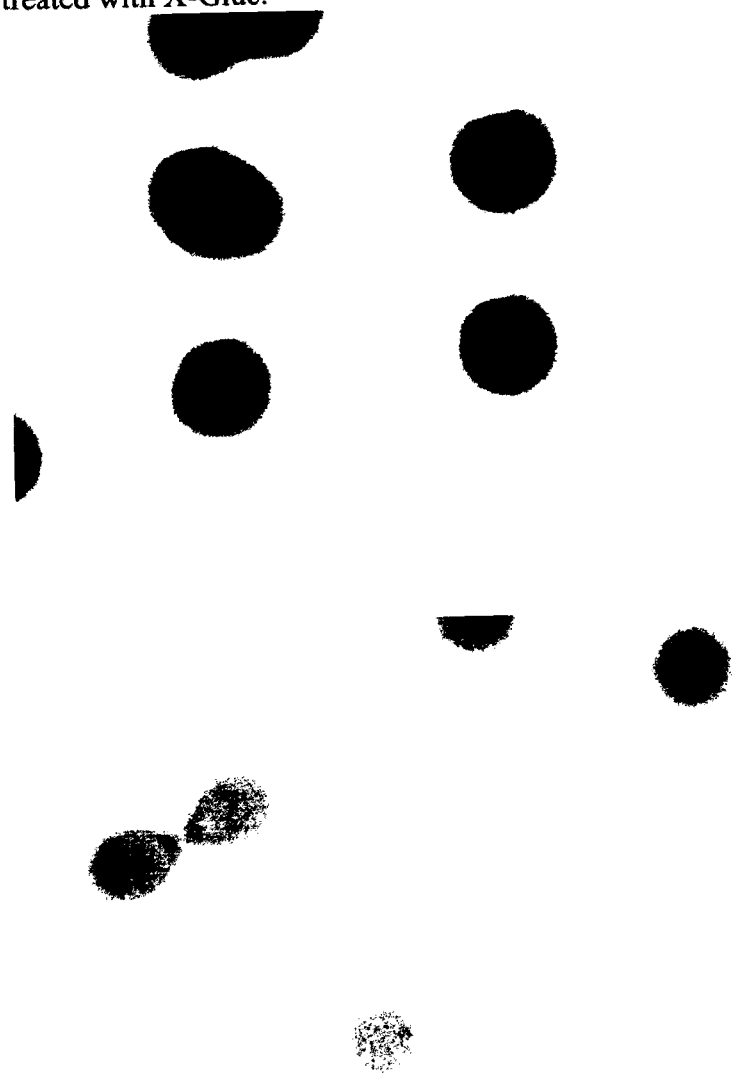


Figure. 11 **Colonies of *Gluconacetobacter diazotrophicus* 5541/PRGS561 strain on solid LGI-P medium (Control I).** The medium contained bromothymol blue but was not treated with X-Gluc.

5.5.2 Assessment of sugar beet infection with GUS-labeled stains of *G. diazotrophicus*

To visualize the infectious sites of sugar beets by *G. diazotrophicus*, beets were inoculated with GUS labeled strains of *G. diazotrophicus*. Visualization was made possible through GUS staining.

Most of seedlings inoculated on MS medium and agar plates looked unhealthy regardless of repetition of the experiment with slight changes of procedures for more than four times. However, alternative seedling rhizosphere inoculation in sand pots generated healthy plants for further staining experiment. Thus, seedling inoculation in sand pots was conclusively carried out in the rest of GUS staining experiments. The GUS-inoculation of sugar beet seedlings was repeated for seven times. More than 80% of plants out of 80 seedlings resulted in positive blue colour. The inoculation with higher OD value of bacteria broth resulted in deeper blue colour. Stained healthy sugar beet seedlings were observed under dissecting microscope using the INFINITY software program.

Based on sites and patterns of blue staining's, both GUS gene labelled strains confirmed that root tips (Figure. 12, Figure. 16 and Figure. 19), the intersection of lateral roots (Figure. 13, Figure. 15 and Figure. 18) and root hairs (Figure. 14, Figure. 17 and Figure. 20) were potential sites for bacteria to enter the plant. Samples stained after seven

days of inoculation showed the same results with stained four days of inoculation, demonstrating that four days was enough for bacteria to infect the host plant.

Interestingly, the leaves of one sugar beet plant that inoculated with GUS strain resulted blue colour (Figure. 21). Considering the fact that those sugar beet seedlings were rhizosphere inoculated, an explanation for GUS activity in sugar beet leaves is that inoculated GUS strains travelled through from roots to all the way up to leaves.

Colonization of leaves by GUS labelled strain has an important biological importance when it comes to nitrogen fixation, however, some studies reported that bacteria number in sugar cane leaves decreases dramatically during vegetative growth stage compared to shoots and roots (Hallman and Berg, 2006). Given the fact that only one out of more than 60 plants had positive GUS activity in leaves, the result is not statistically significant but biologically interesting.

(1) GUS-labeled strain one: *Gluconacetobacter diazotrophicus* UAP-5541/PRGS561

Figure. 12 **Light micrographs of lateral root tips of sugar beet inoculated with GUS-labelled *Gluconacetobacter diazotrophicus* UAP-5541/pRGS561.**
Root tip regions show blue stain from GUS activity associated with colonization by *G. diazotrophicus* UAP-5541/pRGH561. Bars = 1 mm

Figure. 13 **Light micrographs of lateral root of sugar beet inoculated with GUS-labeled *Gluconacetobacter diazotrophicus* UAP-5541/pRGS561.**

Arrows indicating blue stain from GUS activity associated with colonization by *G. diazotrophicus* UAP-5541/pRGH561 at the intersection of lateral roots. Bars = 1 mm.

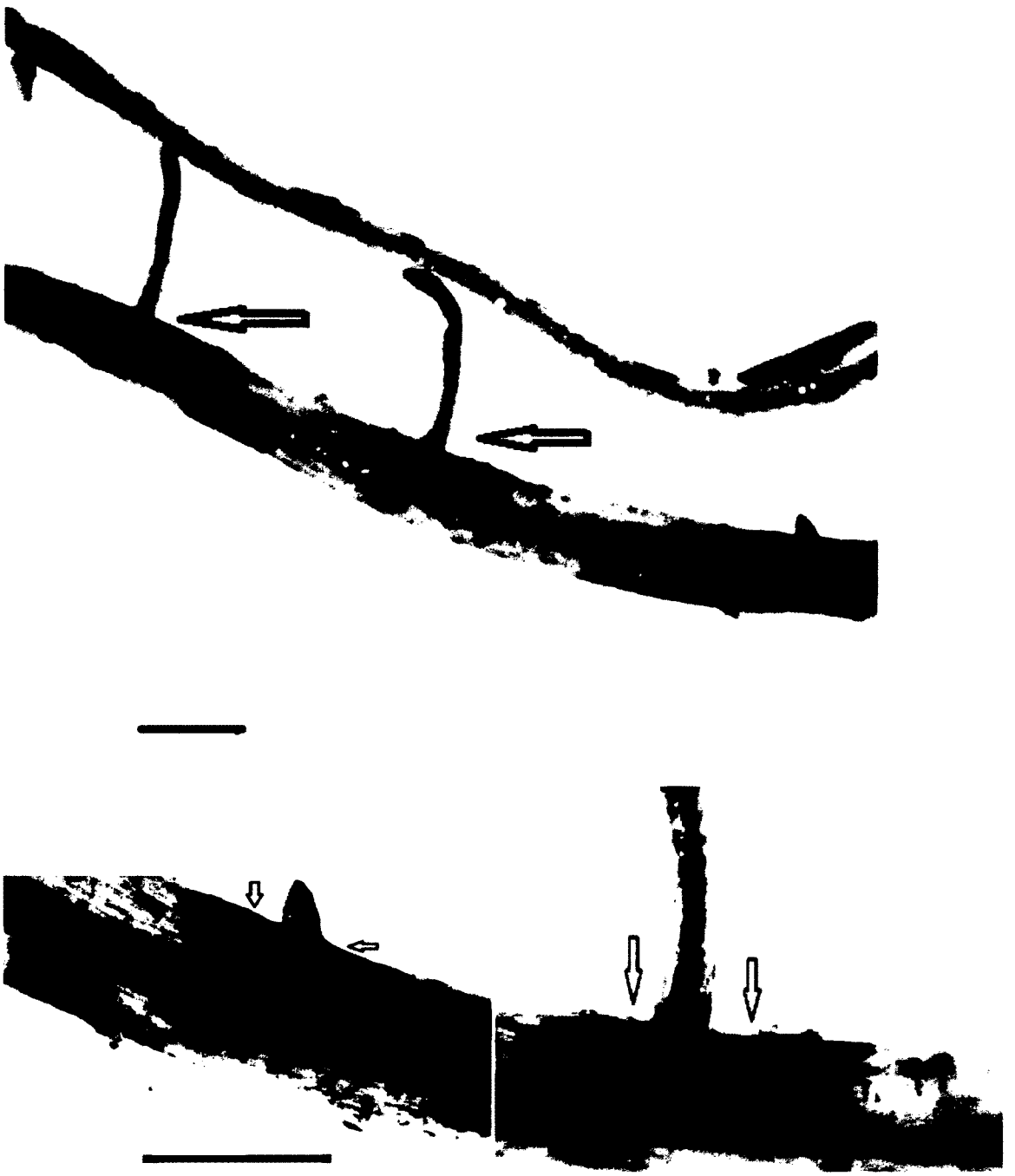
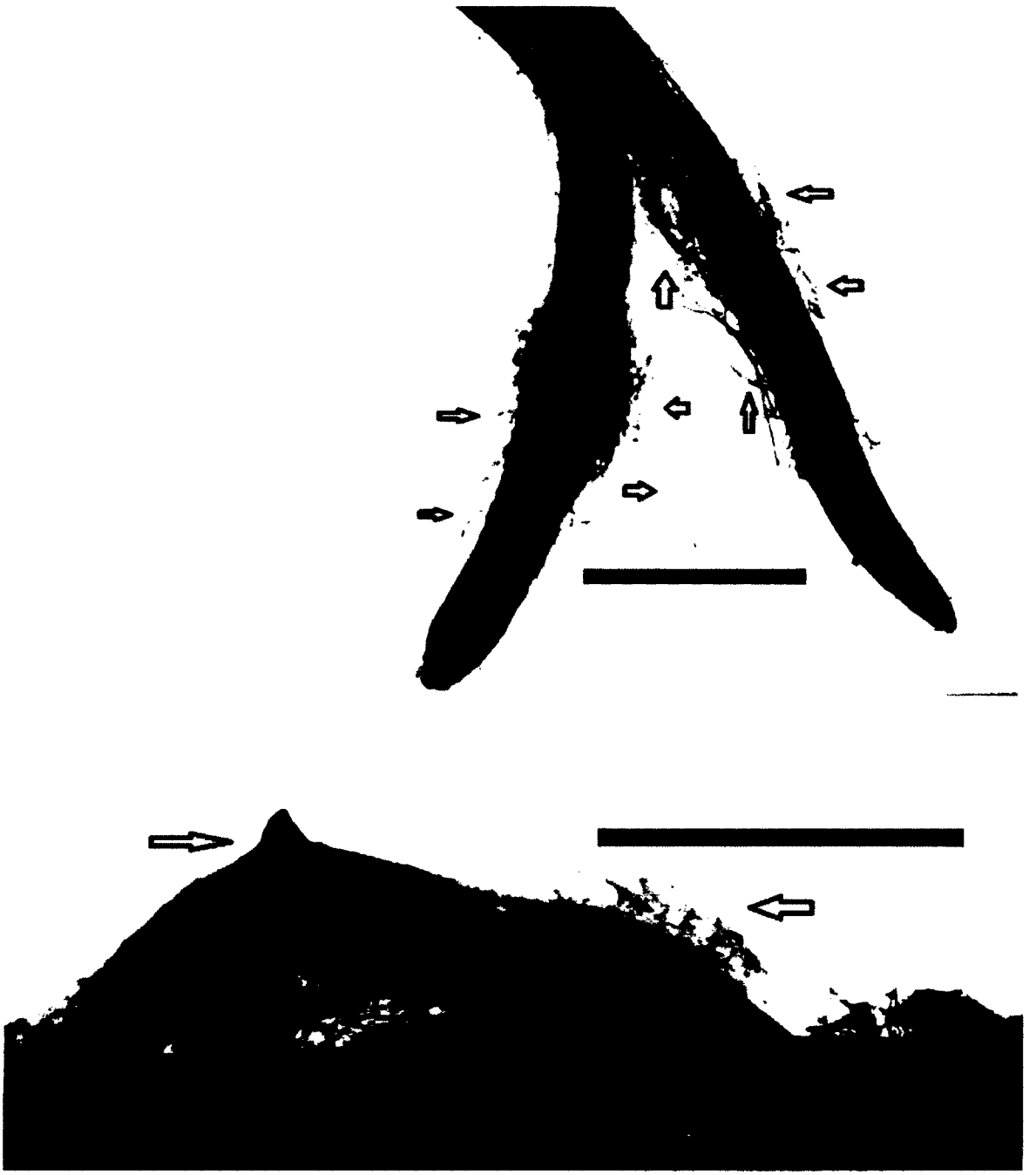


