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THESIS

EFFECT OF AZOTOBACTER APPLICATION AS NITROGEN SOURCE ON GROWTH AND YIELD OF BROCKALELI (*Brassica oleracea*)

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A Thesis Submitted in Partial Fulfillment of
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Marisa Phiromtan 2013: Effect of Azotobacter Application as Nitrogen Source on Growth and Yield of Brockaleli (*Brassica oleracea*). Doctor of Philosophy (Agricultural Research and Development), Major Field: Agricultural Research and Development, Faculty of Agriculture at Kamphaeng Saen. Thesis Advisor: Associate Professor Thongchai Mala, Ph.D. 185 pages.

This study was started from 1) studying the abundance of Azotobacter in the rhizosphere from various crops, 2) screening the high efficiency strains of Azotobacter in the laboratory, 3) evaluating the effect of various kinds of carrier and temperature levels on the survival of Azotobacter, and 4) monitoring effects of various *Azotobacter* spp. on growth and yield of brockaleli in the greenhouse and field conditions, respectively. The results revealed that population of *Azotobacter* spp. from various soil habitats were significant as determined by plant crops, moisture content, organic matter and pH. Various isolates of *Azotobacter* spp. showed significant difference on N_2-fixation, IAA production, and phosphate solubilization. Eight isolates were chosen as the high potential strains. Peat + leaf compost was the most suitable carrier and storing inoculums at 5 ^{o}C was the suitable for Azotobacter inoculum after storage for 90 days. Interaction between types of carrier and temperatures was found. N at 20 kg/rai had the highest effect on growth and yield of brockaleli followed by N at 10 kg/rai, *Azotobacter vinelandii* CK-KS-2, *A. vinelandii* CK-KS-3, mixed cultures, *A. vinelandii* PR-NDD-1, *A. vinelandii* CHI-KK-2, and *A. vinelandii* CK-NDD-1 and control, respecttively. Azotobacter promoted plant growth, yield, day to first flowering and total K in brockaleli flower in greenhouse experiment. N at 10 kg/rai had the most effect on growth and yield of plant in field experiment followed by N at 5 kg/rai, *A. vinelandii* CHI-KK-2, *A. chrooccocum* LET-NDD-3, *A. vinelandii* CH-KS-3, *A. vinelandii* CK-KS-2, and *A. vinelandii* PR-NDD-1, and control, respectively. The result indicated that 44.44 % of bacterial species promoted better plant growth than that of N at 5 kg/rai. *A. vinelandii* CHI-KK-2, *A. vinelandii* CK-KS-3, *A. vinelandii* CK-KS-2, and *A. vinelandii* PR-NDD-1 were high effective strains which promoted plant growth both in greenhouse and field experiments and have shown a potential to reduce the use of nitrogenous chemical fertilization in sustainable agriculture in the future.

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Student's signature Thesis Advisor's signature

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Marisa Phiromtan

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LIST OF ABBREVIATIONS

AAS	=	atomic absorption spectrophotometer
ARA	=	acethylene reduction assay
avai. P_2O_5	=	available P_2O_5
BNF	=	biological nitrogen fixation
CC	=	corn stubble compost
CEC	=	cation exchange capacity
CFU	=	colony forming unit
EC	=	electrical conductivity
exch.K_2O	=	exchangeable K_2O
IAA	=	indole -3- acetice acid
LC	=	leaf compost
MC	=	mushroom media compost
N	=	nitrogen
N_2ase	=	nitrogenase enzyme
N-fixation	=	nitrogen fixation
OM	=	organic matter
Pt	=	peat
PtCC	=	peat with corn stubble compost
PtLC	=	peat with golden flamboyant leaf compost
PtMC	=	peat with mushroom media compost
SMC	=	soil moisture content
SOM	=	soil organic matter
Total K	=	total potassium
Total N	=	total nitrogen
Total P	=	total phosphorous
WHC	=	water holding capacity
µg	=	microgram

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EFFECT OF AZOTOBACTER APPLICATION AS NITROGEN SOURCE ON GROWTH AND YIELD OF BROCKALELI (*Brassica oleracea*)

INTRODUCTION

Nitrogen (N) is a key element as a plant nutrient for plant growth and crop production which is frequently deficient from the soil. Generally, crop yields are often limited by N supply, and application of nitrogenous chemical fertilizer is an important source of N for cropping system (Staff of Soil Science Department, 1998). However the over utilization of fertilizer will be a problem and may lead to undesirable change in the physical, chemical and biological soil properties. Moreover, the price of nitrogenous fertilizer is very expensive (nearly doubled during the last 3-4 years) which increases the cost of plant production. In 2012, Thailand imported 2.436 million tons of nitrogenous fertilizer with a market value of 32.506 billion baht (Office of Agriculture Economics, 2013).

Fortunately, there are many N_2–fixing microorganisms that exist in the rhizospheric soil which can fix atmospheric nitrogen in terms of biological nitrogen fixation (BNF) provide N inputs into agricultural soils and helps replenish the soil N pool. Input of biologically fixed N into agriculture system is derived from symbiotic and free living diazotrophs and plant root (Bohlool *et al*., 1992). Azotobacter is the one of nitrogen fixing bacteria which has many beneficial effects on plant. For example, Azotobacter produces phytohormones (such as auxin, cytokinin and gibberellin which enhance root and plant growth), antifungal substance and more. This bacterium can fix dinitrogen gas (N_2) and convert it into ammonium within microbial cell through BNF process with approximately 10 mg N/mg of carbon assumption (Holt *et al*., 2000).

There are many researchers who study the effect of Azotobacter and its application in many countries like Russia, USA, and India etc. Those studies were conducted with various crops such as rice, wheat, maize, potato, sugar beet,

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sugarcane, and cabbage (Narula, 2000). They attributed the significant influence of Azotobacter on the growth and yield of these crops. However, there are only few reports of Azotobacter utilization in Thailand with rice, maize and sugarcane and no study with vegetable crops. Therefore, Azotobacter is a potential microorganism which can be used as biofertilizer for N source to increase the nitrogen levels in soil, instead of nitrogenous chemical fertilizer, in the organic farming system (Mala, 2003).

Organic farming system is an agriculture method which is characterized by soil-building, exclusion of synthetic pesticide, utilization of organic fertilizer and bio-control or integrated pest management. Moreover, the organic produce market is rapidly growing in many countries such as Germany, United States of America, Argentina, the United Kingdom and Denmark with market value US$ 40 billion in 2005 (Food and Agriculture Organization [FAO], 2005). The major export markets of Thai organic farm produce are the European countries, particularly Germany, the United Kingdom and France. Export markets in the United States of America, Japan and high-income countries in Asia such as Singapore, Hong Kong and Malaysia are also expanding significantly (FAO, 2013). This cropping system is emphasized in the 11th National Economic and Social Development Plan of Thailand (2012-2016) which promotes application of organic fertilizer such as manure, green manure and biofertilizers such as rhizobium, mycorrhiza, cyanobacteria, Azotobacter in the organic farming system and sustainable agriculture.

To conclude, Azotobacter is the one of the biofertilizer that can be used in sustainable and organic agriculture. Particularly the production of high effective Azotobacter inoculum is necessary (Bashan, 1998; Mala, 2003). Therefore this bacteria can be used as N source to enhance the growth and yield of brockaleli particularly in an organic farming system in not far future.

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OBJECTIVES

1. To study the abundance and characteristics of Azotobacter grown in different habitats.

2. To collect, isolate and classify the Azotobacter from rhizospheric soil of different crops and screen the high effective strains in the laboratory.

3. To investigate the efficiency of various inoculum carriers on growth and survival of this bacteria during storage in various temperature levels.

4. To study the effects of various Azotobacter inoculum on growth of brockaleli in the greenhouse and field experiment.

BENEFITS

The expected benefits of this research are as follows:

1. To collect high effective strain of Azotobacter and apply their diversity on inoculum production for agricultural proposes.

2. Agricultural waste can be used as inoculum carriers of Azotobacter with an advantage of reducing the pollutant, and utilizing natural resources. This method can maximise profits and is eco-friendly.

3. The cost and the use of nitrogen based fertilizer in the soil can be reduced by applying efficient Azotobacter strains on vegetable production.

4. To apply Azotobacter inoculum as biofertilizer in a large scale and use it as an important tool for vegetable production.

5. To study the efficiency of Azotobacter for soil nitrogen fixation for a sustainable agriculture.

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LITERATURE REVIEWS

Azotobacter spp.

There are free N_2-fixing microorganisms that exist in the rhizospheric soil which can fix atmospheric nitrogen gas, in term of biological nitrogen fixation, provide N inputs to agricultural soils and helps replenish the soil N pool. Azotobacter is the one of these microorganisms that has many beneficial effects on plant (Mala, 2003). This bacterium belongs to the family of Azotobacteraceae (Breed *et al*., 1948; Thomson and Skerman, 1979; Holt *et al*., 2000). The bacteria can grow well on nitrogen free medium and utilizes atmospheric nitrogen gas for their cell protein synthesis. This cell protein then mineralizes in the soil after the death of Azotobacter cells thereby contributing towards the nitrogen availability for the crop plants. These bacteria also produce growth-promoting substances, thereby enhancing the plant growth and finally the yield (Kamlesh *et al*., 2004).

1. The habitats of *Azotobacter* spp.

Azotobacter chroococcum is reported to occur in parenchymatous cells of root cortex and leaf sheath (Tippannawar and Ramachandra, 1989) and possess several unique features. *Azotobacter* spp. form cysts containing normal lipids. They have more than one type of nitrogenase which is extremely tolerant to oxygen while fixing nitrogen (Narula, 2000) due to respiratory protection of nitrogenase (Robson and Postgate, 1980). Azotobacter can survive in the wide range of conditions from polar region, temperate, warm region, arid, arable areas, forest through to rivers and ocean (Ravikumar *et al*., 2004) This bacteria had been reported in various places such as India (Channal *et al.,* 1989), Canada (Kole *et al.,* 1988), and Poland (Martyniuk and Martyniuk, 2003). Generally, the number of Azotobacter was found of 10^4 cells per dry soil (Narula, 2000). In Thailand, there were few reports which studied the population of Azotobacter. In 1979, report of Satrulee showed that the population of Azotobacter in Sakaerat forest soils ranged from 10^4-10^6 cells per dry soil. Phiromtan *et al.* (2009) showed that population of Azotobacter of various locations in Thailand

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ranged from 2.58 to 4.64 x 10^6 cfu/g. However, population of Azotobacter occurs 10^7 cells in Nile valley and other warm regions of the Middle East in India (Narula, 2000).

2. Morphological and the characteristics of *Azotobacter* spp.

Azotobacter is a gram-negative bacteria (Holt *et al*., 2000; Tchan, 1984). They exist in different sizes and shapes depending on the age in cultural media and gram viable in the old culture. The cell shape is oval, roughly 2x4 μm. Azotobacter are motile and they use their peritrichious flagella as movable organs. The bacterium is encapsulated and this increases its resistance to heat, desiccation and harmful conditions. Under stress conditions, Azotobacter forms cyst (resting cells) which germinates under favorable conditions to give vegetative cells, which are rounded and metabolically dormant (Holt *et al*., 2000; Narula, 2000). These are also larger and often irregularly swollen or filamentous and these cells have played an important role on pleomorphism and are described as ginidia. These arise under unfavorable environmental conditions. Azotobacters produce polysaccharides which are sensitive to acidic pH, high salts, and temperature above 30 ^oC. Appearance of morphologically different cells in culture of Azotobacter depends upon the composition of medium and conditions of cultivation. It established that under these conditions, morphology of Azotobacter could be actively changed. The important characteristic of several Azotobacter strains is the production of extracellular slime or capsular material (Holt *et al*., 2000). Although, it is related to nutritional conditions such as certain type of carbon and nitrogen source, its characteristics are also genetically controlled and are specific for different species. Presence of sugars (glucose, galactose, mannose and rhamnose and sugar acids such as uronic acid, galatouronic acid, mannuronic or gluconic acid) induce production of polysaccharide (Mala, 2003). The slime supplied as a waste product can be used as a storage material for carbon and energy or as protective covering. Slime either protects bacterial cell in desiccation or excludes oxygen for nitrogenase enzyme. The morphological characteristics are the criteria for identification and classification. *Azotobacter chroococcum* is characterized by the formation of an insoluble black pigment (melanin) which arises in aging culture due to the oxidation of tyrosine, a copper containing enzyme. In the case of *A. beijerinckii*

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it forms a yellow pigment, whereas *A. paspali* forms water soluble greenish yellow fluorescent pigment (Mulder and Brotonegoro, 1974).

3. Species of Azotobacter

At present, there are six important species of Azotobacter viz. *Azotobacter chroococcum, A. vinelandii, A. beijerinckii, A. nigricans, A. armeniacus* and *A. paspali,* which *A. chroococcum* is most commonly found in the soils (Mulder and Brotonegoro, 1974; Thompson and Skerman, 1979; Tchan, 1984; Holt *et al*., 2000; Mala, 2003). The specific details of each species describe as follow (Table 1).

3.1 *Azotobacter chroococcum*

Azotobacter chroococcum is the first species of the genus Azotobacter. *A. chroococcum* was first isolated in 1901 from the soil in Holland. It is the most widely distributed species that mainly occurred in neutral and alkaline soil. It is reported to occur also in parenchyma cells of root cortex and leaf sheath (possess several unique features). Since then, many researchers have made a number of significant studies about this genus. For the morphological characteristics, cell size is large ovoid rod approximately 2-5 µm and are found frequently in pairs. It is motile and uses its peritrichious flagella around the cell. Cysts and capsular slime are formed in this strain. These bacteria form a non-water soluble pigment, but the growth on agar media is characterized by a water-insoluble brown pigment which later in some strains become black. The utilization of starch varies, depending on the species. *A. chroococcum* can utilized mannitol, but cannot use rhamnose. This strain usually occurs in soil and water. The G+C content of DNA ranges from 65-66 moles % (Holt *et al*., 2000; Mala, 2003).

3.2 *Azotobacter vinelandii*

Azotobacter vinelandii was first isolated from the soil in North America (Lipman, 1904). *A. vinelandii* was named after the Vineland in New Jersey State,

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United State, where this strain was first obtained. Morphological characteristics is described as below. Cells are large ovoid rods approximately 2-5 µm, frequently in pairs and are motile with peritrichous flagella. It forms cyst as well as a copius capsular slime. A water-soluble fluorescent pigment is produced which appears green under ultraviolet light. It cannot utilize starch, but can utilize both mannitol and rhamnose. This is the only species of this genus that can use rhamnose. The habitat is soil and water. The G+C content of DNA is 66 moles % (Holt *et al*., 2000; Mala, 2003).

3.3 *Azotobacter beijerinckii*

Azotobacter beijerinckii was first isolated by M. W. Beijerinckii of Delft, Holland (Lipman, 1904). Cells of these strains are ovoid rods shape similar to *A. chroococcum*, but they are not motile. Cells occur singly, in pairs and sometimes in chains of several cells that become coccoid. It forms water-insoluble pigment, which turns to yellow or cinnamon. This bacteria forms cysts and are more tolerate under acidic condition than *A. chroococcum.* They are incapable of utilizing starch, mannitol or rhamnose. It can be found in soil and water. The G+C content of DNA is 66 moles % (Holt *et al*., 2000; Mala, 2003).

3.4 *Azotobacter nigricans* (L; nigricans = black)

Azotobacter nigricans is a non-motile bacteria due to they do not have a flagella. These cells produce homopolysaccharide from using sucrose or raffinose depending on the species. It is able to utilize mannitol and malonate as a carbon source and ammonium or nitrate as a nitrogen source for the growing. The G+C content of DNA is 64.5 moles %. This species was found in soil and water (Holt *et al*., 2000; Mala, 2003).

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3.5 *Azotobacter armeniacus*

This strain was isolated by Thompson and Skerman in 1981. *A. armeniacus* was named after Armenian state in the Union of Soviet Socialist Republics (USSR) (Thompson and Skerman, 1981). They have peritricous flagella around the cell and are motile. Colony is shaped round, glittering convex and they adhere to the medium. Some strains can produce homopolysaccharide by using sucrose but it cannot use raffinose. It can also utilize starch by alpha or beta amylase enzyme depending on the species. This bacteria cannot utilize ammonium, nitrate and glutamate as nitrogen source. The G+C content of DNA ranges from 63.5-65.3 moles % (Holt *et al*., 2000; Mala, 2003).

3.6 *Azotobacter paspali*

Azotobacter paspali was first described by Döbereiner in 1966 who isolated it from the rhizospheric soil of *Paspalum notatum,* a tetraploid subtropical grass. The cells are large ovoid rods shaped, and hence it becomes pleomorphic even in young cultures and the cells become coccoid of 2 µm to elongated filaments of 30 µm. Both coccoid and rod shaped cells are motile with peritrichous flagella. They form cysts and capsular slime. They also produce a water soluble fluorescent pigment, which appears green under ultraviolet light and resembles that of *A. vinelandii.* They do not utilize starch, mannitol and rhamnose. The habitat is soil, in particular, the root surface of *Paspalum notatum*. The G+C content of DNA ranges from 63-65 moles % (Holt *et al*., 2000; Mala, 2003).

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Table 1 Differential characteristics of the species of the genus *Azotobacter* spp.

Characteristics	*A. armeniacus*	*A. beijerinckii*	*A. chroococcum*	*A. nigricans*	*A. paspali*	*A. vinelandii*
Motility	+	-	+	-	+	+
Long filament in young culture	-b	-b	-b	-b	+	-b
Water soluble pigments:						
-Yellow-green fluorescent[c]	-	-	-	-	+	+
- Green	-	-	-	-	-	+
- Brown-black	-	-	-	d	-	-
- Brown-black to red violet	+	-	-	+	-	-
- Red-violet	+	-	-	d	+	d
Utilization as carbon source						
- Rhamnose	-	-	-	-	-	+
- Caproate	-	-	+	-	-	+
- Caprylate	+	-	-	-	-	+
- Meso-inositol	d	d	-	-	-	+
- Mannitol	+	d	+	d	-	+
- Malonate	-	+	d	d	-	+

Source: Holt *et al*. (2000)

Remark + = 90% or more of strains are positive.

\- = 90% or more of strains are negative.

b = these species may sporadically produce filamentous forms of different lengths.

c = on iron-deficient medium

d = 11-89% of strains are positive

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4. Factor affecting the growth and activities of *Azotobacter* spp. in rhizosphere

The growth of Azotobacter depends on several factors, such as physical, chemical, (Channal *et al.,* 1989; Mala, 2003) and microbiological properties (Kizilkaya, 2009) – namely, soil type, soil organic matters, soil pH, moisture content, mineral (Ca, P, Mo, Co and Fe) and indigenous microorganisms. These parameters greatly control the population of *Azotobacter* spp. in rhizosphere described as follows. The report of Satrulee (1979) showed that pH, nitrogen and moisture content in the soil were factors controlling the population. In addition, Phiromtan *et al.* (2009) reported that the populations of Azotobacter varied depending on pH, soil organic matters, moisture content, and plant group. However, the locations and types of agricultural practices had no significant effect on the abundance and density of Azotobacter in that study. Azotobacter can also survive in various pH ranges depending on the strains (Mendes *et al.,* 1999). Yasmin *et al.* (2004) reported that Azotobacter survived between pH 4.0 and 8.5 which was similar to the range reported by Channal *et al.* (1989). Islam *et al.* (2008) found that Azotobacter was able to grow at pH 4.2 - 4.7. On the other hand, Agrawal and Singh (2002) reported that Azotobacter could grow in a pH range of 6.0-8.0 but it was unable to grow at pH 3.5 (strongly acidic soil) and only four strains survived at pH 10.

Azotobacter develops more intensively in root zone of plants than in the soil. This finding suggested that the root secretion and root litter of the plants may provide the means of existence of varied microflora including Azotobacter. According to Rovira (1959), the relative populations of Azotobacter depended on plant species. Active nitrogen fixation of *Ammophila arevariae* in rhizosphere which is attributed to Azotobacter associated with the roots, has been observed in South Africa, Middle East and Western Australia. Azotobacter was found in tropical grasses and other crops and contributes largely to the nitrogen economy (Mala, 2003).

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Beneficial effects of *Azotobacter* spp.

There are many beneficial effects of Azotobacter inoculation in agriculture. Mainly, Azotobacter can fix nitrogen from atmosphere which increases availability of nitrogen for soil and plant. This microorganism can also produce growth regulating substance such as phytohormones (auxins, cytokinins, gibberellins) and vitamins which can enhance plant growth. This bacterium enhances availability of phosphate by phosphate solubilization activity. Moreover, it also produces antifungal substances to prevent the pathogenic fungi, and siderophores which can act as chelating agent for increasing the availability of Fe^{+3} and suppress plant pathogens through iron deprivation (Quispel, 1974; Holt *et al.,* 2000; Narula, 2000).

1. Nitrogen fixation

Major beneficial effect of Azotobacter is the biological nitrogen fixation. This bacteria is able to convert N_2 to ammonia which transformed into proteins of microbial cells. It is useful and taken up by plant root. It is available for soil by mineralization process after the death of bacterial cells thereby contributing towards the nitrogen availability of crop plants. Nitrogen fixation of Azotobacter can vary depending on species. They are able to fix at least 10 mg of molecular nitrogen per gram of carbohydrate consumed under aerobic conditions (Holt *et al*., 2000). Rao (1975) reported that all seven strains of *Azotobacter chroococcum* isolated from the rhizosphere of sugarcane can fix nitrogen in pure culture between 6.16 - 10.64 N_2 fixed mg/g sucrose utilized. Some strains of Azotobacter have nitrogen fixing ability as high as 46.5 mg/g glucose utilized (Mulder and Brontonegoro,1974). It has been reported that various strains of Azotobacter that were isolated in Thailand had the nitrogen-fixing rate ranging from 4-421.84 µgN per 10^9 cells per day. Isolated strains from maize, millet and sugarcane gave similar results with the nitrogen fixing rate around 16-24 µgN per 10^9 cells per day (Mala, 2003).

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1.1 Mechanism of biological nitrogen fixation

Biological nitrogen fixation can be represented by the following equation (Mulder and Brotonegoro, 1974; Rudnick and Kennedy, 2002; Mala, 2003), in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions):

$$N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + 16ADP + 16Pi + H_2$$

This reaction is performed exclusively by prokaryotes (bacteria and related organisms), using an enzyme complex termed nitrogenase. This enzyme consists of two proteins - an iron protein and a molybdenum - iron protein (Mulder and Brotonegoro, 1974; Fisher and Newton, 2002; Mala, 2003).

The reactions occur while N_2 is bound to the nitrogenase enzyme complex. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds to ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to $H_2N\text{-}NH_2$, and this in turn is reduced to $2NH_3$. Depending on the type of microorganism, the reduced ferredoxin, which supplies electrons for this process, is generated by photosynthesis, respiration or fermentation (Mulder and Brotonegoro, 1974; Fisher and Newton, 2002; Rudnick and Kennedy, 2002).

1.2 Factor affecting nitrogen fixation

Apart from species and population density of Azotobacter, the following factors are also known to influence the nitrogen fixing ability.

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1.2.1 Oxygen

As an aerobe, oxygen not only plays an important role in the growth of Azotobacter but also affects the efficiency of nitrogen fixation. Higher level of oxygen can reduce nitrogen fixation rate, while lower oxygen concentration promotes nitrogenase activity. This may be due to the fact that oxygen is more competitive in binding with the terminal hydrogen acceptor of Azotobacter than nitrogen (Mulder, 1975; Rudnick and Kennedy, 2002; Mala, 2003).

1.2.2 pH

pH value has a direct impact on the population and nitrogen fixation rate of Azotobacter, both in culture media and natural soil. Azotobacter prefers to grow in neutral or slightly alkaline condition, depending on the species (Mulder, 1975). For example, much lower Azotobactor population is found when soil pH is lower than 6.5 (Jensen, 1965). *Azotobacter beijerinckii*, however, is much more tolerant to wider ranges of pH especially when incubating in pure culture. In turn, it thrives reasonably well in nature and even in acidic soil (Becking, 1961; Tate, 2000; Mala, 2003).

1.2.3 Temperature

The optimum temperature for the growth of Azotobacter is around 30-32 ^{o}C and the lowest temperature that they can survive is at 14 ^{o}C. In the case of *A. vinelandii, A. paspali* and some strains of *A. chroococcum*, the optimum growth temperature is at 37 ^{o}C. Optimal temperature stimulates growth of Azotobacter which helps increasing nitrogen fixing rate while lower temperature greatly reduces their nitrogen fixing capacity (Mala, 2003).

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1.2.4 Organic matters

Organic matters enhance nitrogen fixation because they act as local energy sources for Azotobacter. Generally Azotobacter receives various organic matters, such as sugars and polysaccharides, amino acids, organic acids, growth factors, enzymes and nucleotides, from root exudates of plants (Bolton *et al*., 1992; Pinton *et al*., 2007). Rajaramamohan-Rao (1976) reported that soil fortified with moist rice straw enhanced nitrogen fixation.

1.2.5 Soil moisture content

Soil moisture content (SMC) directly influence the population size of Azotobacter and their activity. Phiromtan *et al.* (2009) reported that the population number of Azotobacter decreased when soil moisture content was greater than 20 %. However, the microorganism still could survive at lower soil moisture content at 5-10 % which was similar to an attribution found by Nijhoff (1982). However, bacterial growth remained constant at 70 % SMC and decreased at over 100 % SMC (Islam *et al*., 2008). It was also reported that nitrogen fixation increases when soil has 70 % moisture content. Higher soil moisture content reduces nitrogen fixation rate (Channal *et al*., 1989; Mala, 2003). Jones and Bangs (1985) and Mala (2003) reported that decreased soil moisture content resulting in high oxygen (O_2) concentration in soil particles. High concentration of oxygen inhibited O_2-sensitive nitrogenase enzymes and required high energy for oxygen protection mechanisms leading to declining of nitrogen fixation (Quispel, 1974).

1.2.6 Presence of inorganic nutrients

Inorganic nutrients are essential for nitrogen fixation of Azotobacter but little is known how these nutrients affect nitrogen fixation activity, particularly soil with trace amount of calcium, magnesium and molybdenum. Acidic soil is harmful to Azotobacter due to calcium deficiency, hence reduces the utilization of Ca^{2+}. Molybdenum exploitation was reported to be very low in iron-stone containing soil found in Europe and Australia. Moreover, it is also recognized that

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phosphorus plays a key role in metabolism process, providing energy (ATP) to the system, such as the nitrogen fixation, which requires a great amount of energy input (Quispel, 1974). However, excess level of inorganic nitrogen in soil can inhibit production of nitrogenase enzymes and nitrogen fixing capacity (Mala, 2003).

1.2.7 Indigenous and other microorganisms

Other microbes may help promoting the growth of Azotobacter in soil. A significant difference was found when Azotobacter was applied in conjunction with Azospirillum in Bermuda grass production (Forlani *et al*., 1995). The utilization of *Azotobacter chroococcum* together with Rhizobium endorsed higher nitrogenase activities and nodulation of chickpea than the application of Rhizobium alone (Yadav *et al*., 1994). However, some of soil microbes can inhibit the growth of Azotobacter, for example *Actinomycete* spp. and *Bacillus mesentericus* (Mala, 2003).

1.2.8 Toxins in soil

Agricultural chemicals have long been reported to affect the development of Azotobactor in the soil. Interestingly, Van Schreven *et al*. (1970) reported that although herbicides were applied on conventional agriculture, no effect on microbial population was found. In addition, it was discovered that some herbicides such as lasso, nitrofen, and simazine could stimulate the growth of Azotobacter in soil (Bopaiah and Rai, 1979). Das and Mukherjee (1998) and Madhuri and Rangaswamy (2006) reported that insecticides was a major factor controlling the number of Azotobacter in rhizosphere. They found that the population of N_2-fixing bacteria was stimulated by the insecticides used in their studies. This may be because some strains of Azotobacter could adapt to the environment (Mendes *et al*., 1999). High concentrations of pesticide limited the proliferation of Azotobacter in soil (Arthur, 1953). Athar and Ahmad (2002) reported that high concentrations of heavy metals accumulated in soil reduced the population size of Azotobacter due to the inactivation of dehydrogenases and proteases in the bacterial cells by those metals (Nada *et al*., 1997).

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2. Phytohormones production by *Azotobacter* spp.

Azotobacter is one of the important heterotrophic bacteria which is able synthesize large amount of biologically active substances such as phytohormones. Azotobacter can produce a variety of phytohormones both in culture media and the soil (Arshad and Frankenberger, 1993). Martinez-Teledo *et al*. (1988) reported the effect of maize (*Zea mays*) root exudates on the liberation of auxins, gibberellins and cytokinins by *A. chroococcum*. Nieto and Frankkenbeger (1989) detected cytokinin in culture filtrates of *A. chroococcum*, *A. vinelandii* and *A. beijernickii.* They were highly effective in producing phytohormones under various growth conditions in liquid media. Sokolova *et al*. (2011) reported that bacterization of *Azotobacter chroococum* stimulated seeds during germination and significantly reduced the number of non-germinated seeds. Generally, the ability to produce plant hormones of Azotobacter depends on the species as briefly described below.

A. chroococcum can produce auxins such as IAA, heteroauxins and auxins-like substances. It can also synthesize cytokinins (e.g. cytokinins-like compounds, N6 adenine, N6 adenosine) and produce gibberellins such as gibberellins-like substance or GA3.

A. vinelandii can produce auxins (e.g. IAA, auxin-like substances) and cytokinins (e.g. zeatin, isopentenyladenosine, isopentenyladenine and gibberellins-like substance or GA3).

A. beijerinckii can produce auxins (e.g. IAA), produce cytokinins (e.g. cytokinins-like compounds) and produce gibberellins (e.g. gibberellins-like substances).

A. paspali can produce phytohormones which are similar to *A. beijerinckii*. However, *A. paspali* can also produce gibberellins-like substance or GA3.

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Various factors affect the production of auxins and phytohormones. Some organisms can synthesize auxins, given that appropriate precursor such as tryptophan is available. Azotobacter can produce more than one type of auxins. Moreover, it has been reported that under natural soil conditions, auxin synthesis is greater than those found in pure culture (Smaly and Bershova, 1975). Report of Gonzalez-Lopez *et al*. (1986) showed that the presence of nitrogen reduced auxin production of *Azotobacter vinelandii* ATCC 12837 in Burk's nitrogen-free medium and Burk's medium with the addition of 0.3 % NH_4NO. Microorganisms isolated from the rhizosphere and rhizophane of various crops are found to be able to produce more auxins than those isolated from root free soil (Brown, 1972), particularly in the areas with high concentration of substrates and microorganisms. For example, it was found that the amount of IAA is greater in the rhizosphere than in non-rhizosphere environments (Arshad and Frankenberger, 1993). Auxins in soil are derived from decomposition of carbonaceous materials from living and dead plant residues. Similarly, soil enzymes can be produced by degradation of tryptophan and converted to IAA in absence of proliferating bacteria. Humic substances control auxin activity while animal manures increase the amount of auxins in soil. The exogenous application of phytohormones may affect the endogenous hormone pattern of the plant, either by supplementation of suboptimal levels or by interaction with the synthesis, translocation, or inactivation of existing hormone levels. The plant response is governed by the rates of hormone uptakes, the active hormone concentrations in the rhizosphere, and the modification of the plant's own pool of hormones because of the exogenous supply (Frankkenbeger and Fitzpatrick, 1984).

3. Phosphate solubilization by *Azotobacter* spp.

Azotobacter helps the utilizing of soil phosphate and makes it bioavailable for plants (Kundu and Gaur, 1980; De Freitas *et al*., 1997). Kumar *et al*. (2001) suggested that *Azotobacter chroococcum* could reduce phosphate fixation in soil fraction. Similar to Rhizobium, Pseudomonas and Bacillus species, Azotobacter can also solubilize phosphate. Recently, Ahmad *et al.* (2008) reported that 74.47 % of Azotobacter is capable to dissolve phosphate compared to only 16.67 % of

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Mesorhizobium. Narula *et al*. (2002) reported that six isolates of *A. chroococcum* isolated from the rhizosphere soil of mustard crop showed solubilization of tricalciumphosphate and rock phosphate activity. Kumar *et al*. (2001) found that mutant strains of *A. chroococcum* could produce growth hormones and phosphate solubilization higher than those isolated from soil. Mechanism of phosphate solubility involves acidification of inorganic acids, organic production, acid phosphatases, releasing of H, reparatory of HCO production, NH_4-N, HS production, exopolysaccharide production and assimilation from liquid which is controlled by several phosphatase-encoding genes (Kumar *et al*., 2001).

4. Biological control of pathogenic fungi by *Azotobacter* spp.

Azotobacter has the ability to inhibit pathogenic fungi (Narula, 2000; Ahmad *et al.,* 2008), depending on species and virulence of soil-borne pathogens. Azotobacter promotes plant growth in early stages of the development by inhibiting seed infectious fungi such as *Fusarium* spp., *Alternaria* spp. and *Penicillium* spp. Kloepper (1993) reported that Azotobacter promotes plant growth by controlling deleterious microorganisms by biological control of parasitic pathogens. Narula (2000) reported that *A. chroococcum* inhibited the growth of pathogenic fungi and prevented seed infections. However, the effectiveness of antagonistic actions varied depending on species. For example, Azotobacter strain 70 has a higher antagonistic action than that of strain 49. Furthermore, application of Azotobacter can also reduce fungi toxicity of *Alternaria* spp. found in pearl millet by producing fungal static compounds. Ahmad *et al.* (2008) concluded that the mechanism against phytopathogenic microorganisms is a production of siderophores (Scher and Baker, 1982), antibiotics (Shanahan *et al*., 1992) and cyanide (Flaishman *et al*., 1996). However, Kloepper (1993) argued that the antagonictic effect was due to antibiosis, competition, parasitism, cyanide and siderophores productions.

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Production of Azotobacter powder inoculum

Azotobacter inoculum have been developed and adapted from Rhizobium inoculum production (Burton, 1984). The Soviet Union was the pioneer of Azotobacter production and was commercialized as azotobacterin. It then became widespread throughout Eastern Europe such as Czechoslovakia, Rumania, Poland, GDR, Bulgaria and Hungary (Rao, 1975) and more recently, it is also well known in India (Mala, 2003).

1. Selection criteria for highly effective Azotobacter as biofertilizer

Mala (2003) outlined that highly effective strains of Azotobacter for biofertilizer production should have the following characteristics.

1) Well-grown strain and large cells.

2) High ability of nitrogen fixation and IAA or plant hormone production.

3) Pure isolate with no contamination of plant pathogens.

4) Great potential for promoting plants' growth for both indoor and outdoor plantation.

2. Type of carriers

Many carriers have been evaluated and used in producing Azotobacter inoculum for seed or soil inoculation purposes. These materials include peat, charcoal, compost, bagasse, sugarcane, filter mud, coir dust, coal, lignite, straws, various compost mixtures, clays, and minerals such as apatite and vermiculite (Date and Roughley, 1977; Burton, 1979; Paczkowski and Berryhill, 1979). Mixing of some carriers are also common such as peat and coconut coir dust (Thungtrakul, 1987), press-mud with charcoal or soil (Jauhri and Philip, 1984), vermicompost and

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farm yard manure (Madan and Singh, 2010), vermicast with lignite (Raja and Karmegam, 2010). Modified charcoal-soil based carrier (Jauhri *et al*., 1979), and a combination of soil and fly ash at 1:1 ratio are also available (Gaind and Gaur, 2004). Carrier helps preserving the quality of inoculants which largely dependent on its chemical and physical properties. Generally, carrier materials must be ground to fine powder of 10-40 μm, or 0.5-1.5 mm in granular form. After carrier materials are disinfected, they should be mixed with limestone to neutralize its pH.

3. Optimal characteristics of carrier for inoculum production

No universal carrier or formulation is presently available for the release of microorganisms into soil (Trevors *et al*., 1992). A good carrier should have one essential characteristic: the capacity to deliver the right number of viable cells in good physiological condition at the right time (Fages, 1992; Trevors *et al*., 1992). Burton (1979) summarized that good carrier materials for seed inoculation should be 1) able to hold great absorption capacity, 2) easy to dry and grind, 3) nontoxic to inoculant bacterial strains, 4) easy to sterilize, 5) free of abrasive minerals, 6) inexpensive, 7) able to provide great adhesion to seeds, 8) non-toxic to plant and 9) able to have large pH buffering capacity.

4. Production of Azotobacter inoculum

4.1 Laboratory and small scale

Laboratory based Azotobacter production can be achieved in various ways. Different types of culture commonly used are Jensen's medium, Burk's medium or Ashby's medium. Ashby's broth is prepared by combining 20 g mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.1 g K_2SO_4, 5 g $CaCO_3$, 0.05 g Na_2MoO_4, and 1 g NH_4Cl in one liter of distilled water. A loopful of Azotobacter pure culture is transferred into a 250 ml erlenmeyer flask containing 100 ml of Ashby's broth and incubated at 28±2 ^oC on 120 rpm rotary shaker for 72 hrs. Afterward, 75 mL of broth culture is then mixed thoroughly with 100 g of each sterile

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carrier which adjusted moisture content to 75 %, packed in polyethylene bags. It can then be sealed and incubated under room temperature for 5-7 days. The inoculums were repacked into sterile polyethylene bags (Phuangsaeng *et al*., 1991). For a smaller scale production, Azotobacter inoculant can be prepared as a starter by incubating pure culture of Azotobacter in Ashby's agar for 2 days. This starter is then used for a larger production by mixing with peat, using 40 mg of starter per one kilogram of peat. The mixture needs to be further incubated at 20-24 ^{o}C. Once the total cell count has reached 10^{8} cells per gram, it is ready for usage (Mala, 2003).

4.2 Azotobacter inoculum production on a large scale

The following is a peat-based commercial formula commonly used in Russia. This formula could produce about a ton of azotobacterin (Azotobacter inoculum). The ingredients of azotobacterin comprises one ton of peat, 10 kg of sugar, 1-2 kg of superphosphate, 20 kg of calcium carbonate and 25 flasks of Azotobacter culture. Firstly, the culture is mixed with half of the above ingredients. The mixture is made into a layer of about 20-40 centimetres thick and incubated at 20-25 ^{o}C for 3 days. Then, the rest of the materials are added, mixed well and further incubated for another 3 days before bagging (Mala, 2003).

5. Type of Azotobacter inoculum

There are 3 basic forms of Azotobacter inoculum commonly used in seeds and soil (Bashan, 1998; Mala, 2003).

1. Liquids form
2. Powers form
3. Granulars form

Although a broth culture or liquid formulation is not commonly used nowadays, it has long been very popular due to its ease of use. These inoculants use broth cultures or liquid formulations, mainly in water, but also in mineral or organic

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oils. The seeds are either dipped into, or evenly sprayed with liquid inoculant by an applicator before sowing (Bashan, 1998). For seeding transplant, it should be dipped in broth culture of Azotobacter for 10-30 minutes before planting. Normally, the instruction is clearly printed and it is simple to follow. It is widely used for planting potatoes by simply soaking potato seeds in 200 ml and potato seedlings in 500 ml of azotobacterin solution, containing 10^9 cells/ml. After 2-3 hours of soaking, the seeds and seedlings are ready to be planted in up to 1 hectare of land.

Powder inoculant is used as a seed coating before planting. The smaller the particle size, the better the inoculant will adhere to the seeds (Burton, 1984; Bashan, 1998). It is important that the Azotobacter powder should be liquidised with minimal amount of water prior to use for further inoculation with selected seeds or seedlings. Once soaked, the seeds must be left to dry in the shed. In addition, it has been reported that 3 and 6 kilograms of Azotobacter powders per hectare are required for the seeds and seedlings of potato due to a lower count of viable Azotobacter (around 5 x 10^7 cells per gram of peat based culture) (Mala, 2003).

It has been suggested that granular inoculant is better than the liquid and power inoculants due to cell aggregation which allows a higher chance of cell survival. The inoculant is applied directly to the furrow together with the seeds. The granular forms can be prepared by blending peat, lime, superphosphate and humus together and then mix them with sawdust and 5-30 % of rice straw. The mixture is then combined with Azotobacter broth culture before kneading into a dough and finally pressed to form 0.5-centimetre tablets. It is then coated with lime or phosphate to accelerate drying process and protect the Azotobacter strains which helps extending their survivability compared to the powder and liquid inoculants (Bashan, 1998).

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Utilization of Azotobacter for agricultural purposes

Azotobacter holds a great potential for agriculture applications due to its ability for nitrogen fixation, phosphate solubilization, soil-borne pathogenic fungi (e.g. *Fusarium* spp., *Alternaria* spp. and *Aspergillus* spp.) inhibition and hormone production (e.g. auxins, gibberellins and cykinins) (Holt *et al*., 2000; Mala, 2003; Ahmad *et al*., 2008). These are very beneficial in promoting plant growth and preventing plant diseases. Its positive attributes are greatly evident in plant production in comparison to non-application of Azotobacter plant (Chahal and Chahal, 2000).

During 1958-1960, there was an investigation of how plant responded to azotobacterin in an experimental field carried out in Russia. It was found that 8 out of 23 plant samples showed significant improvement in production, ranging from 7-12 % (Mishustin and Shilnikova, 1969). By and large, field experiments carried out by many researchers on several plantations showed positive benefit of 7-12 % in grain yields (Lakshmanan, 2000). Azotobacter has been used in many plants such as eggplant, tomato, potato, rice, wheat and soybean. Rao (1975) discovered that using Azotobacter in seed generated greater production of wheat, barley, maize, sugar beet, carrot, cabbage and potato. Study carried out by Indian Agricultural Research Institute, using a laboratory scale-pot model, also found that the growth rates and productivities of wheat, rice and vegetables crops were significantly different. Generally, the weights of Azotobacter-applied plants (both fresh and dried) was found to be higher than those of non-Azotobacter-applied plants (Lakshmanan, 2000; Narula, 2000). In rice study conducted by Dewan and Subba Rao (1979), it was reported that application of Azotobacter together with Azospirillum increased dried weight of rice roots and crop yields compared to non-use of Azotobacter and the single inoculation.

Thailand began experimenting in this area on maize in 1983. The results showed that Azotobacter helped increasing plant growth (determined by height, wet and dry weight) compared to non-inoculation of Azotobacter (Phuangseang *et al*.,

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1991; Mala, 2003). In 1983, four different strains of Azotobacter were applied to the maize plantation in comparison with the use of 20 kg/rai of commercial nitrogen fertilizer in some provinces of Thailand. The results obtained from Kalasin and Nakhon Ratchasima provinces showed that there were significant differences in terms of dry weights and total yields of maize between the two treatments. It was found that using Azotobacter could elevate the maize productivity by 220 kg/ha or 32.5 kg/rai. Surprisingly, the greatest improvement was found in Prajeenburi province during rainy season in 1983 and 1982 when the maize yields were reported to go up by 133 kg/rai and 240 kg/rai, respectively (Tangchum, 1984).

Encouraging results has also been observed with vegetable crops grown in soil. Correspondingly, work conducted in Pakistan (Hussain *et al*., 1987) indicated significant increase in yields in most experiments, and have confirmed the positive effects of this bacterium on vegetables (Brown *et al*., 1964). Pepper, tomato, eggplant, bhindi, chilies, carrot, etc. were the vegetable crops studied.

In the review of Rao (1984) focusing on experiments in India on wheat, rice, onion, brinjal, tomato, cabbage and cardaman under different agro-climatic conditions. It was found that rice, wheat, maize, sorghum, oats, ragi, pearl millet, barley, forage crop (Guinea grass), chilies, sesamum, cotton, sugarcane, poppy, mungbean, lavender, rape seed (*Brassica napus*) and cotton yields increased due to *Azotobacter chroococcum* inoculation (Mala, 2003).