Figure. 14 **Light micrograph of root hairs of sugar beet inoculated with GUS-labelled *Gluconacetobacter diazotrophicus* UAP-5541/pRGS561.**

Arrows indicating mature root hairs showing blue stain associated from GUS activity associated with colonization by *G. diazotrophicus* UAP-5541/pRGH561. Bars = 1 mm.



(2) GUS-labeled strain two: *Gluconacetobacter diazotrophicus* 5541/PRGH562

Figure. 15 **Light micrographs of lateral root of sugar beet inoculated with GUS-labelled *Gluconacetobacter diazotrophicus* UAP-5541/pRGH562**
Arrows indicating blue stain from GUS activity associated with colonization by *G. diazotrophicus* UAP-5541/pRGH561 at the intersection of lateral roots. Bars = 1 mm

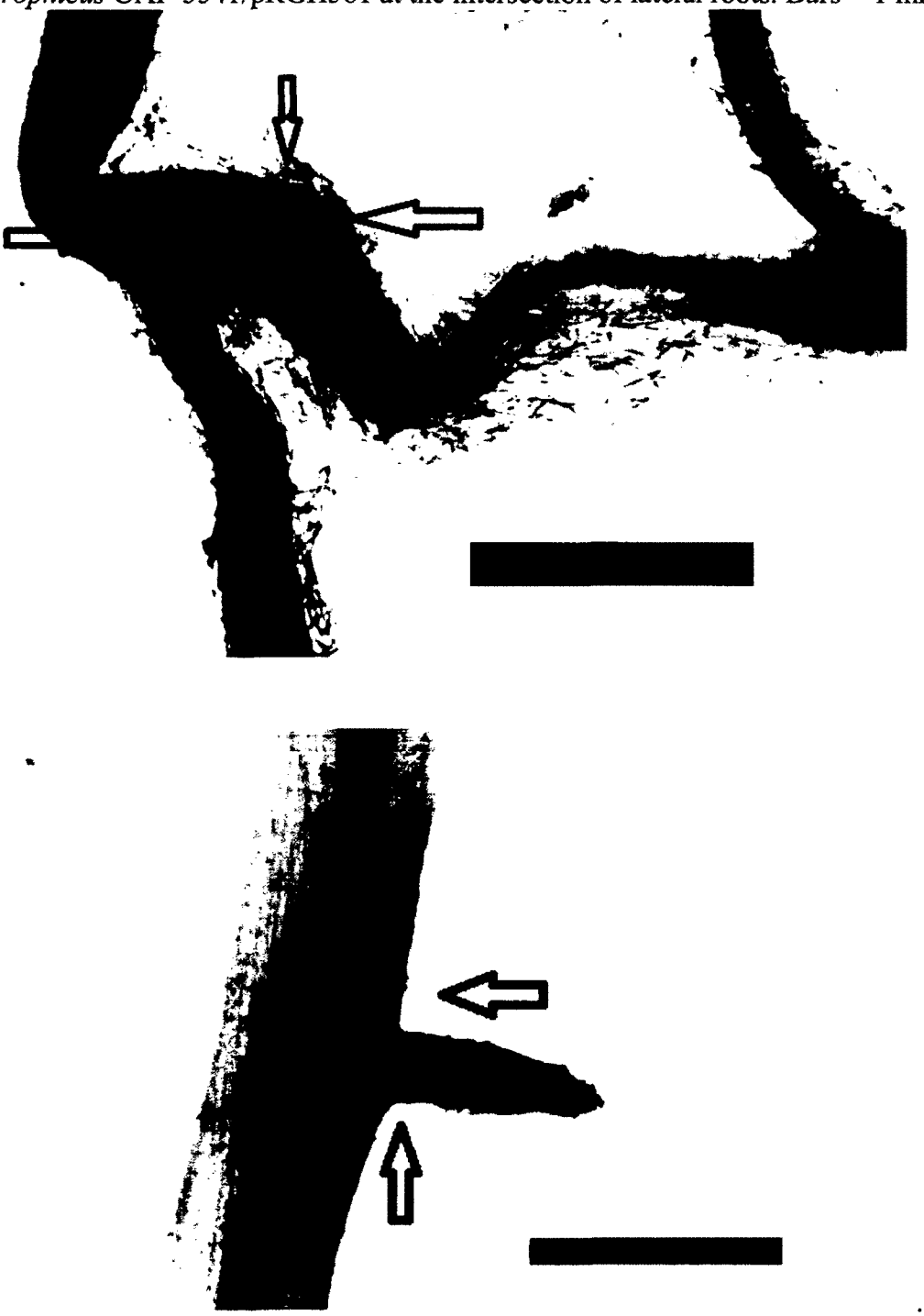


Figure. 16 **Light micrograph of lateral root tips of sugar beet inoculated with GUS-labeled *Gluconacetobacter diazotrophicus* UAP-5541/pRGH562.**

Root tip regions show blue stain from GUS activity associated with colonization by *G. diazotrophicus* UAP-5541/pRGH561. Bars = 1 mm

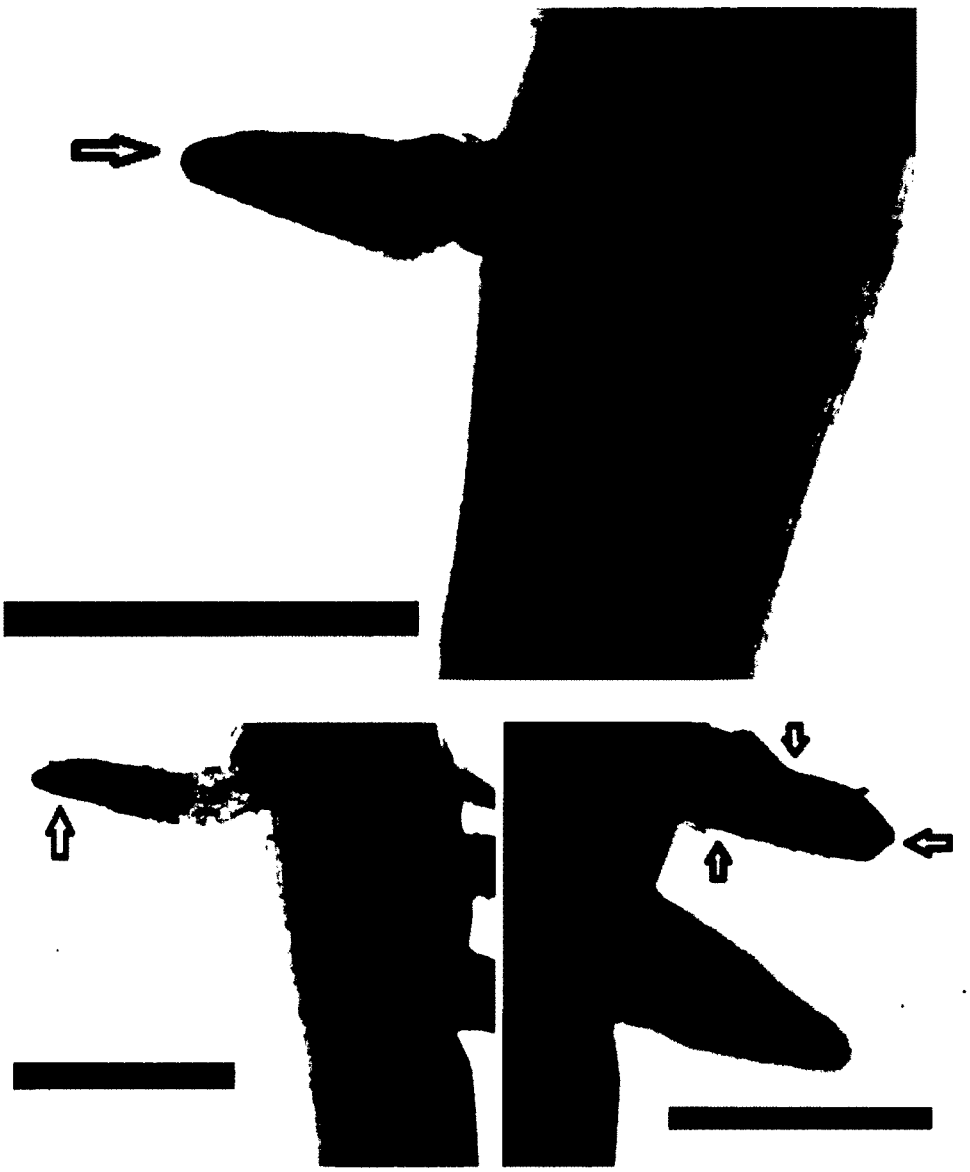
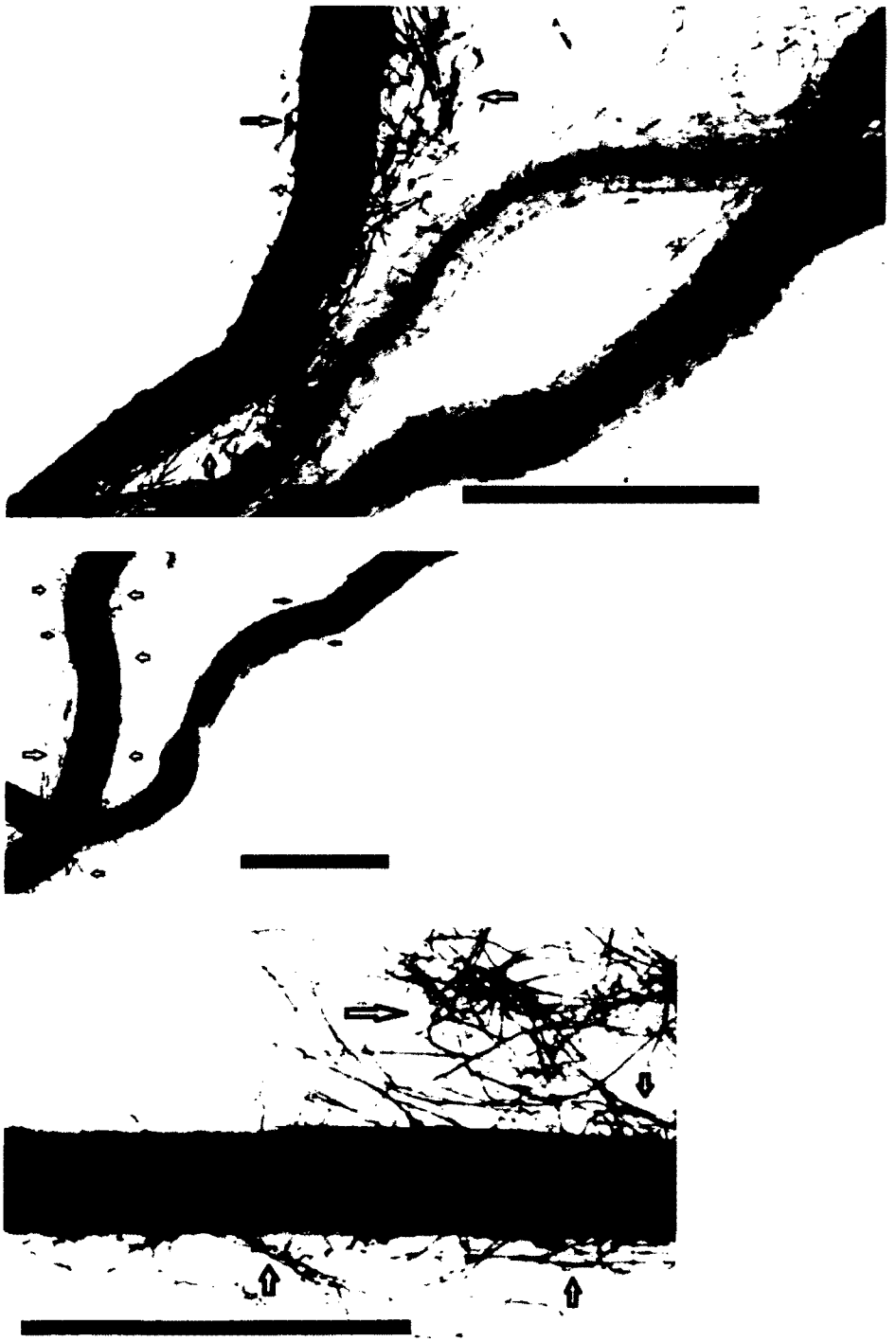


Figure. 17 **Light micrograph of root hairs of sugar beet inoculated with GUS-labelled *Gluconacetobacter diazotrophicus* UAP-5541/pRGH562.**

Arrows indicating mature root hairs showing blue stain associated from GUS activity associated with colonization by *G. diazotrophicus* UAP-5541/pRGH562. Bars = 1 mm



(3) Control strains: GUS parental strain UAP 5541.

Figure. 18 **Light micrographs of lateral root junctions of sugar beet inoculated with parental *Gluconacetobacter diazotrophicus*.**

Inoculation with parental strain gave the same result. Staining did not result in blue colour, indicating lack of B-glucuronidase activity and providing the specificity of the visualization procedures. Bars=1 mm