Application of Azotobacter in experimental rice field increased the productivity to 0.4-0.9 t/ha (7–20 %) and nitrogen accumulation in rice stem to 11–15 kg N/ha. With these effects, panicle length, number of tillers, plant height, number of panicles, and grain yields were also improved (Lakshmanan, 2000; Narula, 2000). Kumar *et al*. (2001) found that mutant strains of *A. chroococcum* showed higher increase in grain (12.6 %) and straw (11.4 %) yields over control as compared to their parent soil isolate (P4). Döbereiner *et al*. (1972) reported that application of Azotobacter improved nitrogen fixation to 90 kg N/ha per year for *Paspalum notatum,* and 20-160 kg N/ha per year for sugar cane. Additionally, inoculation of seeds with

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Azotobacter and Azospirillum using 60 kg N/ha had no negative effects on growth characteristics crop yield, fresh or dry weight of leaves per plant and the number of inflorescences per plant in rape seed (*Brassica napus*). Malik *et al*. (1994) reported that lignite-based cultures of *Azotobacter chroococcum* significantly improved plants' vitality and yield of cotton.

Azotobacter can also be successfully used with tea. It helps enhancing the growth rate with a higher yield, as well as increasing organic matters and nitrogen content in soil. Furthermore, the application of lignite-based cultures of Azotobacter can increase tillers, numbers of panicles, plant vitality and yields of cardamom. Another study concluded that introducing Azotobacter to latex showed an increase in height and productivity while downy mildew was reduced (Chahal and Chahal, 2000).

In summary, Azotobactor is one of the most efficient biofertilizers that can increase quality and productivity of crops as well as decrease production cost on commercial nitrogen-based fertilizer usage. Thus, it is highly recommended for a wider use of Azotobacter to improve soil quality in Thailand.

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The brockaleli, new plant variety in Thailand

1. Background

The brockaleli is a green vegetable similar to broccoli but with smaller florets and longer, thin stalks. It is a natural hybrid of broccoli and kale (gai-lan), a Chinese broccoli, both cultivar groups of *Brassica oleracea* and is often mistakenly identified as young broccoli. Brockaleli is also known as, broccolini, broccoletti, aspiration, sweet baby broccoli and tender stem. Brockaleli has been introduced to Thailand since 1978 by Associate Professor Sucheera Tachavongsathian, a scientist from the faculty of Agriculture, Khon Kean University but it was not until 1985 that Associate Professor Mayuree Sakthong had studied its growth and seed production (Sukthong, 2008). In the USA, Sanbon Incorporated originated a commercial program for asparation in 1994 and first brought it to the U.S. market in 1996. It was developed by the Sakata Seed Company of Yokohama, Japan **(**Anonymous, 2010**)**.

2. Characteristics and nutrition values of brockaleli

Brockaleli is a crisp, slender vegetable which has a long stem like asparagus. The leaf, stem and head of brockaleli are virtually 100 % consumable. This stem is very tender and sweet. The head looks like a mini broccoli with a unique taste of subtle peppery flavour that comes from the Chinese kale. Most common cooking methods include sautéing, steaming, roasting, boiling or stir frying (Sukthong, 2008; Anonymous, 2010).

There are a lot of nutritional values in this vegetable. It is high in fibre, low in calories and rich in vitamins such as vitamin A, vitamin C, and beta carotene. Both vitamin C and beta carotene are antioxidants which are the substance that may protect bodily tissues against oxidative damage by free radicals, and may thus play an important role in preventing chronic diseases and specific cancers. As well, brockaleli contains folate, sulforphanes, gluconotate, folatesul, foraphanes, gluconate,

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iron and polyphenol compounds which are essential for good health (Sukthong, 2008; Anonymous, 2010; Vegetable Research and Extension, 2013).

3. Brockaleli growing and production in Northeast of Thailand

Although brockaleli has recently introduced to Thailand, it is a very interesting plant with a promising future due to its high phytonutrients and vitamin contents that are known to have beneficial health effects and wider planting locations than broccoli (Sukthong, 2008). In addition, brockaleli is superior over plain broccoli and Chinese kale in many ways as described above. Brockaleli can grow in a warmer climate like in the northeast region of Thailand due to its higher heat tolerance than broccoli. The seeds can be stored for the next season. Lastly, its flavor is excellent compared to broccoli (Vegetable Research and Extension, 2013). Therefore, the brockaleli is considered to be a good choice for planting in Thailand. At the present, the plant is not distributed to the Thai commercial market because of the lack of commercial seed production. In 2001, assistant professor Mayuree Sukthong had studied the life cycle of this plant in the field. The results showed that it grew very slowly in the first month, and rapidly over the next two months. Generally, it is ready for harvesting after 90 days after planting; giving a head size around 11-14 cm in diameter and a mature plant has a height of about 51 cm with 11-13 leaves (Sukthong, 2008).

With a good quality plant, it contains up to 58.3 SPAD unit of greenness with the crispness of the flower stalk, leaf stalk and shoots about 7.55 neutrons. Moreover, for 450 g of fresh weight after trimming and discarding the unused parts, the final weight remains about 360 g. According to experiment in Khonkaen province, the average weight, height, node, and flower size per plant are 201.5 g, 29.9 cm, 5.04 nodes and 10.54 cm, respectively. This vegetable is more expensive than asparagus and broccoli. In addition, growing and harvesting brockaleli is very difficult and it is much more labour intensive for agricultural practice. In a regular broccoli field, harvesters may pick a field about 3 times while the harvesters may need to go through the same field for up to 9 times with brockaleli flower. There are only 2 to 3 spears in normal broccoli whereas brockaleli has 18-24 spears that requires

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a lot more cutting and labour (Sukthong, 2008; Anonymous, 2010; Vegetable Research and Extension, 2013).

4. Factor effecting growth and yield of brockaleli

The growth rate of brockaleli depends on the temperature. It prefers a cool climate with the optimum temperature for brockaleli is 22-25 ^{o}C. However, it can grow in a warmer weather more than its broccoli counterpart. It grows better in well-drainage soil with the average pH around 6-6.5. This plant requires more fertilizers and plant nutrients than broccoli due to its higher harvesting times than those of broccoli. Major essential nutrients are nitrogen, phosphorous and potassium, while minor nutrients such as molybdenum and boron are also required. Therefore, Azotobacter can fix nitrogen approximately 20 kgN/rai (Tangchum, 1985). This advantage can reduce or replace the usage chemically produced nitrogenous fertilizers as it is possible to use bio-fertilizer formed by Azotobacter for brockaleli production.

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MATERIALS AND METHODS

Hypothesis

The utilization of effective strains of Azotobacter isolated from different soils as biofertilizer for nitrogen source will increase growth of brockaleli.

Materials and Equipments

1. 100 glass bottles
2. 100 plastic pots (30 cm diameter)
3. 20 kg of cow manure
4. 200 vacuum tubes
5. 400 kg of Nam Pong soil series was used by taking 30 cm of top soil and drying it. The soil was sterilized by heating dry soil in direct sunlight for a week before taking in the pots.
6. 600 kg of beef cattle manure
7. 90 isolates of *Azotobacter* spp. from various rhizospheric crops
8. Agriculture waste composts viz. corn stubble compost (CC), golden flamboyant leaf compost (LC) and mushroom waste compost (MC)
9. Ashby's medium
10. Atomic Absorption Spectrophotometer (AAS)
11. Autoclave
12. Brockaleli seeds
13. Centrifugator
14. Chemical fertilizer (15-15-15), urea (46-0-0), triple superphosphate (0-46-0) and potassium chloride (0-0-60)
15. Chemical for C-source testing, viz. rhamnose, Na-caproate, Na-capyric, Malonic acid
16. Chlorophyll meter (SPAD UNIT)
17. Digesters

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18. Eight effective strains of *Azotobacter* spp. including 3 strains selected from Chinese kale growing soil (*Azotobacter vinelandii* CK-KS-2, *Azotobacter vinelandii* CK-KS-3 and *Azotobacter vinelandii* CK-NDD-1), 2 strains from lettuces growing soil (*Azotobacter beijerinckii* LET-NDD-1 and *Azotobacter chroococcum* LET-NDD-3), 1 strain from chili growing soil (*Azotobacter vinelandii* CHI-KK-2), 1 strain from broccoli growing soil (*Azotobacter vinelandii* BRO-KPS-5) and 1 isolate from Para rubber growing soil (*Azotobacter vinelandii* PR-NDD-1) were selected to produce powder inoculum and mixed inoculant (8 isolates, Mix) in this experiment.

19. Freezer

20. Fruit Firmness Tester Model FT-327

21. Gas chromatograph 'Shimadzu' model GC 14B equipped with H_2 flame ionization detector (FID with Porapak Q column, 1.5 m long and 32 mm diameter

22. Glass wares

23. Hot air oven

24. Incubator

25. Indole acetic acid

26. Kamphaeng Saen Soil Series

27. Knife

28. Laminar air flow

29. Leaf area meter

30. Lime

31. L-tryptophan

32. Micro Kjedahl apparatus

33. Microscope and camera

34. Nam Pong Soil Series

35. N-free Ashby's broth

36. Nutrient agar

37. Peat

38. Pikovskaya's agar

39. Plastic bags

40. Plastic boxes

41. Potassium hydroxide

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42. Red lime
43. Refrigerators
44. Rice straws
45. Rotary shakers
46. Salkowski reagent
47. Soil samples collected from different locations and several crops
48. Spectrophotometer
49. Sprayers
50. Sprinkles irrigation system
51. Tillage equipment such as shovel, and a hoe
52. Tractor
53. Tri-calcium phosphate
54. Volumetric flasks

Methods

This research consisted of five experiments, viz. studying on the abundance of Azotobacter in rhizosphere of various crops, screening of high effective isolates of Azotobacter in the laboratory, evaluating effect of various carriers from agricultural wastes and temperatures on the survival of *Azotobacter* spp. in inoculum, and monitoring the effects of various *Azotobacter* spp. on growth and yield of brockaleli in the greenhouse as well as under field condition.

Experiment I. A study on the abundance of Azotobacter from soils growing with various crops

The experimental design was a Completely Randomized Design (CRD) with 29 treatments and 3 replications. Twenty-nine soil samples from different crops were collected as describes below.

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1. Soil sampling

1) The research was conducted during November 2007 to April 2008 at the Laboratory of Soil Microbiology, Department of Soil Science, Kasetsart University, Kamphaeng Saen, Thailand. All soil samples were taken from twenty nine crops including rose, gerbera, marigold, Job's tear, maize, pineapple, para rubber, pasture grass, sugarcane, lychee, lanzonez, Burmese grape, bamboo, teak tree, tamarind, broccoli, brockaleli, Chinese kale, lettuce, Cantonese, cabbage, chili, coriander, water convolvulus, sweet basil, *Inula polygonat*, *Baliospermum montanum,* bitter gourd and citronella grass from five locations including Loei, Nonthaburi, Kanchanaburi, Nakhon Si Thammarat and Nakhon Pathom provinces. Two types of agricultural practices (conventional agriculture and organic agriculture), six crop groups (flowerings, field crops, fruit trees, trees, vegetables and herbs), five levels of soil moisture contents (5-10, 11-15, 16-20, 21-25 and 26-30 %), six pH levels (4.6-5.0, 5.1-5.5, 5.6-6.0, 6.1-6.5, 6.6-7.3 and 7.4-7.8) and five levels of soil organic matter contents (1.1-1.5, 1.6-2.5, 2.6-3.5, 3.6-4.5 and >4.5 %) were studied in the laboratory.

2) The soil samples were taken from the top 6 inches by clean hand auger and kept in sterile polyethylene bags. The samples were mixed thoroughly to make one composite sample and transferred to another sterile polyethylene bag.

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Table 2 The soil samples under growing various crops, agricultural practices, moisture contents, organic matters and pH.

No.	Plant rhizosphere	Sample locations	Agricultural practice	Crop groups	Moisture contents (%)	Organic matter (%)	pH
1	rose	LRU	conventional agriculture	flowering plants	5-10	1.1-1.5	4.6 - 5.0
2	gerbera	LRU					
3	marigold	KPS					
4	Job's tear	PR		agronomy	11-15	1.6-2.5	5.1 - 5.5
5	maize	KPS					
6	pineapple	NM					
7	para rubber	NDD, LEI					
8	pasture grass	KPS					
9	sugarcane	KPS					
10	lychee	ML		fruit trees	16-20	2.6-3.5	5.6 - 6.0
11	lanzonez	NST					
12	Burmese grape	ML					
13	tamarind	NDD					
14	bamboo	ML		trees			
15	teak tree	ML					
16	broccoli	PR, KPS	organic agriculture	vegetables	21-25	3.6-4.5	6.1 - 6.6
17	brockaleli	LRU					
18	Chinese kale	NDD, KS					
19	lettuce	NDD					
20	cantonese	NDD, ERW					
21	cabbage	KPS					
22	chili	KK, KS					
23	coriander	NDD, ERW					
24	water convolvulus	KS					
25	sweet basil	KS		herbs	26-30	>4.5	7.4 - 7.8
26	*Inula polygonat*	TMK					
27	*Baliospermum montanum*	TMK					
28	bitter gourd	TMK, KS					
29	citronella grass	TMK					

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2. Enumeration on the number of total bacteria and Azotobacter from soil samples

2.1 Add 10 g soil sample to a 90 ml distilled water (10^{-1} dilution).

2.2 Shake the suspension by hand for 30-60 seconds, and then place it on a shaker for 20 mins with at least 150-200 rpm of speed, allow it to stand for approx. 30 s.

2.3 Transfer of 0.1 ml from the middle region of 10^{-1} dilution and into 9 ml distilled water to achieve the 10^{-2} dilution. Dilute the dilution in the same method to 10^{-6} dilution (Mala, 2003; Weaver *et al.*, 1994).

2.4 Transfer 1 ml aliquot of the chosen dilutions (those will likely yield 30-300 colonies) for spread plate method in N-free Ashby's medium plates (containing 20 g manitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.1 g K_2SO_4, 5 g $CaCO_3$, 0.05 g Na_2MoO_4, 20 g agar per liter of distilled water) (Subba Rao, 1984; Weaver *et al.*, 1994; Mala, 2003) and incubate at 28±2 ^{o}C for 7-10 days.

2.5 After 7 to 10 days, the cell numbers of Azotobacter were counted.

2.6 The total number of bacteria was checked at 2 days and the amount of Azotobacter was checked at 7 days. The density of Azotobacter was calculated in term of percentage as compared to total bacteria.

$$\% \text{ density of Azotobacter} = \frac{\text{amount of Azotobacter in 1 g soil}}{\text{amount of total bacteria in 1 g soil}} \times 100$$

2.7 Purify the culture by re-streak on N-free Ashby's agar. After fully grown of the culture, a single colony was picked in to slants and labels the name of isolate.

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3. Study characteristic of *Azotobacter* spp. from various habitats

A loopful culture of pure culture was inoculated in N-free Ashby's agar, and then incubate at 28±2 ^{o}C for 5-7 days. After 2-5 days, each culture was identified by cultural conditions, morphological colony and their characteristics such as form, shape, size, edge, consistency of colony, gram reaction etc. by using standard method (Holt *et al*., 2000).

Experiment II. Screening the high effective strains of various Azotobacter isolates in the laboratory

There were two parts in this experiment including 1) screening the high effective strains of various Azotobacter isolates, and 2) study the characteristics and classification of high effective isolates into species level by using standard method and Bergey's manual determinative of bacteriology (Holt *et al*., 2000). The procedure of these experiments were described below.

1. Screening of high efficiency strains of various *Azotobacter* spp.

In this experiment, nitrogen fixation, IAA production and phosphate solubilization of Azotobacter were determined in order to screen the high effective strains. Completely Randomized Design (CRD) consisted of 90 treatments (Azotobacter isolates) with two replications were applied as follows.

1. 1 Nitrogen fixation of various Azotobacter isolates

A loopful culture of Azotobacter was transferred into 125 ml erlenmeyer flask contained 50 ml of sterile nitrogen–free Ashby's broth, and incubate at 28±2 ^{o}C on 90 rpm rotary shaker for a week. After that, the cultures were sterilized in autoclave and determined for nitrogen content by micro Kjeldahl method (Soil Science Society of America, 1996).

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1.2 The quantity of IAA production from various isolates of Azotobacter

Determining the quantity of IAA production from various isolates of Azotobacter was examined by the method of Brick *et al*. (1991). Each bacteria was transferred into 125 ml erlenmeyer flask containing 25 ml of N–free Ashby's broth including 50 µg/L of tryptophan, and incubated at 28±2 ^{o}C on 150 rpm rotary shaker for 72 hrs. Five millimeters of cultures were transferred in the tube and centrifuged at 3,000 rpm for 30 mins. Then, 3 ml of supernatant were taken and mixed with 2 ml of the Salkowski reagent (2 ml of 0.5 M $FeCl_3$ solution + 98 ml of 35 % perchloric acid ($HClO_4$)). After 30 mins, the intensity of pink color was developed as IAA production. The absorbances of the aliquots were determined at 530 nm with spectrophotometer. The concentration of IAA production of the culture was measured by standard graph of IAA obtained in the range of 0-100 µg/ml. Two independent replicates of each isolates were analyzed.

1.3 Phosphate solubilization by Azotobacter

Ninety isolates of Azotobacter were determined the phosphate solubilization using tricalcium phosphate on Pikovskaya's agar (Gaur, 1990). Each bacterial isolate was steaked in Pikovskaya's agar containing of 10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g NH_4SO_4, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, trace of $MnSO_4$ and $FeSO_4$, 0.5 g yeast extract, 15 g agar per liter of distilled water and incubated for 7 days at 28±2^{o}C. After 7 day, the clear zone on Pikovskaya's agar indicates a positive result while the opaque zone indicates a negative result.

After preliminary investigation into the potential of 90 isolates of Azotobacter in the laboratory were determined. All isolates were streaked on Ashby's agar and incubated for 5-7 days. Then colony size of bacteria were studied and screened for further exploration. Based on colony size, a bacterium with larger colony indicates fast growing bacterium.

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The criterion for screening high effective strains for successful inoculum production was described as reports of Bashan (1998) and Mala (2003). The biofertilizer product must be shown to contain beneficial strains of microorganisms. The microorganisms should have high performance or high activities such as efficiency of nitrogen fixation, IAA production and phosphate solubilization, and large colonies size represented as the fast growing bacteria (the organisms should be as fast-growing as possible) were combined. To screening high efficiency isolates, the high potential strains such outstanding efficiency resulting in Part I were selected to study characteristics and classification into species level by reports of Holt *et al*. (2000) and for greenhouse and field trials.

2. Study the characteristics and classification of high effective isolates of Azotobacter

2.1 Classification and their characteristics on N-free Ashby's agar

A loopful culture of eight high potential isolates of Azotobacter was streaked in nitrogen-free Ashby's agar, and then incubates at 28±2 ^{o}C for 5-7 days. Afterwards, each culture was identified by cultural conditions, morphological colony and their characteristics such as carbon source (C-source), gram reaction etc. by using standard method into species level by Bergey's manual of determinative bacteriology (Holt *et al*., 2000).

2.2 Growth of high potential isolates in Ashby's broth

High potential of eight Azotobacter isolates were studied. A loopful of pure culture was picked up into erlenmeyer flask containing 50 ml of Ashby's broth (20 g mannitol, 1g of NH_4Cl 0.2 g K_2PHO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.1 g K_2SO_4, 5 g $CaCO_3$ and 0.05 g Na_2MoO_4 per liter of distilled water) incubated at 120 rpm on rotary shaker at room temperature for 72 hrs. Then, the cell numbers of all broth isolates were determined by dilution and spread plate method. The fresh culture was taken at 24, 48, 60 and 72 hrs. after inoculated by dilution

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plating method on N-free Ashby's agar and incubated at 28±2 ^{o}C for 5-7 days (Mala, 2003). Number of apparent colonies were counted, calculated into viable cells of Azotobacter, converted to log number value per milliliter of fresh culture, and then used in data analysis.

Experiment III. Effect of various carriers from agricultural wastes and temperatures on the survival of Azotobacter in the inoculum

1. Preparation and analysis on properties of carriers

Four types of materials including peat (Pt), corn stubble compost (CC), golden flamboyant leaf compost (LC) and mushroom media compost (MC) were utilized in this study. The materials were prepared following the manual of Burton (1984). The raw materials were ground, sieved with 0.5 cm mesh screen and dried in a hot air oven at 60 ^{o}C for two days. Then, the materials were prepared into carriers of peat (Pt), Pt mixed with each agricultural waste at a ratio of 1 to 2 and named as peat with corn stubble compost (PtCC), peat with golden flamboyant leaf compost (PtLC), and peat with mushroom media compost (PtMC). The materials were sterilized in autoclave at 121 ^{o}C with steam at a pressure of 15 psi for 30 min. The carriers were analyzed for chemical properties, including total nitrogen (total N) by micro Kjedahl method, total phosphorous (total P), total potassium (total K), organic matter (OM), pH (1:10) and electrical conductivity (EC) by standard method as described by Attanandana and Chanchareonsook (1999).

2. Experimental design

The experimental design was 4 x 5 factorial arrangement of treatments in a Completely Randomized Design (CRD) with three replications. The first factor comprised four carriers viz. Pt, PtCC, PtLC, and PtMC, while the second factor comprised five storage temperature levels, viz. deep freezing (-16 ^{o}C), refrigerating (5 ^{o}C), air conditioning, (25±2 ^{o}C), ambient room temperature (30±2 ^{o}C) and greenhouse temperature (37.5±2.5 ^{o}C).

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3. Production of Azotobacter inoculum

Azotobacter vinelandii NDD-CK-1, a fast growing isolate screened from rhizosphere of Chinese kale at Nadindam village, Loei province, Thailand was chosen based on its high effectiveness. Inoculums were prepared in powder form under aseptic condition. A loopful of NDD-CK-1 strain was transferred into a 250 ml erlenmeyer flask containing 100 ml of Ashby's broth (adding 1 g NH_4Cl per liter of distilled water) and incubated at 28±2 ^oC on 120 rpm on rotary shaker for three days. Seventy-five milliliters of broth culture was mixed thoroughly with 100 g of each sterile carrier, adjusted the moisture content to 75 % water holding capacity, packed in polyethylene bags, sealed and incubated under room temperature for five days. The inoculum were repacked into sterile polyethylene bags, and stored in a cool-dry place away from direct sunlight.

4. Evaluation for survival of the Azotobacter during storage in different temperatures

The numbers of Azotobacter were determined after being subjected to different carriers and temperatures. Ten grams of each sample was taken for estimating viable cells at the initial date, 7, 15, 30, 60 and 90 days after storage using dilution plating method on N-free Ashby's agar and incubated at 28±2 ^oC for 5-7 days (Mala, 2003). The number of apparent Azotobacter colonies were counted, calculated into viable cells and converted to log number value per gram of dry inoculum, and then used in data analysis.

Experiment IV. The greenhouse experiment for testing the effectiveness of various *Azotobacter* spp. on growth enhancement of brockaleli

1. Experimental design

The experiment was conducted in the greenhouse of Department of Soil Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University at

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Kamphaeng Saen campus, Nakhon Pathom, Thailand. The experimental design was Completely Randomized Designed (CRD) consisted of 12 treatments with 4 replications as described below.

Treatment 1: Control (without Azotobacter and chemical fertilizer application)

Treatment 2: Nitrogen fertilizer 10 kg N/rai (N10 (urea 0.418 g/6 kg of soil))

Treatment 3: Nitrogen fertilizer 20 kg N/rai (N20 (urea 0.8365 g/6 kg of soil))

Treatment 4: *Azotobacter vinelandii* CK-KS-3

Treatment 5: *Azotobacter vinelandii* CK-KS-2

Treatment 6: *Azotobacter vinelandii* CK-NDD-1

Treatment 7: *Azotobacter beijerinckii* LET-NDD-1

Treatment 8: *Azotobacter vinelandii* CHI-KK-2

Treatment 9: *Azotobacter vinelandii* PR-NDD-1

Treatment 10: *Azotobacter chroococcum* LET-NDD-3

Treatment 11: *Azotobacter vinelandii* BRO-KPS-5

Treatment 12: Mixed strains (MIX)

2. Soil preparing for planting

Nam Pong Soil Series was used in this experiment. Thirty centimeters of top soil was taken and dried in a cool well-dry place for two weeks. Then, the soil was sterilized by warming in direct sunlight for a week before taking in the pots. Six kilograms of dry soil was taken and mixed thoroughly with planting media including 90 g of raw chaff and 200 g of cow manure. Six-hundred milliliters of water were slowly added (to adjust moisture content at field capacity (FC)) to the mixture. Then, 20 ml of water was added and left to stand for two days before planting.

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3. Production of Azotobacter inoculum

A loopful of bacteria was transferred into the 250 ml Erlenmeyer flask containing 100 ml of Ashby's broth, and then incubated at 28 ±2 ^{o}C on a 120 rpm rotary shaker for three days. After that, 75 ml of each Azotobacter culture was mixed thoroughly with 100 g of sterile peat (adjusting the moisture content to 75 % WHC). Ten milliliters of each broth culture was combined and mixed with 107 g of sterile peat for mixing inoculum. Subsequently, all inoculums were sealed and incubated at room temperature for five days. Inoculants were packed into sterile polyethylene bags, and stored in a cool dry place away from direct sunlight. Ten gram of all inoculants was determined for the initial population.

4. Planting and cultural practices

The seeds were prepared as follows:

4.1 The brockaleli seeds were soaked in warm water for one hour to break the seed dormancy and then the water was poured off the seeds. The seeds were grown in 25 cm diameter polyethylene pots filled with 6 kg soil which had already been mixed with cow compost and watered.

4.2 A soil inoculation method was conducted (Subba Roa, 1984). 1 to 1.5 inches of soil was dug and 1.5 g of inoculum was transferred. Then, five seeds were laid above the inoculum and covered with the soil.

4.3 After inoculation, the seeds were lightly watered to prevent the splashing of seeds from the pot. All pots were covered for three days with sunshade net to decrease exposure of daylight for 3 days. After the seeds germinated, the covering was taken off from the pots. The samples were watered three times a day for seven days – morning (07.00-08.00), afternoon (13.00) and evening (16.00) using 50 ml of water per pot. The plants were harvested ninety to a hundred days after planting.

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4.4 For fertilizer application, nitrogenous fertilizer and Azotobacter biofertilizer were applied as described above. In this study, 35 kg P_2O_5 of triple superphosphate (0-46-0) (1.4643 g/ 6 kg of soil) and 35 kg K_2O of potassium chloride (0-0-60) (1.1217 g/ 6 kg soil) were utilized for all treatments two weeks after planting. At day 69, 1.5 g of chemical fertilizer (15-15-15) per 6 kg of soil and spraying liquid fertilizer 8 cc/ 2-liter water were applied due to plants showing signs of chlorosis.

4.5 Plant disease and insects were managed by using integrated pest management, herbal extracts and wood vinegar when there was the outbreak of cabbage webworm during blooming stage.

5. Data collection

Data collection consisted of three subjects including microbiological properties, some chemical properties of soil and manures, and plant growth and yield components.

5.1 Some properties of soil and cow manure including organic matter, EC, total N, available P (avai. P_2O_5), exchangeable K (exch. K_2O) before planting and after harvesting and cow manure were analyzed following the standard method.

5.1.1 Analysis of soil samples

The soil was prepared according to the manual of Attanadana and Chanchareonsook (1999). Soil samples were dried in a dry open room away from direct sunlight. Following that, the soil was ground by mortar, put through a 2 mm sieve and stored in a plastic box for avai. P_2O_5, exch. K_2O, EC and pH. Also, 0.5 mm of soil was sieved and kept for analysis of OM and total N.

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5.1.2 Analysis of dairy cattle manure

The dairy cattle manure was dried in a hot air oven at 60 ^{o}C for 48 hrs, ground and sieved with a 0.5 cm mesh screen. The dry manure was taken and stored in dry plastic bags before analysis. The sample was taken and analyzed to find out the total N, total P, total K, OM, pH and EC by a standard method.

5.2 Determining the population of Azotobacter in the soil.

Soil samples were dug and taken from four spots around the pot, mixed to make the composite sample, kept in sterile plastic bags. Ten grams of soil sample was taken to estimate the quantity of Azotobacter before planting, at 30, 45, and 65 days after planting by dilution plating method on N-free Ashby's agar and incubated at 28 ±2 ^{o}C for 5 to 7 days. The number of apparent colonies were counted, calculated into the viable cell of Azotobacter, converted to a log number per gram of dry weight soil (Mala, 2003).

5.3 Nitrogenase activity of various *Azotobacter* spp.

5.3.1 Nitrogenase activity by acetylene reduction assay (ARA) activity of *Azotobacter* spp. was applied by the method of Hardy *et al*. (1968). 120 g of soil was placed (about 100 g dry weight) into a 250 ml glass bottle and the details labeled on it, then covered with a silicone stopper. Thereafter, 50 ml of acethylene gas was pierced by needle through the rubber cap into the glass bottles. All bottles were kept in a cardboard box away from direct sunlight and incubated for three hours. After three hours, 10 ml of the inner gas was taken by clean needle and injected into a 10 ml vacuum bottle with the details labeled on it. The gaseous tubes were inverted, wrapped with aluminum foil and stored in the refrigerator before analysis of ethylene by Shimadzu GC 14B with a flame ionized detector (FID) with a 3.2 mm diameter internal glass column, 2.1 meters long containing Porapack QN (80/100 mesh) setting the injection temperature at 100 ^{o}C and 50 ^{o}C for the column.

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5.3.2 Nitrogenase activity per crop was calculated based on nitrogen fixation of *Rhizobium* spp., while 40 % of nitrogenase activity reduced at night for 12 hrs (Abu-baker and Arya, 1991) (µmol C_2H_4/1 kg soil/crop) plus 100 days (crop) and values of *Azotobacter* spp. was estimated by average of 9 Azotobacter treatments from previous data (5.3.1). The equation based on: 1) the nitrogenase activity (mmoles C_2H_4), 2) 312,000 kg soil per rai, 3) 100 days per crop, and 4) a theoretical conversion factor of one-third N_2 fixed for each C_2H_4 formed (Hardy *et al*., 1968) as shown on equation.

$$\frac{(X \text{ mmoles } C_2H_4)}{10^6} \times \frac{28}{3} \times 312{,}000 \text{ kg soil} = Y \text{ kg } N_2 \text{ fixed/rai/crop}$$

Remark: Equation for calculate nitrogenase activity under greenhouse and field trials

X = Quantity of C_2H_4

Y = kg N_2 fixed/rai/crop

5.4 Yield and yield component including number of leaves, height, length of leaf stalk, leaf width, leaf length, fresh and dry weight, number of branches, number of leaves, diameter of stem, number of roots, fresh-dry weight of root, diameter of stems and flowers, root length, were recorded. Percentage of root was calculated by this equation.

$$\text{Percentage of plant root} = \frac{\text{Weight of root x 100\%}}{\text{Weight of entire plant}}$$

5.5 Quantity of total N, total P, total K of stem and flower

Fresh material was cleaned with cotton to remove dust, soil, and fertilizer. Following this, the plants were dried in a hot air oven at 60 ^oC for 48 hrs until constant weight and then allowed to cool to room temperature and prevented from gathering moisture from the atmosphere by keeping the cool-dry plants in a desiccator before weighing and cutting off. Dry materials were separately chopped, ground into very small pieces or powder by putting through the blender, sieved

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through 40 mesh and kept in a paper bag. Additionally, ground plant parts were dried in a hot air oven for four hrs and left to cool in the desiccator before weighing for analysis. Then the plant tissues were digested by wet digestion by sulphuric acid and investigated to find out the total N, total P and total K by the micro-Kjeldahl method, vanadomolybdate yellow color and Atomic Absorption Spectrophotometer, respectively.

Experiment V. Effect of various inoculums of Azotobacter on growth and yield of brockaleli in Kamphaeng Saen Soil Series

1. The experimental design

The experiment was conducted on a farm trial located at Department of Soil Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University at Kamphaeng Saen campus, Nakhon Pathom province, Thailand. The experimental design was Randomized Completely Block Design (RCBD) and consisted of 12 treatments with 4 replications as described below.

Treatment 1: Control (without Azotobacter and chemical fertilizer)

Treatment 2: N5 (60 g urea/plot/44 plants = 1.36 g Urea/plants = 10.87 kg urea/rai)

Treatment 3: N10 (120 g urea/plot/44 plants =2.72 g Urea/plants =21.74 kg urea/rai)

Treatment 4: *Azotobacter vinelandii* CK-KS-3

Treatment 5: *Azotobacter vinelandii* CK-KS-2

Treatment 6: *Azotobacter vinelandii* CK-NDD-1

Treatment 7: *Azotobacter beijerinckii* LET-NDD-1

Treatment 8: *Azotobacter vinelandii* CHI-KK-2

Treatment 9: *Azotobacter vinelandii* PR-NDD-1

Treatment 10: *Azotobacter chroococcum* LET-NDD-3

Treatment 11: *Azotobacter vinelandii* BRO-KPS-5

Treatment 12: Mixed strains (MIX)

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2. Production of Azotobacter inoculums

The production of various Azotobacter inoculums were prepared by the procedure of Experiment IV, inoculum production, section 3.

3. Growing the seedling of brockaleli

3.1 Peat was mixed with burn rice husk in the ratio 3 to 1 and taken into the 100 hole-seed tray. Then, culture media was watered until saturated and left for 1-2 days.

3.2 Brockaleli culture was cultivated during 29-31 January, 2010 as follows. The brockaleli seed was soaked in warm water for 1 hour to break seed dormancy and pour the water out of the seed. One inch of soil was dug and 2 seeds were placed and dropped in the gap. Then, 1.5 g of each Azotobacter inoculum was inoculated as prescribed treatments, and added 1.5 g of peat above the inoculant and the hole filled with planting media, respectively. Additionally, non-bacterial inoculum was inoculated on the control and chemical fertilizer treatments. All seed trays were watered gently.

3.3 After inoculation, the seed trays were covered with sunshade net for 7 days before seed germination. These inoculated seeds were watered gently 2 times a day (7 a.m. and 4 p.m.) preventing splashing of seeds from the tray during watering. The seeds germinated between February 2nd-3rd, 2010. Herbal extract was sprayed preventing the infestation of insects. The seedlings were taken care of for 30 days (March 5th, 2010) before for transplanting to the field.

4. Soil tillage and preparation

4.1 The soil (Kamphaeng Sean Soil series at experimental field of Soil Science Department) was ploughed roughly at the first time and air dried for 2 weeks to eliminate weeds and some pathogens in the soil. After that, the soil was ploughed

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in regular furrows for the second time and spread out thoroughly in the field. The soil was chopped with a shovel again.

4.2 Lift the track to prepare the plot in the range 4.5 meter long and 2 meter wide for a total for of 48 plots. The soil was spitted to smaller size, spread and adjusted, leveling it throughout the plot with a shovel. The soil samples were collected according to the method described by Attanadana and Chanchareonsook (1999) for analysis of the soil before planting.

4.3 A sprinkle irrigation system was installed with one main water pipe with four sub-main line pipes. The amount of moisture per hour and water distribution were tested until the water was distributed evenly throughout the plot and ensured that each plot had similar amount of water.

4.4 Two tons of fermented cow manure were applied by sowing (11 kg/plot), and then mixed thoroughly into the top soil.

4.5 The rice straws were spread around the plot. After the water distribution testing, each plot was watered until saturated for 2 days, and then left to dry for 3 days before planting. The moisture content was checked. Moisture content was suitable for planting, and so watering was not necessary. The spacing was measured at 40 cm x 50 cm and a hole with 4 inch in depth was dug. Then, the plots were watered again for one hour and left for a day.

5. Transplanting the seedling

After the seedlings were 30 days old, the strongest seedlings of similar size were selected. Seedling was transplanted to the field in the late afternoon (3 p.m. - 6 p.m.) for a total of 44 plants/plot (one hole/one plant). Later on, the seedlings were covered with the soil, then smoothed over the surface and covered with rice straw, respectively. Lastly, the seedlings were watered for one hour.

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6. Agricultural practices

6.1 Irrigation

After transplantation, all seedlings were watered for the first time in the evening for 1 hour, then two times a day at 7 a.m. and 4 p.m. for 1 hour. However, during late March to April 2010, the plants required watering three times per day by adding midday (12 p.m.) for one hour due to hot weather and stronger sun than usual in the summer which made the plants wilt.

6.2 Fertilizer application

In this research, 11 kg/plot of fermented cow manure (2 tons/rai or 13 tons/ hectare) were applied once before planting, and nitrogenous fertilizer (urea) were used during 15-30 days after transplanting at the rate 120 g and 240 g per plot for N5 and N10 treatments, respectively, without using potassium and phosphate fertilizers due to the very high levels of both elements in the soil.

6.3 Plant disease and insect management

The integrate pest management method of sustainable agriculture was used in this study and sustainable agriculture. During 30-45 days after transplanting, biological pest management, microbial substances, fermented fertilizer and neem extract were applied every 7 days for protection against harmful insects in the area. In addition, spraying of insecticide every 7 days 2 times a day during the flowering bud-period was necessary, because the severe outbreak of diamondback moth and cabbage webworm had damaged and eaten apical bud until most of the flowers had been destroyed and the yield losses were more than 50 %. Therefore, insecticide was necessary during that time and we stopped using it when the outbreak of the insects was over and started the re-use of natural extracts as usual. In early May, there was the outbreak of the disease. Some plants showed wilt symptoms and there was a need to pull out that plants and water with fungicide in the hole. In

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addition, there was an outbreak of rust, so it had to be harvested before the outbreak became too destructive.

6.4 Weed management

No herbicide was applied in the experiment. Rice straw covered the plot for controlling weed germination after transplanting in the field. After that, the weeds were removed by hand and cut with a spade when the weeds grew up.

6.5 Harvesting

10-14 days after flower budding, the bouquet of flowers were harvested; none of which were too immature or too old. 6-8 inches of bouquet of flowers long were cut. After that, using lime paint on the wound to prevent air and water born pathogen infection that could be found throughout the site, into the plant tissues. In the case of flowers from lateral buds, they were harvested in the same way. According to Sakthong (2006), the brockaleli flowered during 90-100 days after planting.

All plants were harvested on 19 May 2010 when plants were 106 days old due to the heavy rains between late April and mid May 2010 which caused the outbreak of the disease: onset wilting and rust. Moreover, the weather was hot during the day and there was much rain, so the drainage of the soil was poor and water could not drain because the ground was dense and non-absorbent. These conditions were the favor for the spreading of rust on the leaves and wilt disease by soil borne pathogens. Therefore, the hot and rainy evenings in the last 2 weeks before harvesting, the plants needed to be harvested by cutting and separating the flowers, weighed fresh and dried weight, respectively.

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7. Data collections

7.1 Some soil properties and cow manure including OM, EC, total N, avai.P_2O_5, exch.K_2O, before planting and after harvesting and cow manure were determined.

7.1.1 Analysis of soil sample

Soil samples were prepared and analyzed some properties including OM, total N, avai. P_2O_5, exch. K_2O, EC and pH as described by Attanadana and Chanchareonsook (1999).

7.1.2 Analysis of cow manure

Cow manure was prepared by the instruction of Experiment IV, section 5.1.2. The sample was analyzed in reference to OM, total N, total P, total K, pH and EC by standard method (Attanadana and Chanchareonsook, 1999).

7.2 Microbiological properties

7.2.1 Determine viable cell of Azotobacter at 45, 55, and 65 days after planting.

The soil samples were dug and taken from 4 spots by hand auger around the pot, mixed to make the composite sample, kept in sterile plastic bags. The population of Azotobacter at 45, 55, and 65 days after planting was estimated and calculated as described in Experiment IV, section 5.2 (Phuangsaeng, *et al*., 1991; Mala, 2003).

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7.2.2 Nitrogenase activity of various *Azotobacter* spp.

1) 150 g of fresh soil was taken (about 129.32g dry weight) into a 500 ml glass bottle and labeled the details, then covered with a rubber stopper in the middle.

2) Then, the bottles of soil sample were prepared for determining nitrogenase activity by ARA activity as described in Experiment IV, section 5.3.1 and calculating nitrogenase activity per crop (Hardy *et al*., 1968) by an equation on section 5.3.2.

7.3 The yield and yield component including number of leaves per stem, length of leaf stalk, leaf width, leaf length, fresh and dry weight of the entire plant, height, number of branches, leaf number, diameter of stem, fresh-dry weight of root, leaf area, chlorophyll and firmness were recorded in this experiment.

7.4 Analysis of total N, total P, and total K contents in the flower and stem was prepared as described in Experiment IV, section 5.5 (Attanadana and Chanchareonsook, 1999).

STATISTICAL ANALYSIS

The collected data were statistically analyzed and compared between the treatment means by Duncan's Multiple Range Test (DMRT) method at the significance level 0.05.

LOCATIONS AND DURATIONS

These researches were conducted during October 2007 to October 2010 at the Laboratory of Soil Microbiology, greenhouse and field laboratory of Department of Soil Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University at Kamphaeng Saen Campus, Nakhon Pathom province, Thailand.

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RESULTS AND DISCUSSIONS

RESULTS

Experiment I. A study on the abundance of Azotobacter from soils growing with various crops

1. Abundance of *Azotobacter* spp. from various crops

The result revealed that 87 soil samples collected from the rhizosphere of 29 plants as shown in Table 3. In this finding, 90 isolates of large dominant colonies were picked up as follows. A large number of 90 isolates were isolated from vegetables (38) followed by field crops, fruit trees and trees, herbs and flowers, (18, 15, 13 and 6 isolates, respectively). The result indicated that abundance of Azotobacter varied depends on plant types. In this finding, that the number population density of total Azotobacter and its density were statistically significant differences (Table 4). Soil from Chinese kale had the highest in Azotobacter population and its density of 12.89 x 10^6 cfu/g dry soil and 52.60 %, respectively followed by water convolvulus (7.09 x 10^6 cfu/g dry soil) which was higher than those of other plants. While soil sample of pineapple gave the less Azotobacter population of 0.56 x 10^6 cfu/g dry soil. The minimum density of Azotobacter (10.13%) was found in sugarcane soil. The finding suggested that population of Azotobacter varied depends plant types which directly concerned soil amended and root exudates (Rovira, 1959). It revealed that population of Azotobacter were higher in vegetable soils than that of soil from agronomy.

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Table 3 Soil samples from 29 crops, sample locations and number of isolate of *Azotobacter* spp.

No.	Plant groups	Plant rhizosphere	Sample locations	No. of isolate
1	flora	rose	LRU	1
2	plants	gerbera	LRU	2
3		marigold	KPS	3
4	agronomy	Job's tear	PR	1
5		maize	KPS	1
6		pineapple	NM	3
7		para rubber	NDD, LEI	3
8		pasture grass	KPS	4
9		sugarcane	KPS	6
10	trees and	lychee	ML	5
11	fruit trees	lanzonez	NST	1
12		Burmese grape	ML	3
13		bamboo	ML	2
14		teak tree	ML	1
15		tamarind	NDD	3
16	vegetables	broccoli	PR, KPS	7
17		brockaleli	LRU	3
18		Chinese kale	NDD, KS	3
19		lettuce	NDD	3
20		cantonese	NDD, ERW	4
21		cabbage	KPS	4
22		chili	KK, KS	4
23		coriander	NDD, ERW	5
24		water convolvulus	KS	1
25		sweet basil	KS	4
26	herbs	*Inula polygonat*	TMK	5
27		*Baliospermum montanum*	TMK	4
28		bitter gourd	TMK, KS	2
29		citronella grass	TMK	2
		Total		90

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Table 4 Abundance of Azotobacter from 29 crops.

No.	Plants	Total bacteria (10^6 cfu/g dry soil)	Total count (10^6 cfu/g dry soil)	Density (%)
1	rose	17.35 a-d	3.74 b-f	21.84 b-d
2	gerbera	38.30ab	5.82 cb	22.40b-d
3	marigold	27.46 a-d	3.17 b-f	13.84cd
4	Job's tear	17.38 a-d	2.06c-f	12.59cd
5	maize	21.75 a-d	2.84c-f	14.85b-d
6	pineapple	4.33d	0.56f	13.11cd
7	para rubber	11.22cd	1.75c-f	18.54b-d
8	pasture grass	16.11 a-d	1.56ef	12.16cd
9	sugarcane	22.31 a-d	2.15c-f	10.13d
10	lychee	13.84b-d	2.86c-f	29.72b-d
11	lanzonez	25.56 a-d	5.80 b-d	24.85b-d
12	burmese grape	21.23 a-d	4.79b-e	22.40b-d
13	bamboo	9.83cd	2.03c-f	18.37b-d
14	teak	12.51 b-d	1.63c-f	14.92b-d
15	tamarind	16.39 a-d	3.06 b-f	19.97b-d
16	broccoli	21.42 a-d	3.94 b-f	26.05 b-d
17	brockaleli	25.57 a-d	4.09 b-f	20.74 b-d
18	Chinese kale	29.38 a-c	12.89a	52.60a
19	lettuce	10.45cd	2.35c-f	28.06b-d
20	cantonese	20.19 a-d	4.17 b-f	21.02 b-d
21	cabbage	8.61cd	3.27 b-f	37.55ab
22	chili	32.44a-c	4.16 b-f	13.72 cd
23	coriander	20.64 a-d	5.11 b-e	27.31 b-d
24	water convolvulus	17.14 a-d	7.09 b	34.17a-c
25	sweet basil	12.15 b-d	2.75c-f	26.53 b-d
26	*Inula polygonat*	23.26 a-d	5.13b-e	24.52 b-d
27	*Baliospermum*	25.15 a-d	4.82b-e	22.55 b-d
28	bitter gourd	41.91a	2.93c-f	8.123d
29	citronella grass	17.19 a-d	2.54c-f	14.351 b-d
	Mean	19.73	3.71	21.71
	P-value	NS	**	*
	CV(%)	67.10	55.58	53.47

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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2. Effect of various habitats on the abundance of Azotobacter in the rhizosphere

2.1 Effect of soil moisture contents on the abundance of Azotobacter in the rhizosphere

This study indicated that the soil moisture contents (SMC) effected the number of Azotobacter ($P < 0.05$) as illustrated in Figure 1. The highest population of Azotobacter were found (5.33×10^6 cfu/g) at 21-25 % of SMC and the lowest number of this bacteria were found (1.98×10^6 cfu/g) at 5-10 % of SMC. In this research, it revealed that the population of Azotobacter was ranged between 1.98-5.33 x 10^6 cfu/g. The increasing of SMC induced the more population of Azotobacter, and then reached the maximum population at 21-25 % SMC and there were slightly decrease at 26-30 % SMC. Abundance of Azotobacter in soil among 6-10 %, 11-15 %, 16-20 %, 26-30 % SMC were similar to that of 21-25 % SMC. In contrast, no significant results on the density of Azotobacter and on total bacteria were found in this study. Although, the density was in a similar to its abundance.

2.2 Effect of soil organic matter on the abundance of Azotobacter in the rhizosphere

The result shown that the population of Azotobacter in various soil organic matter (SOM) levels were significance ($P < 0.05$) as illustrated in Figure 2. The highest population of Azotobacter (4.06×10^6 cfu/g) was recorded in the soil at 2.6 to 3.5 % OM, while the lowest number of Azotobacter (2.77×10^6 cfu/g) was found at 1.1-1.5 % OM. The study revealed that the abundance of Azotobacter were increased when > 2 % OM were obtained. However, the highest density of Azotobacter (27.29 %) was observed at 3.6-4.5 % OM, while the lowest density (14.35 %) was found at 1.1-1.5 % OM. Meanwhile, no significant effect on total bacteria was found in this study.

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2.3 Effect of soil pH on the abundance of Azotobacter in the rhizosphere

This results indicated that highly significant effect of pH on the abundance of Azotobacter ($P < 0.05$), and significant effect on density of Azotobacter were found as illustrated in Figure 3. The highest number of Azotobacter (4.37×10^6 cfu/g) was recorded in the soil at pH 6.1-6.5, while the lowest number of Azotobacter (6.2×10^5 cfu/g) was found at pH 4.6-5.0. In this study, the highest density of Azotobacter (25.58 %) was found in the soil at pH 5.1-5.5, while the lowest density (8.12 %) was found at pH 7.4-7.8.

2.4 Effect of different crop groups on the abundance of Azotobacter in the rhizosphere

Crop groups had significant effect on the abundance and density of Azotobacter in the soil ($P< 0.05$). The result revealed that the collected soil from vegetables showed the highest population (4.24×10^6 cfu/g) and density (28.78 %) of Azotobacter (Figure 4). Contrary, the field crops shown the lowest number (1.73×10^6 cfu/g), and density (12.57 %). No significant effect on total bacteria was found in this study.

2.5 Effect of agricultural practices on the abundance of Azotobacter in the rhizosphere

Non-significance on the abundance of Azotobacter were found in both types of agricultural practices ($P > 0.05$) (Figure 5). The populations of Azotobacter were ranged from 3.46 - 3.70 $\times 10^6$ cfu/g and the densities were in the range of 21.30 - 23.34 %. Similarly, there was no significant effect on total bacteria in this study. These results were similarity to Wardle *et al*. (1999) which suggested that no indicators effect on microbial population due to agricultural intensification.

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2.6 Effect of soil sampling locations on abundance of Azotobacter in the rhizosphere

Soil samples were taken from five provinces of Thailand; Loei, Nakhon Pathom, Nonthaburi, Kanchanaburi and Nakhon Si Thammarat. The result found that these locations had no significant effect on total count of Azotobacter ($P > 0.05$) (Figure 6.) The population of Azotobacter was ranged from 2.58 - 4.64 x 10^6 cfu/g soil. None significant effect on total bacteria was found. In this study, the density of Azotobacter was in the range of 19.11 - 22.48 %. According to earlier result, the numbers of Azotobacter from various locations were higher than the reported by Islam *et al*. (2008).

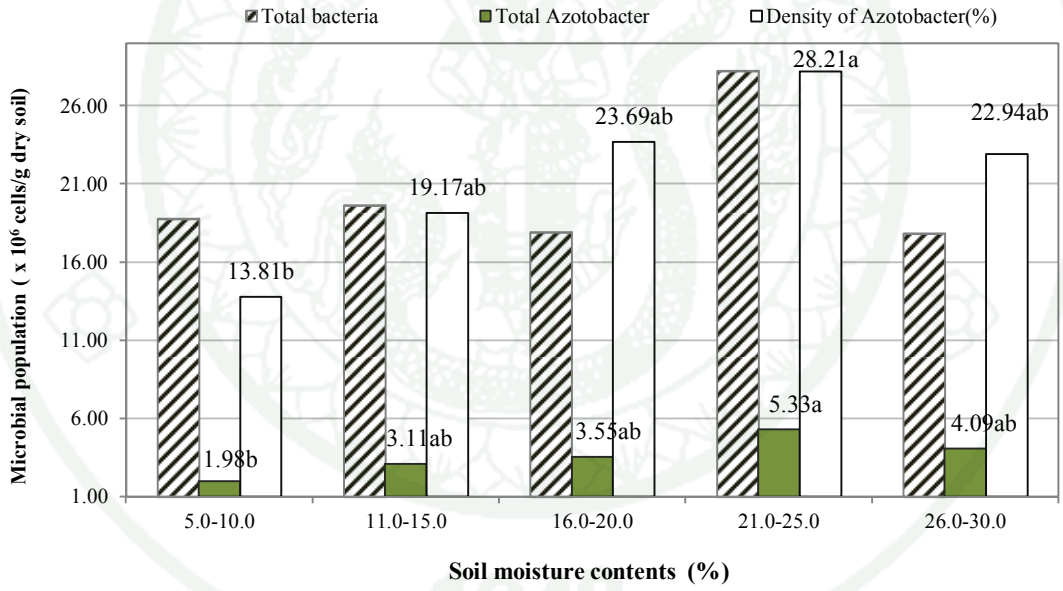

Figure 1 Effect of soil moisture contents on total bacteria, amount and density of Azotobacter in the rhizosphere.

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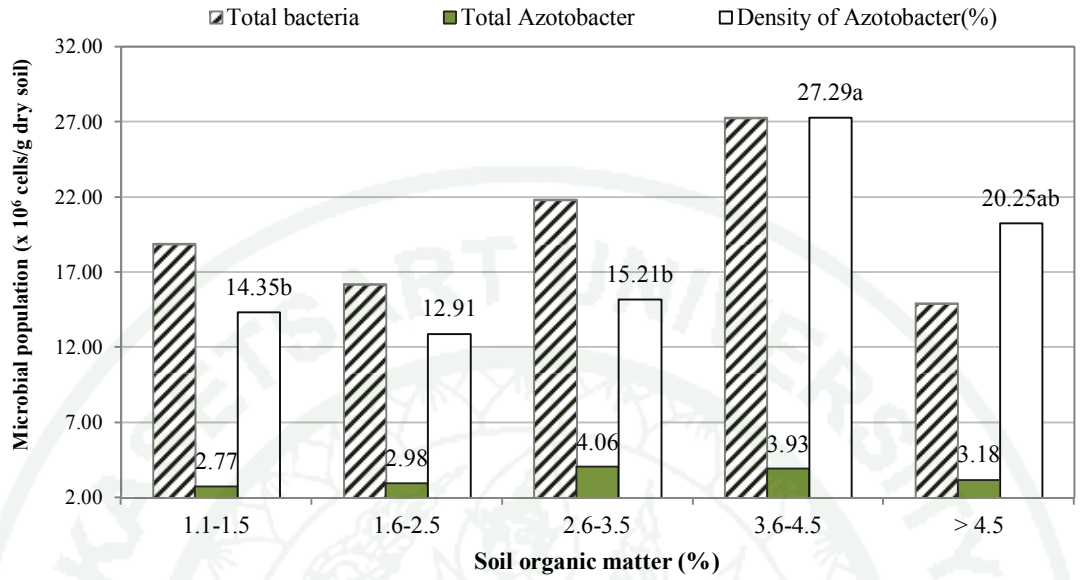

Figure 2 Effect of soil organic matter on total bacteria, amount and density of Azotobacter in the rhizosphere.

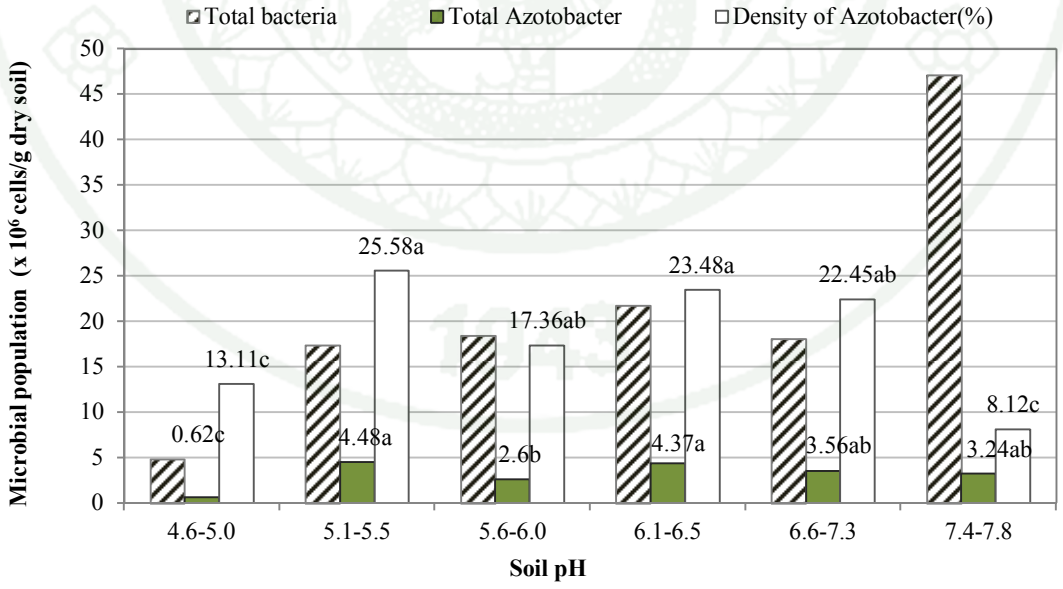

Figure 3 Effect of soil pH on total bacteria, amount and density of Azotobacter in the rhizosphere.

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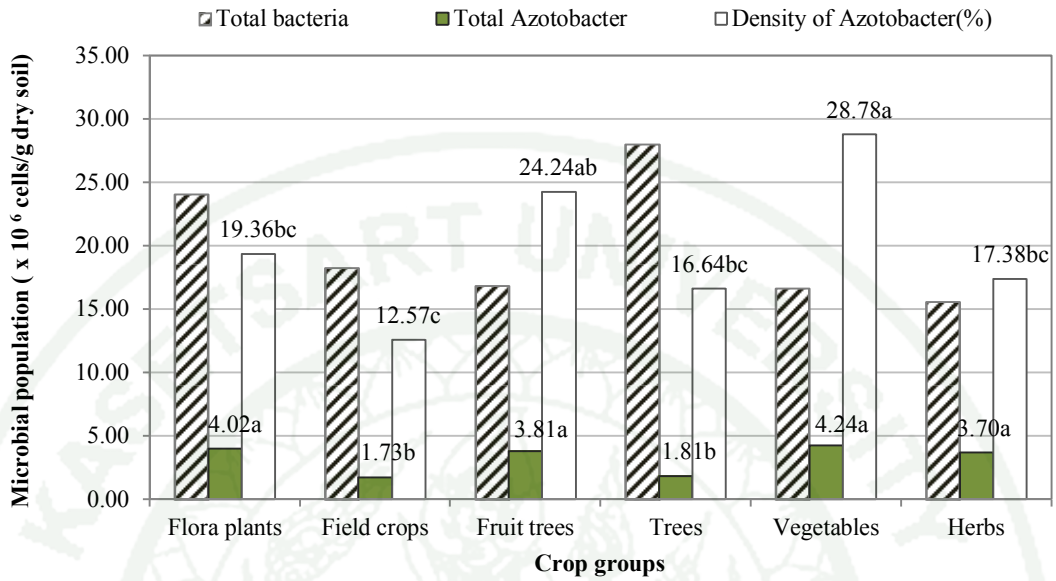

Figure 4 Effect of various crop groups on total bacteria, amount and density of Azotobacter in the rhizosphere.

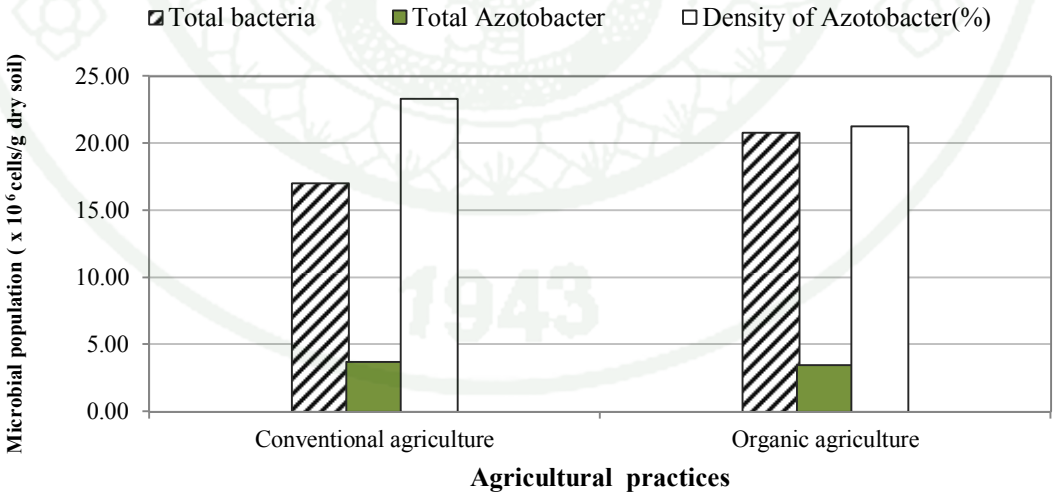

Figure 5 Effect of agricultural practice types on total bacteria, amount and density of Azotobacter in the rhizosphere.

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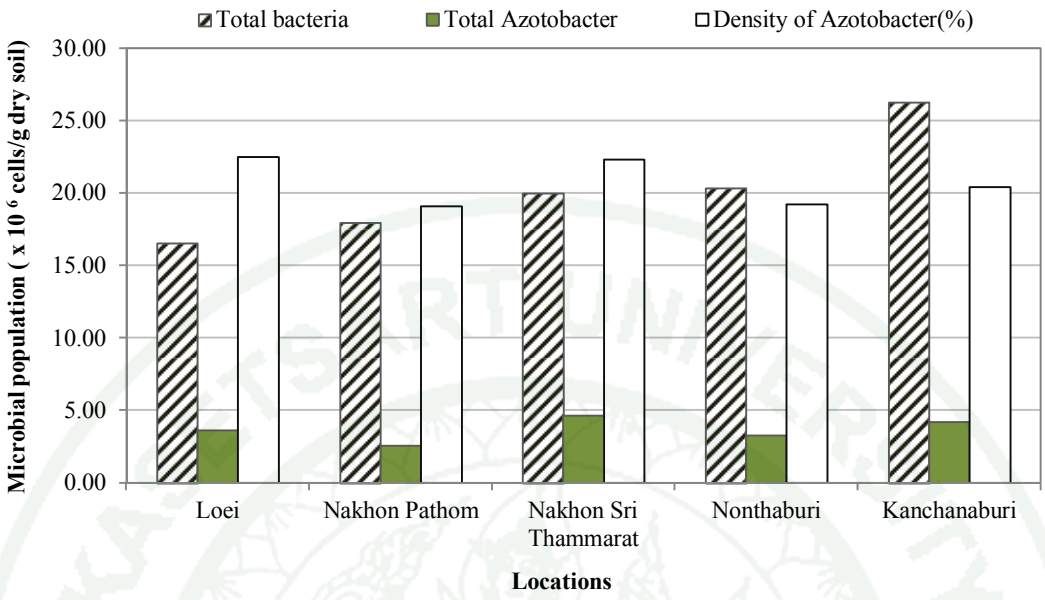

Figure 6 Effect of locations on total bacteria, amount and density of Azotobacter in the rhizosphere.

3. Morphological characteristics of 90 isolates of *Azotobacter* spp. from various crops

Ninety-well separated colonies develop on the surface of the streak plate, represented the growth of single isolate of *Azotobacter* spp. (pure culture) on N-free Ashby's agar, were picked up. These pure cultures were maintained in the N-free Ashby's slant and keep in the refrigerator in order to present moisture loss. Afterward, all gram-negative 90 cultures were named by following method. The first set of letter created by the abbreviation name of the plant followed by abbreviation of soil sampling locations (Table 5) and number of isolates, respectively. In this case, all test isolates were named as shown in Table 6. The colonial characteristics of local 90 pure isolates of *Azotobacter* spp. were shown as Table 6. The appearance colonies were varied depending on isolates.

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Table 5 Name and abbreviation of sample sites and 29 various crops.

Name	Abbreviation	Name	Abbreviation
Erawun district	ERW	teak	TEA
Kamphaeng Saen	KPS	lettuce	LET
Kanchanaburi province	KPT	cantonese	CAN
Khlong Khwang subdistrict	KK	brockaleli	BKL
Khun Si subdistrict	KS	broccoli	BRO
Loei	LEI	sugarcane	SUG
Loei Rajabhat University	LRU	lychee	LIC
Mueang Loei district	ML	lanzonez	LAN
Na Nin Dam village	NDD	Chinese kale	CK
Nakhon Pathom province	NPT	chili	CHI
Nakhon Si Thammarat province	NST	Burmese grape	BG
Nam Mhan village	NM	tamarind	TAM
Nonthaburi province	NBI	bamboo	BAM
Phu Ruea district	PR	para rubber	PR
Sainoi district	SN	cabbage	CAB
Tha Maka district	TMK	coriander	COR
rose	ROS	water convolvulus	WC
gerbera	GER	sweet basil	SB
marigold	MAR	*Inula polygonata*	IP
job's tears	JT	*Baliospermum*	BM
maize	MAI	*montanum*	
pineapple	PIN	bitter gourd	BIT
grass	GRA	citronella grass,	CIT

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Table 6 Colonial characteristics of the 90 isolates of Azotobacter from various crops.

No	Isolates no.	Colonial morphology							
		Shape	Edge	Elevation	Pigment	Colony size (mm)	Opacity	Gram reaction	Slime
1	ROS-LRU-1	round	entire	raised	-	3-4	translucent	-	moderate
2	GER-LRU-1	round	entire	convex	-	2-3	transparent	-	few
3	GER-LRU-2	round	entire	convex	-	2-3	transparent	-	few
4	MAR-KPS-1	round	entire	convex	-	3-4	transparent	-	moderate
5	MAR-KPS-2	curled	fobate	flat	cream	2	opaque	-	abundant
6	MAR-KPS-3	round	entire	convex	-	1.5-2	transparent	-	few
7	JT-PR-1	round	entire	convex	-	4-5	translucent	-	few
8	MAI-KPS-1	round	entire	convex	-	2-4	transparent	-	few-moderate
9	PIN-NM-2	round	entire	raised	-	2-4	translucent	-	moderate
10	PIN-NM-3	round	entire	raised	-	2	translucent	-	moderate
11	PIN-NM-5	round	entire	raised	-	1.5-3	transparent	-	few
12	GRA-KPS-1	round	entire	convex	-	3-4	opaque	-	moderate
13	GRA-KPS-2	round	entire	convex	-	4-6	opaque	-	moderate-abundant
14	GRA-KPS-5	round	entire	convex	-	2-3	opaque	-	moderate
15	GRA-KPS-6	round	entire	convex	-	4-7	opaque	-	moderate

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Table 6 (Continued)

No	Isolates no.	Colonial morphology							
		Shape	Edge	Elevation	Pigment	Colony size (mm)	Opacity	Gram reaction	Slime
16	SUG-KPS-1	round	entire	convex	-	4-6	translucent	-	moderate
17	SUG-KPS-2	irregular	lobate	flat	-	2-5	opaque	-	moderate
18	SUG-KPS-3	round	entire	convex	-	5-8	translucent	-	moderate
19	SUG-KPS-4	entire	round	convex	-	5-6	translucent	-	moderate
20	SUG-KPS-5	entire	round	convex	-	5-7	opaque	-	moderate
21	SUG-KPS-6	entire	round	convex	-	5-6	transparent	-	moderate
22	LIC-ML-1	entire	round	raised	-	2-3	transparent	-	moderate
23	LIC-ML-2	entire	round	raised	-	2.5-3	opaque	-	moderate
24	LIC-ML-3	entire	round	raised	-	3-4	opaque	-	moderate
25	LIC-ML-4	entire	round	convex	-	3-4	translucent	-	moderate
26	LIC-ML-5	entire	round	raised	-	2-3	opaque	-	moderate
27	LAN-NRT-1	lobate	irregular	flat	brown-black	>10	opaque	-	abundant
28	BG-ML-1	entire	round	convex	cream	4-6	opaque	-	moderate-abundant
29	BG-ML-2	lobate	round	flat	light- yellow	>10	opaque	-	moderate
30	BG-ML-3	entire	round	convex	-	3	translucent	-	moderate

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Table 6 (Continued)

No	Isolates no.	Colonial morphology							
		Shape	Edge	Elevation	Pigment	Colony size (mm)	Opacity	Gram reaction	Slime
31	BAM-ML-1	entire	round	raised	-	3-5	opaque	-	moderate
32	BAM-ML-2	entire	round	convex	-	4	translucent	-	moderate
33	TEA-ML-1	entire	round	flat	-	2-3	translucent	-	moderate
34	TAM-NAD-1	entire	round	convex	cream	3-5	opaque	-	moderate
35	TAM-KPS-2	entire	curled	flat	brown-black	>10	opaque	-	abundant
36	TAM-KPS-3	entire	round	raised	-	3-5	paque5	-	moderate
37	PR-NDD-1	round	entire	convex	-	3-4.5	transparent	-	few-moderate
38	PR-LEI-2	round	entire	convex	-	3-4	translucent	-	moderate
39	PR-LEI-3	round	entire	convex	-	3-4	translucent	-	moderate
40	BRO-PR-1	round	entire	convex	-	7-11	transparent	-	moderate
41	BRO-PR-2	round	entire	convex	-	2.5	transparent	-	moderate
42	BRO-PR-3	round	entire	convex	-	2-3	transparent	-	moderate
43	BRO-PR-4	round	entire	convex	-	3-6	transparent	-	moderate
44	BRO-KPS-5	round	entire	raised	-	2-3.5	translucent	-	moderate
45	BRO-KPS-6	round	entire	convex	-	2-3	transparent	-	moderate

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Table 6 (Continued)

No	Isolates no.	Colonial morphology							
		Shape	Edge	Elevation	Pigment	Colony size (mm)	Opacity	Gram reaction	Slime
46	BRO-KPS-7	round	entire	convex	-	4-6	transparent	-	moderate
47	BKL-LRU-2	round	entire	raised	-	2-3	opaque	-	moderate
48	BKL -LRU-3	round	entire	convex	-	2-4	transparent	-	moderate
49	BKL -LRU-4	round	entire	convex	-	3-4.5	opaque	-	moderate
50	CK-NDD-1	round	entire	convex	-	2-3.5	transparent	-	moderate
51	CK-KS-2	round	entire	convex	-	3-4	transparent	-	moderate
52	CK-KS-3	round	entire	convex	-	3-4	transparent	-	moderate
53	LET-NDD-1	round	entire	convex	-	3-5	transparent	-	moderate
54	LET-NDD-2	round	entire	convex	-	2-3	translucent	-	moderate
55	LET-NDD-3	round	entire	raised	-	2.5-3	opaque	-	moderate
56	CAN-NDD-1	round	entire	convex	-	3.5-5	transparent	-	moderate
57	CAN-NDD-2	round	entire	convex	-	3-5	transparent	-	moderate
58	CAN-ERW-3	round	entire	convex	-	3	transparent	-	moderate
59	CAN-ERW-4	round	entire	convex	-	3-4	transparent	-	moderate
60	CAB-KPS-1	round	entire	convex	-	2-4	opaque	-	moderate

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Table 6 (Continued)

No	Isolates no.	Colonial morphology							
		Shape	Edge	Elevation	Pigment	Colony size (mm)	Opacity	Gram reaction	Slime
61	CAB-KPS-2	round	entire	convex	-	4-6	translucent	-	moderate
62	CAB-KPS-3	round	entire	convex	-	3-4	transparent	-	moderate
63	CAB-KPS-5	round	entire	convex	-	4-6	transparent	-	moderate
64	CHI-KK-1	round	entire	convex	-	3-6	translucent	-	moderate
65	CHI-KK-2	round	entire	convex	-	4-6	transparent	-	moderate
66	CHI-KK-3	irregular	lobate	flat	brown-black	>10	opaque	-	abundant
67	CHI-KS-4	round	entire	raised	-	2.5-3.5	translucent	-	moderate
68	COR-NDD-1	round	entire	raised	-	2	transparent	-	few
69	COR-NDD-3	round	entire	convex	-	5-7	translucent	-	moderate
70	COR-NDD-4	round	entire	convex	-	>10	opaque	-	abundant
71	COR-NDD-5	round	entire	flat	-	2-3	transparent	-	moderate
72	COR-ERW-6	round	entire	convex	-	2-3	transparent	-	moderate
73	WC-KS-1	irregular	lobate	flat	brown-black	>10	opaque	-	abundant
74	SB-KS-1	curled	lobate	flat	cream	>15	opaque	-	abundant
75	SB-KS-2	round	entire	flat	-	4	translucent	-	moderate-abundant

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Table 6 (Continued)

No	Isolates no.	Colonial morphology							
		Shape	Edge	Elevation	Pigment	Colony size (mm)	Opacity	Gram reaction	Slime
76	SB-KS-3	curled	lobate	flat	cream became	>10	opaque	-	abundant
77	SB-KS-4	round	entire	flat	-	5-6	opaque	-	moderate
78	IP-TMK-1	round	entire	raised	-	2-4	opaque	-	moderate
79	IP-TMK-2	round	entire	convex	-	2-4	transparent	-	moderate
80	IP-TMK-3	irregular	entire	flat	-	6-9	translucent	-	abundant
81	IP-TMK-4	round	entire	raised	-	3-5	translucent	-	moderate
82	IP-TMK-6	round	entire	flat	-	3	opaque	-	moderate
83	BM-TMK-1	round	entire	convex	-	2-4	transparent	-	moderate
84	BM-TMK-2	round	entire	convex	-	1-2	translucent	-	few
85	BM-TMK-3	round	entire	flat	-	3-4	opaque	-	moderate
86	BM-TMK-4	round	entire	raised	-	3-4	opaque	-	moderate
87	BIT-TMK-1	irregular	lobate	flat	brown-black	>10	opaque	-	abundant
88	BIT-KS-2	round	entire	convex	-	3-5	translucent	-	moderate
89	CIT-TMK-1	round	entire	raised	-	3-4	opaque	-	moderate
90	CIT-TMK-2	round	entire	convex	-	3-4	opaque	-	moderate

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In this finding, 90 isolates of *Azotobacter* spp. were studied their morphology on N-free Ashby's agar. The result revealed that 90 of *Azotobacter* spp. from various crops could characterize and classify into 6 groups (Table 7-8).

Group 1. Large curled, opaque, lobate and flat colony

Morphological colony of group one was determined. Large curled colony (> 10 mm), lobate edge, flat, with medium as illustrated in Figure 7. Color of colony was white or cream at the beginning stage and became brown or yellowish brown in old culture on N-free Ashby's agar. The colony could not emulsify. Abundant of slime was formed by this organism. The consistency was butyrous type. This organism grew very fast which formed abundant of slime. There were 7 isolate belong to this group as shown in Table 7.

Group 2. Round smooth, translucent, entire edge and raised colony

The colony of this group were small (2-3 mm), round, entire, raised on medium. This bacteria could grow moderately. Pigmentation of colony was translucented in N-free Ashby's agar as illustrated in Figure 7. It produced few to medium slime. Consistency was butyrous. The isolates were screened from rhizosphere of lettuce, coriander, cantonese, chlli, and other crops. There were 21 isolate belong to this group as illustrated in Table 7.

Group 3. Smooth, glistering, round, transparent, entire edge and convex colony

The bacteria had medium size (3-5 mm) colony which was transparent clear, smooth, glistering, round, entire and convex elevation in N-free Ashby's agar as illustrated in Figure 7. This group grew rapidly and produced fewer-medium slime. Consistency was viscid, hard to get off medium. This group of Azotobacter was isolated from Chinese kale, pasture grass, Chinese cabbage, etc. There were 38 isolates belong to this group (Table 7).

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Group 4. Round, opaque, raised colony

In this finding, medium size (4-6 mm) which was opaque, round, entire edge and convex colony in N-free Ashby's agar plate as illustrated in Figure 7 was found by this organism. The growth rate was medium and produced fewer-medium slime. Consistency was viscid, hard to get off medium. This group was isolated from bamboo, pasture grass, cabbage, sugarcane, lychee, lettuce, brockaleli, chilli, *Inula polygonat* and *Baliospermum montanum.* There were 19 isolates belong to this group as illustrated in Table 7.

Group 5. Cream, smooth, curled, lobate and flat colony

The morphological colony was large curled (> 10 mm), rugose surface, lobate edge and flat elevation in N-free Ashby's agar as illustrated in Figure 7. The colony was cream at the beginning and became brown black in old culture. It could produce abundant slime by this group. Consistency was butyrous. This group was isolated from sweet basil, lanzonez. Three isolates were belong to this group viz. BG-ML-1, SB-KS-1 and SB-KS-3 (Table 7).

Group 6. Cinnamon, curled colony, lobate and flat colony

This group is cinnamon (light yellow), large, rough curled, irregular-lobate or undulate, flat colony on agar plate. Small colony sized (2-3 mm) was found with light yellow colony at the beginning stage and became cinnamon or yellowish brown when culture age in N-free Ashby's agar as illustrated in Figure 7. Abundant of slime was produced in this bacterium. Consistency was butyrous. There were 2 isolate belong to this group viz. LAN-NRT-1 and COR-NDD-3 (Table 7).

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Table 7 Group of Azotobacter characterized from morphology on N-free Ashby's agar.

Crop group	Plants	Sample sites	No.	Isolate no.	Group
Flora plants	rose	LRU	1	ROS-LRU-1	2
	gerbera	LRU	2	GER-LRU-1	3
		LRU	3	GER-LRU-2	2
	marigold	KPS	4	MAR-KPS-1	1
		KPS	5	MAR-KPS-2	1
		KPS	6	MAR-KPS-3	2
Field crops	job's tears	PR	7	JT-PR-1	3
	maize	KPS	8	MAI-KPS-1	2
	pineapple	NM	9	PIN-NM-2	3
		NM	10	PIN-NM-3	3
		NM	11	PIN-NM-5	4
	grass	KPS	12	GRA-KPS-1	4
		KPS	13	GRA-KPS-2	2
		KPS	14	GRA-KPS-5	4
		KPS	15	GRA-KPS-6	3
	sugarcane	KPS	16	SUG-KPS-1	2
		KPS	17	SUG-KPS-2	2
		KPS	18	SUG-KPS-3	3
		KPS	19	SUG-KPS-4	4
		KPS	20	SUG-KPS-5	4
		KPS	21	SUG-KPS-6	2
Fruit trees	lychee	ML	22	LIC-ML-1	3
		ML	23	LIC-ML-2	3
		ML	24	LIC-ML-3	4
		ML	25	LIC-ML-4	4
		ML	26	LIC-ML-5	4
	lanzonez	NST	27	LAN-NST-1	1
	burmese grape	ML	28	BG-ML-1	5
		ML	29	BG-ML-2	6
		ML	30	BG-ML-3	3

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Table 7 (Continued)

Crop groups	Plants	Sample sites	No.	Isolate no.	Group
Trees	bamboo	ML	31	BAM-ML-1	4
		ML	32	BAM-ML-2	3
	teak	ML	33	TEA-ML-1	3
	tamarind	NDD	34	TAM-NAD-1	3
		KPS	35	TAM-KPS-2	1
		KPS	36	TAM-KPS-3	2
	para rubber	NDD	37	PR-NDD-1	2
		LEI	38	PR-LEI-2	2
		LEI	39	PR-LEI-3	2
Vegetables	broccoli	PR	40	BRO-PR-1	3
		PR	41	BRO-PR-2	3
		PR	42	BRO-PR-3	3
		PR	43	BRO-PR-4	3
		KPS	44	BRO-KPS-5	4
		KPS	45	BRO-KPS-6	3
		KPS	46	BRO-KPS-7	2
	brockaleli	LRU	47	BKL-LRU-2	3
		LRU	48	BKL-LRU-3	3
		LRU	49	BKL-LRU-4	4
	chinese kale	NDD	50	CK-NDD-1	3
		KS	51	CK-KS-2	3
		KS	52	CK-KS-3	3
	lettuce	NDD	53	LET-NDD-1	4
		NDD	54	LET-NDD-2	2
		NDD	55	LET-NDD-3	3
	cantonese	NDD	56	CAN-NDD-1	2
		NDD	57	CAN-NDD-2	3
		ERW	58	CAN-ERW-3	4
		ERW	59	CAN-ERW-4	3
	cabbage	KPS	60	CAB-KPS-1	3

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Table 7 (Continued)

Crop groups	Plants	Sample sites	No.	Isolate no.	Group
	cabbage	KPS	61	CAB-KPS-2	3
		KPS	62	CAB-KPS-3	2
		KPS	63	CAB-KPS-5	3
	chili	KK	64	CHI-KK-1	4
		KK	65	CHI-KK-2	4
		KK	66	CHI-KK-3	1
		KS	67	CHI-KS-4	2
Vegetables	coriander	NDD	68	COR-NDD-1	3
		NDD	69	COR-NDD-3	3
		NDD	70	COR-NDD-4	3
		NDD	71	COR-NDD-5	3
		ERW	72	COR-ERW-6	3
	water	KS	73	WC-KS-1	1
	sweet basil	KS	74	SB-KS-1	5
		KS	75	SB-KS-2	6
		KS	76	SB-KS-3	5
		KS	77	SB-KS-4	2
	Inula	TMK	78	IP-TMK-1	2
	polygonat	TMK	79	IP-TMK-2	3
		TMK	80	IP-TMK-3	3
		TMK	81	IP-TMK-4	4
		TMK	82	IP-TMK-6	4
	Baliospermum	TMK	83	BM-TMK-1	2
Herbs	*montanum*	TMK	84	BM-TMK-2	3
		TMK	85	BM-TMK-3	4
		TMK	86	BM-TMK-4	4
	bitter gourd	TMK	87	BIT-TMK-1	1
		KS	88	BIT-KS-2	2
	citronella grass	TMK	89	CIT-TMK-1	3
		TMK	90	CIT-TMK-2	3

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Table 8 Characteristics of 90 isolates of *Azotobacter* spp. from 29 various crops growing on N-free Ashby's agar for 7 days

Group	Colonial Morphology	Colony Size (mm)	Growth rate on agar	Pigmentation	Homopoly-saccharide (slime)	Consistency	Gram reaction (+/-)	No.of Isolates
1	large curled, lobate, flat	>10	quickly	opaque/cream become brown when culture age	abundant	bytyrous	-	7
2	smooth, round, entire, opacity: translucent, elevation: raised	2-3	medium	translucent (alomost clear, but distorted vision)	none	bytyrous	-	21
3	smooth, round, entire, transparent, convex	3-5	quickly	transparent, glistering	few	viscid	-	38
4	smooth round, opaque, entire, medium raised	4-6	medium	opaque /white cream and become cinnamon when culture age	medium	viscid	-	19
5	smooth curled, irregular: lobate, flat	>15	medium-quickly	opaque become brown culture age	abundant	bytyrous	-	3
6	light yellow/cinnamon, curled colony, fobate, flat elevation	2-3	medium-quickly	cream/cinnamon become yellowish when culture age	abundant	bytyrous	-	2

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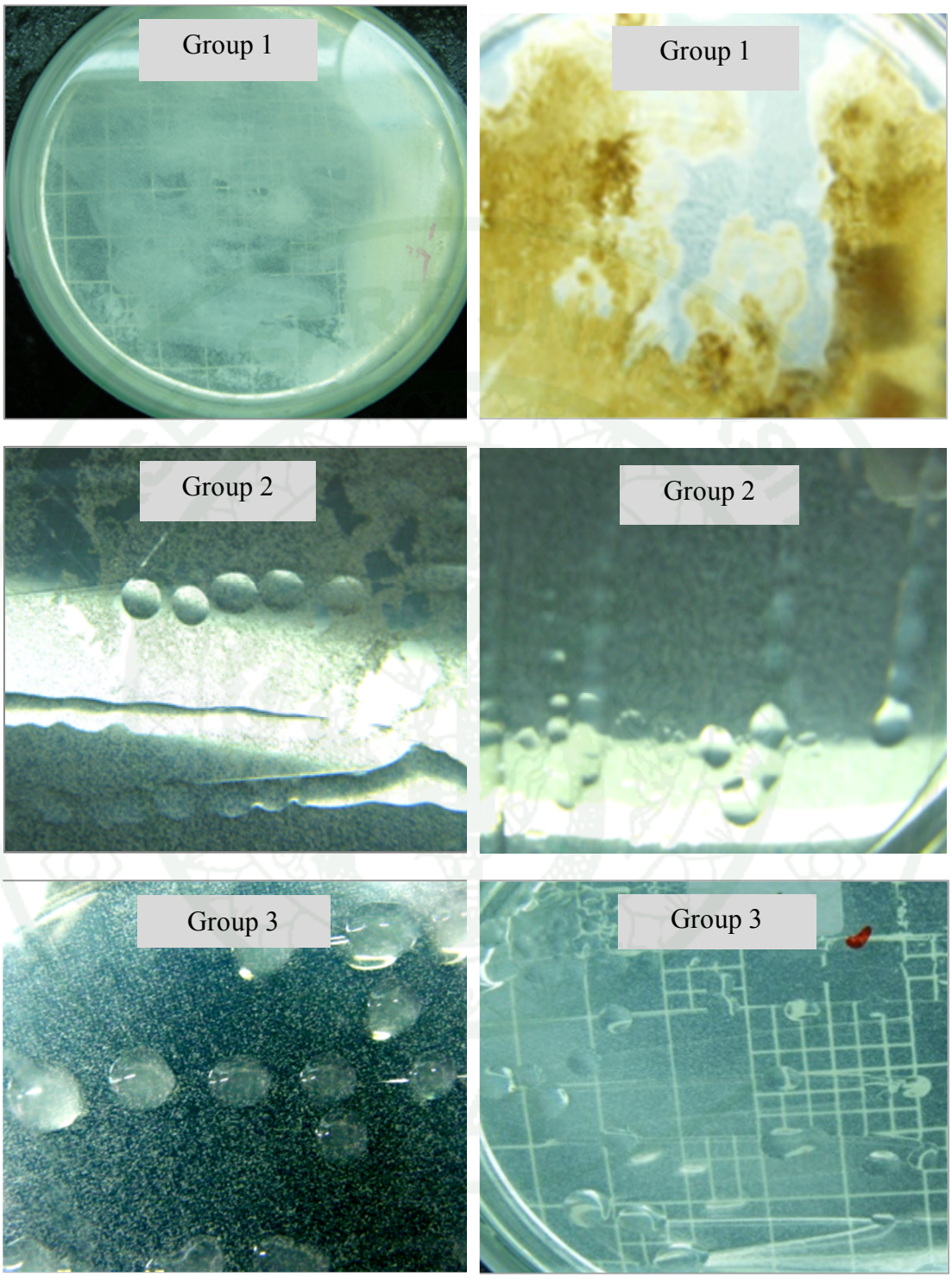

Figure 7 Colonial characteristics of Azotobacter groups.