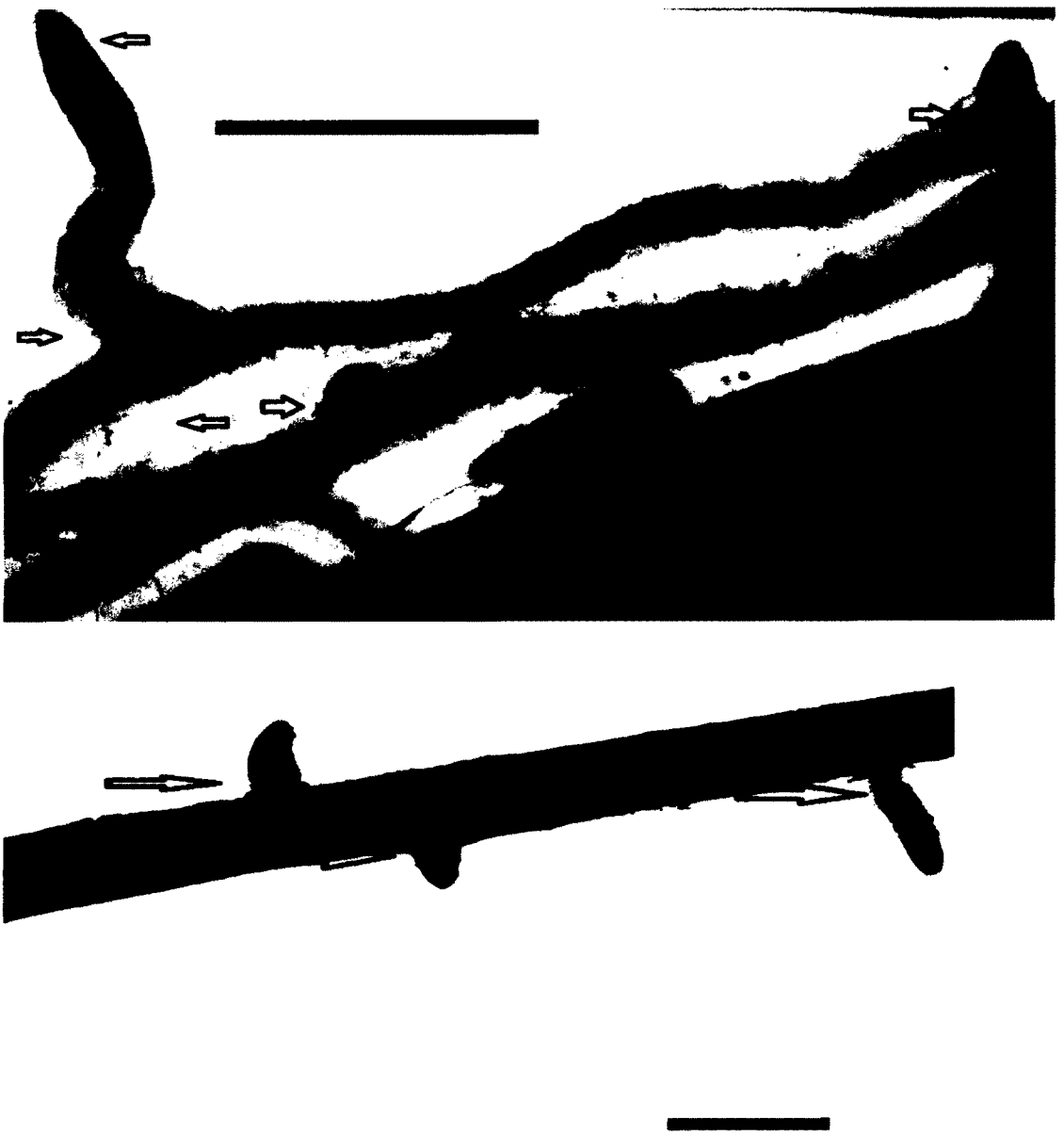


Figure. 19 **Light micrographs of lateral root tips of sugar beet inoculated with parental *Gluconacetobacter diazotrophicus*.**

Inoculation with parental strain resulted in the same colourless staining. Arrows indicating staining did not result in blue colours at root tip zone, proving only GUS lablled strains were capable of giving blue colour from GUS activity. Bars=1 mm

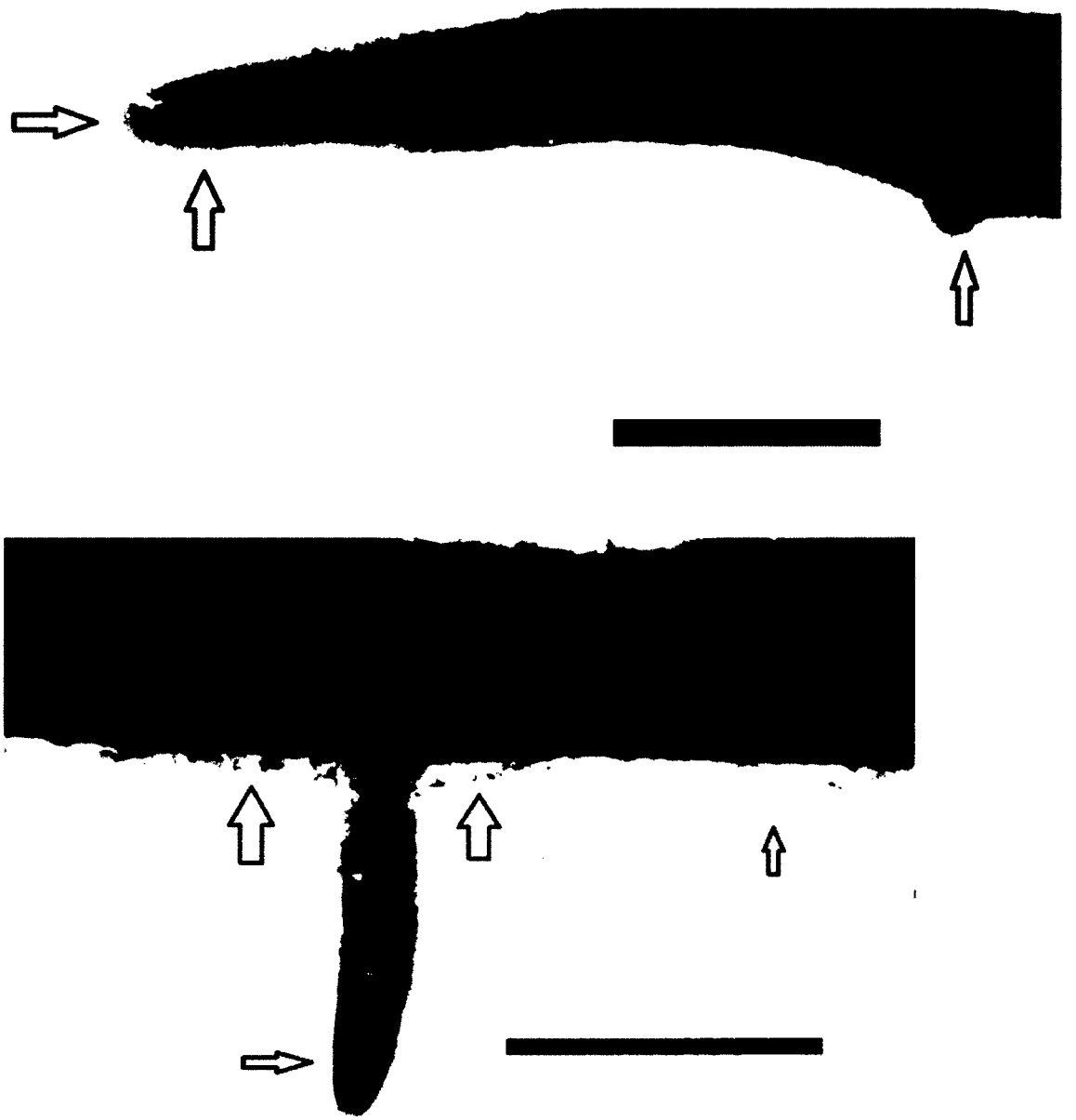
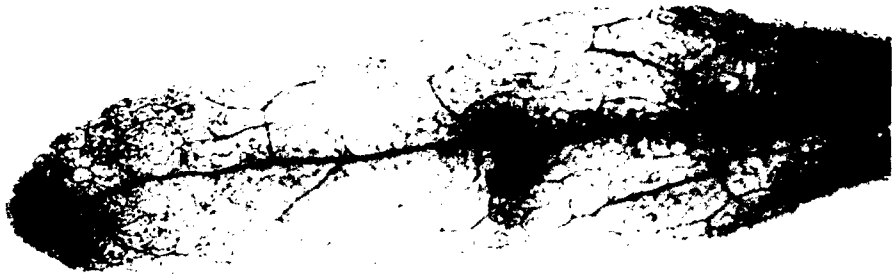


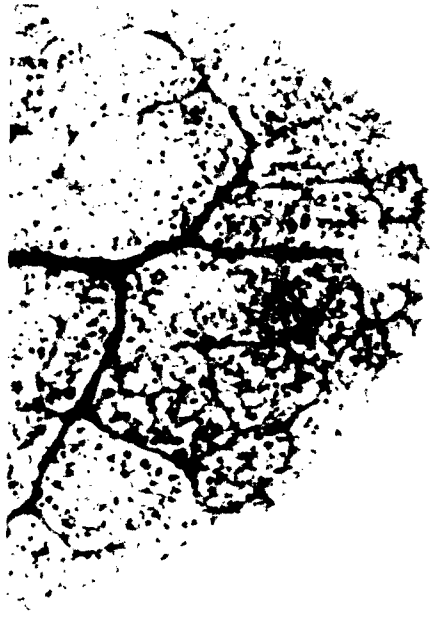
Figure. 20 **Light micrographs of root hairs of sugar beet inoculated with parental *Gluconacetobacter diazotrophicus*.**

Inoculation with parental strain generated the same result. Arrows indicating staining did not result in blue colours on root hairs, proving only GUS lablled strains were capable of giving blue colour from GUS activity. Bars=1 mm

Figure. 21 **Light micrographs of sugar beet leaf inoculated with GUS labeled strain.**

Blue colours indicating GUS activity associated with colonization of GUS labelled *G. diazotrophicus*.





5.6 Isolation of *G. diazotrophicus* (V)

To confirm that bacteria isolated from beet tissues were *G. diazotrophicus*, the polymerase chain reaction (PCR) was applied.