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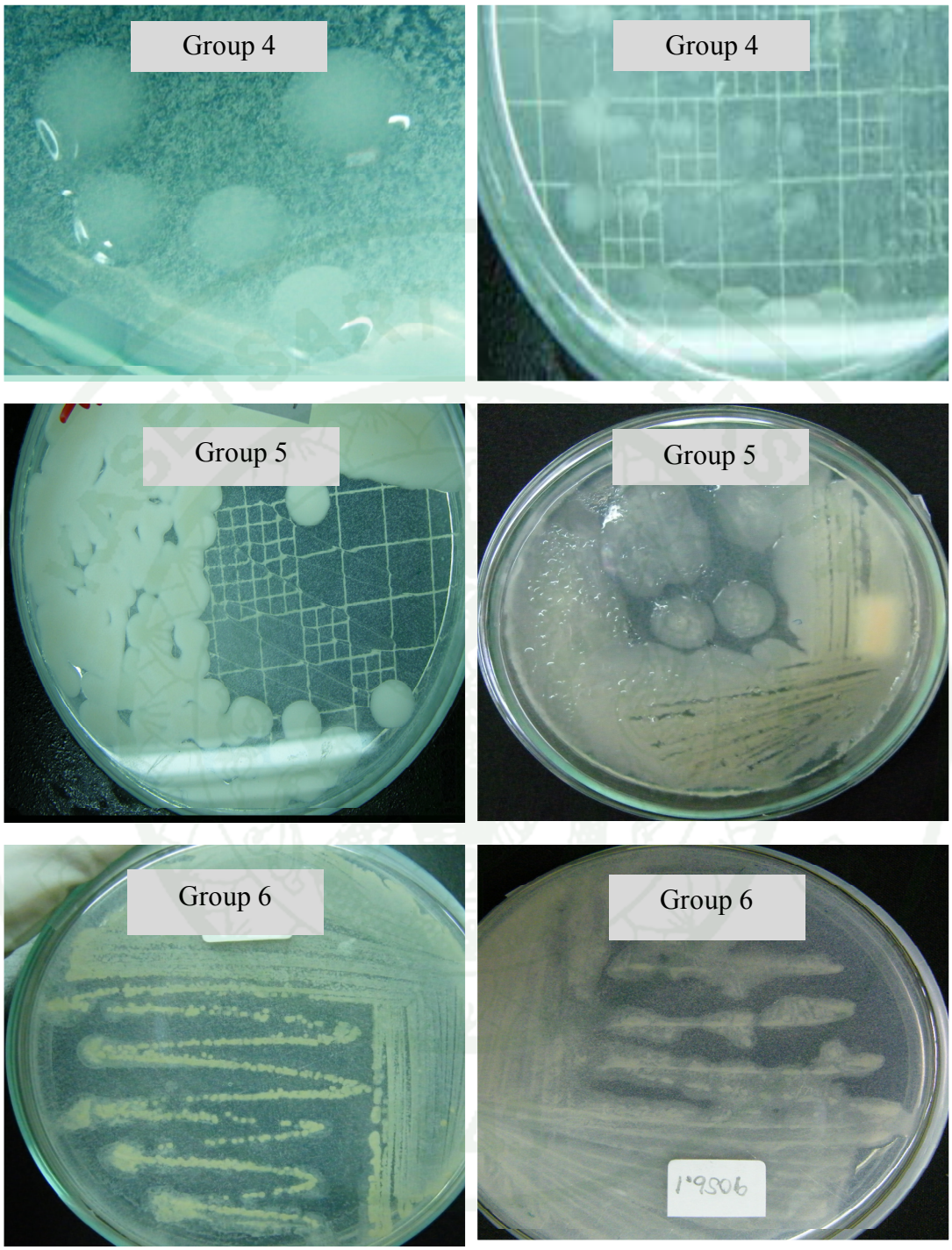

Figure 7 (Continued)

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Experiment II. Screening of high efficiency strains of various Azotobacter isolates in the laboratory

1. Screening of high efficiency strains of various *Azotobacter* spp.

1.1 Nitrogen fixation of various Azotobacter isolates

The capability of nitrogen fixation among 90 isolates of *Azotobacter* spp. screened from the rhizosphere of twenty-nine crops was examined (Table 9). There was a highly significant effect on nitrogen fixation presented as fixed-N among 90 isolates. The fixed-N was in the range of 4.0-6.5 mg/L. The results revealed that isolates no. CK-KS-2, CK-KS-3, CK-NDD-1 and TAM-NDD-1 had the highest of 6.5 mg/L, followed by LET-NDD-1 similar to those of CHI-KS-4, LIC-ML-1, PR-LEI-2, TAM-KPS-2, SUG-KPS-6, BG-ML-2, CHI-KK-2 and LET-NDD-2, respectively which were higher than the rest (77 isolates). This finding suggested that 27 isolates had the lowest fixed-N (4.0 mg/L) including BRO-PR-4, CAN-ERW-3, KPS-GRA-5, KPS-GRA-4, SUG-KPS-2, etc.

1.2 The quantity of IAA production by various Azotobacter isolates

The results show that the quantity of IAA produced by various Azotobacter isolates was highly statistical difference ($P< 0.0001$) (Table 9). The potential of IAA synthesis by these organisms ranged from 0 to 44.87 µg/ml. Our study established that PR-ND D-1 was the highest of IAA production at 44.87 µg/ml, higher than those of BRO-KPS-6, CAN-ERW-4, BRO-KPS-7, and COR-ERW-6 (41.65, 41.17, 40.70, and 40.23 µg/ml, respectively) which were also higher than those of the other 85 isolates. Whereas, KPS-SUG-4, KPS-GRA-1, KPS-GRA-2, KPS-GRA-5, and NM-PIN-5 could not detect IAA in the culture.

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Table 9 Performance testing of N-fixation, IAA and phosphate solubilization of 90 isolates *Azotobacter* spp.

No.	Plants	Isolate No.	Fixed-N (mg/L)	IAA production (µg/ml)	Phosphate solubilization
1	rose	ROS-LRU-1	4.0d	2.72 y-A	+
2	gerbera	GER-LRU-1	4.5cd	36.01 f-l	-
3		GER-LRU-2	4.5cd	37.98 d-g	-
4	marigold	MAR-KPS-1	4.0d	36.38 e-k	-
5		MAR-KPS-2	4.0d	1.96 z-B	-
6		MAR-KPS-3	4.0d	33.80 l-n	-
7	job's tears	JT-PR-1	4.5cd	0.60 AB	+
8	maize	MAI-KPS-1	4.0d	38.59 c-e	+
9	pineapple	PIN-NM-2	4.0d	0.58 AB	-
10		PIN-NM-3	4.0d	0 B	-
11		PIN-NM-5	5.0b-d	0 B	-
12	grass	GRA-KPS-1	4.5cd	0 B	-
13		GRA-KPS-2	4.0d	0 B	-
14		GRA-KPS-5	4.0d	0 B	-
15		GRA-KPS-6	4.5cd	37.30 e-j	-
16	sugarcane	SUG-KPS-1	5.0b-d	0.34 B	+
17		SUG-KPS-2	4.0d	2.38 z-B	-
18		SUG-KPS-3	4.5cd	0 B	+
19		SUG-KPS-4	4.0d	0 B	+
20		SUG-KPS-5	5.0b-d	7.19 v	-
21		SUG-KPS-6	5.5a-c	0.75 AB	-
22	lychee	LIC-ML-1	5.5a-c	19.82 s	+
23		LIC-ML-2	5.0b-d	2.13 z-B	-
24		LIC-ML-3	4.5cd	1.15 AB	-
25		LIC-ML-4	4.5cd	35.67 g-l	-
26		LIC-ML-5	4.5cd	5.97 vw	-
27	lanzonez	LAN-NST-1	4.5cd	16.98 t	-
28	Burmese	BG-ML-1	5.0b-d	4.79 v-x	-
29	grape	BG-ML-2	5.5a-c	1.23 AB	-
30		BG-ML-3	4.5cd	26.75 q	+

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$ (a-z-AB).

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Table 9 (Continued)

No.	Plants	Isolate No.	Fixed-N (mg/L)	IAA Production (µg/ml)	Phosphate solubilization
31	bamboo	BAM-ML-1	4.0d	0 B	-
32		BAM-ML-2	4.0d	0.61 AB	-
33	teak	TEA-ML-1	4.5cd	0 B	-
34	tamarind	TAM-NAD-1	6.5a	5.16 v-x	-
35		TAM-KPS-2	5.5a-c	5.14 v-x	-
36		TAM-KPS-3	5.0b-d	37.14 e-k	-
37	para rubber	PR-NDD-1	5.0b-d	44.865 a	+
38		PR-LEI-2	5.5a-c	0 B	+
39		PR-LEI-3	4.5cd	1.26 ab	+
40	broccoli	BRO-PR-1	4.0d	0 B	-
41		BRO-PR-2	4.5cd	36.29 f-k	+
42		BRO-PR-3	4.5cd	36.52 e-k	-
43		BRO-PR-4	4.0d	35.30 i-m	-
44		BRO-KPS-5	4.5cd	36.17 f-k	-
45		BRO-KPS-6	4.5cd	41.65 b	-
46		BRO-KPS-7	4.0d	40.695 b	+
47	brockaleli	BKL-LRU-2	4.0d	35.60 h-l	-
48		BKL-LRU-3	4.5cd	1.17 AB	+
49		BKL-LRU-4	4.0d	35.78 g-l	-
50	Chinese kale	CK-NDD-1	6.5a	37.74 e-h	+
51		CK-KS-2	6.5a	35.61 h-l	-
52		CK-KS-3	6.5a	36.08 f-k	-
53	lettuce	LET-NDD-1	6.0ab	30.64 o	-
54		LET-NDD-2	5.5a-c	1.36 AB	-
55		LET-NDD-3	5.0b-d	37.60 e-i	-
56	cantonese	CAN-NDD-1	5.0b-d	32.92 n	+
57		CAN-NDD-2	5.0b-d	34.85 k-n	+
58		CAN-ERW-3	4.0d	36.49 e-k	+
59		CAN-ERW-4	4.5cd	41.165 b	+
60	cabbage	CAB-KPS-1	5.0b-d	1.13 AB	-

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$ (a-z-AB).

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Table 9 (Continued)

No.	Plants	Isolate No.	Fixed-N (mg/L)	IAA Production (µg/ml)	Phosphate solubilization
61		CAB-KPS-2	5.0b-d	11.33 u	-
62		CAB-KPS-3	4.5cd	0.63 AB	-
63		CAB-KPS-5	4.5cd	1.87 z-B	-
64	chili	CHI-KK-1	4.5cd	39.92 b-d	+
65		CHI-KK-2	5.5a-c	33.23 mn	-
66		CHI-KK-3	5.0b-d	0	+
67		CHI-KS-4	5.5a-c	1.09 AB	+
68	coriander	COR-NDD-1	4.5cd	2.92 y-A	+
69		COR-NDD-3	4.5cd	0 B	+
70		COR-NDD-4	5.0b-d	3.81 x-z	+
71		COR-NDD-5	4.5cd	0 B	-
72		COR-ERW-6	4.5cd	40.225 bc	+
73	water	WC-KS-1	5.0b-d	30.28 o	+
74	sweet basil	SB-KS-1	4.0d	0 B	-
75		SB-KS-2	4.5cd	26.48 q	-
76		SB-KS-3	4.0d	35.46 h-l	-
77		SB-KS-4	4.5cd	23.42 r	-
78	*Inula*	IP-TMK-1	4.5cd	12.56 u	+
79	*polygonata*	IP-TMK-2	4.0d	35.20 j-m	-
80	DC.	IP-TMK-3	4.5cd	38.32 c-f	+
81		IP-TMK-4	4.0d	29.16 op	+
82		IP-TMK-6	4.5cd	28.23 op	-
83	*Baliospermum*	BM-TMK-1	4.0d	37.42 e-j	-
84	*montanum*	BM-TMK-2	4.0d	2.88 y-A	-
85		BM-TMK-3	4.5cd	37.14 e-k	-
86		BM-TMK-4	4.0d	35.86 g-l	-
87	bitter gourd	BIT-TMK-1	4.5cd	11.64 u	+
88		BIT-KS-2	4.5cd	0.08 B	-
89	citronella grass	CIT-TMK-1	4.0d	1.83 z-B	-
90		CIT-TMK-2	4.0d	0 B	-
	Mean		4.62	17.70	-
	P-value		**	**	-
	CV(%)		14.95	5.64	-

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$ (a-z-AB).

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1.3 Phosphate solubilization of various Azotobacter isolates on Pikovskaya medium

Ninety isolates of Azotobacter were streaked on Pikovskaya's agar for studying of solubility of tri-calcium phosphate (Table 9). It was found that 31 isolates (33.3%) of all isolates could dissolve tri-calcium phosphate resulting in a clear zone (positive result) in Pikovskaya agar viz. ROS-LRU-1, JT-PR-1, MAI-KPS-1, SUG-KPS-1, SUG-KPS-3, SUG-KPS-4, LIC-ML-1, BG-ML-3, PR-NDD-1, PR-LEI-2, PR-LEI-3, BRO-PR-2, BRO-KPS-7, BKL-LRU-3, CK-NDD-1, CAN-NDD-1, CAN-NDD-2, CAN-ERW-3, CAN-ERW-4, CHI-KK-1, CHI-KK-3, CHI-KS-4, COR-NDD-1, COR-NDD-3, COR-NDD-4, COR-ERW-6, WC-KS-1, IP-TMK-1, IP-TMK-3, IP-TMK-4, and BIT-TMK-1, respectively. Whereas 59 isolates did not solute tricalcium phosphate, resulting in a negative result.

A B C

Figure 8 IAA production of various Azotobacter culturing in Ashby's medium: developing of pink color (A and C) showed IAA production, whereas non-pink color (B) showed the negative result.

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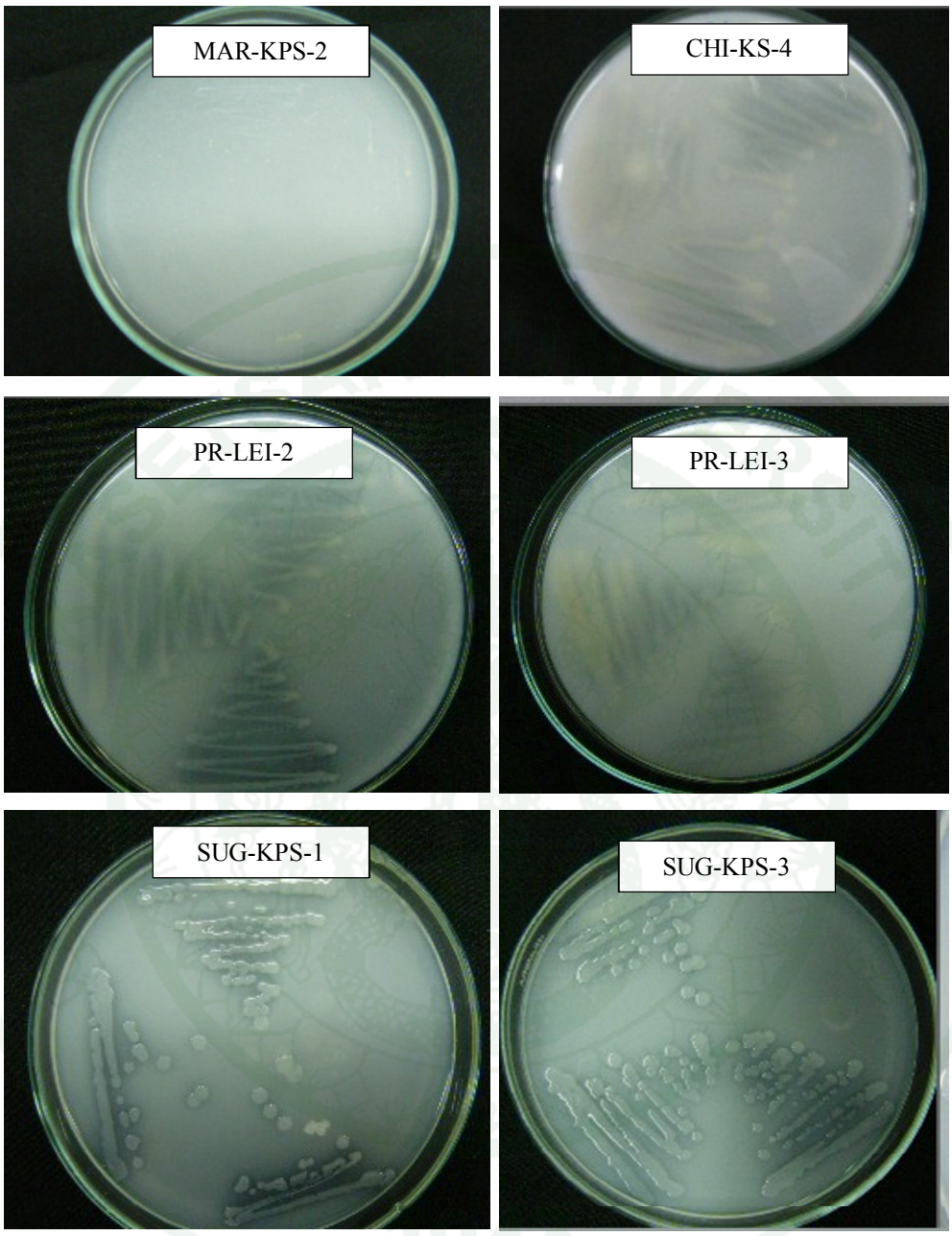

Figure 9 Phosphate solubilization of various Azotobacter showed clear zone (positive result) around the streak line on Pikovskaya's medium, except MAR-KPS-2 showed negative result (non-clear zone).

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Based on colony size, a bacterium with larger colony indicates the faster growing strains. In this finding, 44 isolates of 90 isolates had a larger colony than those of others which were continued to screen as the fast growing bacterium by streaking method on N-free Ashby's agar and analyzed the statistical difference. The result revealed that there was a highly difference on colony size among 44 isolates ($P<.0001$) as shown in Table 10. The data presented here showed that BRO-KPS-5 had the largest colony (5.67 cm) similar to those of PR-LEI-3 (5.50 cm) and IP-TMK-3 (5.33 cm), whereas WC-KS-1 (1.27. cm) was the lowest in colony size similar to those of BM-TMK-2 (1.50 cm), TAM-NAD-1 (1.67 cm) and BRO-PR-3 (1.70 cm), respectively.

For the criteria of high effective strains after screening the fast growing isolates, including 1) selection of high performance isolates viz. potential of nitrogen fixation (fixed-N), IAA production and phosphate solubilization (Table 9) and 2) the size of colony indicating the fast growing strains were combined (Tables 10). Eight of forty-four isolates of *Azotobacter* spp., viz. BRO-KPS-5, CHI-KK-2, CK-KS-2, CK-KS-3, CK-NDD-1, LET-NDD-1, LET-NDD-3 and PR-NDD-1 were selected for further experiment (Table 11).

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Table 10 Colony diameters of various isolates of Azotobacter on N-free Ashby's agar at 10 days after streaking.

No.	Isolate no.	Diameter of colony (cm)	No.	Isolate no.	Diameter of colony (cm)
1	CK-KS-3	4.00b-d	23	BRO-PR-3	1.70pq
2	CK-KS-2	3.37e-k	24	GER-LRU-2	2.83i-o
3	CK-NDD-1	2.25op	25	CAN-ERW-4	2.50m-o
4	TAM-NAD-1	1.67pq	26	IP-TMK-3	5.33a
5	LET-NDD-1	4.00b-e	27	IP-TMK-6	3.00h-n
6	LIC-ML-1	3.17f-m	28	SB-KS-4	4.00b-e
7	CHI-KK-2	3.60d-g	29	COR-ERW-6	3.80c-f
8	PR-NDD-1	3.50d-i	30	BKL-LRU-4	2.70k-o
9	LET-NDD-3	4.00b-e	31	BKL-LRU-2	2.75j-o
10	CAN-NDD-2	2.40n-o	32	MAR-KPS-3	2.25op
11	CAN-NDD-1	2.53l-o	33	BRO-PR-4	4.67b
12	WC-KS-1	1.27q	34	BRO-KPS-7	4.17c-d
13	TAM-KPS-3	2.83i-o	35	MAR-KPS-1	3.00h-n
14	CHI-KK-1	3.50d-i	36	SB-KS-3	2.67k-o
15	LIC-ML-4	2.40n-o	37	MAI-KPS-1	3.10g-n
16	GER-LRU-1	3.25f-l	38	IP-TMK-2	3.83c-f
17	BM-TMK-3	3.83c-f	39	IP-TMK-4	4.33bc
18	BRO-KPS-5	5.67a	40	BM-TMK-1	3.43e-j
19	SB-KS-2	2.60l-o	41	BM-TMK-2	1.50q
20	GRA-KPS-6	3.00h-n	42	CAN-ERW-3	3.00h-n
21	BRO-PR-2	4.00b-e	43	BM-TMK-4	2.60l-o
22	BRO-KPS-6	2.73j-o	44	PR-LEI-3	5.50a
	Mean			3.233	
	P-value			<.0001	
	CV (%)			11.51	

Remark: Values in each column followed by the same letter are not significantly different by DMRT at $P \leq .01$.

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Table 11 Potential and colony size of eight effective Azotobacter isolates after steak on N-free Ashby's agar for 10 days.

No.	Isolates no.	Fixed-N (mg/L)	IAA production (µg/ml)	Phosphate solubilization
1	CK-KS-2	6.5	35.61	-
2	CK-KS-3	6.5	36.08	-
3	CK-NDD-1	6.5	37.74	+
4	LET-NDD-1	6.0	30.64	-
5	CHI-KK-2	5.5	33.23	-
6	PR-NDD-1	5.0	44.865	+
7	LET-NDD-3	5.0	37.60	-
8	BRO-KPS-5	4.5	36.17	-

2. Study the characteristics and classification of high effective isolates of Azotobacter

2.1 Growth and morphological characteristics of high effective isolates of *Azotobacter* spp. on N-free Ashby's agar

Every isolate of Azotobacter was streaked in N-free Ashby's agar and incubated at room temperature for 7-10 days for the colonial morphology, carbon source, gram straining reaction, pigmentation, slime production and consistency for species classified level were determined using Bergey's manual of determinative bacteriology (Holt, *et al*., 2000) (Figures 10-11, Tables 12 and 13).

1) Azotobacter isolate no. BRO-KPS-5

BRO-KPS-5 was isolated from soil under growing broccoli at the field laboratory at Kasetsart University, Kamphang Saen campus, Nakhon Pathom province. Characteristics of this bacterium were shown in Tables 12 and 13. It is a gram-negative bacteria with large ovoid cells of 2-3.5 µm in diameter under

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microscopic. After growing on N-free Ashby's agar, the colony size is 2-3.5 mm in diameter after incubation for 7-10 days. Smooth, transparent, round shape, entire edge and raised elevation was exhibited by this isolate. Little capsular slime is formed. The consistency is viscid. Moreover, rhamnose, caproate and mannitol were utilized as C-source by this organism. According to Bergey's manual of determinative bacteriology, BRO-KPS-5 was classified as *Azotobacter vinelandii.*

2) Azotobacter isolate no. CHI-KK-2

The characteristics of CHI-KK-2 are described in Table 12 and 13. It was isolated from red-chili rhizospheric soil at Khong Khwang subdistict, Sainoi district, Nonthaburi province. A large ovoid cell was 1-1.5 μm in diameter. Stanning reaction showed gram negative bacteria. It had a large colony sized 4-6 mm during the 7-10 days on N-free Ashby's agar. Green smooth glistening, round, entire edge with raised elevation on N-free Ashby's agar. This isolate produced few polysaccharides/slime on agar. The consistency of bacteria was mucoid. It was able to utilize rhamnose, caproate and mannitol as C-source for growth. According to Bergey's manual of determinative bacteriology and characteristics of colony, this isolate was classified as *Azotobacter vinelandii*.

3) Azotobacter isolate no. CK-NDD-1

This microorganism was isolated from Chinese kale rhizosphere at Na Din Dam village, Loei province. It is gram negative bacterium. Ovoid cell of 1-1.5 μm in diameter was exhibited. After growing on N-free Ashby's agar, colony size was 2-3.5 mm in diameter. The colony was round green smooth, glistening, entire edge and raised colony on agar plate. This isolate produced polysaccharides/slime. The consistency of the colony was viscid, sticks to loop and hard to get off. This organism was capable to utilize rhamnose and mannitol as C-source for its growth. According to Bergey's manual of determinative bacteriology, this bacterium was identified as *Azotobacter vinelandii* (Table 12 and 13).

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4) Azotobacter isolate no. CK-KS-2

This strain was first isolated from the rhizosphere of Chinese kale soil at Khunsri village, Sainoi district, Nonthaburi province. This is a gram negative bacterium which had large oval rods of 2-3 μm in diameter. Colony size was 3-4 mm in diameter after inoculated on N-free Ashby's agar. The colony was green, smooth glistening by changing color in reflected light, round shape, entire edge and convex elevation. Polysaccharides/slime was produced. The consistency of the colony was viscid. This strain could utilize rhamnose and mannitol as C-source for bacterial growth. According to Bergey's manual of determinative bacteriology and the morphology, this organism was identified as *Azotobacter vinelandii* (Tables 12 and 13).

5) Azotobacter isolate no. CK-KS-3

This bacterium was isolated from Chinese kale soil at Khunsri village, Sainoi district, Nonthaburi province. It showed gram negative property. The cell shape was small ovoid cells with diameter of 1-1.5 μm. Colony size was 3-4 mm in diameter after growing for 7-10 days on N-free Ashby's medium. The colony surface was green smooth round, glistening, entire edge and raised elevation on the agar. Little slime was found. The consistency of the bacterium was viscid which stuck to loop and was hard to get off. This isolate could utilize rhamnose and mannitol as C-source for bacterial growth. According to Bergey's manual of determinative bacteriology, this bacterium was identified to *Azotobacter vinelandIi* (Tables 12 and 13).

6) Azotobacter isolate no. LET-NDD-1

LET-NDD-1 was isolated from lettuce rhizosphere soil at Na Din Dam village. It was gram negative bacterium. The cell shape was oval with a diameter of 1-1.5 μm, whereas colony size was 3-5 mm in diameter after inoculate on N-free Ashby's agar for 7-10 days. The colony was round, smooth, transparent and

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raised colony on the agar. Few polysaccharides/slime was found by this isolate. The consistency of the bacterium was strong with mucoid type. This strain could utilize mannitol as C-source for bacterial growth. According to Bergey's manual of determinative bacteriology, this bacterium was identified as *Azotobacter beijerinckii* (Tables 12 and 13).

7. Azotobacter isolate no. LET-NDD-3

Characteristics of isolates no. LET-NDD-3 is shown in Table 12 and 13. This strain was first isolated from rhizosphere of lettuce, Na Din Dam village, Loei province. Strain reaction was gram negative. The cell shape was ovoid cells of 1-1.25 μm in diameter under the microscopic. The colony size of 2.0-3.0 in diameter appeared after incubation for 7-10 days on N-free Ashby's agar. Transparent, round, smoothing colony with entire edge and raised colony was found. This bacterium could produce few to medium polysaccharides. The consistency was mucoid. This isolate utilized mannitol and caproate as C-source for bacterial growth, except rhamnose. According to Bergey's manual of determinative bacteriology, this bacterium was classified into *Azotobacter chroococcum*.

8. Azotobacter isolate no. PR-NDD-1

This bacterium was screened from Para rubber soil at Na Din Dam village, Loei province. This was gram-negative bacteria which had large ovoid cells of 1.5-2.0 μm diameter under microscopy. Colony size of 3-4.5 mm in diameter was formed on N-free Ashby's agar. It had green smooth, round, glistening, entire edge, with raised colony on the medium. This isolate produced abundant slime. The consistency of visible colony was viscous and sticks to loop. This microorganism could utilize rhamnose and mannitol as C-source for the growing. According to Bergey's manual of determinative bacteriology, it was classified as *Azotobacter vinelandii* (Table 12 and 13).

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Table 12 Colonial morphology of various isolates of Azotobacter.

No.	Isolate no.	Shape of cell	Colonial morphology							Micro-morphology		
			Cell Size (µm)	Diffusible	Colony Size (mm)	Opacity	Edge	Shape	Elevation	Gram reaction	Slime	Consistency
1	BRO-KPS-5	large ovoid	2.25	-	2-3.5	transparent	entire	round	raised	-	few	viscid
2	CHI-KK-2	ovoid	1-1.25	-	4-6	translucent	entire	round	convex	-	few-moderate	mucoid
3	CK-NDD-1	ovoid	1-1.25	-	2-3.5	transparent	entire	round	convex	-	few	viscid
4	CK-KS-2	large ovoid	2-3	-	3-4	transparent	entire	round	convex	-	few	viscid
5	CK-KS-3	large ovoid	1-1.5	-	3-4	transparent	entire	round	convex	-	few	viscid
6	LET-NDD-1	ovoid	1-1.5	-	3-5	translucent	entire	round	convex	-	few	viscid
7	LET-NDD-3	ovoid	1-1.25	-	2.5-3	opaque	entire	round	raised	-	few	mucoid
8	PR-NDD-1	large ovoid	1-1.5	-	3-4.5	translucent	entire	round	convex	-	few-moderate	mucoid

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Table 13 The utilization of C-source of various isolates of Azotobacter from various crops.

No.	Isolaten no.	Reference strains	Origins	Pigmentation	Gram-reaction	Utilization as carbon source				
						Rhamnose	Caproate	Caprylate	Mannitol	Malonate
1	BRO-KPS-5	*A. vinelandii*	broccoli	green fluorescent	-	+	+	-	+	-
2	CHI-KK-2	*A. vinelandii*	chili	green fluorescent	-	+	+	-	+	+
3	CK-NDD-1	*A. vinelandii*	Chinese kale	green fluorescent	-	+	-	-	+	-
4	CK-KS-2	*A. vinelandii*	Chinese kale	green fluorescent	-	+	-	-	+	-
5	CK-KS-3	*A. vinelandii*	Chinese kale	green fluorescent	-	+	-	-	+	-
6	LET-NDD-1	*A. beijerinckii*	lettuce	-	-	-	-	-	+	+
7	LET-NDD-3	*A. chroococcum*	lettuce	-	-	-	+	-	+	-
8	PR-NDD-1	*A. vinelandii*	para rubber	green fluorescent	-	+	-	-	+	-

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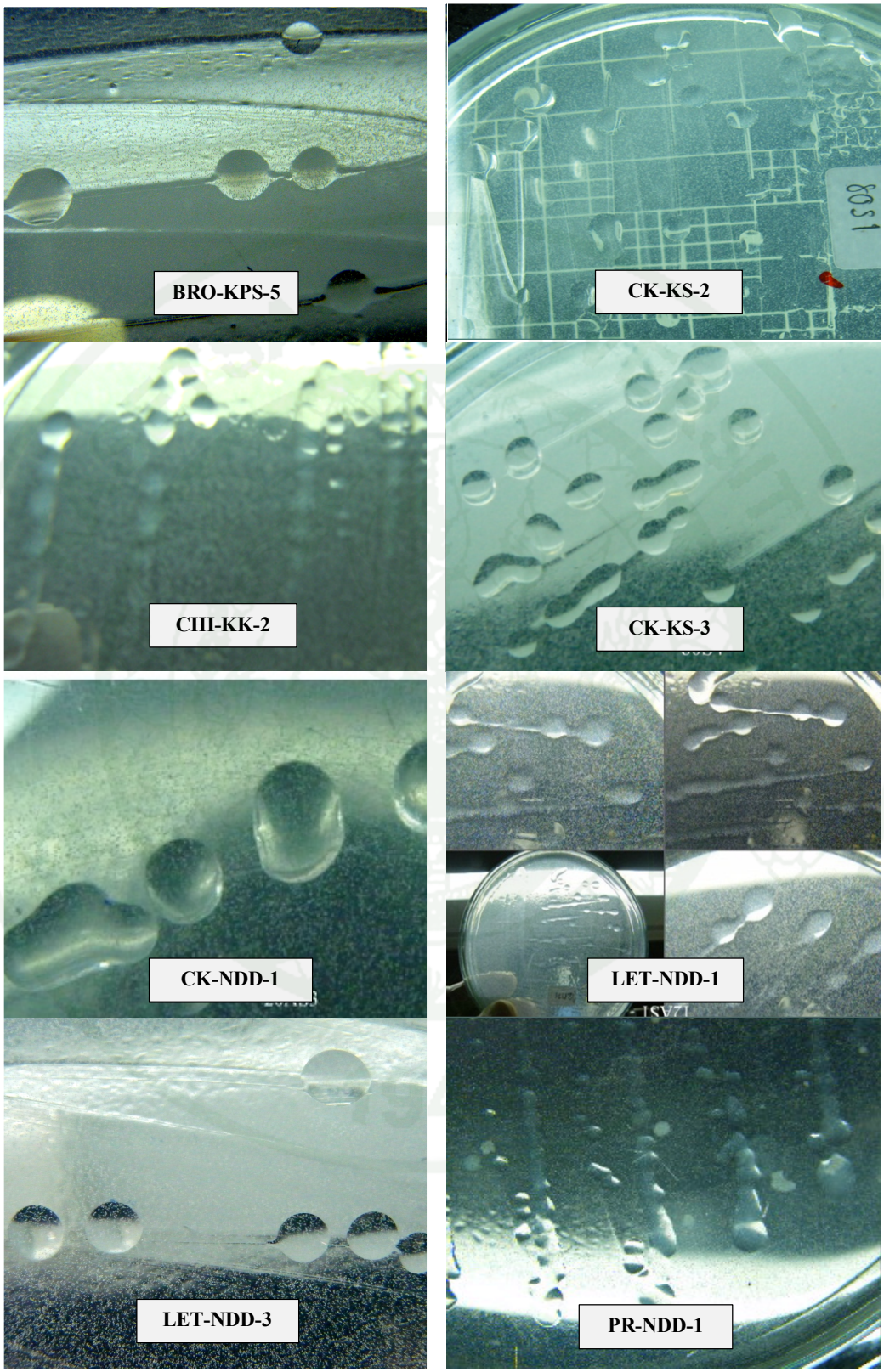

Figure 10 Colonial morphology of various isolates of Azotobacter on N-free Ashby's agar.

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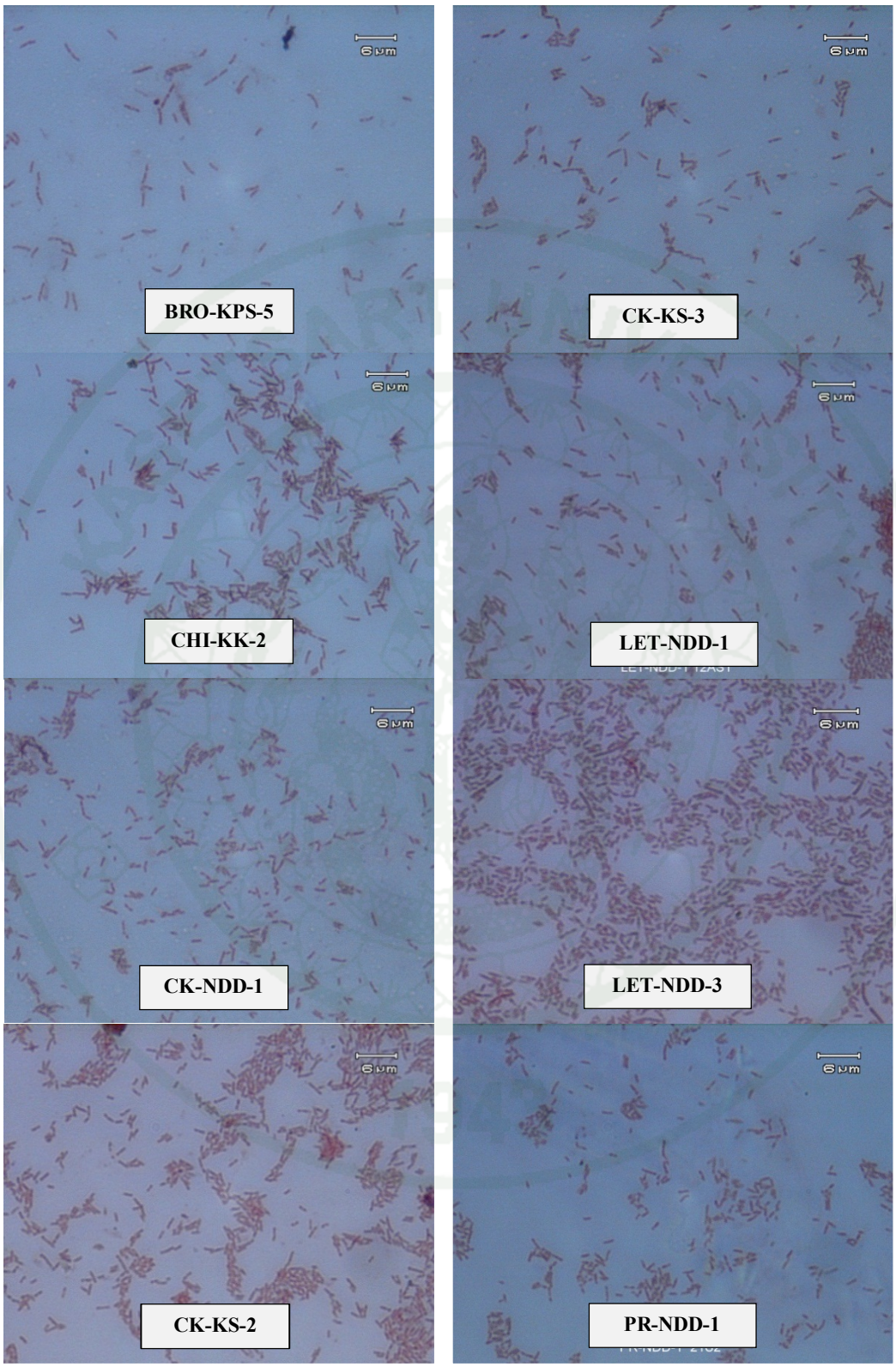

Figure 11 Gram negative strain of various isolates of *Azotobacter* spp. under a light microscope (100x).

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2.2 Growth of high potential isolates in Ashby's broth

Growth rate of high potential isolates are shown in Table 14. The populations of these bacteria were varied depending on species (Figure 13). During 24 hours, *Azotobacter vinelandii* PR-NDD-1 tend to have the highest cell numbers (9.22 log cfu/ml) followed by *A. vinelandii* CK-NDD-1 and those of the others. The microbial densities gradually increased to the highest point at 48 hours. In this finding, the highest total viable counts were observed at *A. beijerinckii* LET-NDD-1 (11.02 log cfu/ml), whereas *A. vinelandii* BRO-KPS-5 showed the lowest in cell count (6.02 log cfu/ml). During 60 hours, proliferation of various *Azotobacter* spp. slightly dropped and decreased to the lowest bacterial cells at 72 hours (9.06 log cfu/ml). This finding suggests that *A. vinelandii* CK-NDD-1 (9.69 log cfu/ml) had the highest growth followed by CHI-KK-2 (9.37 log cfu/ml), and *A. vinelandii* PR-NDD-1 (9.37 log cfu/ml), whereas *A. vinelandii* BRO-KPS-5 showed the lowest growth rate (7.48 log cfu/ml) by comparison with those of others during incubation for 72 hours.

Table 14 Growth of 8 high efficiency isolates of *Azotobacter* spp. inoculated in Ashby's broth adding 1 g of NH_4Cl under continuous culture for 72 hours.

No.	Isolates	Population density (log cfu/ml)			
		24 hrs	48 hrs	60 hrs	72 hrs
1	*A. vinelandii* BRO-KPS-5	5.30	6.02	7.26	7.48
2	*A. vinelandii* CHI-KK-2	8.60	9.26	9.30	9.37
3	*A. vinelandii* CK-KS-2	9.02	9.25	9.16	9.06
4	*A. vinelandii* CK-KS-3	7.23	8.83	9.16	9.11
5	*A. vinelandii* CK-NDD-1	9.22	9.67	9.63	9.69
6	*A. beijerinckii* LET-NDD-1	9.00	11.02	9.57	9.24
7	*A. chroococcum* LET-NDD-3	9.16	9.33	9.00	9.13
8	*A. vinelandii* PR-NDD-1	9.22	9.36	9.39	9.37

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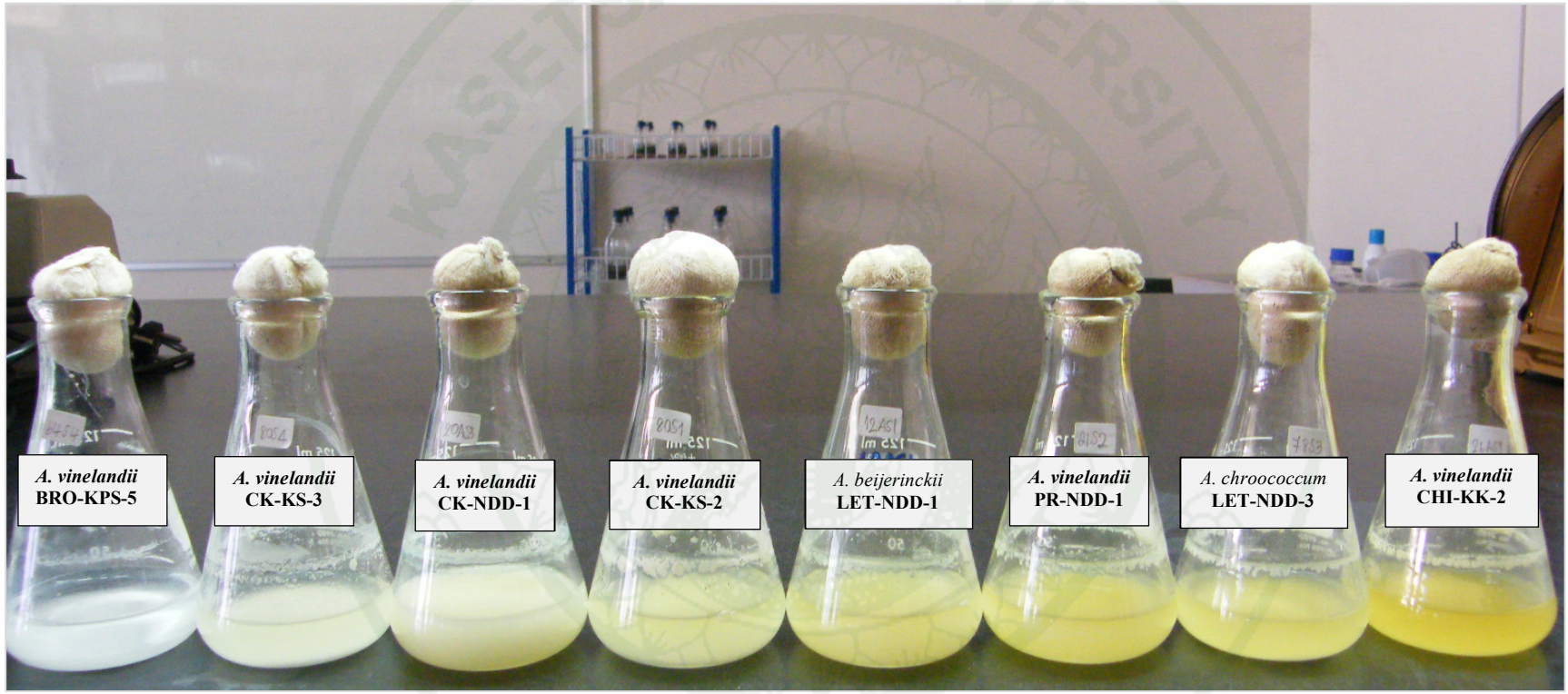

Figure 12 Growth rate determination of various strains of Azotobacter in Ashby's broth adding 1g of NH_4Cl under continuous condition for 72 hours.

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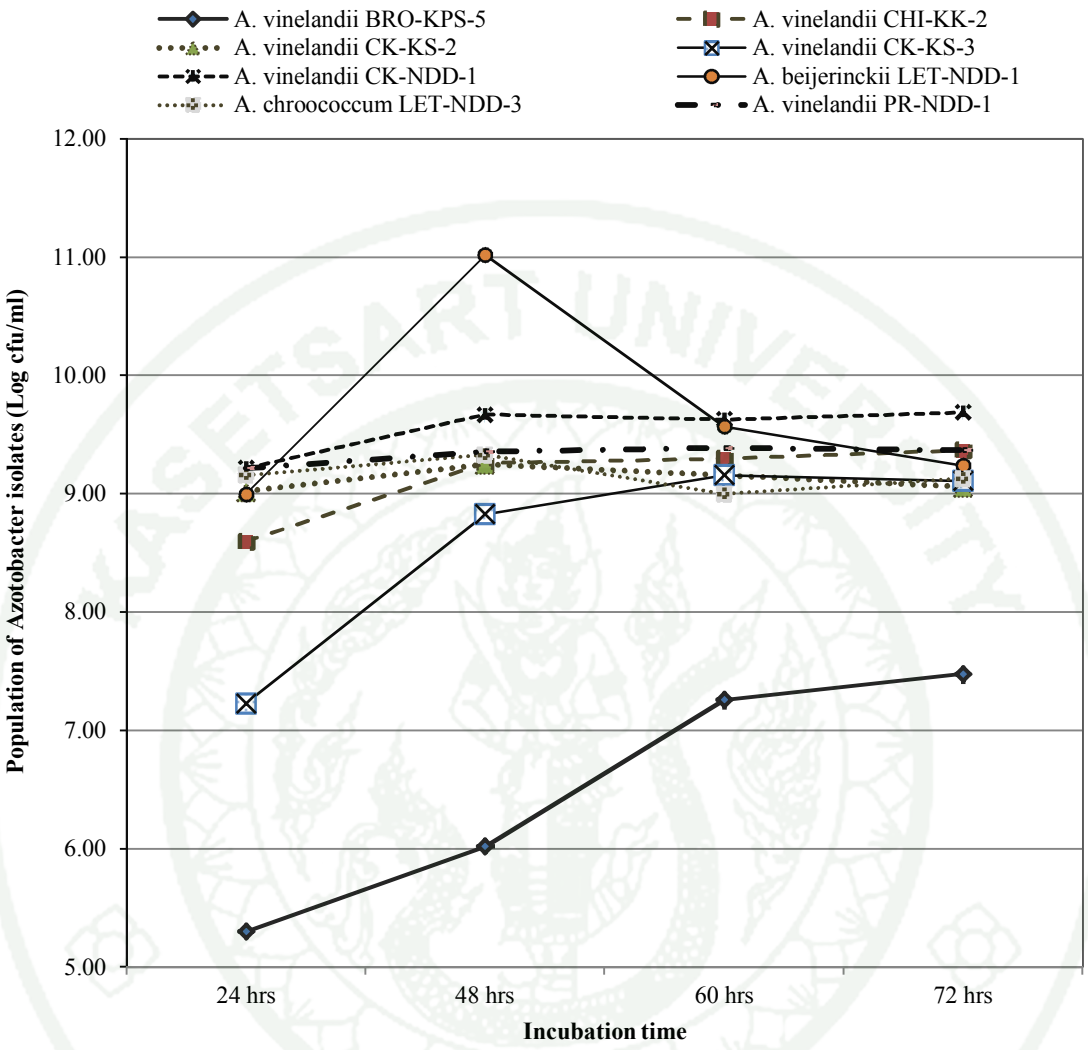

Figure 13 Growth of various *Azotobacter* spp. in Ashby's broth adding 1 g of NH_4Cl under continuous culture for 72 hours.

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Experiment III. Effect of various carriers from agricultural wastes and temperatures on the survival of Azotobacter in the inoculum

1. The properties of various carriers

Some chemical properties of the carriers are shown in Table 15. There was a signigificantly difference among chemical properties of the carriers. Total N of the carriers were considered low. PtMC had the highest total N of 1.40 %, while PtCC and PtLC were 0.83 % and 0.86 %, respectively. For total P content, PtMC was the highest (0.47 %) while that of Pt was the lowest (0.07 %). PtMC had the highest total K at 2.21 % while those of PtCC and Pt were the lowest at 0.24 % and 0.27 %, respectively. The pH of all carriers showed high acidity (< 4.5). The highest pH was found in PtLC and PtMC (3.63 and 3.56, respectively), whereas that of Pt was the lowest at 2.80. EC of PtMC was the highest at 3.12 dS/cm, while that of PtLC was the lowest at 0.45 dS/cm. All materials were high in OM, with the highest in Pt (39.87 %) and the lowest in PtLC (31.44 %).

Table 15 Some properties of Pt, PtCC, PtLC and PtMC used as carriers for inoculum production of Azotobacter.

Carriers	**Properties**					
	Total N (%)	Total P (%)	Total K (%)	OM (%)	pH (1:10)	EC (dS/cm)
Pt	1.00b	0.07d	0.27c	39.87a	2.80c	1.19b
PtCC	0.83c	0.39b	0.24c	37.18ab	3.38b	0.60c
PtLC	0.86c	0.14c	0.98b	31.44c	3.63a	0.45d
PtMC	1.40a	0.47a	2.21a	34.26bc	3.56ab	3.12a
Mean	1.02	0.27	0.93	35.69	3.34	1.34
P-value	<.0001	<.0001	<.0001	0.0303	<.0001	<.0001
CV (%)	2.60	2.11	7.13	7.89	2.87	4.33

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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2. Survival of Azotobacter in various carriers during storage in different temperatures

There were significant differences in survival of *Azotobacter vinelandii* NDD-CK-1 among carriers, storage temperatures at initial date to 90 days and interaction between carriers and temperatures during 7 to 90 days after storage (Figure 14 to 17). At the initial date, PtCC gave the highest bacterial number followed by Pt, PtLC and PtMC with 12.48 (Figure 15), 12.40 (Figure 14), 11.02 (Figure 16), and 10.96 of log cfu/g (Figure 17), respectively. During 7, 15, 30, 60 and 90 days across five temperature regimes (Figure 18), PtLC had the highest bacterial count (10.04, 9.36, 8.88, 7.36 and 6.41 of log cfu/g), followed by PtCC (9.60, 9.18, 8.86, 7.32 and 6.02 of log cfu/g, PtMC (9.63, 9.00, 8.4, 6.78 and 5.67 of log cfu/g) and Pt (8.20, 7.76, 7.56, 6.15 and 5.50 of log cfu/g), respectively.

For storage temperatures, storing in deep freeze gave the highest population of Azotobacter at 7 to 15 days after preservation. During 7 days after storage, the survival of bacteria in deep freeze was 9.72 of log cfu/g, while those stored in the refrigerator, green house, ambient temperature, and air-conditioned temperature had viable cells at 9.44, 9.33, 9.22 and 9.14 of log cfu/g, respectively (Figure 19). At 15 days after storing, keeping the inoculum in deep freezer gave the highest number of Azotobacter (9.35 of log cfu/g) similar to that in refrigerator, but higher than those under air-conditioning temperature, ambient temperature and greenhouse condition which gave 8.59, 8.49 and 8.47 of log cfu/g, respectively (Figure 19). While, at 30, 60 and 90 days after storage, preserving the inoculum in refrigerator gave the highest survival rate of 9.47, 8.03 and 7.52 of log cfu/g, respectively, followed by those kept in deep freezer (9.15, 7.97, and 7.30 of log cfu/g), air conditioning temperature (8.17, 6.22 and 5.19 of log cfu/g), ambient temperature (7.90, 6.21 and 5.17 of log cfu/g) and greenhouse condition (7.46, 6.08 and 4.34 of log cfu/g) as shown in Figure 19.

The interaction between carriers and storage temperatures during 7 to 90 days showed that PtLC stored at 37.5 ±2.5 ^{o}C gave the largest microbial population

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(10.37 of cfu/g) (Figure 16), similar to that of PtCC at -16 ^{o}C (Figure 15), PtLC at 5 ^{o}C (Figure 16) and PtLC at -16 ^{o}C (Figure 16) with 10.32, 10.15 and 10.14 of log cfu/g, respectively. While, Pt at 5 ^{o}C and at 25±2 ^{o}C had the lowest survival rates at one week after storage (Figure 14). At 15 days after storage, PtCC stored at -16 ^{o}C gave the highest survival (9.98 of log cfu/g) (Figure 15), similar to PtCC at 5 ^{o}C (Figure 15), PtLC at -16 ^{o}C (Figure 16), and PtLC at 5 ^{o}C (Figure 16) (9.92, 9.85 and 9.77 of log cfu/g, respectively). Whereas maintaining Pt carrier at 37.5±2.5 ^{o}C gave the lowest number of Azotobacter (7.26 of log cfu/g) (Figure 14). At 30 days after preservation, maintaining the inoculum in PtCC at 5 ^{o}C showed the highest bacterial number (10.08 of log cfu/g) (Figure 15), similar to those maintained in PtLC at 5 ^{o}C and PtLC at -16 ^{o}C (Figure 16) (10.03 and 10.00 of log cfu/g, respectively). In contrast, Pt at 30±2 ^{o}C had the lowest bacterial population (6.69 of log cfu/g) (Figure 14), which was close to PtMC at 37.5±2.5 ^{o}C (Figure 17), PtLC at 37.5±2.5 ^{o}C (Figure 16), Pt at 25±2 ^{o}C and Pt at 37.5±2.5 ^{o}C (Figure 14) at 7.65, 7.48, 7.43 and 6.88 of log cfu/g, respectively. Interaction between carriers and temperatures had a significant effect on the survival of Azotobacter stored for 60 days. The maximum number of bacteria was obtained from PtCC stored at 5 ^{o}C (Figure 15), similar to PtLC at 5 ^{o}C (Figure 16) and PtCC at -16 ^{o}C (Figure 15), gave 8.93, 8.88 and 8.70 of log cfu/g, respectively. While, Pt at 37.5±2.5 ^{o}C gave the lowest microbial population 5.70 of log cfu/g (Figure 14). At 90 days of preservation, the highest survival of Azotobacter was found in PtLC at 5 ^{o}C (8.42 of log cfu/g) (Figure 16) and PtCC at 5 ^{o}C (8.37 of log cfu/g) (Figure 15) followed by PtCC at -16 ^{o}C (7.91 of log cfu/g) (Figure 15). While PtCC at 37.5±2.5 ^{o}C (Figure 15) and PtMC at 25±2 ^{o}C (Figure 17) had the lowest survival cells at 3.69 and 3.71 of log cfu/g dry inoculant, respectively.

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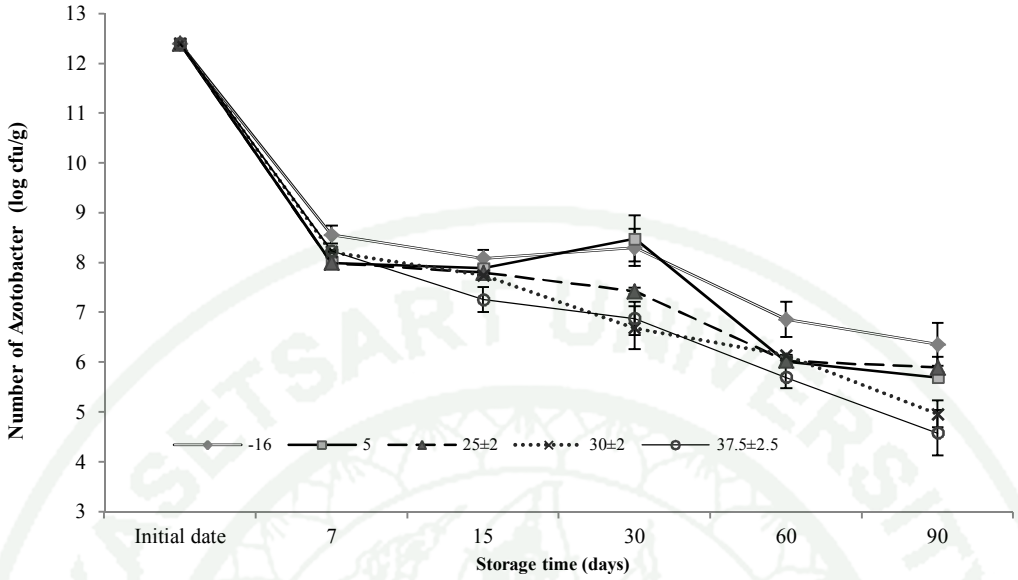

Figure 14 Population of *Azotobacter vinelandii* NDD-CK-1 existed in Pt carrier during initial date to 90 days after storage in different temperatures.

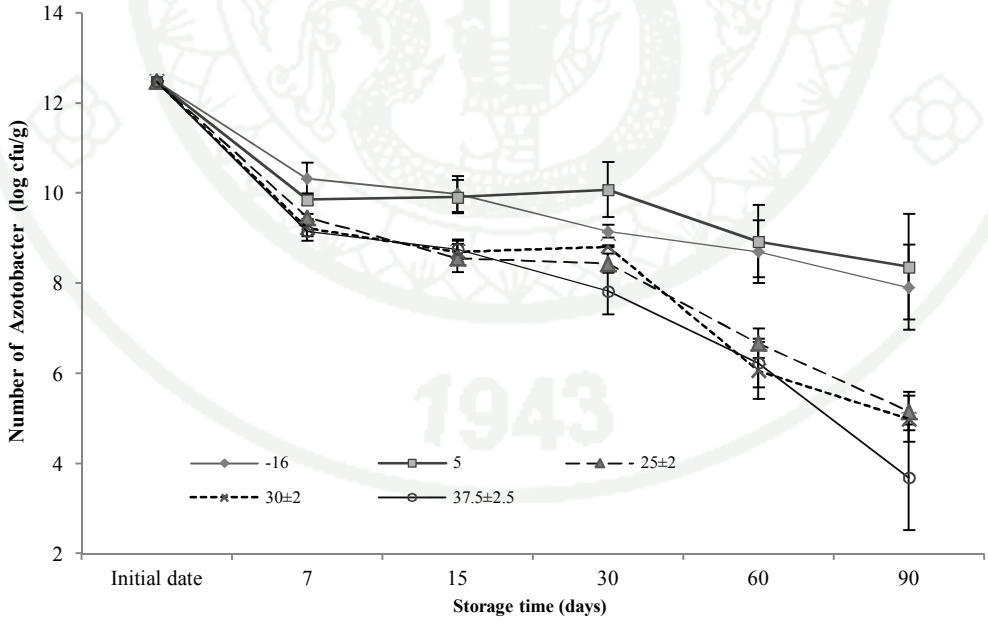

Figure 15 Population of *Azotobacter vinelandii* NDD-CK-1 existed in PtCC carrier during initial date to 90 days after storage in different temperatures.

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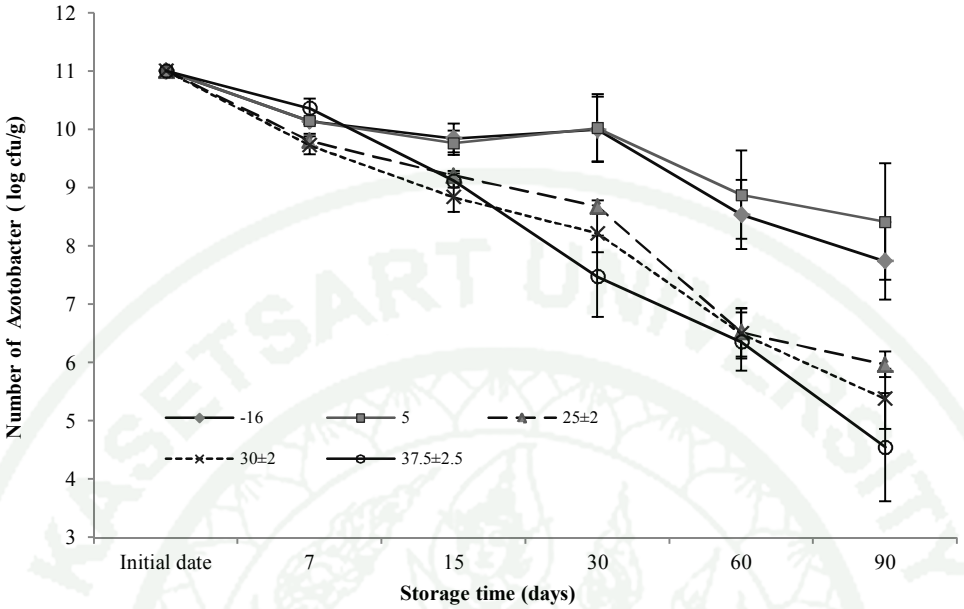

Figure 16 Population of *Azotobacter vinelandii* NDD-CK-1 existed in PtLC carrier during initial date to 90 days after storage in different temperatures.

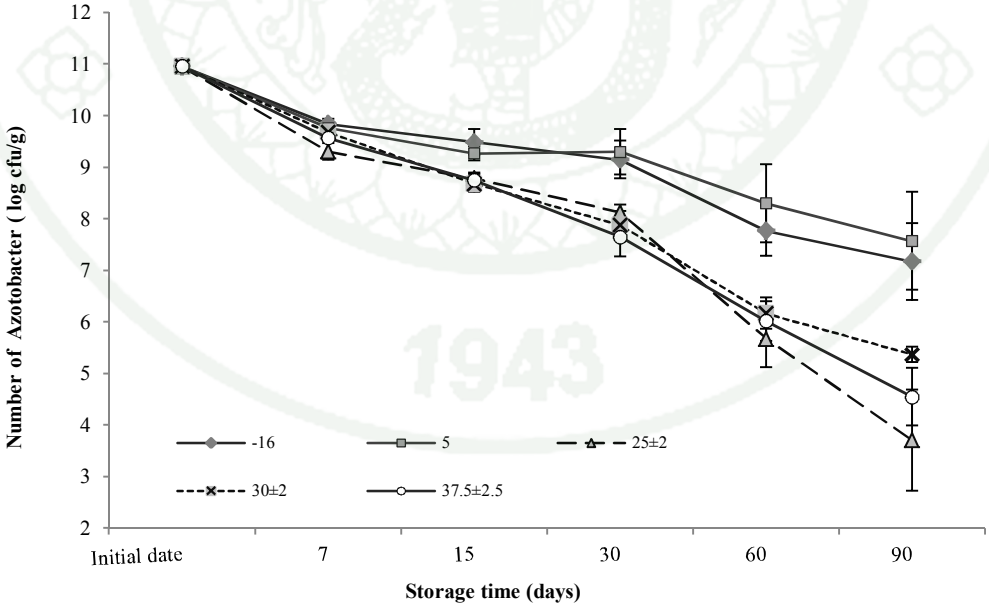

Figure 17 Population of *Azotobacter vinelandii* NDD-CK-1 existed in PtMC carrier during initial date to 90 days after storage in different temperatures.

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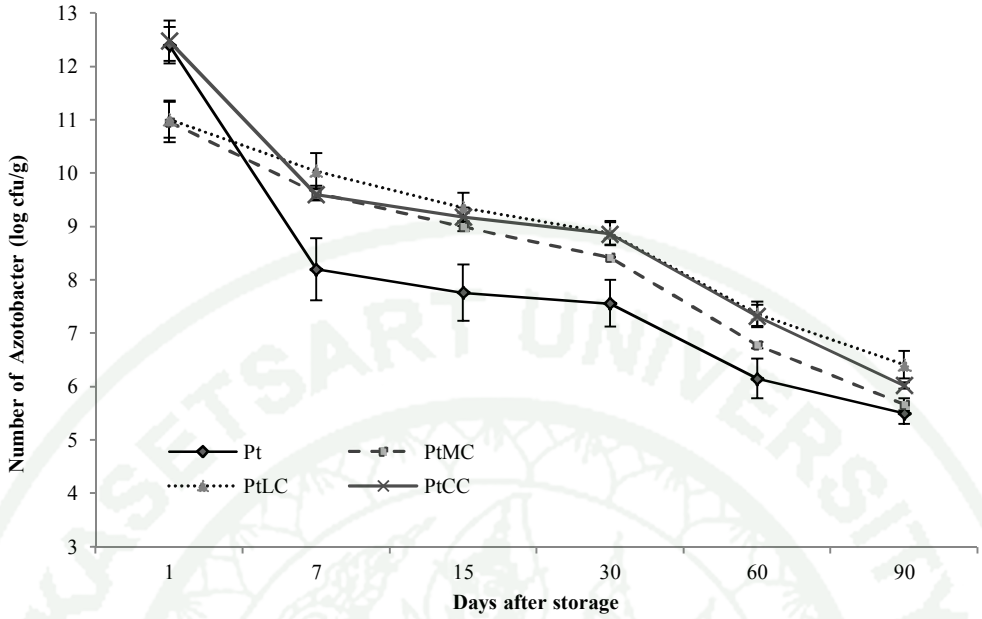

Figure 18 Effect of P, PMC, PtLC, and PCC carriers on mean number of *Azotobacter vinelandii* NDD-CK-1 averaged across temperatures.

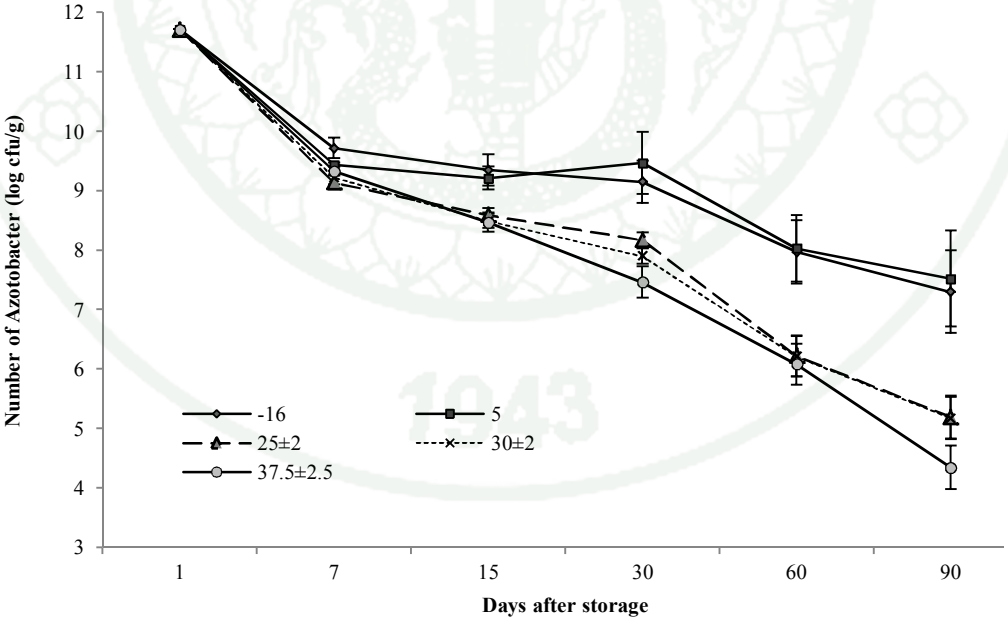

Figure 19 Effect of various storage temperature on mean number of *Azotobacter vinelandii* NDD-CK-1 averaged across carriers.

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Experiment IV. The greenhouse experiment for testing the effectiveness of various *Azotobacter* spp. on the growth enhancement of brockaleli

1. Some properties of soil before planting and dairy cattle manure

Chemical analysis in dairy cattle manure and soil before planting was assessed as Table 16. Total N in the manure was 1.49 %, while that of the soil was 0.05 %. Available P_2O_5 in the manure was very low (0.43 mg/kg), while that of the soil was low (4.16 mg/kg). Exchangeable K_2O of the manure and the soil were very low (1.63 mg/kg) and low (37.48 mg/kg), respectively. OM in the manure was very high at 7.28 %, while that of the soil was low at 0.69 %. EC in the cow manure and soil were 3.81 and 0.09 dS/m, respectively. However, both pH of manure and soil were very strongly acid (2.19 and 4.90, respectively.) In case of dairy cattle manure manure, the total N (1.541 %) was higher than a standard at 0.8 – 1.2 %, whereas total P (0.933 %) was in range of the standard of 0.5 to 0.9 %, and the total K (0.558 %) was in the standard of 0.5 to 3.7 % (Staff of Soil Science, 1998).

Table 16 Some properties of dairy cattle manure and Nampong Soil Series before planting.

Properties	Nam Pong Soil Series	Properties	Dairy cattle manure
Total N (%)	0.05	Total N (%)	1.50
Avai. P_2O_5(mg/kg)	4.16	Total P (%)	0.44
Exch. K_2O (mg/kg)	37.48	Total K (%)	1.63
OM (%)	0.69	OM (%)	37.28
EC (1:5) (dS/m)	0.09	EC (1:10) (dS/m)	3.81
pH (1:1)	2.19	pH (1:1)	4.90

2. Some properties of soil after application of various Azotobacter

No significantce effect on some properties of soil after application of various *Azotobacter* spp. was found (Table 17). However, it indicated that more than

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50 % of utilization of Azotobacter increased soil chemical properties such as avai. P_2O_5, exch. K_2O, EC and pH similar to N10 which were higher than that of control. This finding appears to imply that inoculated soil showed the highest values of some soil properties. In this case, *A. vinelandii* CK-KS-3, *A. vinelandii* CHI-KK-2, *A. vinelandii* CK-NDD-1, MIX, and *A. vinelandii*. PR-NDD-1 treatments were the highest in total N, avai. P_2O_5, exch. K_2O, OM and pH, respectively. In addition, total N ranged of 0.071 % - 0.100 %. Avai.P_2O_5 and exch.K_2O of all treatments were high in the range of 47.82 - 65.23 and 203.93 - 289.65 mg/kg, respectively, while OM was quite low ranging from 1.06 to 1.30 %. EC and pH values of inoculated soils were 0.178 dS/m and 5.91, respectively.

Table 17 Properties of soil inoculated of *Azotobacter* spp. after harvesting in greenhouse.

Treatments	Total N (%)	Avai. P_2O_5 (mg/kg)	Exch. K_2O (mg/kg)	Organic matter (%)	EC (1:5) dS/m	pH (1:1)
Control	0.088	47.82	203.93	1.13	0.15	5.95
N10	0.086	53.71	259.35	1.06	0.17	5.30
N20	0.071	56.13	218.35	1.23	0.16	5.75
A. vinelandii CK-KS-3	0.100	56.58	253.92	1.06	0.18	5.60
A. vinelandii CK-KS-2	0.084	51.20	277.32	1.13	0.19	6.30
A. vinelandii CK-NDD-1	0.086	52.81	289.65	1.06	0.19	5.40
A. beijerinckii LET-NDD-1	0.075	62.46	228.76	1.06	0.17	6.20
A. vinelandii CHI-KK-2	0.082	65.23	266.20	1.20	0.20	6.10
A. vinelandii PR-NDD-1	0.085	61.60	259.48	1.27	0.19	6.35
A. chroococcum LET-NDD-3	0.083	60.34	273.20	1.20	0.17	5.85
A. vinelandii BRO-KPS-5	0.081	64.56	258.74	1.06	0.17	6.20
MIX	0.085	61.20	274.70	1.30	0.20	5.95
Mean	0.084	57.80	255.29	1.15	0.18	5.91
P-value	NS	NS	NS	NS	NS	NS
CV(%)	11.07	13.42	9.78	10.75	15.35	11.70

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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3. Number of Azotobacter of various inoculums

The initial population of Azotobacter in the inoculums were in the ranged of 9.45 - 11.30 of log cfu/g depending on the strains (Figure 20). The result showed that *Azotobacter vinelandii* CK-NDD-1 gave the highest bacterial density followed by *A. vinelandii* CK-KS-3, *A. beijerinckii* LET-NDD-1, *A. vinelandii* BRO-KPS-5, *A. vinelandii* PR-NDD-1, MIX, *A. vinelandii* CK-KS-2, *A. vinelandii* CHI-KK-2 and *A. chroococcum* LET-NDD-3, respectively.

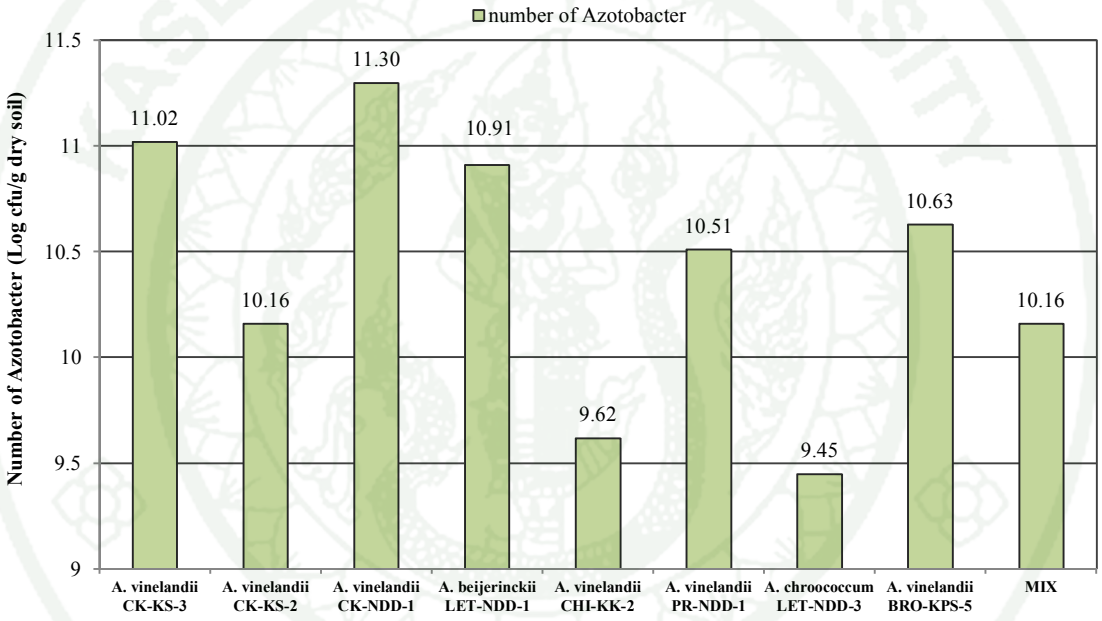

Figure 20 Number of Azotobacter in various inoculums at initial date.

4. Number of various *Azotobacter* spp. in soil after planting

After various Azotobacter inoculums were introduced into the soils by soil application method. The number of Azotobacter was determined during 35, 45, and 65 days after planting (Figure 21). The result revealed that there was the significant effect on the population of Azotobacter at 65 days after planting. *A. vinelandii* CK-KS-2 had the highest bacterial density (6.74 of log cfu/g) followed by MIX, *A. vinelandii* PR-NDD-1, *A. beijerinckii* LET-NDD-1, *A. vinelandii* BRO-KPS-5 which were higher than those of N10, N20 and control. In contrast, no significant effect on

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the population at 35 and 45 days were found. In this case, it would seem that *A. vinelandii* PR-NDD-1 and *A. vinelandii* BRO-KPS-5 had the highest survival at 35 and 45 days, respectively. Anyhow, the result appeared that 88.89 % of Azotobacter treatments showed the larger cell counts than that of control, whereas 50 % of bacterium had higher population than those of N10 and N20, respectively.

5. Nitrogenase activity in soil after planting

The data presented here showed that nitrogenase activity of various Azotobacter was significantly different at 45 days ($p<0.0327$) except at 65 days after planting as shown in Figure 22. It showed that *A. vinelandii* BRO-KPS-5 treatment was the highest in nitrogenase activity (0.849 µmol C_2H_4/100 g soil/hr) similar to those of others except that of the control (0.315 µmol C_2H_4/100 g soil/hr). At 65 days, the nitrogenase activity of all treatments was closely in the range of 0.186 - 0.444 C_2H_4/100 g soil/hr. However, the data appeared to suggest that PR-NDD-1 was the highest in nitrogenase activity, whereas N20 soil showed the lowest nitrogenase activity.

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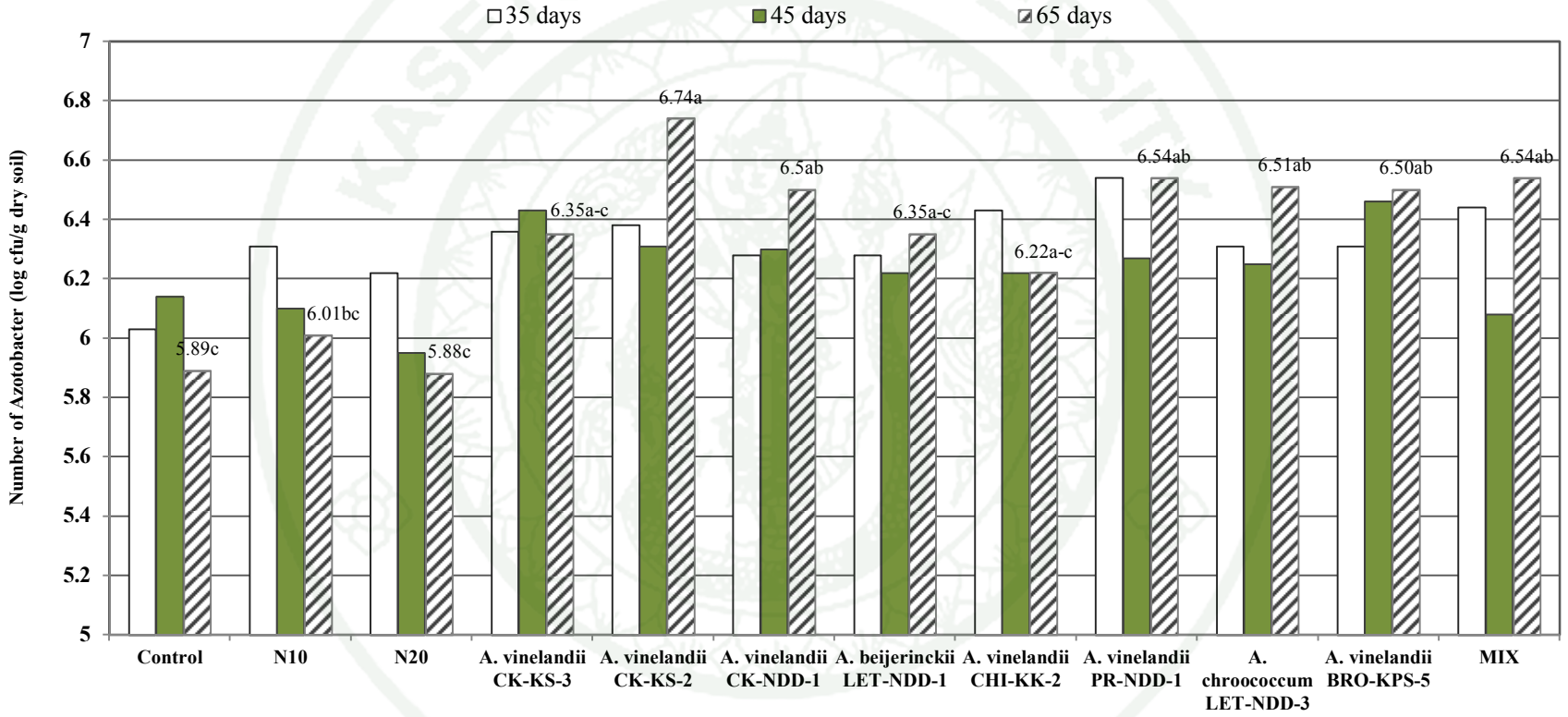

Figure 21 Population of Azotobacter after inoculation into the soil at 30, 45, and 65 days.

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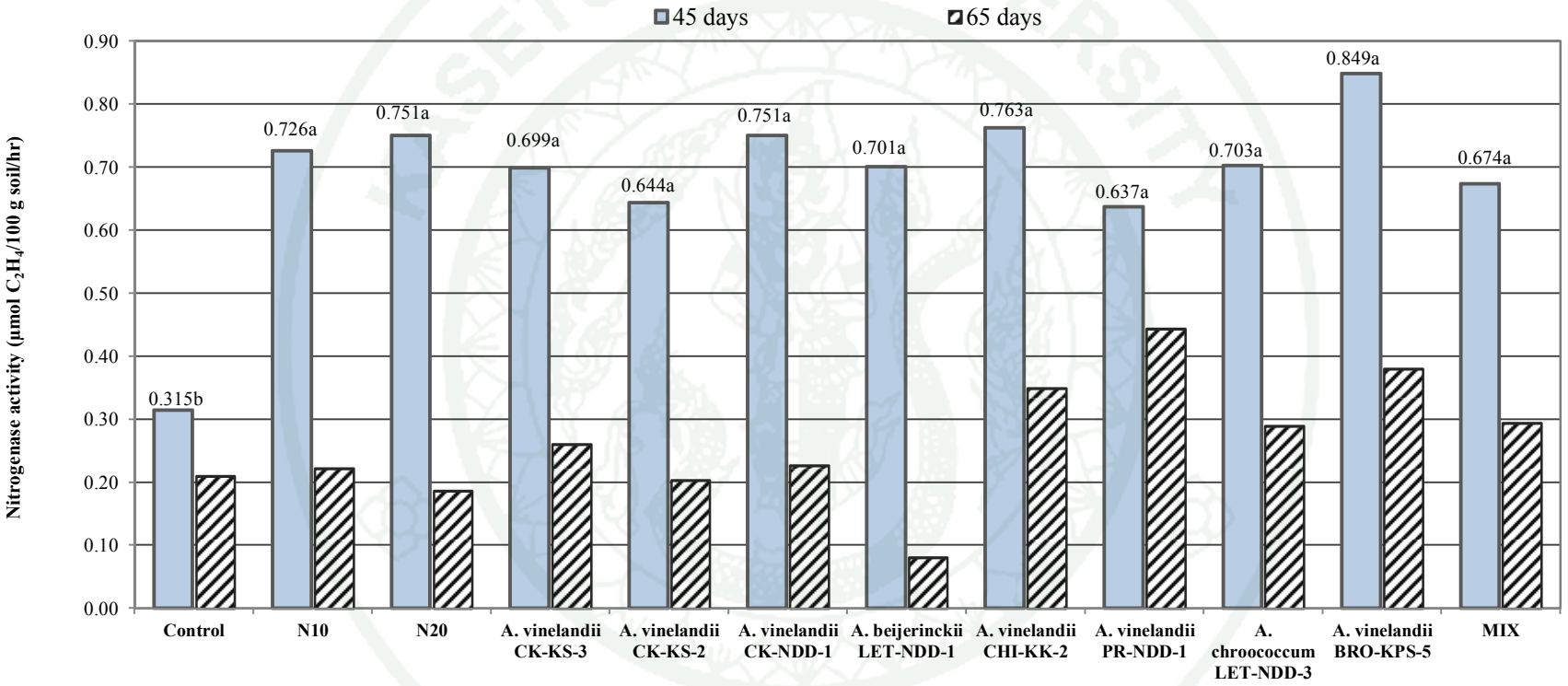

Figure 22 Nitrogenase activity of various *Azotobacter* spp. at 45, and 65 days after planting.

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6. Brockaleli growth after application of various *Azotobacter* spp. under greenhouse condition

The result showed that there was significant difference in the length of leafstalk at 15 days after planting, while no differences on height, leaf number, leaf width were found as illustrated in Table 18-22. In case of length of leaf stalk, *A. vinelandii* CK-KS-2 and PR-NDD-1 were the highest in the length of 15 days (2.25 and 2.31 cm) similar to those of *A. vinelandii* CK-KS-3, N10, N20 and control which were higher than that of MIX inoculum (1.26 cm). During 30 days, 45, and 65 days after planting, the mean of length of leaf stalk were at 4.14, 6.84, and 8.66 cm, respectively (Table 18). This tendency showed that *A. vinelandii* CK-NDD-1 had the highest length during 30 and 45 days, whereas *A. chroococcum* LET-NDD-3 gave the highest length of leaf stalk at 65 days after planting.

Height of brockaleli was measured in the greenhouse experiment for testing the effectiveness of various strains of *Azotobacter* spp. (Table 19). The application of *A. vinelandii* PR-NDD-1 had the highest height during 15, 30 and 45 days, while that of *A. chroococcum* LET-NDD-3 was the highest at 65 days. In addition, it seemed that 33.33 %, 55.56 %, 66.67 % and 44.44 % of various *Azotobacter* spp. showed plant height than that of control during 15, 30, 45 and 65 days after planting, respectively.

Leaf number of brockaleli was showed in Table 20. The average leaf number at 15, 30, 45, and 65 days after planting were 2.25, 5.63, 8.88 and 10.29 leaves/plant, respectively. It would seem that N20 had the highest leaf number at 15 days, whereas *A. vinelandii* PR-NDD-1, *A. vinelandii* CK-KS-3 and control gave the most leaf number at 30 days. At 65 days, *A. vinelandii* CHI-KK-2 tend to showed highest plant leaves.

Leaf width of brockaleli was varied depends on species. In Table 21, it seemed that *A. vinelandii* CK-NDD-1 had the largest leaf during 30 and 45 days after planting, whereas *A. vinelandii* CK-KS-2 and *A. beijerinckii* LET-NDD-1 showed the

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highest leaf width at 15 and 65 days after planting. In any case, approximately 45 % of Azotobacter application tended to promote leaf width similar to N10 which was better than that of control.

In case of leaf length of brockaleli, the result appears to suggest that precisely 60 % of Azotobacter would have larger leaf length than that of control, whereas 45 % of Azotobacter could increase leaf length more than the N10 (Table 22).

Table 18 Length of brockaleli leaf stalk in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	Length of brockaleli leaf stalk (cm)			
	15 days	30 days	45 days	65 days
Control	1.85a-c	4.60	6.50	9.19
N10	1.68a-c	4.00	5.95	8.74
N20	1.86a-c	4.20	6.35	8.77
A. vinelandii CK-KS-3	1.95ab	4.29	5.90	8.67
A. vinelandii CK-KS-2	2.25a	4.16	6.20	6.66
A. vinelandii CK-NDD-1	1.84a-c	5.15	8.47	9.13
A. beijerinckii LET-NDD-1	1.60bc	4.40	7.63	8.99
A. vinelandii CHI-KK-2	1.48bc	3.30	7.03	8.24
A. vinelandii PR-NDD-1	2.31a	3.82	8.08	8.86
A. chroococcum LET-NDD-3	1.50bc	4.14	6.28	10.22
A. vinelandii BRO-KPS-5	1.40bc	4.12	7.22	8.65
MIX	1.26c	3.51	6.45	7.82
Mean	1.75	4.14	6.84	8.66
P-value	0.0110	0.7820	0.2040	0.2910
CV(%)	12.47	19.13	13.71	11.82

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Table 19 Height of brockaleli in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	Height of brockaleli (cm)			
	15 days	30 days	45 days	65 days
Control	7.23	22.55	36.15	44.00
N10	7.32	20.38	37.75	43.50
N20	7.42	22.38	36.00	44.65
A. vinelandii CK-KS-3	7.55	23.18	35.25	39.00
A. vinelandii CK-KS-2	7.40	21.38	34.50	40.65
A. vinelandii CK-NDD-1	7.13	24.15	41.75	45.00
A. beijerinckii LET-NDD-1	6.55	22.88	35.75	41.75
A. vinelandii CHI-KK-2	6.75	22.20	38.25	40.75
A. vinelandii PR-NDD-1	8.75	24.60	42.80	45.75
A. chroococcum LET-NDD-3	6.73	21.25	40.25	46.65
A. vinelandii BRO-KPS-5	6.28	21.48	40.00	43.50
MIX	6.68	20.00	37.00	44.50
Mean	7.15	22.20	37.70	43.30
P-value	NS	NS	NS	NS
CV(%)	14.68	11.01	37.95	9.40

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Table 20 Leaf number of brockaleli in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	Leaf number of brockaleli (cm)			
	15 days	30 days	45 days	65 days
Control	2.50	6.25	9.00	9.50
N10	2.25	5.25	9.00	10.00
N20	3.50	5.75	10.00	11.00
A. vinelandii CK-KS-3	2.25	6.25	10.0	10.00
A. vinelandii CK-KS-2	1.75	5.25	7.50	10.00
A. vinelandii CK-NDD-1	2.25	5.25	8.50	10.50
A. beijerinckii LET-NDD-1	2.25	5.50	8.50	10.00
A. vinelandii CHI-KK-2	2.00	6.00	9.00	12.00
A. vinelandii PR-NDD-1	2.25	6.25	9.50	10.50
A. chroococcum LET-NDD-3	2.00	5.00	9.00	10.00
A. vinelandii BRO-KPS-5	2.00	5.25	8.00	9.50
MIX	2.00	5.50	8.50	10.50
Mean	2.25	5.63	8.88	10.29
P-value	NS	NS	NS	NS
CV(%)	14.00	17.78	9.48	13.31

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Table 21 Leaf width of brockaleli in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	Leaf width of brockaleli (cm)			
	15 days	30 days	45 days	65 days
Control	2.08	5.33	8.28	11.36
N10	2.05	5.47	8.76	12.47
N20	2.28	5.67	7.73	12.80
A. vinelandii CK-KS-3	2.18	5.59	9.95	11.63
A. vinelandii CK-KS-2	2.45	5.76	8.89	11.59
A. vinelandii CK-NDD-1	2.19	6.05	10.18	10.17
A. beijerinckii LET-NDD-1	1.81	5.07	8.13	10.98
A. vinelandii CHI-KK-2	1.83	3.97	8.79	10.82
A. vinelandii PR-NDD-1	2.01	4.02	9.00	10.59
A. chroococcum LET-NDD-3	1.83	5.00	8.40	10.72
A. vinelandii BRO-KPS-5	1.65	5.14	9.76	11.53
MIX	1.87	4.69	9.35	12.20
Mean	2.02	5.15	8.68	11.40
P-value	0.0740	0.450	0.4930	0.6780
CV(%)	16.70	25.12	21.50	11.42

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Table 22 Leaf length of brockaleli in greenhouse as affected by various strains of Azotobacter.