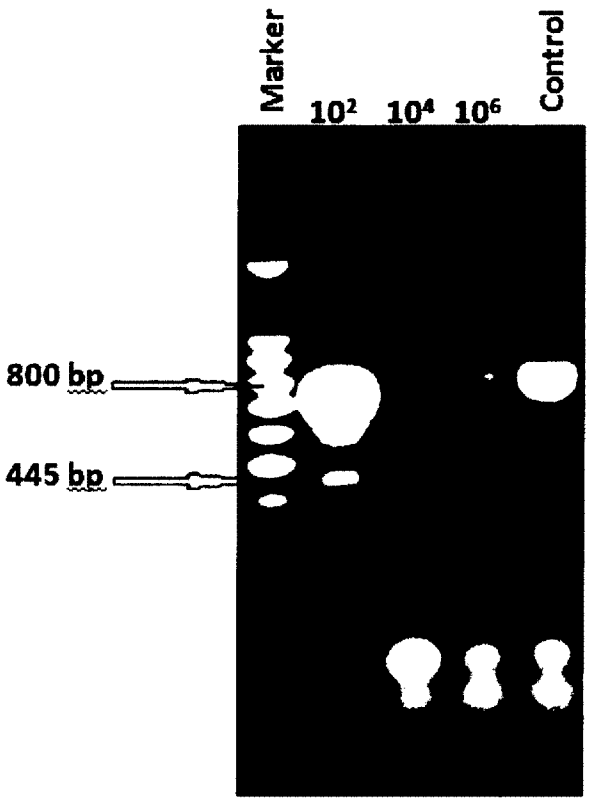
Two weeks after incubation of isolated strains in semi-solid LGI-P medium at 30 ^{0}C, the yellowish colour started to appear. The semi-solid LGI-P medium did not contain ammonium sulphate, nor the yeast extract. Appearance of a yellow colour indicated increasing acidity of the semi-solid medium, confirming the isolated bacteria was acid producing bacteria. Additionally, the medium did not contain much mineral nitrogen except the small amount of biotin (0.1 mg L^{-1}), which further supports that the isolated bacteria were most probably nitrogen fixing bacteria. Then, the bacteria were plated on standard LGI-P solid medium for PCR testing. After a week of incubation at 30 ^{0}C, yellow colonies appeared on the LGI-P solid medium, which were directly used for PCR. It is worth mentioning that the morphology of some of the isolated colonies differed from typical *G. diazotrophicus*, such as colonies in colour and the shape.

5.5 Confirmation of *G. diazotrophicus* by PCR (VI)

PCR products were visualized by electrophoresis in a 1% agrose gel containing ethidium bromide for 30 min and viewed under UV light. The correct PCR products from colonies of isolates show bands at 800 pb region, which is common to all bacteria, and at 445 pb region that is specific to the *G. diazotrophicus*.

In seed inoculation titre experiment (inoculated once), the homogenized sugar beet tissues suspended in 10% sucrose were filtered and directly used for PCR. The PCR generated positive bands at 455 bp and 800 bp regions in 10^2 treatment (Figure. 22), indicating the isolated bacteria was *G. diazotrophicus*. The same bands were not observed in rest of treatments, 10^4 and 10^6. As a band at 800 bp region on gel indicates that control sample was contaminated with unspecific bacteria. A repeatition of the experiments showed similar results. However, purified bacteria DNA generated better results (*pers. comm.* Dr. H. Fei, December, 2011).

Figure. 22 **PCR products from isolates of seed inoculation (inoculated twice) (I).** Left to right (seed inoculation- twice):10^2,10^4,10^6 and control. Each marker is 100 bp. PCR products are showing 445 bp and 800 bp.

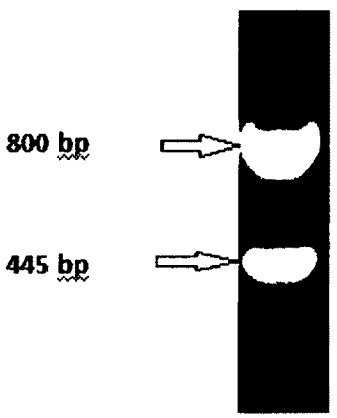


Yellow colonies of isolates from the seed inoculation (inoculated once) treatment were cultured in liquid medium for purification and treated to the PCR analsis. Culturing of the bacteria took 10-15 days, 5-10 days more than the regular time to culture *G. diazotrophicus*. OD value of broth culture did not increase above 0.2, A=600 nm.

However, all 10^2 CFU ml^{-1},10^4 CFU ml^{-1}, 10^6 CFU ml^{-1} samples resulted 800 bp bands but 445 bp bands were completely missing (data not shown).

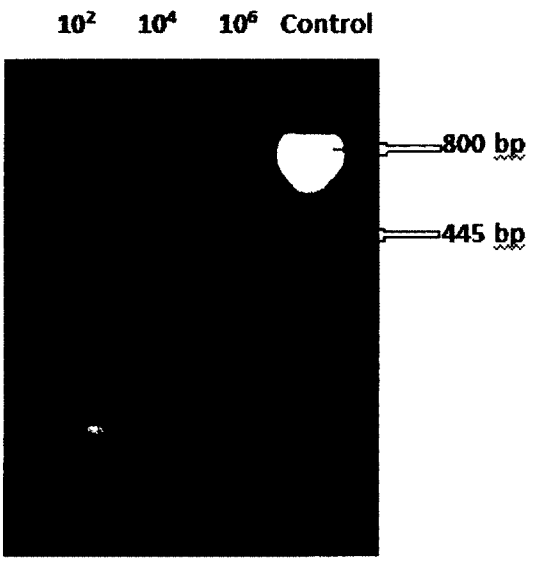
However, re-cultured bacteria broth from stock gave proper PCR results at 445 bp and 800 bp when the same PCR procedures were applied (Figure. 23), indicating a problem might have occurred during isolation or culturing process.

Figure. 23 **PCR products from cultured cells from stock.**



In the seedling inoculation titre experiment (inoculated once), the homogenized sugar beet tissues suspended in 10% sucrose were filtered and directly used for PCR. Results show some controversial data (Figure. 24). As bands on gel indicate that control sample of seedling experiment was contaminated with the same bacteria, PAL5T . It might be possible that contamination might have happened at greenhouse or during PCR process. Since inoculation did not significantly increase the biomass of beets in treatments, further conducting PCR was abandoned.

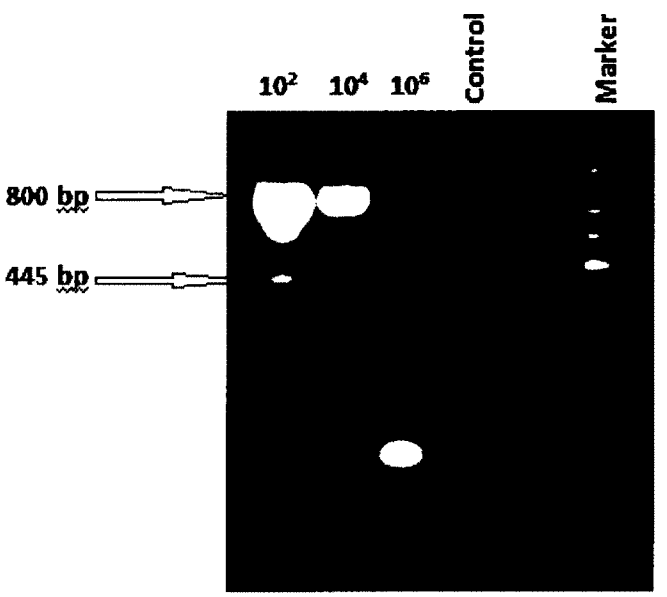
Figure. 24 **PCR products from isolates of seedling inoculation.** Left to right: 10^2,10^4 and10^6. PCR products were showing at 800 bp.



However, in the seed inoculation titre study (inoculated twice), positive bands were observed (Figure. 22). Contamination of control might have caused either from isolation or during conducting PCR considering the seed inoculation treatment resulted positive biomass increased data.

In the second seed- titer study (inoculated once), even though control was not contaminated and had positive band from one of treatment samples (Figure. 25), however, there was no significant biological difference in dry biomass. Thus, importance of PCR result as for the twice-seed inoculation experiment is negligible.

Fig. 25 **PCR products from isolates of seed inoculation (inoculated once) (V).** Left to right (seed inoculation- twice): 10^2,10^4 and10^6. Each marker is 100 bp. PCR products are showing at 800 bp.



6. Discussion

6.1 Screening of 14 strains of *Gluconacetobacter spp.* (I)

The greenhouse screening experiment showed that 12 of 14 strains of *Gluconacetobacter spp.* improved sugar beet growth to different extents. The increase was higher at lower concentrations of applied nitrate. This result was most probably due to the fact that higher concentrations of nitrate limited the infection of bacteria (Fuentes-Ramirez *et al.* 1993). The infection level of sugar cane by *G. diazotrophicus* was diminished by application of high nitrogen fertilization, due to possible change in the plant's physiology, which further negatively affected the association between host and endyphote (Fuentes-Ramirez *et al.* 1999). Further, the limitation of high levels of N-fertilization on population of *G. diazotrophicus* was reported in sugar cane plants (Muthukumarasamy, 1999; Reis Junior *et al.* 2000). Thus, the result that higher growth effect of endophytic nitrogen fixing bacteria at lower nitrogen concentration supports the finding that higher concentration of nitrogen limits the growth of the bacteria (Caballero-Mellado *et al.* 1995). Logically, fewer bacteria at the higher nitrogen concentration may explain the biomass differences between two treatments. Therefore, a moderate level of nitrogen concentration was used for later study, i.e; 5 mM NO_3^-.

Screening of nitrogen fixing bacteria experiment also confirmed that infection of sugar beet by *Gluconacetobacter* spp. can be achieved. As for the strain of *G. diazotrophicus*, one of the main components for establishment of symbiosis with sugar beet might be

attributed to the fact that the optimal growth of bacteria requires higher levels of sugar (10%) in the host plant (Cavalcante and Dobereiner, 1988) and sugar beet is capable of providing the *G. diazotrophicus* with 25% more sugar than sugar cane (Martin *et al.* 2006; World Bank, 1998). Establishment of sugar beet infection by *Gluconacetobacter* spp. indicates that there is a huge potential for developing bio-fertilizers, which will reduce the dependence on costly chemical fertilizers. Thus, the application of bio-fertilizers reduces the costs on chemical fertilizers, making the sugar beet more cost-effective biofuel stock in Canada. Therefore, greenhouse gas emissions can be further reduced.