Treatments	Leaf length of brockaleli (cm)			
	15 days	30 days	45 days	65 days
Control	2.58	7.22	11.43	16.53
N10	3.00	7.56	11.87	16.21
N20	3.06	7.92	11.74	17.45
A. vinelandii CK-KS-3	2.84	7.63	12.67	15.37
A. vinelandii CK-KS-2	3.23	7.66	13.30	17.18
A. vinelandii CK-NDD-1	2.83	7.96	14.84	15.05
A. beijerinckii LET-NDD-1	2.50	7.36	14.52	18.12
A. vinelandii CHI-KK-2	2.66	5.67	12.33	14.25
A. vinelandii PR-NDD-1	2.99	5.84	13.52	16.42
A. chroococcum LET-NDD-3	2.45	7.10	12.76	16.56
A. vinelandii BRO-KPS-5	2.31	7.15	14.84	17.83
MIX	2.66	6.42	12.94	17.17
Mean	2.76	7.126	13.06	16.51
P-value	NS	NS	NS	NS
CV(%)	17.32	25.95	9.65	8.10

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

7. Yield and yield component of brockaleli after application of various strains of Azotobacter

7.1 Yield and yield components of brockaleli at harvesting date were shown in Table 23. Day to first flowering was significantly different, whereas no significant effects in days to harvesting, number of buds, number of branches, and diameter of flower were found in this study. Application of *A. vinelandii* CK-KS-2 inoculum could induced flowering stage of brockaleli followed by *A. vinelandii* CK-KS-3, *A. vinelandii* CK-NDD-1 treatments which had faster flowering stage than those of, N20, N10 and the control, respectively. The data suggested that most of

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Azotobacter (88.89 %) showed the effect in early blooming stage date than those of others, control, N20 and N10. Although not significant were found on number of bud, number of branch and diameter of stem at harvesting period, the tendency indicated that 80 % of Azotobacter application, showed similar effect that of N20, showed higher number of bud and branched than that of control. However, the result appeared to found that only *A. beijerinckii* LET-NDD-1 and *A. chroococcum* LET-NDD-3 showed larger flower than those of N20, N10 and control. Whereas MIX, *A. vinelandii* CK-KS-3, *A. chroococcum* LET-NDD-3, *A. vinelandii* CHI-KK-2, *A. vinelandii* CK-NDD-1 trend to have higher stem size than that of N20.

7.2 Fresh and dry weight of brockaleli

After application of various strains of Azotobacter under the pot trial, the result showed that there were the significantly differences on root fresh weight, dry weight of flower and root dry weight (Table 24), whereas those of others did not statically difference. In case of root fresh weight, the result showed that *A. vinelandii* BRO-KPS-5 gave the highest root fresh weight (70.74 g/plant) followed by those of *A. vinelandii* CHI-KK-2, *A. chroococcum* LET-NDD-3, *A. vinelandii* CK-KS-2. For flower dry weight, the result exhibited that most of Azotobacter (66.67%) utilization had a trend to give higher dry matter than those of N10 and control. In this study, *A. vinelandii* CK-KS-2 had the highest dry matter that similar to those of *A. beijerinckii* LET-NDD-1, *A. vinelandii* CK-NDD-1, *A. chroococcum* LET-NDD-3 which were higher than those of other strains, N10, N20 and control treatments. In term of root biomass of brockaleli, *A. vinelandii* BRO-KPS-5 gave the highest root dry weight (24.78 g/plant) followed by those of *A. vinelandii* CHI-KK-2, N10, *A. chroococcum* LET-NDD-3. For the conclusion, more than 80 % of Azotobacter utilization enhanced the brockaleli to have higher both fresh weight and dry weight.

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Table 23 Day to first flowering, harvest date and yield components of brockaleli in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	Day to first flowering	Harvesting date	Buds/ stem	Branches/ stem	Diameter of flower (cm)	Diameter of stem (cm)
Control	71.50bc	92.33	2.67	0.50	1.15	1.46
N10	84.00a	98.00	2.33	0.25	0.99	1.44
N20	76.5ab	91.00	2.50	0.50	1.22	1.45
A. vinelandii CK-KS-3	65.00c	87.33	5.67	1.25	1.10	1.58
A. vinelandii CK-KS-2	63.50c	79.00	6.50	1.00	1.05	1.38
A. vinelandii CK-NDD-1	65.50c	86.33	5.0	1.00	1.07	1.47
A. beijerinckii LET-NDD-1	67.00bc	87.67	4.00	1.00	1.38	1.40
A. vinelandii CHI-KK-2	66.00c	86.33	5.00	1.25	1.03	1.47
A. vinelandii PR-NDD-1	66.50c	83.667	3.00	1.75	1.03	1.26
A. chroococcum LET-NDD-3	69.00bc	90.67	5.00	1.00	1.25	1.56
A. vinelandii BRO-KPS-5	84.50a	96.67	4.67	0.00	1.12	1.45
MIX	65.50c	79.67	4.00	2.50	0.93	1.63
Mean	70.38	88.22	4.18	1.00	1.10	1.47
P-value	*	NS	NS	NS	NS	NS
CV(%)	5.82	8.03	55.41	133.00	17.68	9.87

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Table 24 Fresh and dry weight of brockaleli in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	Fresh weight (g/plant)				Dry weight (g/plant)			
	Stem	Flower	Root	Whole plant	Stem	Flower	Root	Whole plant
Control	126.98	52.15	12.61c	191.10	26.92	6.63bc	15.787b	39.33
N10	141.11	51.35	41.94b	234.40	29.53	7.56a-c	19.14ab	56.23
N20	172.97	61.98	38.36bc	273.13	33.99	8.10a-c	17.66ab	59.74
A. vinelandii CK-KS-3	165.32	70.52	37.12bc	272.95	33.43	9.41ab	13.31bc	56.16
A. vinelandii CK-KS-2	161.81	81.63	44.45ab	289.33	29.21	11.28a	17.87ab	57.79
A. vinelandii CK-NDD-1	151.28	76.63	38.37bc	266.27	33.45	10.19ab	14.77bc	58.41
A. beijerinckii LET-NDD-1	164.90	68.02	36.00bc	263.59	30.57	10.40ab	14.02bc	52.17
A. vinelandii CHI-KK-2	159.98	55.63	47.47ab	263.09	30.75	7.20bc	19.81ab	57.76
A. vinelandii PR-NDD-1	182.12	53.64	31.49bc	267.25	35.30	7.28a-c	13.10bc	55.67
A. chroococcum LET-NDD-3	154.21	56.42	46.78ab	257.41	30.88	9.79ab	18.72ab	59.39
A. vinelandii BRO-KPS-5	145.04	40.13	70.74a	255.90	24.37	4.75c	24.78a	53.91
MIX	149.56	73.56	31.30bc	254.43	30.57	8.81ab	12.27bc	51.64
Mean	156.12	61.04	40.62	258.28	30.79	8.31	16.29	55.31
P-value	NS	NS	*	NS	NS	*	*	NS
CV(%)	23.30	19.66	35.54	18.99	23.24	23.96	19.64	17.12

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Moreover, more than 65 % of bacterial application could promote higher fresh and dry weight of plant than that of N10, whereas only *A. vinelandii* CK-KS-2 showed a trend on dry weight of entire plant than that of N20. In case of Azotobacter application, the treatment of *A. chroococcum* LET-NDD-3 tended to have highest dry matter of entire plant.

7.3 Percentage root weight of brockaleli

The significant differences were found in root fresh and dry weight percentage of brockaleli. *A. vinelandii* BRO-KPS-5 had the highest in the fresh and dry weight ratio (37.60 % and 84.63 %, respectively), whereas control had the lowest (7.57 % and 18.74 % respectively) (Table 25). The result suggested that brockaleli had more percentage of dry and fresh weight than that of control after using of various *Azotobacter* spp., whereas only *A. vinelandii* BRO-KPS-5 treatment showed higher percentage of dry and fresh weight than that of N20.

8. Quantity of total N, P and K contents of stem and flower of brockaleli at harvesting date

There was the significant effect on total K in flower after applying of various strains of *Azotobacter* spp., whereas others did not as illustrated in Table 26. The result revealed that N10 had the highest total K in flower (3.85 %) followed by control, *A. vinelandii* CK-KS-2, *A. vinelandii* BRO-KPS-5 and *A. vinelandii* PR-NDD-1, which were higher than those of the others. In any case, application of Azotobacter showed the lower of total N, total P and total K of stem and total N, total P of the flower than chemical fertilizers and control. In contrast, *A. vinelandii* CK-KS-2 seem to have highest in total N of stem, whereas MIX tended to show the high level of total N in flowers.

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Table 25 Percentage root weight of brockaleli in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	% Root weight	
	Fresh weight	Dry weight
Control	7.57c	18.74c
N10	22.81b	57.76bc
N20	17.17bc	43.85bc
A. vinelandii CK-KS-3	16.09bc	31.96bc
A. vinelandii CK-KS-2	18.46bc	41.11bc
A. vinelandii CK-NDD-1	16.69bc	34.45bc
A. beijerinckii LET-NDD-1	16.38bc	37.96bc
A. vinelandii CHI-KK-2	21.82b	50.92bc
A. vinelandii PR-NDD-1	13.44bc	31.93bc
A. chroococcum LET-NDD-3	22.38b	48.02bc
A. vinelandii BRO-KPS-5	37.60a	84.63a
MIX	13.73bc	30.99bc
Mean	19.19	43.61
P-value	**	*
CV(%)	15.12	18.65

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Table 26 Total N, total P and total K contents of the stem and flower of brockaleli at harvesting date in greenhouse experiment as affected by various strains of Azotobacter.

Treatments	Stem			Flower		
	Total N (%)	Total P (%)	Total K (%)	Total N (%)	Total P (%)	Total K (%)
Control	1.00	0.51	3.65	3.27	0.57	3.58ab
N10	1.23	0.60	3.54	3.68	0.53	3.85a
N20	1.11	0.53	.3.016	3.93	0.59	3.21bc
A. vinelandii CK-KS-3	0.93	0.45	3.27	2.80	0.48	3.14bc
A. vinelandii CK-KS-2	1.16	0.55	3.32	2.96	0.56	3.29bc
A. vinelandii CK-NDD-1	0.77	0.34	2.88	2.36	0.45	3.12bc
A. beijerinckii LET-NDD-1	0.92	0.45	2.99	2.98	0.49	2.90c
A. vinelandii CHI-KK-2	1.03	0.49	3.55	3.11	0.53	3.11bc
A. vinelandii PR-NDD-1	0.82	0.39	3.22	2.71	0.47	3.21bc
A. chroococcum LET-NDD-3	0.95	0.42	2.62	2.62	0.46	3.17bc
A. vinelandii BRO-KPS-5	0.79	0.42	2.95	2.83	0.53	3.27bc
MIX	1.11	0.57	3.38	3.34	0.54	3.07c
Mean	0.98	0.48	3.20	3.05	0.52	3.24
P-value	NS	NS	NS	NS	NS	**
CV(%)	17.78	19.78	19.52	20.18	15.23	7.45

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Experiment V. Effect of various inoculums of Azotobacter on growth and yield of brockaleli in Kamphaeng Saen Soil Series

1. Some properties of soil before and after planting, and cow manure

1.1 Some properties of soil before planting and cow manure

The properties of the soil were shown in Table 27. It was found that total N in soil was very low (0.025 %), while avai. P_2O_5 and exch. K_2O were very high (182.28 and 1593.82 mg/kg, respectively). The OM was moderately high of 1.80 %, while the EC was very low of 0.35 dS/m and the pH was neutral of 7. In case of cow manure, total N (1.541 %) was higher than a standard of 0.8 - 1.2 %, whereas total P (0.933 %) and total K (0.56 %) were in the range of standard (0.5 to 0.9 % and 0.5 to 3.7 %, respectively) (Staff of Soil Science, 1998).

Table 27 Some properties of Kamphaeng Saen Soil Series and cow manure before planting.

Properties	Kamphaeng Saen Soil Series	Properties	Cow manure
Total N (%)	0.025	Total N (%)	1.54
Avai. P_2O_5 (mg/kg)	182.28	Total P (%)	0.93
Exch.K_2O (mg/kg)	1593.82	Total K (%)	0.56
OM (%)	1.80	OM (%)	27.38
EC (1:5) (dS/m)	0.35	EC (1:10) (dS/m)	3.34
pH (1:1)	7	pH (1:1)	4.27

1.2 Some properties of Kampheang Sean Soil Series after planting.

No significances were found on total N, avai. P_2O_5, exch. K_2O, OM, EC, except pH among treatments in the field as illustrated in Table 28. The average total N of all treatment soils was very low (0.14 - 0.17 %), but was higher than those of before planting. The result established that *A. vinelandii* CHI-KK-2

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had a trend to show the highest in total N (0.17 %). In case of avai. P_2O_5, the analysis values were very high ranging from 108.11 - 168.27 mg/kg which lower than that of before planting (182.28 mg/kg). It seemed to indicate that *A. vinelandii* CK-KS-2 had the highest avai. P_2O_5. Exchangeable K_2O of soil had been reduced at 50 % in comparison with soil before planting which ranged from 523.04 - 758.17 mg/kg. It appeared that *A. beijerinckii* LET-NDD-1 had the highest of exch. K_2O. Most of OM ranged between 1.80 - 2.40 % which were higher than that of before planting. *A. vinelandii* CHI-KK-2 showed a trend to give the highest OM, whereas the lowest was found in control soil. In addition, EC of soils ranged from 0.10 to 0.13 dS/m which were lower than that of before planting. The significant effect on pH was found in this study. N5 and *A. vinelandii* CK-NDD-1 had the highest pH (7.08, neutral) and *A. vinelandii* CK-KS-3 had the lowest of 6.80. It may be suggested that the application of Azotobacter stimulated the plant growth and then the uptake of phosphorous, potassium, and also organic matter accumulation will be higher than that of control.

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Table 28 Some soil properties as affected by various strains of Azotobacter in field trial.

Treatments	Total N (%)	Avai. P_2O_5 (mg/kg)	Exch. K_2O (mg/kg)	OM (%)	EC (1:5) dS/m	pH (1:1)
Control	0.14	108.11	523.04	1.80	0.10	6.98a
N5	0.15	145.91	651.35	1.83	0.11	7.08a
N10	0.15	116.71	661.85	1.99	0.11	6.95a
A. vinelandii CK-KS-3	0.14	113.52	555.93	1.90	0.10	6.80b
A. vinelandii CK-KS-2	0.16	168.27	610.17	2.33	0.10	6.93a
A. vinelandii CK-NDD-1	0.16	144.65	673.85	2.14	0.10	7.08a
A. beijerinckii LET-NDD-1	0.15	136.32	758.17	2.10	0.13	6.93a
A. vinelandii CHI-KK-2	0.17	114.08	656.70	2.40	0.11	6.93a
A. vinelandii PR-NDD-1	0.16	124.13	577.42	2.20	0.11	6.98a
A. chroococcum LET-NDD-3	0.14	110.56	540.53	1.90	0.11	6.90a
A. vinelandii BRO-KPS-5	0.15	127.98	549.88	2.06	0.10	6.95a
MIX	0.15	165.33	653.69	2.00	0.11	6.93a
Mean	0.15	131.13	619.73	2.07	0.11	6.95
P-value	NS	NS	NS	NS	NS	*
CV(%)	15.11	31.93	21.72	22.94	15.37	1.59

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

2. Population of *Azotobacter* spp. and nitrogenase activity in field condition

2.1 The quantity of Azotobacter population

Populations of various Azotobacter inoculums are shown in Figure 23. The bacterial numbers ranged from 9.36 to 10.22 log cfu/ 1.5 g fresh inoculant. In result found that *A. vinelandii* CK-KS-3 gave the highest population (10.22 log cfu/1.5 g fresh inoculant), followed by *A. vinelandii* CHI-KK-2, *A.*

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vinelandii PR-NDD-1, *A. vinelandii* CK-KS-2, *A. beijerinckii* LET-NDD-1which were higher than that of *A. vinelandii* BRO-KPS-5 (9.36 log cfu/1.5 g inoculant).

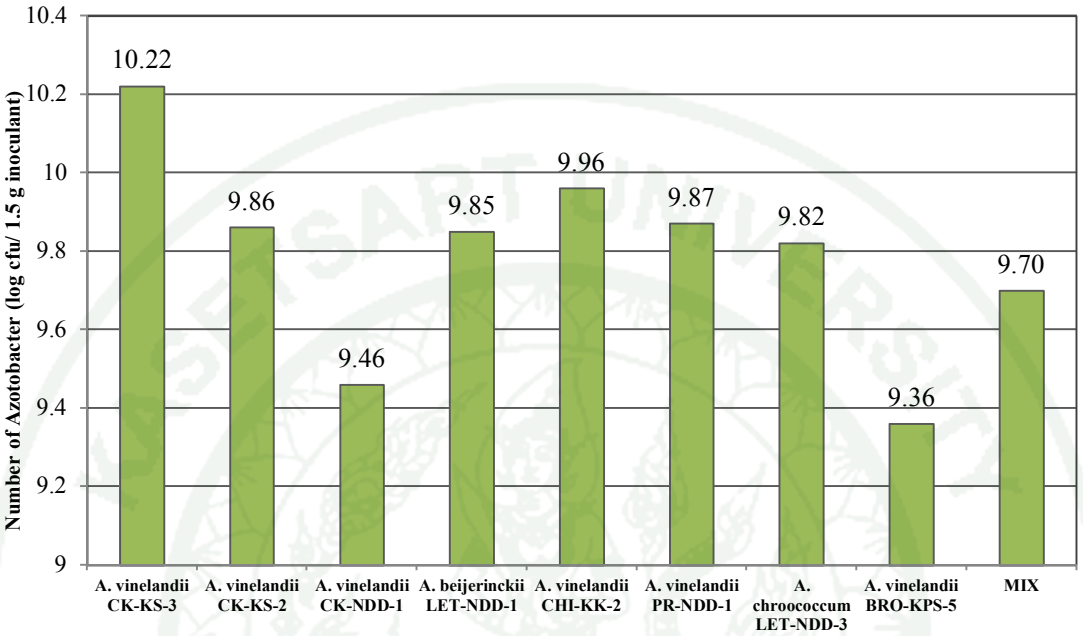

Figure 23 The population of Azotobacter in the inoculums.

2.2 Number of various *Azotobacter* spp. in the rhizosphere at 45, 55, and 65 days in field trial

Although, there was not significant different in the population of Azotobacter in the rhizosohere during 45, 55 and 65 days after planting as shown in Figure 24. The abundance of Azotobacter decreased approximately 50 % at 45 days after planting by comparison with initial population. It seemed that number of Azotobacter continuously decreased until at 65 days after planting. At 45 days. *A. vinelandii* BRO-KPS-5, *A. beijerinckii* LET-NDD-1 and *A. vinelandii* CK-KS-3 seemed to have the highest bacterial density (4.97 of log cfu/g), while *A. vinelandii* CHI-KK-2 was the lowest cell number (4.63 of log cfu/g). However, *A. vinelandii* CK-KS-3 and *A. chroococcum* LET-NDD-3 had the highest survival at 4.93 of log cfu/g, while the mix treatment gave the smallest at 4.57 of log cfu/g at 55 days after

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planting. At 65 days after planting, N10 had the most population at 4.96 of log cfu/g, while *A. vinelandii* CHI-KK-2 gave the fewest at 4.56 of log cfu/g.

2.3 Nitrogenase activity in soil at 45, 55 and 65 days after planting in field trial

The data appeared that nitrogenase activity of soil in various treatments during 45, 55 and 65 days after planting was not significant different (Figure 25). At 45 days, N10 tended to have the highest nitrogenase activity at 0.587 µmol C_2H_4/100 g soil/hr, while *A. vinelandii* PR-NDD-1 had the lowest level of 0.241 µmol C_2H_4/100 g soil/hr. After 55 days after planting, nitrogenase activity of *A. vinelandii* CK-NDD-1 seem to be the highest nitrogenase activity of 0.419 µmol C_2H_4/100 g soil/hr, while N5 was the lowest of 0.197 µmol C_2H_4/100 g soil/hr. While, the activities of almost treatments decreased at 65 days. It would seem that *A. vinelandii* CK-KS-2 tended to be the highest activity of 0.418 µmol C_2H_4/ 100 g soil/hr, whereas *A. vinelandii* PR-NDD-1 tended to have the lowest activity of 0.162 µmol C_2H_4/100 g soil/hr.

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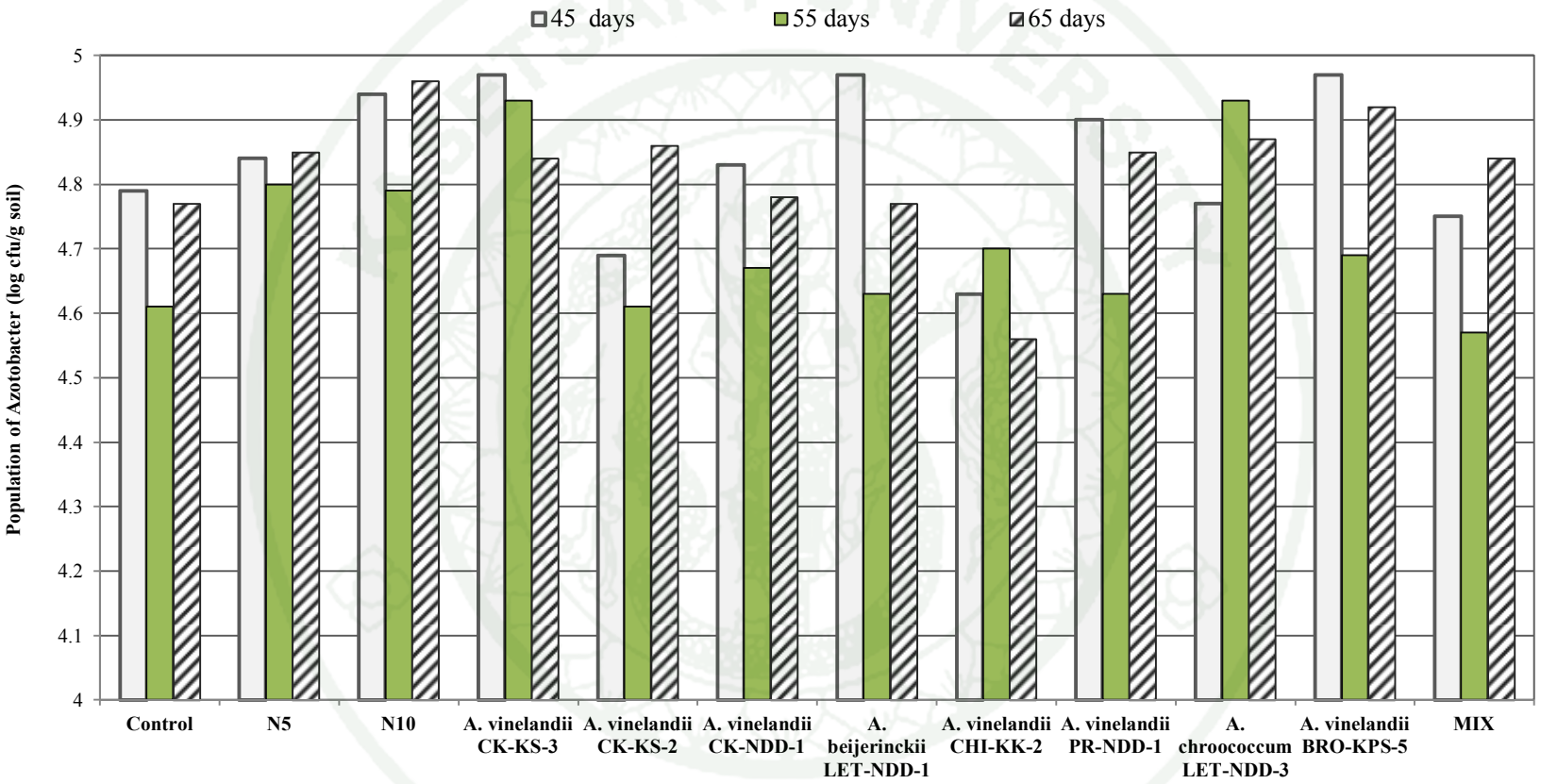

Figure 24 Population of Azotobacter in soil at 45, 55, and 65 days after application of various *Azotobacter* spp. inoculums on field trial.

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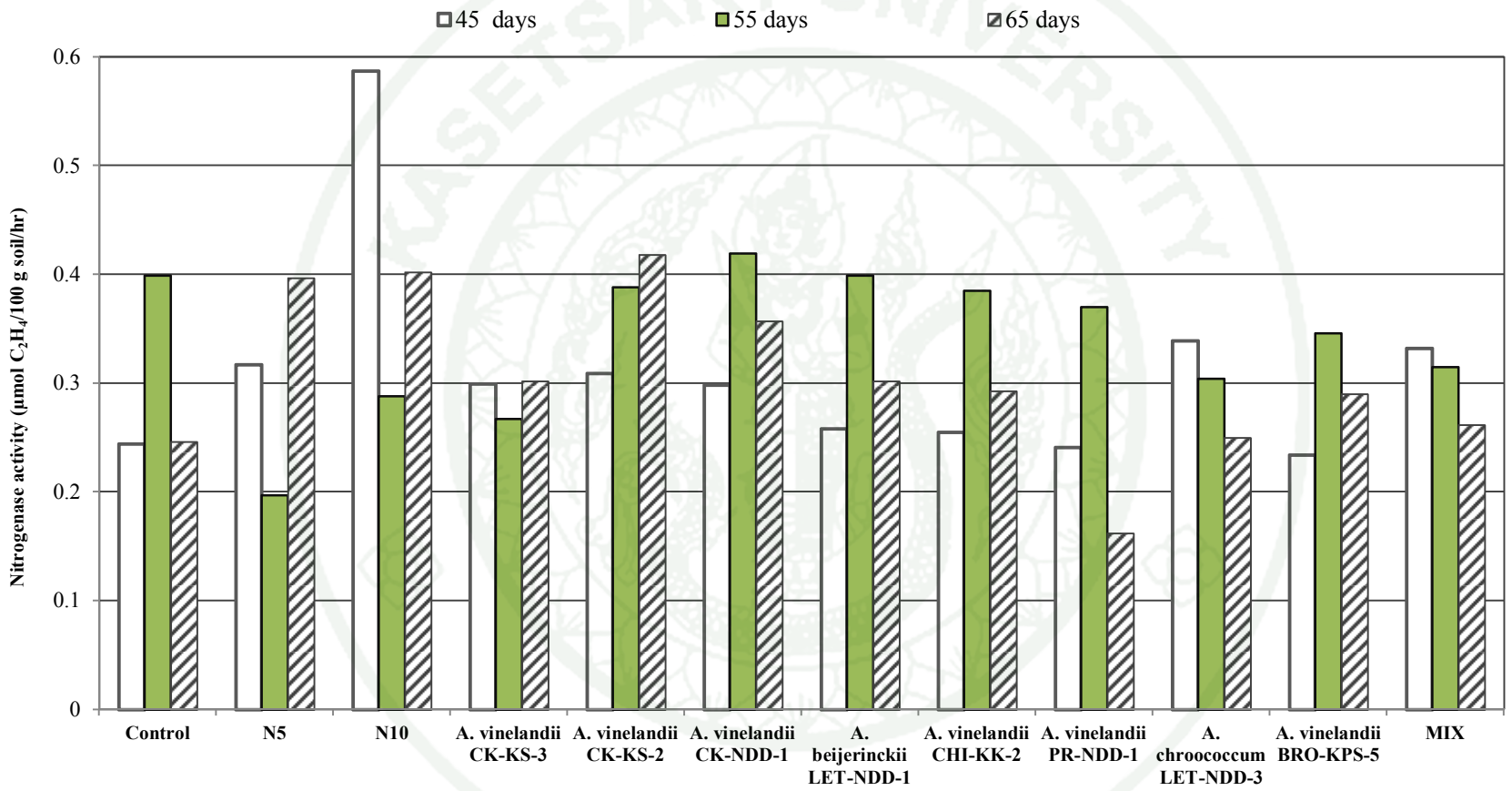

Figure 25 Nitrogenase activity of various Azotobacter treatments at 45, 55 and 65 days after planting.

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3. Growth and yield components of brockaleli

There were significant effect of various treatments on length of leaf stalk during 45, 55 and 65 days, and fresh weight of entire plant at 55 days, whereas other yield components did not significant (Table 29-34).

3.1 The effect on length of brockaleli leaf stalk

Application of various Azotobacter and chemical fertilizer affected the length of petiole of brockaleli as illustrated in Table 29. At 45 days after planting, *A. vinelandii* CHI-KK-2 had the highest length of leaf stalk (3.69 cm) followed by *A. vinelandii* CK-KS-2, *A. vinelandii* CK-NDD-1, MIX and *A. chroococcum* LET-NDD-3 (3.11, 2.97, 2.94 and 2.79 cm, respectively). This finding suggested that plant inoculated with Azotobacter responded better than those of control, N5 and N10. After planting for 55 days, inoculated plant had length of leaf stalk more than those of chemical fertilizers at 55 days. The result revealed that *A. vinelandii* PR-NDD-1 gave the highest length of leaf stalk, similar to those of *A. vinelandii* CHI-KK-2, *A. beijerinckii* LET-NDD-1 and *A. vinelandii* CK-KS-3, which were higher than those of control, N5 and N10. At 65 days, *A. vinelandii* CK-KS-3 plant had the highest at 5.17 cm similar to those of N5 and N10 which the leaf stalk was longer than that of control. Moreover, N5 had the longest of petiole at harvest period.

3.2 The brockaleli height of field experiment

In case of plant height, Azotobacter inoculation seemed to give higher plant than those of in control, N5 and N10 at 45 and 55 days (Table 30). This result suggested that application of *A. chroococcum* LET-NDD-3 tended to give the highest plant at 45 days, whereas *A. vinelandii* CHI-KK-2 showed the highest plant at 55 days. Similarly, *A. vinelandii* CHI-KK-2 treatment gave the highest at 65 days and

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harvesting period. From earlier result, the data tended to support the hypothesis that Azotobacter application showed more height than the control, except that of *A. vinelandii* CK-NDD-1 at harvesting. In addition, 88.89 % of Azotobacter promoted more plant height than those of N5 and N10 at 45-65 days, whereas application of *A. vinelandii* CHI-KK-2*, A. chroococcum* LET-NDD-3, MIX and *A. beijerinckii* LET-NDD-1) promoted more plant height than that of N5 and N10.

Table 29 Length of brockaleli leaf stalk in various treatments of field experiment.

Treatments	Length of brockaleli leaf stalk (cm)			
	45 days	55 days	65 days	Harvest
Control	2.56b	3.13cd	3.99b	4.37
N5	2.30b	3.46a-d	4.22ab	5.93
N10	2.56b	3.38b-d	4.82ab	5.15
A. vinelandii CK-KS-3	2.75b	4.02a-c	5.17a	5.82
A. vinelandii CK-KS-2	3.11ab	3.34b-d	4.86ab	4.84
A. vinelandii CK-NDD-1	2.97ab	3.83a-d	3.90b	4.20
A. beijerinckii LET-NDD-1	2.77b	4.08ab	4.21ab	5.46
A. vinelandii CHI-KK-2	3.69a	4.24ab	4.42ab	5.04
A. vinelandii PR-NDD-1	2.76b	4.35a	4.51ab	5.82
A. chroococcum LET-NDD-3	2.79ab	3.06d	4.35ab	5.47
A. vinelandii BRO-KPS-5	2.57b	3.82a-d	4.62ab	5.54
MIX	2.94ab	3.74a-d	5.07a	5.69
Mean	2.82	3.70	4.51	5.28
P-value	*	*	*	NS
CV(%)	18.29	15.04	13.84	19.94

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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3.3 The effect of various treatments on leaf numbers of brockaleli

Although leaf number of brockaleli at 45-65 days after planting was not statistical difference, *A. beijerinckii* LET-NDD-1 tended to give the highest leave number at 45 and 55 days. Moreover, Mix and *A. vinelandii* CHI-KK-2 treatment showed the highest leaf number at 65 days and harvesting date, respectively (Table 31).

Table 30 Height of brockaleli in various treatments of field experiment.

Treatments	Height of brockaleli (cm)			
	45 days	55 days	65 days	Harvest
Control	13.48	22.23	29.55	37.28
N5	12.75	21.25	34.03	39.99
N10	13.20	23.25	34.35	43.67
A. vinelandii CK-KS-3	16.40	26.38	38.63	43.16
A. vinelandii CK-KS-2	15.83	24.28	35.50	39.51
A. vinelandii CK-NDD-1	15.30	23.30	31.38	37.17
A. beijerinckii LET-NDD-1	15.08	26.45	37.00	46.83
A. vinelandii CHI-KK-2	17.63	27.45	39.38	47.41
A. vinelandii PR-NDD-1	15.20	26.85	33.50	42.75
A. chroococcum LET-NDD-3	18.55	26.55	38.88	46.83
A. vinelandii BRO-KPS-5	13.63	24.30	34.63	42.33
MIX	16.43	26.25	39.00	45.50
Mean	15.29	24.88	35.48	42.70
P-value	NS	NS	NS	NS
CV(%)	21.48	16.94	12.58	13.36

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Table 31 Leaf number of brockaleli as affected by various treatments of field experiment.

Treatments	Leaf number of brockaleli (number/plant)			
	45 days	55 days	65 days	Harvest
Control	4.25	5.75	7.75	11.90
N5	3.75	6.25	8.98	10.84
N10	4.50	6.25	9.25	11.17
A. vinelandii CK-KS-3	4.50	7.00	9.00	11.58
A. vinelandii CK-KS-2	4.25	6.25	9.00	11.17
A. vinelandii CK-NDD-1	4.00	5.75	8.25	10.67
A. beijerinckii LET-NDD-1	5.25	7.50	9.25	12.09
A. vinelandii CHI-KK-2	4.50	6.25	9.75	12.42
A. vinelandii PR-NDD-1	4.25	5.25	8.75	11.66
A. chroococcum LET-NDD-3	4.75	5.75	9.50	11.75
A. vinelandii BRO-KPS-5	4.50	6.25	9.25	10.75
MIX	4.75	6.00	10.25	11.92
Mean	4.44	6.19	9.08	11.49
P-value	NS	NS	NS	NS
CV(%)	20.20	16.81	14.06	12.00

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

3.4 The effect of various treatments on leaf width of brockaleli of field experiment

In case of leaf width, inoculated treatments tended to have more effect than those of control, N5 and N10 at 45 and 55 days, whereas application of Azotobacter showed similar result as chemical fertilizers at 65 days (Table 32). The result indicated that plant applying with *A. chroococcum* LET-NDD-3, *A. vinelandii* CK-KS-3 , MIX, and *A. chroococcum* LET-NDD-3 had the widest leaf width at 45, 55, and 65 days and harvesting date, respectively.

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Table 32 Leaf width of brockaleli as affected by various treatments in field experiment.

Treatments	Leaf width of brockaleli (cm)			
	45 days	55 days	65 days	Harvest
Control	2.99	4.49	7.11	9.63
N5	3.24	5.05	8.06	10.61
N10	3.14	4.99	9.07	10.95
A. vinelandii CK-KS-3	3.40	6.16	8.79	11.46
A. vinelandii CK-KS-2	3.34	5.15	8.04	10.68
A. vinelandii CK-NDD-1	3.58	4.95	7.59	9.07
A. beijerinckii LET-NDD-1	3.25	6.04	8.33	10.87
A. vinelandii CHI-KK-2	3.61	6.02	9.26	12.26
A. vinelandii PR-NDD-1	3.46	5.95	8.26	11.31
A. chroococcum LET-NDD-3	3.67	5.05	8.49	12.80
A. vinelandii BRO-KPS-5	3.25	5.28	8.36	10.59
MIX	3.55	5.88	9.50	11.62
Mean	3.37	5.42	8.40	10.99
P-value	NS	NS	NS	NS
CV(%)	25.78	17.34	17.26	17.31

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

3.5 The effect of various treatments on leaf length of brockaleli

Leaf length of all treatment plants was shown in Table 33. In this study, inoculated plants tended to have similar result as N5 and N10, which were higher than that of control at 45 to 65 days.Application of *A. vinelandii* CHI-KK-2, *A. beijerinckii* LET-NDD-1, *A. vinelandii* CHI-KK-2 and *A. chroococcum* LET-NDD-3 showed the highest leaf length at 45, 55, and 65 days, and harvesting date (5.94, 9.27, 13.69 and 18.54 cm, respectively).

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Table 33 Leaf length of brockaleli as affected by various treatments in field experiment.

Treatments	Leaf length of brockaleli (cm)			
	45 days	55 days	65 days	Harvest
Control	4.71	6.27	9.63	15.31
N5	4.91	7.29	10.90	15.68
N10	4.81	7.75	12.67	15.62
A. vinelandii CK-KS-3	5.43	8.79	13.10	15.95
A. vinelandii CK-KS-2	5.19	7.54	10.57	16.58
A. vinelandii CK-NDD-1	5.71	7.97	10.46	14.55
A. beijerinckii LET-NDD-1	5.30	9.27	12.38	15.09
A. vinelandii CHI-KK-2	5.94	8.98	13.69	17.67
A. vinelandii PR-NDD-1	4.79	9.08	12.06	15.92
A. chroococcum LET-NDD-3	5.82	7.51	11.67	18.54
A. vinelandii BRO-KPS-5	5.30	8.03	11.81	15.20
MIX	5.37	8.67	13.97	16.53
Mean	5.27	8.09	11.91	16.05
P-value	NS	NS	NS	NS
CV(%)	24.08	15.06	18.66	18.69

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

3.6 The effect of various treatments on stem diameter

Diameter of brockaleli stem in various treatments did not significant difference as illustrated in Table 34. The result revealed that brockaleli stem size rapidly increased at 55 days then gradual raised until harvesting stage. In this finding, the data seem to imply that stem size of inoculated plants were similar to those of chemical plants (N5 and N10) which were larger than the control. Anyway, plant inoculated with *A. chroococcum* LET-NDD-3, *A. vinelandii* CHI-KK-2, *A. vinelandii* PR-NDD-1 and *A. vinelandii* CHI-KK-2 trend to showed highest diameter of stem during at 45, 55, 65 days and harvesting date, respectively.

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Table 34 The brockaleli stem size in various treatments in field experiment.

Treatments	Diameter of brockaleli (cm)			
	45 days	55 days	65 days	Harvest
Control	0.19	0.73	0.96	1.54
N5	0.23	0.75	1.24	1.58
N10	0.21	0.79	1.39	1.76
A. vinelandii CK-KS-3	0.24	0.79	1.34	1.75
A. vinelandii CK-KS-2	0.23	0.76	1.18	1.86
A. vinelandii CK-NDD-1	0.22	0.78	1.10	1.42
A. beijerinckii LET-NDD-1	0.27	0.90	1.33	1.72
A. vinelandii CHI-KK-2	0.27	0.90	1.34	2.07
A. vinelandii PR-NDD-1	0.28	0.83	1.59	1.73
A. chroococcum LET-NDD-3	0.31	0.73	1.24	1.93
A. vinelandii BRO-KPS-5	0.23	0.75	1.15	1.69
MIX	0.29	0.89	1.34	1.71
Mean	0.25	0.80	1.26	1.73
P-value	NS	NS	NS	NS
CV(%)	40.96	23.06	19.95	15.42

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

3.7 The effect of various treatments on fresh and dry weight of brockaleli in field experiment.

Various treatments significantly affected fresh weight of whole plant at 55 days ($p<0.05$) (Table 35). In this case, applying of *A. vinelandii* CK-KS-3 had the highest fresh and dry weight of plants (32.28 and 3.12 g/plant) similar to those of *A. beijerinckii* LET-NDD-1, MIX, *A. vinelandii* CHI-KK-2, *A. vinelandii* PR-NDD-1 and *A. vinelandii* BRO-KPS-5 (27.87, 26.37, 25.05, 21.95 and 21.57 g/plant, respectively) which were higher than those of N5 and N10 and control treatments. Azotobacter did not affect fresh weight and dry weight of brockaleli at 45, 65 days and harvesting date. The fresh and dry weight of plant increased rapidly during 65 days to harvesting days. It seemed that 77.78% of application of Azotobacter tended

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to had more fresh weight and dry weight than that of control. At 45 days after planting, the tendency suggested that using *A. chroococcum* LET-NDD-3 had the highest fresh and dry weight of plant. In this study, that of *A. vinelandii* CHI-KK-2 gave the highest of fresh weight at 65 days (14.22 g/plant), whereas MIX gave the highest of dry weight (14.43 g/plant). At the harvesting date, plant treated with *A. vinelandii* CK-KS-3 showed the highest fresh and dry weight at 305.38 g/plant, while N20 was the highest dry weight at 54.35 g/plant.

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Table 35 Fresh and dry weight of brockaleli of various treatments at 45, 55, 65 days and harvest date in field experiment.

Treatments	Fresh and dry weight brockaleli (g/plant)							
	45 day		55 day		65 day		Harvest	
	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight
Control	4.35	0.50	13.18d	1.33	40.03	6.22	187.10	37.87
N5	5.44	0.67	17.26b-d	1.74	65.76	9.95	241.86	45.92
N10	4.46	0.51	18.74b-d	2.11	69.45	10.93	293.26	54.35
A. vinelandii CK-KS-3	4.75	0.58	32.28a	3.12	92.22	12.81	305.38	50.61
A. vinelandii CK-KS-2	4.32	0.53	18.95b-d	1.93	56.88	10.22	269.18	49.27
A. vinelandii CK-NDD-1	4.75	0.63	17.74b-d	1.91	46.58	8.21	163.04	33.22
A. beijerinckii LET-NDD-1	5.26	0.64	27.87ab	3.11	70.71	11.35	220.43	44.31
A. vinelandii CHI-KK-2	5.42	0.67	25.05a-d	2.61	92.94	14.22	295.29	52.21
A. vinelandii PR-NDD-1	4.90	0.62	21.95a-d	2.32	67.68	10.26	258.34	46.64
A. chroococcum LET-NDD-3	6.70	0.76	14.49cd	1.60	73.23	11.30	300.91	52.95
A. vinelandii BRO-KPS-5	4.16	0.52	21.57a-d	2.26	60.54	9.05	195.37	37.38
MIX	5.57	0.69	26.37a-c	2.66	91.66	14.43	248.44	47.89
Mean	5.01	0.61	21.29	2.23	68.97	10.74	248.21	46.05
P-value	NS	NS	*	NS	NS	NS	NS	NS
CV (%)	26.49	28.11	24.48	24.87	29.67	27.63	22.23	25.78

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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4. Yield and yield components of brockaleli at harvesting date

4.1 Days to first flowering, harvest date, shoot length and branches, leaf greeness, leaf area and firmness of leaf stalk, stem and flower of brockaleli

In this work, various treatments affected the firmness of plant stem, while those of other yield components were not significant (Table 36). In case of firmness of plant stem, *A. chroococcum* LET-NDD-3 had the lowest firmness (5.15 N/cm^2) followed by *A. vinelandii* CK-NDD-1, MIX, *A. vinelandii* PR-NDD-1, N5 and N10 (5.85, 6.50, 6.85, 7.20 and 7.20 N/cm^2, respectively) which were lower than those of *A. vinelandii* CK-KS-2, *A. vinelandii* BRO-KPS-5, control, *A. vinelandii* CK-KS-3 and *A. beijerinckii* LET-NDD-1 plants (7.53, 7.78, 7.58, 8.60 and 8.70 N/cm^2, respectively).

For the days to first flowering and days to harvesting, application of these Azotobacter species tend to induce earlier flower buds than chemical and control plants (Figure 36). Most of Azotobacter (67 %) showed rapid flowering and harvesting stages during 75-83 days after planting. SPAD unit of leaves were similar among the treatments. Anyhow, inoculated leaves seemed to have more greenness higher than that of control and chemical fertilizer (Figure 36). About 28.89 % of inoculated plants had the higher greenness than those of N5, N10 and control. In this case, plant inoculated with *A. vinelandii* CK-KS-3 was the highest greenness with SPAD unit of 78.05.

Utilization of Azotobacter increased leaf area of plant and larger than control and N5 (Figure 36). Leaf areas of inoculated plants were larger than that of N10. It seemed that plant had the widest leaf area (3297.30 cm^2/plant) after application of *A. vinelandii* CHI-KK-2, while control plant was the lowest (939.30 cm^2/plant).

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4.2 Yield and dry weight of brockaleli

Various treatments affected on fresh weight of shoot and dry weight of root, whereas the others did not effect as illustrated in Table 37. For shoot fresh weight, the result showed that N10 plants had the highest weight at 1,202.2 kg/rai followed by *A. vinelandii* CHI-KK-2 (1,196 kg/rai), *A. vinelandii* CK-KS-3, and *A. chroococcum* LET-NDD-3 which gave higher plant yield than those of the others. For root dry weight, plant treated with *A. vinelandii* PR-NDD-1 showed the highest dry matter at 47.2 kg/rai that similar to those of *A. vinelandii* CHI-KK-2, N10, and *A. vinelandii* CK-NDD-1. However, *A. vinelandii* CK-NDD-1 tended to have less fresh and dry weight of root, shoot, and whole plant than those of other Azotobacter isolates, chemical fertilizers and control. Among various strains, *A. chroococcum* LET-NDD-3 gave the highest of flower fresh and dry weight (1106.6 and 151.7 kg/rai, respectively). Fresh weight of entire plant of *A. vinelandii* CK-KS-3 showed the highest (2388.8 kg/rai), whereas N10 had the highest dry weight of whole plant (425.1 kg/rai).

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Table 36 Yield components of brockaleli in various treatments of field experiment.

Treatments	Days to first flowering (days)	Harvest date (days)	Shoot length (cm)	Branches	Leaf greenness (SPAD unit)	Leaf area (cm^2)	Firmness(N/cm^2)		
							Leafstalk	Stem	Flower
Control	86	95	24.22	6.74	54.15	939.30	5.00	7.58ab	4.75
N5	88	96	25.44	7.98	62.25	1223.50	5.40	7.20a-c	5.30
N10	84	96	25.81	8.50	64.75	1831.50	5.00	7.20a-c	5.03
A. vinelandii CK-KS-3	83	95	25.82	8.40	78.05	1460.30	4.46	8.60a	4.43
A. vinelandii CK-KS-2	83	94	27.69	7.41	71.78	1384.20	5.43	7.53ab	5.18
A. vinelandii CK-NDD-1	86	92	21.40	5.38	63.58	1243.00	4.68	5.85bc	*4.25*
A. beijerinckii LET-NDD-1	85	93	25.45	7.58	61.83	1555.40	5.18	8.70a	4.75
A. vinelandii CHI-KK-2	75	85	28.12	8.25	64.28	3297.30	4.98	8.03ab	4.98
A. vinelandii PR-NDD-1	87	95	26.32	7.66	62.50	2130.80	4.50	6.85a-c	4.30
A. chroococcum LET-NDD-3	80	92	25.37	8.25	67.78	2263.60	4.43	5.15c	4.50
A. vinelandii BRO-KPS-5	81	91	26.97	7.20	66.10	1972.50	4.65	7.78ab	4.48
MIX	81	93	25.81	6.18	70.25	1884.30	4.95	6.50a-c	3.90
Mean	83	93	25.70	7.46	65.61	1765.48	4.89	7.25	4.65
P-value	NS	NS	NS	NS	NS	NS	NS	*	NS
CV(%)	10	8	16.30	32.27	15.11	34.43	14.39	18.596	16.52

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Table 37 Fresh and dry weight of brockaleli at harvesting date in various treatments of field experiment.

Treatment	Fresh weight (kg/rai)				Dry weight (kg/rai)			
	Flower	Shoot	Root	Whole plant	Flower	Shoot	Root	Whole plant
Control	668.1	693.3bc	102.2	1463.5	110.9	148.3	37.0a-c	296.2
N5	787.1	994.3a-c	110.5	1891.9	113.8	206.7	38.6a-c	359.2
N10	958.8	1202.2a	133.0	2293.9	138.0	241.2	45.9a	425.1
A. vinelandii CK-KS-3	1068.2	1170.4ab	150.1	2388.8	144.6	208.3	42.9ab	395.9
A. vinelandii CK-KS-2	1028.5	965.4a-c	111.6	2105.6	144.8	205.1	35.6a-c	385.4
A. vinelandii CK-NDD-1	595.2	603.3c	76.7	1275.3	97.8	134.7	27.5c	259.9
A. beijerinckii LET-NDD-1	740.4	881.3a-c	102.7	1724.3	111.6	200.3	34.7a-c	346.6
A. vinelandii CHI-KK-2	980.1	1196.0a	133.7	2309.8	119.4	242.1	47.0a	408.4
A. vinelandii PR-NDD-1	936.9	953.1a-c	130.8	2020.8	115.5	202.2	47.2a	364.8
A. chroococcum LET-NDD-3	1106.6	1115.7ab	131.5	2353.8	151.7	221.7	40.9a-c	414.2
A. vinelandii BRO-KPS-5	622.6	816.5a-c	89.2	1528.2	94.8	167.6	30.1bc	292.4
MIX	851.1	949.7a-c	142.7	1943.4	124.5	209.3	40.9a-c	374.6
Mean	861.9	961.7	117.9	1941.6	122.3	199.0	39.0	360.2
P-value	NS	0.038	NS	NS	NS	NS	*	NS
CV(%)	45.99	30.19	31.09	32.23	41.55	28.12	20.91	25.78

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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5. Total N, total P and total K contents of brockaleli

The result showed that total P contents of flower was highly significant different, whereas those of total N, total P and total K contents in shoot and total N and total K in flower did not different in various treatments (Table 38). In this finding, application of *A. vinelandii* CK-KS-2 gave the highest total P content of flower (0.71 %) similar to those of *A. vinelandii* CK-KS-3 , N5, N10, *A. beijerinckii* LET-NDD-1 and *A. vinelandii* CK-NDD-1 (0.64, 0.64, 0.64, 0.62 and 0.61 %, respectively), which were higher than those of others and control. In case of total N content in shoot, the result tended to show that *A. vinelandii* CHI-KK-2 had highest value, whereas N10 had the lowest in total N. For total shoot P, it appeared that *A. vinelandii* BRO-KPS-5 gave the highest total shoot P. The total shoot K ranged from 3.34 % to 4.04 %, which the highest value was showed in *A. vinelandii* PR-NDD-1 treatment. In the case of mineral contents in flowers of brockaleli, the tendency showed that the highest in total N and total K contents in the flowers were found in *A. vinelandii* CK-KS-2 and *A. vinelandii* CHI-KK-2 treatments.

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Table 38 Total N, P and K contents of brockaleli in various treatments of field experiment.

Treatments	Stem			Flower		
	Total N (%)	Total P (%)	Total K (%)	Total N (%)	Total P (%)	Total K (%)
Control	2.50	0.49	3.36	2.63	0.59bc	3.35
N5	2.35	0.55	3.51	2.77	0.64ab	3.72
N10	2.18	0.49	3.36	2.69	0.64ab	3.83
A. vinelandii CK-KS-3	2.38	0.51	3.61	2.79	0.64ab	3.83
A. vinelandii CK-KS-2	2.62	0.55	3.34	2.92	0.71a	3.90
A. vinelandii CK-NDD-1	2.39	0.54	3.34	2.59	0.61a-c	3.64
A. beijerinckii LET-NDD-1	2.31	0.45	3.69	2.74	0.62ab	4.00
A. vinelandii CHI-KK-2	2.73	0.59	3.61	2.48	0.60bc	3.57
A. vinelandii PR-NDD-1	2.72	0.58	4.04	2.73	0.51cd	3.98
A. chroococcum LET-NDD-3	2.37	0.53	3.92	2.74	0.48d	3.97
A. vinelandii BRO-KPS-5	2.40	0.60	3.85	2.55	0.48d	3.80
MIX	2.45	0.51	3.51	2.56	0.45d	3.93
Mean	2.45	0.53	3.59	2.68	0.58	3.79
P-value	NS	NS	NS	NS	**	NS
CV(%)	18.31	15.60	14.75	9.80	10.95	8.56

Remark: Mean values in each column followed by the same letter are not significantly different by DMRT at $P \leq .05$.

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Discussion

Effect of various habitats on the abundance of *Azotobacter* spp. from various rhizospheric soils and its diversity

This experiment showed that population of *Azotobacter* spp. in rhisosphere from various crops ranged of 10^6 cells/g soil similar to the report of Satrulee (1979). It revealed that population of Azotobacter in the Sakaerat forest soils ranged from 10^4-10^6 cells/g soil. In this study, the numbers of Azotobacter were higher than the reported by Islam *et al*. (2008) and Narula (2000). They found that population of *Azotobacter* spp. was generally found to be 10^4 cells in dry soil. However, this result had lower bacterial population (10^7 cells/g) than in the Nile valley and other warm regions of the Middle East in India. In contrast, the report of Barnes *et al*. (2007) showed that cell numbers of Azotobacter were found generally in the soil approximately a few thousand cells per gram dry soil.

The result suggested that population of *Azotobacter* spp. in the rhizosphere from various soil habitats were controlled by plant crops, moisture content, organic matter and pH similar to numerous reports (Channal *et al*., 1989; Mala, 2003). They confirmed that the abundance of Azotobacter depends on several factors such as soil properties including microbiological (Kizilkaya, 2009), chemical and physical properties (Channal *et al*., 1989; Mala, 2003) which were governed by soil types.

Water absorption capacity of the soil varies depending on soil types which are directly controlled by organic matter, humus and clay mineral contents. In this observation, the result indicated that soil moisture content is one of major limiting factor controlling the population of *Azotobacter* spp. in the rhizosphere similar to the reports of Mala (2003) and Islam *et al*. (2008). Soil moisture content is directly controlled by organic matter, humus and clay mineral contents which varies depending on soil types. Barnes *et al*. (2007) demonstrated that there was the significant correlation between proliferation of Azotobacter and soil moisture content.

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In this sense, soil moisture content governs soil temperature, gas exchange capacity, availability of nutrient, transportation of bacterial wastes and decomposing of organic matter in the soil (Tate, 2000) which associates on microbial communities. In this finding, rising of soil moisture content promoted the abundance of *Azotobacter* spp. which was varied depends on soil types, temperature, and plant species. Proliferation of *Azotobacte*r spp. slightly declined at over 20 % soil moisture content as illustrated in Figure 1. The result indicated that *Azotobacter* spp. can exist, although there was low soil water content (5-10 % soil moisture content) which was similar to the attribution of Nijhoff (1982). This bacterium can survive in dry soil more than 24 years (Moreno *et al.,* 1986) due to the dormancy mechanism of cyst formation which will develop (Holt *et al*., 2000; Orville *et al*., 1961) new cell under the favorable environments. However, bacterial growth remained at 70 % soil moisture content and decreased at over 100 % soil moisture content (Islam *et al*., 2008).

In addition, soil organic matter had significant influence on the appearance of Azotobacter in the rhizosphere (Figure 2). Soil organic matter provided an essential carbon and energy source for Azotobacter communities (Tate, 2000) in the rhizosphere. Increasing of soil organic matter, the decomposition rate, organic carbon content and enzyme activities (like alkaline-phosphates, urease, and invertase and protease) had increased. In this study, adding soil organic matter content, the proliferation and density of Azotobacter had increased similar to report of Mala (2003). However, the density of Azotobacter reached to the maximum at 3.6-4.5 % organic matter and was gradually declined at > 4.5 % organic matter. In this case, rising of organic matter over 4.5 % caused the increasing of fungi which was most capable microorganisms of decomposing the majority of organic material, and other microbial decomposer populations. This observation was confirmed by Hopkins and Dungait (2010) that the presence of natural compounds in soil resulted in enhancement of decomposer microbial communities which led to deficiency of organic carbon source and some microorganisms against the growth of *Azotobacter* spp. in the rhizosphere.

According to this study, it may be suggested that soil pH was a crucial effect on the population of Azotobacter in soil (Figure 3) which varied depending on pH

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ranges and species. Soil pH affected soil enzyme activities which was denatured under acid conditions. Moreover, pH influences on solubility of plant mineral and toxic compounds, ionization of inorganic and organic components under acid condition (Eldor, 2007). Under acid condition, molybdenum, necessary mineral for nitrogen fixation, was precipitated, whereas organic acid and toxic compounds were released and inhibited growth of Azotobacter (Tate, 2000). Basically, the optimum pH of Azotobacter was neutral to slightly alkaline soil pH (Narula, 2000; Mala, 2003) which was similar to this study. However, in this study, density of *Azotobacter* spp. sharply depleted to the lowest at pH 7.4 - 7.8 due to rising of bacterial populations and depleting C-source. In another case, most soil samples in this pH range are sandy soils which lacks of moisture contents and limits the growth of Azotobacter (Mala, 2003; Martyniuk and Martyniuk, 2003; Islam *et al*., 2008). Azotobacter can survive in wide pH ranges depending on the strains (Mendes *et al.,* 1999). Yasmin *et al.* (2004) reported that Azotobacter existed between pH 4.0-8.5 which similar the attribution of Channal *et al*. (1989). While Islam *et al*. (2008) found that Azotobacter grew at pH 4.2 - 4.7 which conformed to this study. In addition, at pH 4.6-5.0 is rather more favorable for fungal density than bacterium in the rhizosphere. In contrast, Agrawal and Singh (2002) reported that Azotobacter can grow at pH 6.0-8.0 but it cannot grow at pH 3.5 (strongly acid soil) and only four strains survived at pH 10.

The previous data showed that crop groups had significant effect on the abundance of Azotobacter as illustrated in Figure 4. In this finding, the proliferation of Azotobacter among soil of six crop groups were ranged from 1.73 - 4.24 $x10^6$ cfu/g which higher than previous report of Karthikeyan *et al*. (2008) and Kizilkaya (2009). The data presented here showed that population of Azotobacter in soils among flower crops, fruits and herbs were similar to the vegetable soil, which higher than those of soil samples taken from trees and field crops. For any assumption, it may come from the high soil organic matter contents of these soils due to intensive application of organic matter during cropping and high amount of crop residues in the area and more suitable for Azotobacter growth (Kautz *et al*., 2004) than those of soil from trees and field crops, similar to the report of Kole *et al*. (1988). The data presented here

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suggested that abundance of *Azotobacter* spp. concerned with root litter and variation of root exudates presented in the rhizosphere (sugars, polysaccharides, amino acids, organic acid, growth factors, enzymes and nucleotides etc. (Bolton *et al*., 1992; Pinton *et al*., 2007), the major source of carbon and energy for Azotobacter activities, which different exudation patterns promote different microbial densities. It indicated that large amount and various pattern of root exudates were produced by vegetables, flower crops, fruits and herbs more than those of trees and agronomy crops. This assumption was confirmed by numerous authors (Rovira, 1959; Bolton *et al*., 1992) that there was the variation of different exudation patterns from different plant species. For instance, oats growing in the sand excreted 14 amino compounds, whereas 22 amino compounds were released by Peas (Rovira, 1956). It also seems that soil properties were the main cause of Azotobacter population in rhizosphere (Channal *et al*., 1989) of field crops. Most of agronomy soils are sandy soils which have less soil moisture content, soil organic matter and plant residues returning into the soils than those of other crop group soils (Ibomcha Singh and Yadava, 2006). However, quantities and pattern of root exudate varied depending on stage of plant development.

In the point of agricultural practices, the population and density of Azotobacter between conventional and organic agricultural practices was not significant (Figure 5.) which was similar to Wardle *et al*. (1999). This research suggested a non-significant effect on the microbial population by agricultural practices. Although herbicides and insecticides were applied during cropping on conventional agricultural practices, no effects on microbial population were found. In addition, these substances can stimulate abundance of Azotobacter in rhizosphere. On the contrary, high concentration of pesticide can limit the occurrence of Azotobacter in the soil. In this case, soil temperature during sampling period also was a factor which controlled the number of Azotobacter and resulted in a non-significant effect. This probably due to the organic farming soils which were taken during low temperature (18-20 ^{o}C.), while conventional agricultural soils were taken during high temperature (30-35 ^{o}C.). At low temperature condition, Azotobacter growth rates had declined (Slonnezewski and Foster, 2009).

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However, non-significant effects on the proliferation of Azotobacter were found in Loei, Nonthaburi, Kanchanaburi, Nakhon Si Thammarat and Nakhon Pathom locations (Figure 6). In this observation, it suggested that the organic matter contents and soil pH of these sites were not different. Although Loei soil had shown more soil organic matter than those of other locations, plant crops including tree and field crops were limiting factors which indicated non-significant result. Moreover, Kanchanaburi soil in which herbs were planted, the population of Azotobacter remained the same as that of other areas. In this case, soil moisture content of Kanchanaburi soils were the lowest level (5-10 % soil moisture content), which was a limiting factor and inhibited the appearance of Azotobacter as described above. According to earlier result, it appears that the distribution of *Azotobacter* spp. in various locations varied depending on many factors, viz soil types and soil properties (organic matter, moisture, pH and temperature (Channal *et al*., 1989; Mala, 2003). Moreover, the microbiological properties (microbial interaction properties) are limiting factors for Azotobacter growth (Kizilkaya, 2009).

Chracteristics of *Azotobacter* spp. from various crops

Diversity and origin of 90 isolates were shown in Table 7 and 10. In case of classification, ninety isolates showed difference morphological colony depends on strains and origins. It revealed that 90 dominant isolates of Azotobacter were screened from 29 crops belonging to 5 provinces which were preliminary classified by their morphology into 6 groups as described. The result suggested that most of 42.2 % (38 isolates) were found on group three which obtained from vegetable soils followed by group two (23.33 %), group four (21.11 %), group one (7.78 %), group five (3.33 %) and group six (2.22 %).

The appearance of colonial morphology and other homopolysaccharide/slime varied depending on species and genetically control. This study showed that most of 90 strains had transparent and translucent colony on N-free Ashby's agar as shown in Table 8. In addition, it exhibited that colony size of Azotobacter collecting by vegetable and flower crops had higher population and larger colony than those of

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other crops while culturing on N-free Ashby's agar. This group could had large curled colonies and grown very fast. It had higher heat tolerant capacity than those of other groups when preserved in N-free agar. Number of cells and distribution depends on many factors as described above (Channal,1989; Phiromtan *et al.*, 2009). However, group one, five and six were formed abundant slime on N-free Ashby's agar.

Effect of various carriers from agricultural wastes and storage temperatures on survival of *Azotobacter* spp. in the inoculum

Population of *Azotobacter vinelandii* NDD-CK-1 decreased over time depending on types of carrier (Figure 18) and storage temperatures (Figure 19), similar to earlier reports (Youssef *et al*., 1997; Saleh *et al*., 2001). Initial microbial populations of those materials were higher than those reported by Muthuselvam and Tholkappian (2008). The density of bacteria sharply dropped at 7 days due to lack of moisture and nutrients of the carriers. In this case, OM, total N, total P, and total K as well as moisture content of inoculum carriers were almost depleted by time due to bacterial activities and storage conditions while transitioning from logarithmic to stationary phase during incubation (Neidhardt *et al*., 1990; Tate, 2000). The bacterial population slightly declined until 15 days. During 7-30 days of storage the death rate was the smallest but greatly decreased from 30 to 60 days and leveled off at 90 days of storage. In this study, the survival of Azotobacter in PtLC, PtCC and PtMC carriers were satisfactory in comparison with the biofertilizer standard, except in Pt carrier after storage for 90 days. However, the microbial population was less than those reported by many workers (Wangaruro *et al*., 2000; Madan and Singh, 2010; Raja and Karmegam, 2010). They found that populations of *A. chroococcum* did not reduce below 10^8 cfu/g within three months.

Effect of carriers on the population of Azotobacter

The results indicated that PtLC was the most suitable carrier for production of Azotobacter inoculum, followed by PtCC, PtMC and Pt (Figure 14 -17). LC had more nutrients, especially N content than the others possibly from leaves of the

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legume yellow flamboyant. PtLC also had the highest pH but lowest EC (Table 15) that was suitable for promotiing microbial growth. PtLC may also release less toxic compounds during sterilization as compared to peat (Burton, 1984; Bashan, 1998; Anonymous, 2006). This compost also had high clay mineral derived from adding of clayey soil during composting process which played a critical function in promoting physical and biochemical environment for the microbial population. The increase in high specific surface area of PtLC can promote adsorption of organic and inorganic substances, cation exchange capacity, and water holding capacity. In addition, clay particles also encourage microbial catabolism by increasing adherence and tolerance capacity of Azotobacter in the PtLC under hot condition (Tate, 2000). This finding is similar to the report of Bashan (1998) that survival of bacteria was increased by adding clay to the alginate beads as compared to alginate beads alone. This finding showed that Pt gave poor survival of Azotobacter during 90 days, similar to the report of Muthuselvam and Tholkappian (2008) and Thananusont (1993). Pt had lower quality and higher acidity than that of the others because over 50 % of the peat was mixed with coconut husk which is considered a poor source of carrier. Thus, adding plant leaf compost and corn stubble compost to peat can improve the inoculum quality and longevity of Azotobacter. Many researchers have suggested to use various combinations of carriers to prolong shelf life of bacterium inoculant (Jauhri *et al*., 1979; Jauhri and Philip, 1984; Thungtrakul, 1987; Gaind and Gaur, 2004; Madan and Singh, 2010; Raja and Karmegam, 2010).

PtCC had the highest bacterial number at the initial date possibly due to its fine structure, which gave rise to higher specific surface area, more water content and nutrients spreading more thoroughly and easily adhered to microorganisms than those of the other carriers. This condition promoted bacterial growth within a few days after inoculation as compared to the condition in larger particle size carriers as in PtLC and PtMC. In contrast, PtMC had the lowest bacterial population due to the remaining of lignin and tannin from Para rubber saw dust, the major material of MC. The saw dust blocked bacterial respiration and reduced water absorption of MC resulting in less available water and inhibiting the bacterial population during incubation period.

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Effect of storage temperature on population of Azotobacter

The storage temperature affected proliferation of Azotobacter spp. in the carriers. This study suggested that population of Azotobacter was decreased by increasing temperatures (Figure 19). The survival of bacteria considerably dropped at 7 days and slightly declined during 15 to 30 days, similar to the result of Somasegaran (1985). After one month, number of Azotobacter continuously declined until reaching the lowest number of viable cells at 60 and 90 days after storage. The suitable temperature for preservation of Azotobacter until 90 days was 5 ^{o}C, followed by -16 ^{o}C which gave more viable cells than that stored at over 20 ^{o}C. The extreme temperatures reduced the survival rate of bacteria and its metabolism (Slonnezewski and Foster, 2010). Our finding is similar to the reports of Ben Rebah *et al*. (2002) and Kibunja (1991). At the refrigerating temperature, the bacteria had lower metabolism and physiological activity which maintained high mineral contents and more available moisture than that stored over 20 ^{o}C, similar to the report of Bozida and Vladimir (1995). Inoculum stored at 5 ^{o}C had longer shelf life without formation of ice crystal as that stored at -16 ^{o}C. However, storing carriers at -16 ^{o}C gave the highest bacterial population at 7 to 15 days and sharply declined afterwards. Preservation at -16 ^{o}C for over a month caused water to become ice in the microbial cells and carriers, thus reduced the microbial density by initial killing at the time of freezing and afterwards (Neidhardt *et al*., 1990).

Interaction between carriers and temperatures

The interaction between types of carrier and storage temperatures was similar to the report of Thungtrakul (1987), except at the initial storing period**.** At 7 days, the number of Azotobacter in PtLC stored at 37.5±2.5 ^{o}C was higher than those at other temperatures. It may be due to the increasing of temperature from 17-19 ^{o}C during incubation to 37.5±2.5 ^{o}C in storage which promoted population of bacteria during the first week without limitation of water and nutrients, and then fell down sharply afterwards. A similar result was reported by Saleh *et al*. (2001) that the population of *Azotobacter vinelandii* A1 in rice husk carrier rose up to 128 % from the initial population after storing at 30 ^{o}C. At 30 days after storage, the bacterial population

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increased slightly in Pt and PtLC stored at -16 ^{o}C, and Pt, PtCC, PtMC and PtLC at 5 ^{o}C. During 7-15 days, PtCC stored at -16 ^{o}C gave the maximum survival rate. The explaination may be that PtCC had fine particle which acted as the protective insulation from the damage of ice crystal under sub-zero temperature. In the case of Pt carrier, population of Azotobacter was maximum at -16 ^{o}C, followed by 5 ^{o}C (Figure 18). Among types of carrier, Pt showed a robust property in keeping high population after storage at 37.5±2.5 ^{o}C, similar to PtLC and PtMC, but was higher than PtCC. Pt has lighter particle which protected bacterial cells from extreme temperatures better than the other carriers during 3 months. However, interaction between bacteria, carrier and temperature was a complicated mechanism limiting number of Azotobacter under various environments. The survival ability of the bacteria varies depending on species and strains of Azotobacter which are determined by gene expression under stressful conditions, habitat, and duration of preservation period in the medium.

Nitrogen fixation of various Azotobacter isolates in the laboratory

In case of nitrogen fixation of various *Azotobacter* spp., the study confirmed that N-fixation activity of 90 isolates varied depend on species (Table 9) similar to numerous authors. This finding was similar to several researchers (Holt *et al*., 2000; Narula, 2000; Ahmad *et al.,* 2008). From earlier result, we found that 31.11% of all isolates showed higher fixed N than mean which 13 isolates had the N-fixing ability of 5.5-6.5 mgN/L. Nonetheless, most of 68.89% (62 isolates) showed lower value than mean (Table 9). In addition, the study had shown that nitrogen fixing ability associated with their plant origins. Each of plant isolates expressed the difference nitrogen fixation abilities. The isolates from Chinese kale soil (CK-NDD-1, CK-KS-2 and CK-KS-3) had the most fixed N (6.5 mg N/L), whereas isolates from citronella grass was the lowest on fixed-N (4.0 mg N/L). Moreover, the data presented here showed that most of vegetable isolates had higher N-fixing activities by comparison with that of flora isolates. Nitrogen fixation capability of *Azotobacter* spp. are control by several constituents, viz. microbial properties which is genetically control associated with the gene expression, maintaining of nitrogen fixing ability under subculture condition, oxygen tolerance capacity, nitrogenase enzyme activity and

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homopolysaccharide production. Microenvironment, viz. oxygen concentration, pH (Jones and Bangs, 1985; Mala, 2003), temperature, molybdenum (Holt *et al*., 2000) and the present of nitrite and nitrate in the culture inhibit nitrogenase activity result in reducing N-fixation (Cejudo and Paneque, 1987). From the research that has been carried out, various Azotobacter isolates showed the different nitrogen fixation capacity depending on their species and their plant origins. In order to validate the work, a more in-depth in investigation into the association of subculture maintaining and their nitrogen fixing expression is needed.

Production of IAA by various isolates of Azotobacter

Various isolates of Azotobacter showed different quantities of IAA production, which varied depends on species and type of bacteria similar to numerous authors (Arshad and Frankenberger, 1997; Ahmad *et al*., 2008). The study showed that 82.32 % of the isolates are able to synthesize IAA, whereas 17.78 % could not (Table 9). Among IAA producing isolates, 5.55 % synthesized more than 40 μg/ml, whereas most of bacterium (34.44%) produced IAA in the range of 30-39 μg/ml. Additionally, 31.11 % of them produced IAA below 1 μg/ml. In this study, amount of IAA produced by various isolates of *Azotobacter* spp. were higher than those report of Ahmad *et al*. (2008). They found that *Azotobacter* spp. could produce IAA in the range of 1.27-3.6 μg/ml. In this study, *A .chroococcum, A. vinelandii and A. paspali* have ability to produce IAA similar to this study. It may suggest that over 30 μg/ml of IAA was produced by 8 efficective isolates (Table 9 and Table 11).

This result similar to that of Arshad and Frankenberger (1993) that Azotobacter can produce a variety of plant hormone or phytohormones both in culture media. Nieto and Frankkenbeger (1989) detected cytokinin in culture filtrate of *A. chroococcum*, *A. vinelandii* and *A. beijernickii.* In addition, Martinez-Teledo *et al.* (2000) found that phytohormon production has been reported for strains belonging to the three species of *A. chrooccoccum, A. bejerinckii* and *A. vinelandii* which have been observed in association with plant. In this study, the result confirmed that 41 Azotobacter isolates produced IAA in higher quantity than research of Ahmad *et al*.

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(2008). In this case, IAA was produced by *A. chroococcum* LET-NDD-3 isolated from lettuce soil could produce IAA of 37.60 μg/ml. Martinez-Teledo *et al*. (1988) reported the effect of maize (*Zea mays*) root exudates on the liberation of auxins, gibberellins and cytokinins by *A. chroococcum* in order to elucidate the interactions established between Azotobacter and maize root.

The potential of IAA production varied depends on the isolates, strains and species. In this study, although PR-NDD-1, CK-NDD-1 and CK-KS-2 isolates belong to *Azotobacter vinelandii*, their ability of IAA synthesis expresses in differences values at 44.87, 37.74 and 35.61 μg/ml, respectively (Table 9 and 11). Moreover, difference species showed various activities. LET-NDD-1 (*A. beijerinckii*) could generate IAA of 30.64 μg/ml, whereas 37.60 μg/ml of IAA was produced by LET-NDD-3 (*A. chroococcum*). This finding is similar to many reports (Arshad and Frankenberger, 1997; Ahmad *et al*., 2008) (Table 9 and 11). In addition, it may be suggested that there was an association with IAA production capacity and microbial habitats. Azotobacter isolated from Chinese kale soils had the most IAA (36.48 μg/ml) followed by cantonese isolates (36.36 μg/ml) which were higher than that of pineapple isolates (0.19 μg/ml). The result revealed that 80 % of Azotobacter isolated from vegetable soils could produce higher IAA than average, whereas 57.89 % could produce IAA more than 30 μg/ml.

Capability of IAA production by these bacteria was controlled by viz. species, type of organisms, incubation time, the substrate (tryptophan) (Lee *et al.,* 1970). While Ahmad *et al*. (2008) reported that IAA production of PGPR bacteria depends on the microorganism types and concentration of tryptophan biosynthesis in the culture. High IAA production is found when tryptophan is increased. Finally, it is worth pointing out that IAA production ability of Azotobacter could be criteria for screening high efficiency strains which apply and improved plant growth. Anyway, the optimize condition for this activity is needed.

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Phosphate solubilization of various isolates of Azotobacter

Phosphate solubilization by Azotobacter was difference action dependent on species, microbial origins (rhizosphere) and bacterial types similar to report of Kumar *et al*. (2011). They demonstrated that *A. chroococcum* show the ability of solubilizing phosphate as well as *Rhizobium* spp., *Pseudomonas* spp. and *Bacillus* spp. There was an association between the ability of phosphate solubilization and plant groups. The study revealed that IAA ability was varied among plant types. Various isolates from the same plant soil established the difference activities. It exhibited that isolates from vegetables showed a positive result of 17.78 % of all soil samples, whereas other plant soils had activity below the average (Table 9). Among phosphate solubilizing strains, isolates from tree soils had larger of the clear zone (a sign of phosphate solubilization) around colony than those of other crops. In contrast, all isolates of para rubber trees and cantonese soils could solute tricalcium phosphate. The potential of phosphate solubilization by *Azotobacter* spp. showed a closely association with plant type. For instance, SUG-KPS-1 showed the positive result, while SUG-KPS-2 could not do, even they were selected from sugarcane soil. In addition, 4 in 5 of isolates of coriander soil could solute phosphate, except COR-NDD-5 could not do. Although, most of broccoli soil isolates could not solubilize tricalcium phosphate, that of BRO-PR-2 and BRO-KPS-7 were. For the reason, local strains have higher tolerance capacity on their plant origins than those of other plants depending on their genetic and adaptation. Acidification, inorganic acids, organic production, acid phosphatases, releasing of H^+, HS production, exopolysacchride production and assimilation from liquid, are the mainly mechanism of phosphate solubilization by *Azotobacter* spp. which were control by several phosphatase-encoding genes. Therefore, *Azotobacter* spp. can be applied to improve the usefulness of available forms of soil P and play an important role in providing of P to plants which influence plant nutrition and yield. Eventually, effect of the variation of P form like the soil on phosphate solubilization activity on agar have to be discussed.

Consequently, 90 isolates of *Azotobacter* spp. collected from twenty nine crop soils were capable of fixed nitrogen, IAA production, and phosphate solubilization

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(Table 9) in different activities depend on species similar to numerous authors (Narula, 2000; Ahmad *et al.,* 2008). In this study, the result found that each trait has different isolate of *Azotobacter* spp. Some strains showed more than two and three high beneficial effect in the laboratory.

Effect of various *Azotobacter* spp. on the growth enhancement of brockaleli

1. Population of Azotobacter in the inoculum and soil after inoculation under greenhouse condition

The potential and adaptation capacity for growing in the carrier both in greenhouse and field condition were varied depending on species, times and soil properties (Figure 21 and 24). In this case, declining of Azotobacter population was found both of greenhouse (Figure 26) and field condition (Figure 27) after introduced into the soil. The proliferation of Azotobacter in the pot was found higher than in field condition. In the greenhouse condition, the proliferation of Azotobacter remarkably declined to 6.03 to 6.54 of log cfu/g after introduced into the soil for 35 days after planting similar to report of Phiromtan *et al.* (2009), whereas the growing of *Azotobacter* spp. was found in the range of 4.63-4.97 log of cfu/g in field trial. In case of abundance of *Azotobacter* spp. under greenhouse condition, this study indicated that microbial density was reduced at 45 days and gradually raised during 65 days after planting. There were the variations of temperatures during exploration. At 45 days, the temperature was sharply dropped to 19 ^{o}C that cause of reducing bacterial density. Later on, the temperature rising till 65 days (over 30 ^{o}C) could induce of Azotobacter (66.67 %). Nevertheless, *Azotobacter* spp. was found in control, N10 and N20 treatments even could not detect at the beginning. In this sense, it may due to the occurrence of Azotobacter in the soil before the experiment is very rare (less than 10^3 cells) which could not be detected in Ashby's medium at dilution 10^{-2} to 10^{-3} fold. Raising the microbial population was found due to adding cow manure and water in the soil. In addition, the chemical fertilizer could promote plant growth and microbial density. Moreover, application of Azotobacter induced the higher survival than that of control, N10 and N20.

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After inoculation of various Azotobacter on field trial, it seems that most of Azotobacter (78 %) declined during 55 days, except those of *A. vinelandii* CHI-KK-2 and *A. vinelandii* LET-NDD-3 and slightly increased at 65 days after planting similar to that of greenhouse experiment. This may be due to the weather during 55 days was very hot (40 ^{o}C) and dried period (end of April, 2010) resulting in the decreased of microbial growth. However, during 65 days bacterial population was raised due to raining and cooler of soil temperature.

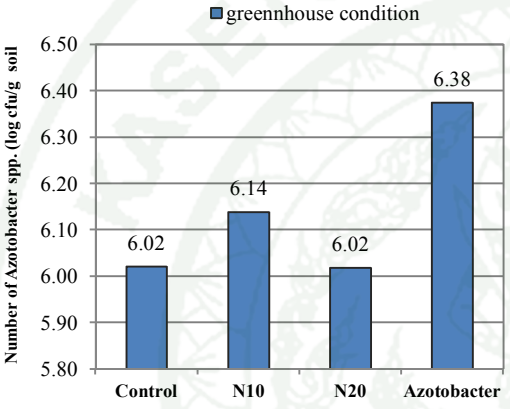

Figure 26 Population of Azotobacter under greenhouse condition.

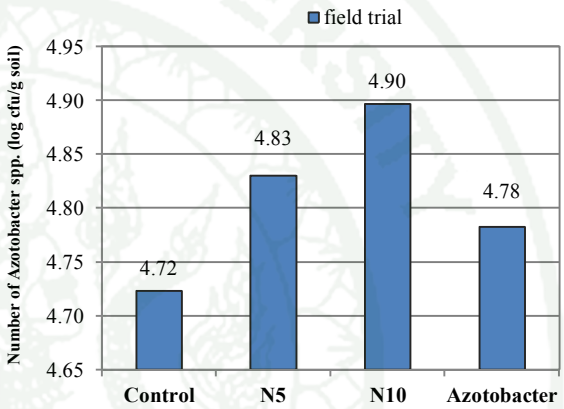

Figure 27 Population of Azotobacter under field condition.

It is unclear that higher population of Azotobacter in the carrier had indicated the high rate of bacterial survival in the soil condition. Although, there were the remarkably of initial population in the inoculum, the actual bacterial survival did not difference after inoculation in the rhizosphere. Although, some isolates grew very slowly in broth culture (Table 14) and carrier material (Figure 23), the population in the soil nearby other strains which gave high population density in such conditions (Figure 24). *A. vinelandii* CK-NDD-1 showed a rapid growth in culture and carrier material, meanwhile it populations in the soil bacteria lower than those of other isolates. This finding is similar to many reports (Chanway and Holl, 1993; Zhender *et al*., 1999). This is complicated interaction between microbial, soil particle and their interaction which is not clearly understood. However, this study could not confirm certainly. Lastly, further studies are need to understand how microbial survived in field which controlled by several factors.

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2. Nitrogenase activity of various strains of Azotobacter

This study found that nitrogenase activity of Azotobacter varied depends on species (Figure 28) which was similar to many reports (Rao, 1975; Narula, 2000; Mala, 2003). In this sense, *A. vinelandii* CK-NDD-1, *A. vinelandii* CK-KS-2 and *A. vinelandii* CK-KS-3 showed the highest nitrogen fixation ability in the laboratory (Table 9), but their nitrogenase activities were lower than those of others under greenhouse condition due to their characteristics, maintaining nitrogen fixing capacity under storage in the culture, soil properties, and interaction between microorganisms and plant response. In this study, the nitrogenase activity of various *Azotobacter* spp. (100 days) was higher than those of N10, N20 and uninoculated control in the ranged of 9,545, 8,995, 9,101 and 5,040 μmol C_2H_4/1 kg soil/crop. This nitrogenase activity were calculated based on the nitrogen fixing ability of *Rhizobium* spp. which nitrogenase activity rate reduced by 40 % during the night (12 hours) by report of (Abu-baker and Arya, 1991) under greenhouse trial. In contrast, nitrogenase activity on field condition (Figure 29) was lower than that observation in the greenhouse (Figure 28). In this case, N10 revealed the most of nitrogenase activity of 8,173 μmol C_2H_4/1 kg soil/crop which was higher than those of Azotobacter application, N5 and uninoculated control (5,969, 5,830 and 5,690 μmol C_2H_4/1 kg soil/crop, respectively). The data suggested that *A. vinelandii* CK-KS-2 had the highest in nitrogenase activity followed by *A. vinelandii* CK-NDD-1, *A. beijerinckii* LET-NDD-1, *A. vinelandii* CHI-KK-2 and MIX which were higher than those of other isolates. For the reason, it may due to several factors on the farm viz. soil environments and soil properties, indigenous microorganisms, predator, substrate and interaction between microorganisms and soil particle, microbe and plant interaction limited and interfered the nitrogen fixing ability of *Azotobacter* spp. in the field (Figure 29) more than soil environment in the pot (Figure 28).

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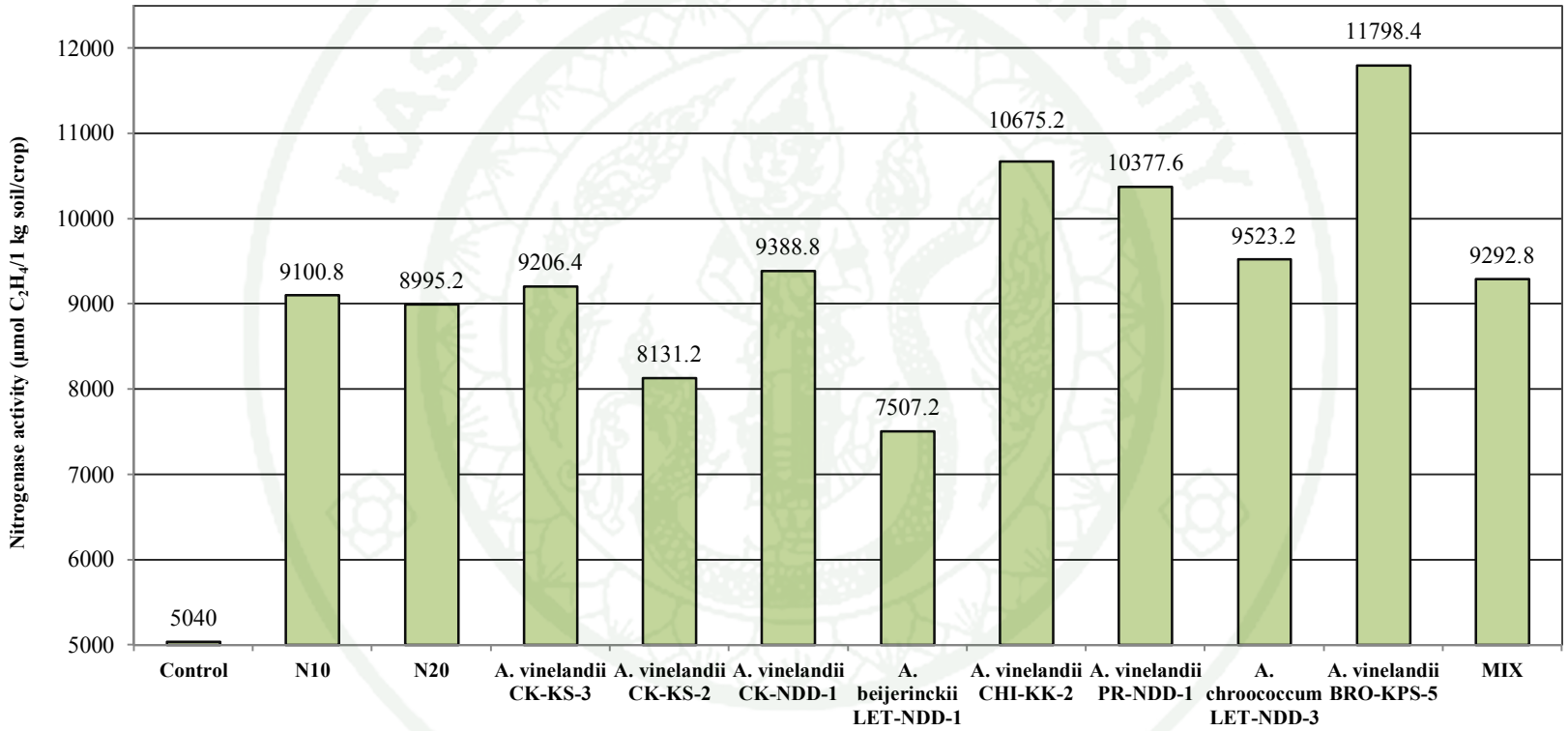

Figure 28 Nitrogenase activity of various Azotobacter in greenhouse (µmol C_2H_4/1 kg soil/crop) calculated from the equation of Abu-baker and Arya (1991).