Comparison of beet biomass increase induced by inoculation of different bacteria strains at different nitrogen treatments showed that the strain *G. d PAL5T LsdB*$^{++}$ performed best among all the strains which were treated with 1 mM NO_3^-, reaching to 110.6 % of beet biomass increase (Table. 2). Even though, the shoot and fibrous biomass increase generated by the strain *G. d PAL5T LsdB*$^{++}$ was not the highest at 1 m M NO_3^- treatment (Table 3 and Table 4), the fixed nitrogen in beets, shoots and fibrous roots were the highest in the same treatment group (Table. 11). Interestingly, the inoculation of this strain negatively influenced the shoot biomass increase at 10 mM NO_3^- treatment (Table. 6). Additionally, nitrogen fixation observed in defferent parts of the host plant was not the highest in the same group (Table. 9).

Analysis of different sugar beet parts for occurrence of ^{15}N demonstrated that the biomass increase is highly associated with biological nitrogen fixation (Table. 8 and Table. 9). As it is shown on the table (Table. 8), the overall biomass increase with 14

strains of *G.diazotrophicus* at 10 mM treatment was significantly higher than overall biomass increase at 1 mM treatment. However, the percentage of nitrogen derived from atmosphere (%Ndfa) was significantly higher at 1 mM NO_3^- treatment than those that at 10 mM NO_3^- treatment (Table. 11). Having significantly higher %Ndfa at the 1 mM NO_3^- treatment indicates that the inoculation is more effective at lower nitrogen concentration. It can also be concluded that the significant biomass increase at 10 mM NO_3^- treamtment may largely induced by the higher concentration of NO_3^-, which likely inhibits the bacteria population. In order to further confirm the conclustion above, it is highly recommended to quantify the bacteria number in different tissues of the sugar beet grown at different levels of NO_3^-.

The similar biomass increase and nitrogen recovery was reported in sugarcane plants inoculated with *G. diazotrophicus* (Archna *et al.* 2005). It was confirmed that inoculation resulted in those differences. Thus, results proving the improvement of biomass accumulation in sugar beet might be resulted from activity of introduced nitrogen fixing endophytes. However, it is also possible that plant hormones produced by *Gluconacetobacter spp.* might attribute to this increase by stimulating root growth in sugar beets (Sevilla *et al.* 1998; Bastian *et al.* 2000; Fuentes- Ramirez *et al.* 1993). Thus, morphological changes of roots, such as elongation and generating more root hairs, might have played a positive role in increasing acquisition of plant nutrition. Further, the capability of solubilising mineral nutrients by bacteria cannot also be neglected in this biomass increase (Saravanan *et al.* 2007a; Mowade and Bhatta-charyya, 2000). Lastly, the defence mechanisms of bacteria species against pathogens and nematodes might also

have played a role during plant growth (Ariel et al. 2006; Blanco et al. 2005; Saravanan *et al.* 2007b)

Most exiting result was that the highest valued for increase in sugar beet biomass induced by *Gluconacetobacter* spp. was occurred in beet tissues (Table. 2 and Table. 5). Considering beet is the main harvesting part of sugar beet, the result consists with expectation of making the sugar beet more viable biofuel feed stock. It is also interesting that the nitrogen derived from atmosphere in beet tissues were increased from shoot, beet to root in both nitrogen concentration treatments (Table. 9 and Table. 10). This result was well correlated with the study of population dynamics of enyphytic bacteria, *G. diazotrophicus*, in sugarcane plant (Archna *et al.* 2005). In which, the bacteria number was higher in roots and shoots. However, in order to make correct assumption, the quantification of bacteria in different tissues of sugar beet is highly recommended.

6.2 Assessment of best inoculation stage (II)

Results from the best inoculation growth stage experiment indicated that there were no significant increases in dry beet biomass (Figure. 2). However, it might be possible that plants might have suffered from heat shock, a serious threat to crop production (Hall, 2001), due to high temperatures in the greenhouse. The greenhouse temperature reached to above 50 °C for couple hours some summer days, which was 10-15 °C higher than ambient temperature and enough to cause heat stress. The heat stress might have caused

irreversible damage to plant physiology and development (Schoffl *et al.* 1999; Howarth, 2005), which further negatively impacted on the plant growth and yields (Wahid *et al.* 2007). Additionally, the similar reduction has been reported in sugar cane production (Ebrahim et al. 1998). Further, the heat stress has been reported as one of most important detrimental factors of reduction in yield in corn (Giaveno and Ferrero, 2003) and that may be the case for the sugar beet in this study. Even though the plant response to high heat stress is varying with plant species and developmental stages, the reproductive phases reported to be the most markedly affected by high temperatures (Foolad, 2005).

In our previous experiment of screening fourteen strains for nitrogen fixation, the growth effect of PAL5T on beet was proportionally higher at lower (1 mM NO_3^-) nitrogen treatment compared to the higher (10 mM NO_3^-) treatment. Nevertheless, under 1 mM nitrogen concentration, the beet increase was not statistically significant compared to control plants. Therefore, these results support the hypothesis that it might be the nitrogen concentration level that caused current result of no significant differences between control and treatment plants were observed under 5 mM NO_3^- concentration. Beet samples were not grinded for further analysis of nitrogen fixation simply because inoculation did not have significant effect on growth of beets.

Both in shoots and roots, significant differences of dry beet biomass were not found among treatments, and no significant biomass differences were observed between treatment and control plants (Figure. 2). Based on positive results both in 1 mM NO_3^- and 10 mM NO_3^- concentrations of our previous screening experiment, theoretically, sugar

beets treated with 5 mM NO_3^- should generate positive shoot biomass results. Interestingly, negative significant difference was found between shoots of fourteen-leave stage and control plants, indicating the inoculation did reduce the shoot biomass at fourteen-leves stages (Figure. 2). Nevertheless, this experiment was conducted at different times of the year compared to previous one and greenhouse temperature was as high as 50 °C. Thus, some different factors, such as temperature and lights, might have played a role in resulting different shoot and root biomass.

It is also possible that the heat stress also had detrimental impacts on plant-microbe interaction. It was noted that plants closer to greenhouse door, where better air circulation occurred, appeared to have detrimental impact on plants. Although, it is reported that microorganisms can play a role in mitigating resistance to abiotic stresses in plant-microbe interactions through variety of mechanisms, such as triggering osmotic response and induction of novel genes in plants (Minakshi *et al.* 2010), we were unable to measure the magnitude of effect of inoculated bacteria on plant heat stress. More importantly, nitrogenase activity can be inhibited by excessive heat (Ortega *et al.* 2001). Further, *G.diazotrophicus* is very sensitive to heat and dryness, decreasing bacteria population more than 60% between 40 ^{0}C -50 ^{0}C (Tejera et al. 2003). Based on these reports, high temperature at greenhouse, coupled with dryness of sandy soil, might have been detrimental to the survivability of *G. diazotrophicus*. Thus, these abiotic factors might have resulted in the lack of positive effects of inoculation. Additionally, since endophytes depend on nutritional supply provided by host plant, any changes in any of those

parameters that affect plant nutritional status could consequently influence the endophyte growth and population (Hallmann *et al.* 1997).

It should also be noted that some of the plants were damaged by grazing by rats at the seedling stage. This may have been another compounding factor that contributed to the lack of a positive effece of inoculation and growth of the beets.

6.3 Titer experiment (III)

In the titre experiment, sugar beets were inoculated at different concentrations (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}) at seed and seedling stages.

6.3.1 Seed inoculation- inoculated twice

The data showed that there were no significant differences in beet dry weight among the treatments (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1}) in seed inoculation (Table. 12). However, significant biomass differences between treatments and the un-inoculated control plants were observed (Figure. 3). This suggests that an inoculant number as low as 10^2 CFU ml^{-1} had the same growth effect as the 10^6 CFU ml^{-1} treatment. It was reported that a rice response to the high levels of rhizosphere bacteria inoculation, 10^8-10^{11} CFU

ml^{-1}, was negative and suggested the optimal inoculant number was 10^5-10^6 CFU/ml (Kapulnik *et al.* 1985; Bashan, 1986). Also studies have shown that increasing the rhizobium population from 10^2/seed to 10^5/seed increased the grain yield of soybean (Kurundkar *et al.* 1991). However, more recently, Luna *et al.* (2010) reported that the inoculum level of *G. diazotrophicus* as low as 10^2 CFU ml^{-1} per seed were enough for root endophytic colonization and for further spreading in aerial tissues in sorghum and rice plants. Based on this report, the finding that 500 CFU was enough to infect a sugar beet seed is logical. However, given the result that different bacteria concentrations did not result in any significant increase in beet dry biomass within the treatment, it can be concluded that it was not increasing bacteria number that induced the positive biomass increase in treatment plants. Further, considering the fact that the seed germination was higher in double inoculated seeds, it might be the seed imbibition played a major role during the seed dipping process, in which water uptake by the dry seed awakened the seed dormancy and activated the metabolic processes (Atia et al., 2011). Therefore, the seed dipping method both created an opportunity for the endophytes to obtain a direct close contact with the host plant seeds and activated the dormant embryo.

Nevertheless, seeds were first dipped into broth for half hour, and then the topsoil of seed planting area was inoculated with additional 5 ml broth. Thus, considering a certain amount of bacteria would definitely attach on the surface of seeds during the dip-inoculation, the actual number of bacteria used for inoculating seeds exceeded 500 CFU per seed. It is impossible to know the number of bacteria that would attached to seed surface during dip inoculation. In some studies, the host was inoculated twice (Albrecht

et al. 1981) or even three times (Millet *et al.* 1984). However, for practical reasons, most inoculation experiments were only inoculated once (Kloepper, 1983; Smith *et al.* 1984). Bashan (1986) found a significant plant growth in response to four successive inoculations. Although some multiple inoculation experiments resulted a marginal effect on plant growth (Bashan, 1986), the current successive seed inoculation experiment promoted the beet growth at significant level (Figure. 3, Figure. 4 and Figure. 5).

The biological nitrogen fixation was unable to be assessed by analyzing the ground samples with mass spectrometry due to missing data. Thus, it was not possible to speculate if the beneficial growth effect of inoculation on plant growth originated from biological nitrogen fixation. Previous screening experiment in sand culture supports this point. The bacteria-induced plant hormone production, such as indole-acetic acid (IAA) and gibberellins (Sevilla *et al.* 1998; Bastian *et al.* 2000; Fuentes- Ramirez *et al.* 1993), might also have contributed to enhanced plant growth. Those plant hormones affected plant morphology and enhanced plant nutrient uptake (Bastian *et al.* 1998; Sevilla *et al.* 1998; Bastian *et al.* 2000). It was also reported that *G. diazotrophicus* contributes to solubilisation of plant macro and micronutrients like P and Zn, which might be another plant growth promoting factor (Saravanan *et al.* 2007).

Interesting fact that germination rate of inoculated seeds was higher than un-inoculated seeds, roughly 30%, (data not shown). Nejad and Johnson (2000) reported isolates of endophytic bacteria that significantly improved seed germination and plant growth of

oilseed rape and tomato. This can be explained that endophytic bacteria positively affect plant growth by pathogen defence mechanism.

The biomass of shoots and roots of treatments were also increased at significant level (Figure. 4 and Figure. 5), indicating inoculation also had positive growth effect both on shoots and roots. The result was consistent with data from previous experiment of screening *Gluconacetobacter* spp. strains, in which both shoots and roots of treatments from 1 mM NO_3^- (Table. 3 and Table. 4) and 10 mM NO_3^- (Table. 6 and Table. 7) concentration experiments resulted the significant increase in dry biomass.

6.3.2 Seedling inoculation

The seedling inoculation study found that no significant affects on beet biomass between treatments and control (Table. 13). It might be because of the fact that the experiment either failed due to some confounding factors or inoculation at the seedling stage did not have any positive growth effects on plant growth. Interestingly, there were also no significant differences in terms of beet biomass among treatments (Table. 13), showing the inoculum number did not have any effect on increasing plant biomass in seedling stage inoculation.

In our previous experiment of screening the fourteen strains of *Gluconacetobacter spp.*, the same strain, PAL5T, resulted in a significant increase in beet biomass in 10 mM

NO_3^- treatment (Table. 5). However, the statistical significant increase in beet biomass was not observed at 1 mM NO_3^- treatment, which was inoculated with the strain PAL5T (Table. 2). Further, the screening of fourteen strains was also inoculated at seedling stage. Considering those results and ruling out other confound factors, it can be extrapolated that it might be the nitrogen level that limited the effect of inoculation on plant growth. Nevertheless, the PCR result shows that control plant was contaminated with the strain of *G. diazotrophicus* PAL5T (Figure. 22) and this fact well explains the unobserved biomass difference between treatment and control plants.

Most previous publications argued that the effect of inoculation is higher at low N level due to the alteration of physiological state of plant by high nitrogen, which further affects the association of the plant with the endophyte (Fuentes-Ramirez *et al.* 1993, 1999; Muthukumarasamy 1999; Reis Junior *et al.* 2000; Archma *et al.* 2005). However, some studies showed that some strains still have higher significant positive effect at higher nitrogen level (Archna *et al.* 2005). The similar results have been reported for *Azospirillum* that some strains of *Azospirillum* are very efficient at high levels of nitrogen concentration (Millet and Fieldman, 1986; Gunarto *et al.* 1999). Additionally, it has been reported that some *G. diazotrophicus* strains are efficient both at high and low nitrogen conditions (Oliveira *et al.* 2002). Thus, the result from current study can be well accepted. To further confirm the nitrogen was the limiting factor for inoculation, the association of isolated bacteria number in the beet tissues with ^{15}N content of should be assessed in future work.

6.3.3 Seed inoculation - inoculated once

The study of a single inoculation of seed generated similar results as the seedling inoculation experiment that had no significant biomass increase in beets found between treatment and control plants (Table. 13 and Table. 14). Nor did the different inoculant numbers resulted in any difference in beet biomass. The results indicate that not only inoculation had no any impact on plant growth, but different levels of inoculants also did not result in any difference in beet biomass. Interestingly, in previous experiment of screening *Gluconacetobacter* spp., the strain PAL5T generated significant increase at beet of 10 mM NO_3^- treatment. However, such significant increase was not obtained in 1 mM NO_3^-treatment.

Compared to the previous double seed inoculation study, sugar beets were only inoculated once using rhizosphere inoculation method. Thus, one might onclude that the factors that caused the different test results may be attributed to a higher number of accumulated bacteria dur to double inoculation. However, int the double inoculation treatments, higher inoculant number (10^6 CFU ml^{-1}) had no significantly increase the beet biomass compared to lower inoculant numbers (10^4 CFU ml^{-1} and 10^2 CFU ml^{-1}). Thus, it can be concluded that it was not the bacteria number from the double inoculation that caused the significant increase of beet biomass.

It might also be possible that the nitrogen level of 5 mM NO_3^-might be the one of the limiting factors for the plant-microbe interactions (Muthukumarasamy, 1999; Reis Junior

et al. 2000). Given the fact that certain strains perform better at higher concentrations of nitrogen (Archna *et al.* 2005; Oliveira *et al.* 2002) and the strain PAL5T resulted significant positive increase in beet biomass at high level of nitrogen (10 mM NO_3^-), the response of strain PAL5T to high nitrogen conditions might be the another factor that influences inoculation effect.

Most inoculations are performed before, during or shortly after sowing (Baldani *et al.* 1983; Millet *et al.* 1984) or a few days after seedling emergence (Okon *et al.* 1983; Thomas-Bauzon *et al.* 1982). However, Kapulnik *et al.* (1985) concluded that the optimal inoculation was the first 24 h after seed imbibition and 20 days after emergence was ineffective. Those studies well explains the significant growth differences between single and successive-double inoculation of beet seeds. In the latter experiment, seeds were washed in sterilized water for five minutes and were dipped into the different concentrations of broth for half hour. Thus, the seed imbibition might have occurred at this stage, creating an opportunity for bacteria to easily colonize the seed. Then the second inoculation might have further enhanced the colonization. The published studies also confirmed that the early inoculation increases the root colonization and the bacteria effect on seed germination, while the affect was less at root colonization stage and much lower in three or four leaf stages (Kapulnik *et al.* 1985; Bashan, 1986). Those results clearly proves that inoculation of seeds twice at seed stage with strain PAL5T was major threshold to have positive biomass increase in experiments treated with 5 mM NO_3^-; most probably because of seed imbibition happened during the dipping period of the seeds into broth.

Lastly, it has been reported that the nitrogen, especially in the form of NH_4^+, inhibited the colonization and acetylene reduction activity while the NO_3^- form did not show any significant suppression (Muthukumarasamy *et al.* 2002). Thus, the nitrogen source, $(NH_4)_2SO_4$, might be another confound factors that influences that plant-microbe interactions. It is also worth mentioning that infection of the host plant by *G. diazotrohicus* is affected by the plant genotype (de Silva *et al.* 1995). Thus, the testing the infection level with different strains of *G. diazotrophicus* and as well as with other cultivars of sugar beet is also recommended.

Our results clearly shown that inoculation of seeds twice at seed stage with strain PAL5T was major factor to have positive increase in experiments treated with 5 mM NO_3^-, possibly because of seed imhibition during the period that the seeds were dipped into broth.

6.4 Assessment of sugar beet infection with GUS labeled stains of *G. diazotrophicus* (IV)

Microscopic observation of GUS stained samples showed that root tips, root hairs and junctions of lateral root emergence were the most possible infectious sites for bacteria. The entry of bacteria via lateral root emergence sites indicating that the colonization pattern of *G. diazotrophicus* in sugar beet is similar to that of sugar cane (James and Olivares, 1997; Reis *et al.* 1995), which also was confirmed in sorghum and rice plantlets (Luna *et al.* 2010; Rouws *et al.* 2010). In sugarcane, the cracks at root branching sites and

wounds on roots were suggested to be the possible entry points for *G.diazotrophicus* (Sevilla *et al.* 1998, 2001; James *et al.* 2001).

Penetration of bacteria through root tips, mainly the cells of root cap and meristem, is a major entry site for endophytes (Hurek *et al.* 1994). James *et al.* (1994) reported that some bacteria entered the root tips through infection thread-like structures, and stated that these bacteria most probably were lysed by the host. But then Bellone *et al.* (1997) reported the similar infection-threads and proposed that the *G. diazotrophicus* cells were not lysed upon entering the host, however, were encapsulated by a host cell membrane. More recently, Rouws *et al.* (2010) confirmed that lateral root emergence and root apices were major entry sites for *G. diazotrophicus* in rice, which were also heavily colonized sites in previous several studies conducted with different microorganisms (Hurek *et al.* 1994; James *et al.* 2002; Govindarajan *et al.* 2008). Colonization of root hairs suggests that *G. diazotrophicus* could also enter the host plant through fibrous root-hairs. James *et al.* (1994) and Reis *et al.* (1995) have found no such infection sites. However, Bellone *et al.* (1997) showed that root-hairs are also one of main infections sites. Further, it has been reported in sorghum and rice plants as well as with other some species of endophytic *Azospirillum* (Hallmann *et al.* 1997; Luna *et al.* 2010).

6.5.1 Isolation of *G. diazotrophocus* (V)

The aseptically isolated bacteria strains from sterilized sugar beet tissues were able to grow in nitrogen free, semi-solid medium, which was first confirmation that the sugar beet isolated endophytic strains were nitrogen fixing bacteria (Vladimir *et al.* 1998). Further, the acidification of the semi-solid LGI-P medium confirmed that the isolated bacterium was an acid producing bacteria (Vladimir *et al.* 1998). Additionally, all the isolated strains in the semi-solid LGI-P medium grew approximately 1 cm from the surface area, indicating it is the mico-aerobic bacteria and also motile (Vladimir *et al.* 1998). Further, the forming of pellicles on the surface of semi-solid LGI-P medium further confirmed that the isolated strains might be *G. diazotrophicus* (Vladimir *et al.* 1998). Also, those characteristics are necessary for the *G. diazotrohpicus* to fix nitrogen and growth in semi-solid medium (Dobereiner, 1992).

The formation of orange bacteria colonies on solid LGI-P medium containing 10% sucrose and round colony shapes were the morphological characteristics of *G. diazotrophicus* (Cavalcante and Dobereiner, 1998). Interestingly, re-culturing of those colonies in liquid LGI-P medium failed to induce the bacteria growth. Muthukumarasamy *et al.* (2002) reported that high levels of nitrogen, especially NH_4^+, induced the morphological changes on cells of *G. diazotrophicus*, resulting in un-culturable state of long pleomorphic cells. Thus, it might be possible that *G. diazotrophicus* cells had already become pleomorphic during treatment of beets with 5 mM NO_3^-.

6.5.2 Confirmation of *G. diazotrophocus* by PCR (VI)

Identification of isolates from inoculated sugar beet plants was confirmed with PCR assays based on species-specific 16 S rRNA fragments. The specific primer AC and DI targeted the 445 amplicon, which is specific to the *G. diazotrophicus* (Sievers *et al.* 1998). The primer pair of RB and RM, however, amplified the 800 bp region that was common to all the bacteria (Madhaiyan *et al.* 2004). In seed-titre experiment, only the lowest concentration (10^2 CFU ml^{-1}) treatment generated PCR products at 800 bp and 445 bp regions. Based on the fact that separation of amplicon at 445 bp is a typical trait of *G. diazotrophicus*, the isolated strain was identified as *G. diazotrophicus,* proving that it was the inoculated bacteria that contributed to plant biomass increase. Although, the PCR products from 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1} treatments of twice seed inoculation experiment failed to give proper bands on gel, the same significant biomass increase of those treatments compared to 10^2 CFU ml^{-1} treatment already confirmed that it was the PCR procedures caused the loss of 445 bands.

In later trials, the 445 pb band was totally lost, generating only 800 bp PCR products. Since mixed-template PCRs and the frequent cycling of template reanealing easily induces the bias in the final products (Ishii and Fukui *et al.* 2001; Suzuki and Giovannoni, 1996), the optimization of PCR protocol is highly recommended, such as lowering the annealing temperature and running a low number of PCR cycles (Ishii and Fukui *et al.* 2001; Suzuki and Giovannoni, 1996). Also, it might be possible that DNA band was

denatured during the PCR process. Further, the purification of bacteria DNA before amplification is suggested in future PCR experiments.

In the latest PCR work, the cultured PAL5T bacterial broth was added to homogenized sugar beet tissues, however, positive bands at 445 bp and 800 bp were not observed after the samples were filtered through syringe-filter (*pers.comm.* Dr. Vessey's lab). On the other hand, the unfiltered cultured bacterial broth generated the positive bands on gel. This result might be attributed to the fact that bacteria were blocked by filter along with homogenized tissues.

Interestingly, the certain morphology traits of some isolates were unlike the characteristics of *G. diazotrophicus*. However, those morphological characteristics, such as the colony coulor and colony shape, fit the previously described recovered strains of nitrogen fixing acetobacters from *Coffea Arabica L* (Jimenez-salgado *et al.* 1997). The strain PAL5T was also isolated from the same coffee plant (Jimenez-salgado *et al.* 1997). It might be possible that 800 bp bands were products of those acetobacters. However, molecular sequencing and biochemical studies are needed for further confirmation of those unknown strains. Nevertheless, those facts indicate that endophytic diazotrophic bacteria may be more prevalent in nature, and possibly there are more potential nitrogen fixing bacteria which can be isolated from sugar beet.

Conclusion

The results of screening 14 strains of *Gluconacetobacter* spp. demonstrated that inoculation with nitrogen fixing endophytes did increase plant dry biomass. Further, the biomass increase in screening experiment was correlated with quantified nitrogen fixation in both 1mM and 10 mM NO_3^- treatments. All of those results indicating that infectious of sugar beet by 14 strains of *Gluconacetobacter* spp. establishes, however, the significant effect on inoculation on biomass is contingent to NO_3^- level and strain specificity. The higher concentration of NO_3^- most likely inhibits the population of *Gluconacetobacter* spp.. Therefore, reduces the biological nitrogen fixation in the plant.

Based on the results of different growth stage inoculation experiment, it was concluded that the level of nitrogen concentration (5 mM NO_3^-) play a determining role in having successful infection as for the certain specific strains of *Gluconacetobacter* spp.. Even though inoculation at different growth stages of sugar beets treated with 5 mM NO_3^- did not result in any difference in dry biomass, the result supports the previous data from screening of fourteen strains of *Gluconacetobacter* spp. experiment that inoculation with PAL5T strain did not significantly contributed to plant biomass increase in 1 mM NO_3^- treatment. Therefore, conducting the experiment in different nitrogen concentrations with a few more strains is highly recommended.

The titre experiments clearly agreed the results of different-stage inoculation experiment that inoculation at seed and seedling stages did not influence the plant growth in terms of biomass increase. Further, different inoculant concentrations (10^2 CFU ml^{-1}, 10^4 CFU ml^{-1}, 10^6 CFU ml^{-1}) did not contribute to different plant biomass increase. In addition, results also support the hypothesis that 5 mM NO_3^- concentration might be the limiting factor to have positive effects on plant growth. However, double inoculation of sugar beets at seed stage clearly showed significant biomass increase in beets, shoots and roots compared to control plants. Considering the fact that the different levels of bacteria inoculum did not result in any differences in beet biomass, it is concluded that seed imbibition induced by seed dipping inoculation method may have been the main factor that contributed to the positive plant growth effects at 5 mM NO_3^- treatment plants. For future studies, it is highly recommended that titre experiment should be conducted under different nitrogen concentrations at different growth stages with different inoculation methods along with different bacteria strains. It is also interesting to look at if double inoculation results better plant growth effects than single inoculation at seedling growth stage.

Also synergic effects of different nitrogen fixing endophytes on sugar beet growth is biologically important, including synergic effects with phosphate and zinc solubilising nitrogen fixing bacteria. Sugar beet being a VAM-negative plant, testing for synergic effects with VAM is not recommended. However, co-inoculation with other nitrogen fixing bacteria strains to test synergic effects of combined nitrogen fixing bacteria strains on plant growth has both biological and economical importance in future research. Given

the fact that the optimum growth condition for *G. diazotrophicus* needs higher percentage of sucrose, 10%, it is promising to introduce this bacterium to other sugar rich crops, such as sweet potato and sweet sorghum. Finally, the soil experiments are necessary to further confirm the bacteria effect on plant growth in the field.

GUS staining experiments with GUS labelled stains clearly proved that root tips, root hairs and the intersection of lateral roots were possible sites of infection of sugar beet by *G. diazotrophicus*. Further microscopic studies are necessary to determine the bacteria localization in the plant to elucidate if *G. diazotrophicus* colonize the sugar beet tissues intracellularly or intercellularly.

Even though PCR results did not show absolute confirmation from each isolate of PAL5T strain, the DNA bands from the isolate of 10^2 CFU ml^{-1} double-seed-inoculation experiment matched with PCR result of stocked PAL5T strain. Given the fact that treatments of 10^4 CFU ml^{-1} and 10^6 CFU ml^{-1} seed inoculation gave the similar biomass increase as with 10^2 CFU ml^{-1} treatment compared to un-inoculated plants, it might be the technical problem in PCR process that caused disappearing of 445 bp band. As filtering the homogenized tissues might be one of the reasons accounting for disappeared the right bands on gel, filtering procedure should be removed or appropriately modified. Also, purification of bacteria DNA before amplification is highly recommended for future molecular confirmation of isolated strains. Quantification of bacteria numbers in different parts of sugar beet using real time PCR has biological importance in future research.

In future work, it is highly suggested not to freeze the plant tissue samples, isolating the bacteria when plant tissue is fresh recommended. Quantifying isolated bacteria numbers from different titre experiment has a significant biological importance in future work. Additionally, quantifying the bacteria in different plant parts at different growth stages is even more interesting, and provides more detailed information regarding dynamics and distributions of bacteria population.

In conclusion, those results further suggested that *G. diazotrophicus* colonizes the sugar beet plant other than its original host of sugar cane. Also, the current studies confirmed that certain strains of *Gluconacetobacter* spp. were quite effective in fixing nitrogen at a lower concentration of NO_3^-. Root tips, root hairs and lateral root junctions were the infection sites. To further confirm its endophytic association with sugar beet, transverse section of roots should be analyzed for its specific localization in the plant.

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