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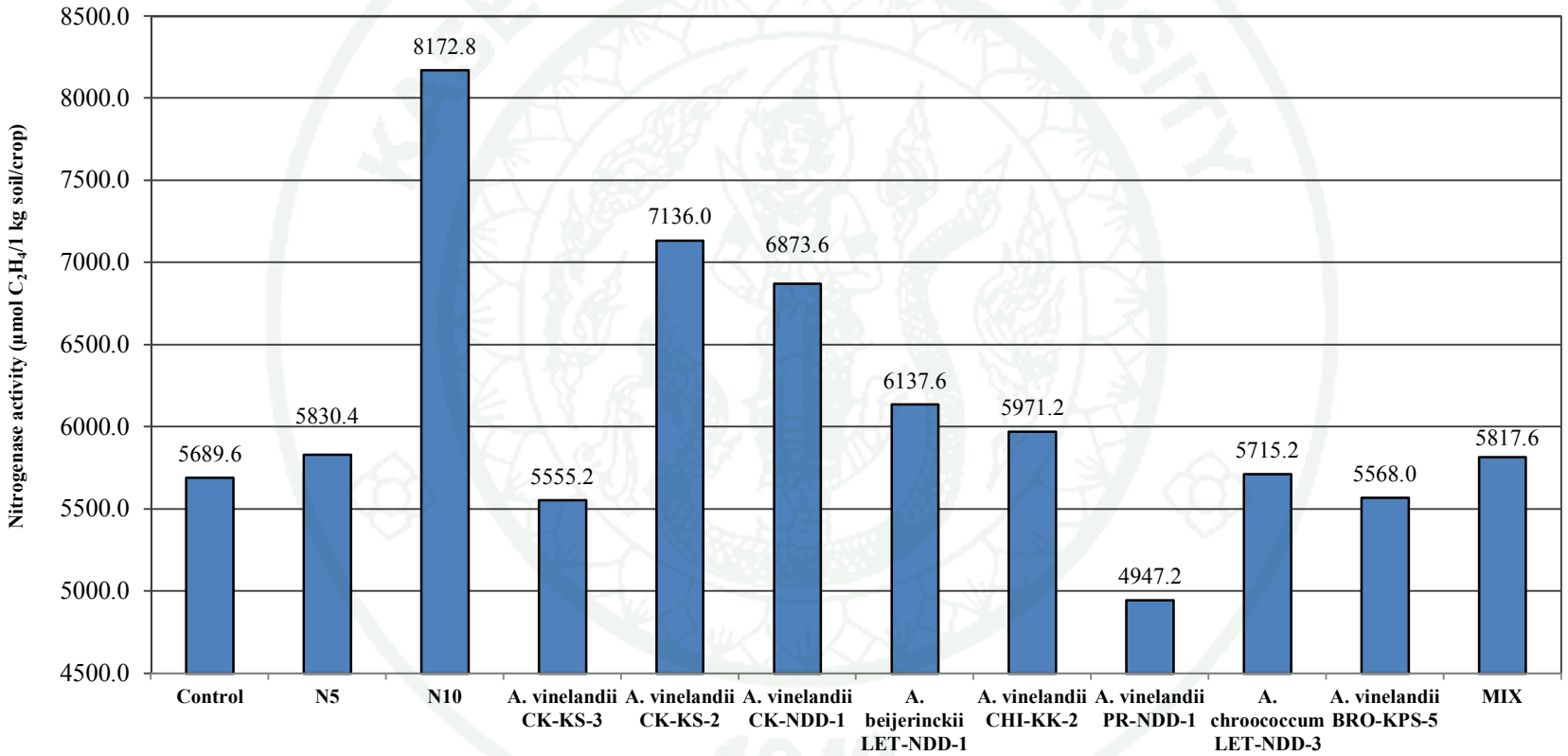

Figure 29 Nitorgenase activity of various Azotobacter in field experiment (µmol C_2H_4/1 kg soil/crop) calculated from Abu-baker and Arya (1991).

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The summation of average of nitrogenase activity indicated that 5.04 to11.80 mmoles C_2H_4/ 1 kg soil/ crop were fixed under greenhouse conditions (Table 39), whereas 4.95 -8.17 mmoles C_2H_4/ 1kg soil/crop were found in field experiment (Table 40). The calculated nitrogenase, was 15 to 34 kg N fixed/rai/crop (92 to 215 kg N fixed/ha/crop) as shown in Table 39, while nitrogenase in field trial ranged from 14 to 24 kg N fixed/rai/crop (90 to 149 kg N fixed/ha/crop) (Table 40) based on: 1) the nitrogenase activity (mmoles C_2H_4), 2) 312,000 kg soil per rai, 3) 100 days per crop, and 4) a theoretical conversion factor of one-third N_2 fixed for each C_2H_4 formed (Hardy *et al*., 1968) as shown on equation (1) and (2). This finding was higher than the report of Hardy *et al*. (1968) that nitrogen fixed rate of soybean nodule range from 30 to 33 kg N fixed/acre/season.

$$\frac{(5.04 \text{ to } 11.80 \text{ mmoles } C_2H_4)}{3} \times \frac{28 \times 312{,}000 \text{ kg soil}}{10^6} = 15\text{-}34 \text{ kg N2 fixed/rai/crop} \quad (1)$$

$$\frac{(4.95 \text{ to } 8.17 \text{ mmoles } C_2H_4)}{3} \times \frac{28 \times 312{,}000 \text{ kg soil}}{10^6} = 14\text{-}24 \text{ kg N2 fixed/rai/crop} \quad (2)$$

Remark: (1) Equation for calculating nitrogen activity under greenhouse condition
(2) Equation for calculating nitrogen activity in field trial
Average values from Figure 28 and 29

Nitrogen fixation required higher energy for nitrogenase and their oxygen protection mechanisms (Quispel, 1974; Rudnick and Kennedy, 2002). In this mechanism, 16 mmoles ATP is required for synthesis of 100 mg of bacterial cell, whereas 8 mmoles of ATP is required for nitrogen fixation of 10 mgN_2. Another reasons, it may come from rising temperature over 35 ^oC (Jones and Bangs, 1985; Mala, 2003) decreased soil moisture content resulting in high oxygen (O_2) concentration in soil particles. In this situation, high oxygen situation inhibited O_2-sensitive nitrogenase enzyme and require high energy for oxygen protection mechanisms lead to declining of nitrogen fixation at that time (Quispel, 1974). Additional points, Azotobacter may form cyst due to hot weather, then their physiology was slow down in this study.

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Table 39 Nitrogenase activity of various *Azotobacter* spp. under greenhouse condition.

Treatments	Nitrogenase activity		
	mmoles C_2H_4/1 kg soil/crop	kg N fixed/ rai/crop	kg N fixed/ ha/crop
Control	5.04	15	92
N10	9.10	27	166
N20	9.00	26	164
A. vinelandii CK-KS-3	9.21	27	168
A. vinelandii CK-KS-2	8.13	24	148
A. vinelandii CK-NDD-1	9.39	27	171
A. beijerinckii LET-NDD-1	7.51	22	137
A. vinelandii CHI-KK-2	10.68	31	194
A. vinelandii PR-NDD-1	10.38	30	189
A. chroococcum LET-NDD-3	9.52	28	173
A. vinelandii BRO-KPS-5	11.80	34	215
MIX	9.29	27	169

Remark: Average values of nitrogenase activity from Figure 22 and 28 at 45 and 65 days.

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Table 40 Nitrogenase activity of various *Azotobacter* spp. in field trial.

Treatments	Nitrogenase activity		
	mmoles C_2H_4/1kg soil/crop	kg N fixed/ rai/crop	kg N fixed/ ha/crop
Control	5.69	17	104
N5	5.83	17	106
N10	8.17	24	149
A. vinelandii CK-KS-3	5.56	16	101
A. vinelandii CK-KS-2	7.14	21	130
A. vinelandii CK-NDD-1	6.87	20	125
A. beijerinckii LET-NDD-1	6.14	18	112
A. vinelandii CHI-KK-2	5.97	17	109
A. vinelandii PR-NDD-1	4.95	14	90
A. chroococcum LET-NDD-3	5.72	17	104
A. vinelandii BRO-KPS-5	5.57	16	101
MIX	5.82	17	106

Remark: Average values of nitrogenase activity from Figure 25 and 29 at 45, 55 and 65 days.

3. Effect of *Azotobacter* spp. on growth and yield of brockaleli

The effects of various Azotobacter inoculum on growth of brockaleli in the green house and field experiment were determined. It is reasonable to assume that utilization of various Azotobacter promoted the growing of brockaleli under greenhouse and field condition similar to numerous reports (Subba Rao, 1984; Gaskins *et al*., 1985; Narula, 2000). This finding confirms that application of Azotobacter enhances growth of brockaleli by increasing fresh and dry root and dry weight of flowers (Prabhjeet and Bhargava, 1994). Azotobacter induced early blooming stage of plant. Moreover, it appeared that these bacterium showed higher fresh weight of stem and flower and increased the number of lateral buds of plant than

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those of N10, N20 and control. According to Experiment II, *Azotobacter* spp. is a free living nitrogen fixing bacteria which had many beneficial effect on plant grown. It is able to fix-N through nitrogen fixation activity which enhances growth and dry matter of plant like the present study and several reports (Brown *et al*., 1964; Subba Rao, 1984; Milosevic *et al*., 1994).

Under greenhouse condition, the result indicated that significant fresh and dry weight of plant were found on N20 followed by *Azotobacter* spp., N10 and uninoculated control (Figure 30). The data had shown that root biomass was significant both in greenhouse and field trial. While the highest of fresh and dry weight of brockaleli was found on N10 followed by *Azotobacter* spp., N5 and uninoculated treatment, respectively in field trial (Figure 31). In this finding, fresh and dry weight of brockaleli increased 39 % and 42.1 % due to application of Azotobacter in the pot (Figure 30,Table 41), whereas 34 % and 21.6 % of fresh and dry weight of plant were gained in field trial (Figure 31, Table 42) in comparison with uninoculated control. In addition, 28.9 % of plant yield were increased higher than that of control in field trial.

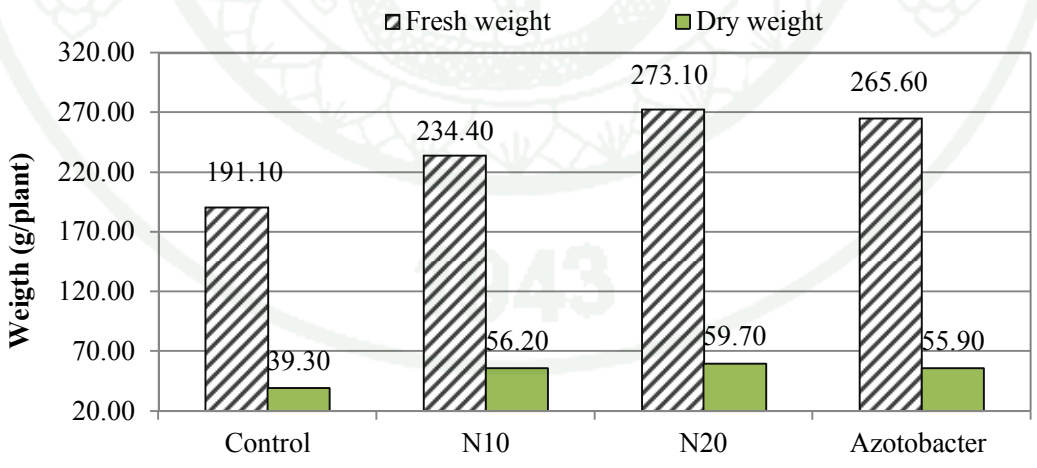

Figure 30 Fresh and dry weight of brockaleli after application of various Azotobacter treatments under greenhouse condition.

Remark: Value of Azotobacter was estimated by average of 9 Azotobacter treatments from previous data.

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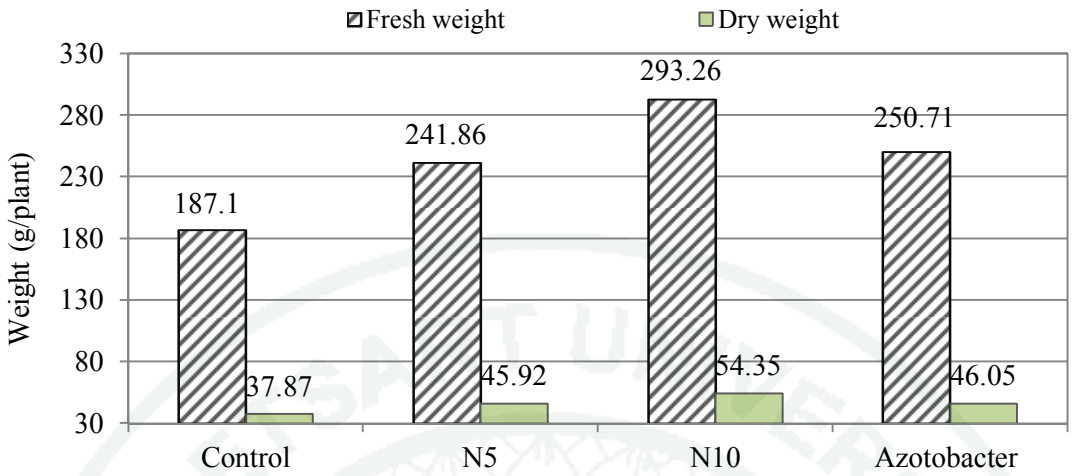

Figure 31 Fresh and dry weight of brockaleli after application of various Azotobacter treatments in field trial.

Remark: Value of Azotobacter was estimated by average of 9 Azotobacter treatments from previous data.

Table 41 Effect of Azotobacter inoculation on growth of brockaleli under greenhouse condition.

Treatments	Fresh weight(g)				Dry weight(g)			
	Stem	Flower	Root	Whole plant	Stem	Flower	Root	Whole plant
Control	127.0	52.2	12.6	191.1	26.9	6.6	15.8	39.3
N10	141.1	51.4	41.9	234.4	29.5	7.6	19.1	56.2
N20	173.0	62.0	38.4	273.1	34.0	8.1	17.7	59.7
Azotobacter	159.4	64.0	42.6	265.6	30.9	8.8	16.5	55.9
% Increasing compared to control	25.5	22.8	238.1	39.0	15.0	32.6	4.6	42.1
% Average	81.3				23.6			

Remark: Value of Azotobacter was estimated by average of 9 Azotobacter treatments from previous data as shown in Table 24.

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Table 42 Effect of Azotobacter inoculation on growth of brockaleli in field trial.

Treatments	Fresh weight of whole plant (g/plant)				Dry weight of whole plant (g/plant)			
	45 days	55 days	65 days	Harvest	45 days	55 days	65 days	Harvest
Control	4.35	13.18	40.03	187.1	0.50	1.33	6.22	37.87
N5	5.44	17.26	65.76	241.86	0.67	1.74	9.95	45.92
N10	4.46	18.74	69.45	293.26	0.51	2.11	10.93	54.35
Azotobacter	5.09	22.92	72.49	250.71	0.63	2.39	11.32	46.05
% Increasing compared	17.0	73.9	81.1	34.0	26.0	79.7	82.0	21.6
% Average	51.5				52.3			

Remark: Value of Azotobacter was estimated by average of 9 Azotobacter treatments from previous data as shown in Table 35.

Table 43 Effect of Azotobacter inoculation on yield of brockaleli in field trial.

Treatments	Fresh weight (kg/rai)			Dry weight (kg/rai)		
	Flower	Shoot	Root	Flower	Shoot	Root
Control	668.1	693.3	102.2	110.9	148.3	37.0
N5	787.1	994.3	110.5	113.8	206.7	38.6
N10	958.8	1202.2	133.0	138.0	241.2	45.9
Azotobacter	881.1	961.3	118.8	122.7	199.0	38.5
% Increasing compared to control	31.9	38.7	16.2	10.7	34.2	4.1
% Average	28.9			16.3		

Remark: Value of Azotobacter was estimated by average of 9 Azotobacter treatments from previous data as shown in Table 37.

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This finding indicated that 28.9 % and 16.3 % of yield and dry matter were found after inoculation with Azotobacter in comparion with unioculated control (Table 41) in field experiment. The result is similar to those of numerous researchers. They used *Azotobacter chroococcum* to increase the yield of several crop plants. Those experiments carried out on various vegetables and field crops. Rice, wheat, maize, sorghum, oats, ragi, pearl millet, barley, forage crop (Guinea grass), chilies, sesamum, cotton, sugarcane, mungbean, lavender, rape seed (*Brassica napus*) and cotton have shown increases in yield due to inoculating with *Azotobacter chroococcum* (Subba Rao, 1984).

The application of Azotobacter affected plant biomass and yield similar to report of Hussain *et al*. (1987) and Brown *et al*. (1964). However, increasing of plant yield and plant biomass was higher than reported by Lakshmanan (2000). They found that field experiments carried out by many researchers on several plantations with the positive benefit of 7-12 % in grain yield is consistent with research. The leaf area of plant after inoculation of Azotobacter were increased than those of others similar to Mohandas (1987). For the reason, due to the trait of Azotobacter like phytohormones production such as IAA, cytokinin and gibberellin were produced by this organisms which may impact of plant growth also similar to my report. Eight local strains could produce IAA in the range of 30.64 - 44.87 µg/ml. IAA could increase biomass of root result in high percentage of root and increasing mineral uptake to the top plant. In case of flowering stage, production of gibberellin by this bacteria result in early flowering similar to report of Arshad and Frankenberger (1997); Martinez-Teledo *et al.* (2000). In this study, the lateral bud of inoculated plant seem to have more bud than those of uninoculated treatments due to synthesis of cytokinin or cytokinin like substance (Nieto and Frankenberger, 1989; Arshad and Frankenberger, 1997). Moreover, the phytohormones help plants for nitrogen assimilation.

The result demonstrated that population of Azotobacter and their nitrogen fixation ability varied by season, plant development and soil properties depends on several factors (Channal, 1989; Phiromtan *et al.*, 2009). The data supported the hypothesis that Azotobacter application influenced plant growth, and yield more than

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34 % both in greenhouse and field trial. Application of this mircroorganism revealed the similar result with that of N5, which was higher than unioculated control on field experiment. This assumed that utilization of Azotobacter could reduce some nitrogenous chemical usage of 10.87 kg urea (5 kg N) while amending of 2 tons/rai of cow manure were applied. The data presented here suggested that isolates *A. vinelandii* CK-KS-2 had the highest potential strain followed by *A. vinelandii* CK-KS-2, MIX, *A. vinelandii* PR-NDD-1, *A. vinelandii* CHI-KK-2, and *A. vinelandii* CK-NDD-1, respectively, which influenced plant growth and yield similar to those of N10 which were higher than control. In case of field trial, the high potential isolates were *A. vinelandii* CHI-KK-2 followed by LET-NDD-3, *A. vinelandii* CH-KS-3, *A. vinelandii* CK-KS-2, and *A. vinelandii* PR-NDD-1 which could be produced as high potential microorganisms.

Consequently, fixed-N by various *Azotobacter* spp. ranged from 137- 215 kgN fixed/ha/crop (22 - 34 kgN fixed/rai/crop). *A. vinelandii* CK-KS-2 was the highest effective strain followed by MIX, *A. vinelandii* PR-NDD-1, *A. vinelandii* CHI-KK-2, *A. vinelandii* CK-KS-3 and *A. vinelandii* CK-NDD-1, respectively under greenhouse condition. Whereas nitrogenase activity were in the range of 90-130 kgN fixed/ha/ crop (14 - 21 kgN fixed/rai/crop). *A. vinelandii* CHI-KK-2, LET-NDD-3, CK-KS-3, *A. vinelandii* CK-KS-2, and *A. vinelandii* PR-NDD-1 were the effective organisms as biofertilizer on field trial. In conclude *A. vinelandii* CHI-KK-2, *A. vinelandii* CK-KS-3, *A. vinelandii* CK-KS-2, and *A. vinelandii* PR-NDD-1 were high effective species which promoted plant growth both in greenhouse and field experiments and provide N which can be apply for plant crops and reduce some part of chemical fertilization in organic farming systems for long term sustainable development.

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CONCLUSION AND RECOMMENDATION

Conclusion

The population of *Azotobacter* spp. in various soil habitats were controlled by plant crops, moisture content, OM and pH. Ninety isolates of Azotobacter were isolated from 29 crops. They were identifying into six groups. Various isolates of *Azotobacter* spp. showed different potential on N_2-fixation, IAA production, and phosphate solubilization. Eight strains including CK-KS-2, CK-KS-3, CK-NDD-1, CHI-KK-2, PR-NDD-1 and BRO-KPS-5 belongs to *Azotobacter vinelandii,* while LET-NDD-1 and LET-NDD-3 belongs to *A. beijerinckii* and *A. chrooccocum,* respectively showed high potential for plant growth enhancement. PtLC was the most beneficial carrier for *Azotobacter* spp. after storage for 90 days. Storing inoculums at 5^{o} C had the largest microbial population. Azotobacter promoted plant growth, yield, first flowering and total K in flower. *Azotobacter vinelandii* CK-KS-2 was the highest effective isolate followed by *A. vinelandii* CK-KS-2, MIX, *A. vinelandii* PR-NDD-1, *A. vinelandii* CHI-KK-2, and *A. vinelandii* CK-NDD-1, respectively. Fixed-N by various *Azotobacter* spp. ranged from 137 to 215 kgN fixed/ha/crop (22 to 34 kgN fixed/ rai/crop). In field trial 44.44% of *Azotobacter* spp. could promote plant growth more than that of N5. *A. vinelandii* CHI-KK-2, *A. chroococcum* LET-NDD-3, *A. vinelandii* CH-KS-3, *A. vinelandii* CK-KS-2, and *A. vinelandii* PR-NDD-1 were the effective organisms. Nitrogenase activity of these bacterium were in the range of 90 to 130 kgN fixed/ha/crop (14 to 21 kgN fixed/rai/crop). *A. vinelandii* CHI-KK-2, *A. vinelandii* CK-KS-3, *A. vinelandii* CK-KS-2, and *A. vinelandii* PR-NDD-1 were high effective strains which promoted plant growth both in greenhouse and field experiments. These *Azotobacter* spp. provided N which can reduce some part of nitrogenous chemical fertilization equal to 5-10 kgN/rai (10.87-21.74 kg of uear/rai) in organic farming systems in near future.

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Recommendation

Adding clayey soil in the inoculum or during composition process is a beneficial technique for improving the quality of bioinoculant and protecting target microorganisms by serving as a micro-environment. An appropriate proportion of clay is necessary for improving product quality. To investigate effect of various habitats, researcher should be done as follows. The study on the factors associated with distribution of Azotobacter should be conducted based on soil type (mainly factor governing the physical, chemical and biological properties of soil), and a plant, which could reduce the variation of the data and obtain deep knowledge on the distribution of Azotobacter and its activities in the agricultural environments. The researcher should study the optimum conditions for *Azotobacter* spp. growth and activities such as pH, salinity, oxygen, substrates (Ca, Mo, etc), fungicides, insecticides and organic carbon, and the capacity of cyst formation and cyst development of Azotobacter in the laboratory. Moreover, a study under greenhouse conditions in order to obtain deep information of tolerant capacity and distribution of *Azotobacter* spp., in the pot have to be discussed. Study the adaptability of *Azotobacter* spp. under various soil plantations and soil communities in field trial in order to predict the distribution and the adaptation ability to various field closely to the actually soil environments is needed. The further research should investigate the effect of *Azotobacter* in different crop and locations in order to gain in deep knowledge which can be apply for various crops in sustainable agriculture development.

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LITERATURE CITED

Abbass, Z. and Y. Okon. 1993. Plant growth promotion by *Azotobacter paspali* in the rhizosphere. **Soil Biol**. **Biochem**. 25(8): 1075-1083.

Abu-baker, M.S, and K.B. Arya. 1991. Nitrogen fixation in Peanut Nodules during dark period and detopped conditions with special reference to lipid bodies. **Plant Physiol**. 95: 896-899.

Agrawal, N. and H.P. Singh. 2002. Selection of Azotobacter strains for growth and survival under stress conditions, pp. 74-81. *In* R.C. Rajak, ed. **Biotechnology of Microbes and Sustainable Utilization**. Scientific Publishers.

Ahmad, F., I. Ahmad and M.S. Khan. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. **Microbiol**. **Res**. 163(2): 173-181.

Anonymous. 2006. **Biofertilizer Manual**. Japan Atomic Industrial Forum (JAIF).

Anonymous. 2010. **Broccolini**. Available Source: http://en.wikipedia.org/wiki/ Broccolini, May 4, 2013.

Arshad, M. and W.T. Frankenberger. 1993. Microbial production of plant growth regulators, pp. 307-347. *In* B.F. Metting., ed. **Soil Microbial Ecology: Applications in Agricultural and Environmental Management**. Marcel Dekker, Inc. New York.

Arshad, M, and W.T. Frankenberger. 1997. Plant Growth-Regulating Substances in the Rhizosphere: Microbial Production and Functions, pp. 45-151. *In* L.S. Donald, ed. **Advance Agronomy**. Academic Press.

Copyright by Kasetsart University All rights reserved

Arthur, R.C. 1953. The action of 2, 4-D upon the Azotobacter of some sugarcane soils. **Appl**. **Microbiol**. 1(4): 184-187.

Athar, R. and M. Ahmad. 2002. Heavy metal toxicity: Effect on plant growth and metal uptake by wheat, and on free living Azotobacter. **Water Air Soil Poll**. 138: 165-180.

Attanandana, T. and J. Chanchareonsook. 1999. **Exercise and Handbook of Laboratory of Soil and Plant Analysis.** Department of Soil Science, Kasetsart University, Bangkok.

Barnes, R.J., J.B. Samantha and R.M. Lark. 2007. Spatial covariation of Azotobacter abundance and soil properties: A case study using the wavelet transform. **Soil Biol**. **Biochem**. 39: 295-310.

Bashan, Y. 1998. Inoculants of plant growth promoting bacteria for use in agriculture. **Biotechn**. **Adv**. 16(4): 729-770.

Becking, J.H. 1961. Studies on nitrogen-fixing bacteria of the genus *Beijerinckia*. **Plant Soil** 14: 49-81.

Ben Rebah, F. and, R.D.D. Tyagi Prévost. 2002. Wastewater sludge as a substrate for growth and carrier for rhizobia: the effect of storage conditions on survival of *Sinorhizobium meliloti*. **Biores**. **Technol**. 83: 145-151.

Bohlool, B.B., J.K. Ladha and D.P. Garrity. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. **Plant Soil** 141(1-2): 1-11.

Bolton, H., J.K. Frederickson and L.F. Elliott. 1992. Microbial Ecology of the Rhizosphere. *In* B.F. Metting, ed. **Soil Microbial Ecology: Applications in Agricultural and Environmental Management**. Marcel Dekker, Inc., New York.

Copyright by Kasetsart University All rights reserved

Bopaiah, B.M. and P.V. Rai. 1979. Effect of four herbicides on the population of microorganisms in red sandy soil. **Plant Soil** 52: 451-452.

Bozidar, S. and V. Maric. 1995. Temperature and dissolved oxygen concentration as parameters of *Azotobacter chrooococcum* cultivation for use in biofertilizer. **Biotechnol**. **Lett**. 17(4): 453-458.

Breed, R.S., E.G.D. Murray and A.P. Hitchens. 1948. **Bergey's Manual of Determinative Bacteriology**. The William & Wilkins Company.

Brick, J.M., R.M. Bostock and S.E. Silverstone. 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. **Appl**. **Environ. Microbiol**. 57: 535-538.

Brown, M.E. 1972. Plant growth substances produced by microorganisms of soil and rhizosphere. **J**. **Appl**. **Bacteriol**. 35: 443-451.

Brown, S.K. Burlingham and R.M. Jackson. 1964. Studies on Azotobacter species in soil. III. Effects of artificial inoculation on crop yields. **Plant Soil** 20: 194-214.

Burton, J.C. 1979. New developments in inoculating legumes, pp. 380-405. *In* N.S. Subba Rao, ed. **Recent Advances in Biological Nitrogen Fixation**. Oxford & IBH Publishing Co., New Delhi.

Burton, J.C. 1984. **Legume Inoculant Production Manual**. NifTal Center-MIRCEN, University of Hawaii, Department of Agronomy and Soil Science, College of Tropical Agriculture and Human Resources, Hawaii, USA.

Cejudo, F.J. and A. Paneque. 1987. Correlation between nitrate uptake rate and nitrate inhibition of nitrogenase activity in *Azotobacter chroococcum*. **FEMS Microbiol**. **Lett**. 43(1): 5-7.

Copyright by Kasetsart University All rights reserved

Chahal, V.P.S. and P.P.K. Chahal. 2000. Application of Azotobacter, pp. 74-80. *In* N. Narula, ed. **Azotobacter in Sustainable Agriculture**. CBS Publishers & Distributors, New Delhi.

Channal, H.T., A.R. Ahagawadi, T.D. Bharama Gowder and S.G. Udupde. 1989. Azotobacter population as influence by soil properties in some soils of northern Karmataka. **Curr**. **Sci**. 58: 70-71.

Chanway, C.P. and F.B. Holl. 1993. First year yield performance of spruce seedlings inoculated with plant growth promoting rhizobacteria. **Can**. **J**. **Microbiol**. 39: 1084–1088.

Das, A.C. and D. Mukherjee. 1998. Insecticidal effects on soil microorganisms and their biochemical processes related to soil fertility. **World J**. **Microb**. **Biot**. 14: 903-909.

Date, R.A. and R.J. Roughley. 1977. Preparation of Legume Seed Inoculants, pp. 277-310. *In* R.W.F. Hardy and A.H. Gibson, eds. **A Treatise on Dinitrogen Fixation**, **Sec**. **IV**. John Kiley & Sons, New York.

De Freitas, J.R., M.R. Banerjee and J.J Germida. 1997. Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). **Biol**. **Fertil**. **Soil** 24: 358–364.

Dewan, G.I. and N.S. Subba Rao. 1979. Seed inoculation with *Azospirillum brasilense* and *Azotobacter chroococcum* and root biomass of rice (*Oryza sativa*). **Plant Soil** 153: 295-302.

Döbereiner, J., J.M. Day and P.J. DART. 1972. Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum-Azotobacter paspali* association. **Microbiolol**. 71(1):103-116.

Copyright by Kasetsart University All rights reserved

Eldor, A.P. 2007. **Soil Microbiology, Ecology, and Biochemistry**. Elsevier Inc., Canada.

Fages, J. 1992. An industrial view of Azospirillum inoculants: formulation and application technology. **Symbiosis** 13: 15-26.

Fisher, K. and W.E. Newton. 2002. Chapter 1 - Nitrogen Fixation - A General Overview, pp 1-34. *In* G.J. Leigh., ed. **Nitrogen Fixation at the Millennium**. Elsevier Science, Amsterdam.

Flaishman, M.A., Z.A. Eyal, A. Zilberstein, C. Voisard and D. Hass. 1996. Suppression of Septoria tritici blotch and leaf rust of wheat by recombinant cyanide producing strains of *Pseudomonas putida*. **Mol**. **Plant Microbe Interact**. 9: 642–645.

Food and Agriculture Organization of the United Nations. 2005. **Global Trends in Organic Agriculture Markets and Countries' demand for FAO assistance**. Global Learning Opportunity - International Farming Systems Association Roundtable: Organic Agriculture. Food and Agriculture Organization of the United Nations, Rome.

Food and Agriculture Organization of the United Nations. 2013. **Organic Supply Chains for Small Farmer Income Generation in Developing Countries – Case Studies in India, Thailand, Brazil, Hungary and Africa**. Food and Agriculture Organization of the United Nations, Rome.

Forlani, G., R. Pastorelli, M. Branzoni, and F. Favilli. 1995. Root colonization efficiency and potentially related properties in plant associated bacteria. **J**. **Gent**. **Plant Breed**. 49(4): 343-41.

Copyright by Kasetsart University All rights reserved

Frankkenbeger, W.T. and K.L. Fitzpatrick. 1984. Exogenous hormones production in soil-root systems. *In* **Improving Efficiencies In Crop Production Systems**. **Proc**. **Calif**. **Plant Soil Conf**.

Gaind, S. and A. Gaur. 2004. Evaluation of fly ash as a carrier for diazotrophs and phosphobacteria. **Biores**. **Technol**. 95: 187-190.

Gaskins, M.H., S.L. Albrecht, and D.H. Hubbell. 1985. Rhizosphere bacteria and their use to increase plant productivity: A review. **Agri. Ecosys**. **& Environ**. 12(2): 99-116.

Gaur, A.C. 1990. **Phosphate Solubilizing Microorganisms as Biofertilizer**. Omega Scientific Publishers, New Delhi.

Gonzalez-Lopez, J., V. Salmeron., M.V. Martinez-Toledo and F. Ballesteros. 1986. Production of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* ATCC 1837 in chemically defined media and dialysed soil media. **Soil Biol**. **Biochem**. 18: 119-120.

Hardy, R.W.F., R.D. Holsten, E.K. Jackson, and R.C. Burns. 1968. The acethylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. **Plant Physiol**. 43: 1185-1207.

Holt, J.G., R.K. Novel, H.A.S. Peter, T.S. James and T.W. Stanley. 2000. **Bergey's Manual of Determinative Bacteriology**. Lippincott Willaims and Wilkins. USA.

Hopkins, D.W, and J.A.J. Dungait. 2010. Soil Microbiology and Nutrient Cycling, pp. 436. *In* G.R. Dixon and E.L. Tilston, ed. **Soil Microbiology and Sustainable Crop Production**. New York, Springer.

Copyright by Kasetsart University All rights reserved

Hussain, A., M. Arshad., A. Hurasani and F. hussain. 1987. Response of maize (*Zea mays*) to Azotobacter inoculation under fertilized and unfertilized conditions. **Biol**. **Fertil**. **Soils** 4: 73-77.

Ibomcha Singh, L. and P.S. Yadava. 2006. Spatial distribution of microbial biomass in relation to land use in subtropical systems of north-east. **India Trop**. **Ecol**. 47(1): 63-70.

Islam M.Z., D.I. Sharif and M.A. Hossain. 2008. A comparative study of *Azotobacter* spp. from different soil samples. **J**. **Soil Nature** 2(3): 16-19.

Jauhri, K.S. and K. Philip. 1984. Pressmud: a potential carrier for Rhizobium and Azotobacter II. Influence of storage temperature on the survival of Rhizobium and Azotobacter in different carriers and nodulation efficiency of rhizobial inoculants. **Zentralblatt für Mikrobiologia** 139: 97-107.

Jauhri, K.S., R.S. Bhatnagar and V. Iswaran. 1979. Survival of bacterial inoculants in a cheaper carrier material. **Cur**. **Sci**. 48: 170-171.

Jensen, L. 1965. Non-symbiotic nitrogen fixation, pp. 436-480. *In* W.V. Bartholomew and F.E. Clark., ed. **Soil Nitrogen**. American Society of Agronomy Inc., Madison.

Jones, K. and Bangs, D. 1985. Nitrogen fixation by free-living heterotrophic bacteria in an oak forest: The effect of liming. **Soil Biol**. **Biochem**. 17(5): 705-709.

Kamlesh, K., S. Suneja, S. Goyal and N. Narula. 2004. Phytohormone production by Azotobacter- A Review. **Agric**. **Rev**. 25(1): 70-75.

Karthikeyan, B., C. Abdul Jaleel, G.M.A. Lakshmanan and M. Deiveekasundaram. 2008. Stiduies on rhizosphere microbial diversity of some commercially important medicinal plant. **Colloid**. **Surface**. **B**. 62: 143-145.

Copyright by Kasetsart University All rights reserved

Kautz, T., S. Wirth and F. Ellmer. 2004. Microbial activity in a sandy arable soil is governed by the fertilization regime. **Eur**. **J**. **Soil Biol**. 40: 87–94.

Kibunja, C.N. 1991. **A Local Carrier for Rhizobium Inoculants in Kenya**. M. E. Thesis, University of Nairobi.

Kizilkaya, R. 2009. Nitrogen fixation capacity of *Azotobacter* spp. strains isolated from soils in different ecosystems and relationship between them and the microbiological properties of soils. **J**. **Environ**. **Biol**. 30(1): 73-82.

Kloepper, J.W. 1993. Rhizobacteria as biological control agents, pp. 255-274. *In* B.F. Metting, ed. **Soil Microbial Ecology: Applications in Agricultural and Environmental Management**. Marcel Dekker, Inc., New York.

Kole, M.M., W.J. Page, and I. Altosaar. 1988. Distribution of Azotobacter in Eastern Canadian soils and in association with plant rhizospheres. **Can**. **J**. **Microbiol**. 34(6): 815-817.

Kumar, V., B.R. Kumar and N. Narula. 2001. Establishment of phosphate-solubilizing strains of *Azotobacter chroococcum* in the rhizosphere and their effect on wheat cultivars under greenhouse conditions. **Microbiol**. **Res**. 156(1): 87-93.

Kumar, K., N. Amaresan and S. Bhagat. 2011. Isolation and characterization of rhizobacteria associated with coastal agricultural ecosystem of rhizosphere soils of cultivated vegetable crops. **World J**. **Microb**. **Biot**. 27(7): 1625-1632.

Kundu, B.S. and A.C. Gaur. 1980. Establishment of nitrogen fixing and phosphate solubilizing bacteria in rhizosphere and their effect on yield and nutrient uptake of wheat crop. **Plant Soil** 57: 223–230.

Copyright by Kasetsart University All rights reserved

Lakshmanan, M. 2000. Azotobacter inoculation and crop productivity, pp. 109-123. *In* N. Narula, ed. **Azotobacter in Sustainable Agriculture**. CBS Publishers & Distributors, New Delhi.

Lee, M., C. Breckenridge, and R. Knowles. 1970. Effect of some cultural conditions on the production of indole-3-acetic acid and gibberellin like substances by *A. vinelandii*. **Can**. **J**. **Microbiol**. 16: 1325-1329.

Lipman, J.G. 1904. Soil bacteriological studies. **Report of the New Jersey Agricultural Experiment Station** 25: 237-289.

Madan, K. and A.K. Singh. 2010. Relative nitrogen fixing efficiency of Azotobacter isolates and shelf life of different organic carriers. **Environ**. **Ecol**. 28: 1655-1659.

Madhuri, R.J., V. Rangaswamy. 2006. Effect of selected insecticides on population and nitrogen fixing efficiency of *Azospirillum* sp. in groundnut soils. **J**. **Ecotoxicol**. **Environ**. **Monit**. 16(2): 157-169.

Mala, T. 2003. **Organic Fertilizer and Biofertilizer: Production Techniques and Usage**. Kasetsart University Press, Bangkok, Thailand.

Malik, S.T., S.T. Shende and M. Singh. 1994. Effect of *Azotobacter chroococcum* inoculation on yield and yield attributes of Egyptian cotton (*Gossypium barbadense*). **Indian J**. **Agric**. **Sci**. 64(8): 552-544.

Martinez-Teledo, M.V., L. Gonzales-Lopez., T. de la Rubia and J. Moreno. 1988. Grain yield response of *Zea mays* (HbidAE703) to *A. chroococcum*. H. 23. **Biolo**. **Fert**. **Soils**. 6: 352-353.

Copyright by Kasetsart University All rights reserved

Martinez-Teledo, M.V., B. Rodelas, V. Salmeron, and J. Gonzalez-Lopez. 2000. Production of phytohormones by free-living nitrogen fixing bacteria, pp. 53-63. *In* N. Narula, ed. **Azotobacter in Sustainable Agriculture**. CBS Publishers & Distributors, New Delhi.

Martyniuk, S. and M. Martyniuk. 2003. Occurrence of *Azotobacter* spp. in some Polish soils. **Pol**. **J**. **Environ**. **Stud**. 12(3): 371-374.

Mendes, I.C., A.K. Bandick, R.P. Dick and P.J. Bottomley. 1999. Microbial biomass and activities in soil aggregates affected by winter cover crops. **Soil Sci**. **Soc**. **Am**. **J**. 63:873–881.

Milosevic, N., M. Govedarica and M. Jarak. 1994. Specific relationship between *Azotobacter chroococcum* and some pepper varieties. **Savremena-pljoprivreda (Yugoslavia**) 42(11): 270-276.

Mishustin, E.N. and V.K. Shilnikera. 1969. Free living nitrogen fixing bacteria of the genus Azotobacter, pp. 72-124. *In* **Soil Biology**, **Reviews of Research**, Unesco Publication.

Mohandas, S. 1987. Field response of tomato (*Lycopersicon esculentum* "Pusa Ruby") to inoculation with VA mycorrhiza fungus *Glomus fasculatum* and with *Azotobacter vinelandii*. **Plant Soil** 89: 295-297.

Moreno, J., J. Gonzalez-Lopez, and G.R. Vela. 1986. Survival of *Azotobacter* spp. in dry soils. **Appl**. **Environ**. **Microbiol**. 51(1): 123–125.

Mulder, E.G. 1975. Physiology and ecology of free-living nitrogen-fixing bacteria, pp. 1-28. *In* W.D.P. Stewart, ed. **Nitrogen Fixation by Free-Living Microorganisms**. Cambridge University Press, Cambridge, UK.

Copyright by Kasetsart University All rights reserved

Mulder, E.G. and S. Brotonegoro. 1974. Free living heterotrophic nitrogen-fixing bacteria, pp. 37-84. *In* A. Quispel, ed. **The Biology of Nitrogen Fixation**. North-Holland Publishing Company, Amsterdam.

Muthuselvam, K. and P. Tholkappian. 2008. Vermicompost: a potential carrier material for bacterial bioinoculants. **Plant Arch**. 8: 895-898.

Nada, M., M. Govedarica, J. Mirjana and N. Petrovic. 1997. The effect of heavy metals on total soil microbiology activity in Lettuce. **Acta Hort**. 462: 133-137.

Narula, N. 2000. Azotobacter as an organism, pp. 1-12. *In* N. Narula, ed. **Azotobacter in Sustainable Agriculture**. CBS Publishers and Distributors, New Delhi.

Narula, N., K. Kukreja and V. Kumar. 2002. Phosphate solubilization by soil isolates of *Azotobacter chroococcum* and their survival at different temperatures. **J**. **Agric**. **Trop**. **Subtrop**. 103(1): 81-87.

Neidhardt, F.C., J.L. Ingraham, and M. Schaechter. 1990. **Physiology of the Bacterial Cell: A Molecular Approach**. Sinauer Associates, Sunderlands, Massachusetts.

Nieto, K.F. and W.T. Frankenberger. 1989. Biosynthesis of cytokinins by *Azotobacter chroococcum*. **Soil Biol**. **Biochem**. 21(7): 967-972.

Nijhoff, M. 1982. Distribution of Azotobacter in arid soils. **Plant Soil** 64: 355-361.

Office of Agricultural Economics. 2013. **The quantity and value of imported important chemical fertilizer in 2008-2012**. Available Source: http://www.oae.go.th/download/FactorOfProduct/Fertilizer_value49-54.html, May 19, 2013.

Copyright by Kasetsart University All rights reserved

Orville W., M.G. Neumann and M.D. Socolofsky. 1961. Development and germination of the Azotobacter cyst. **J**. **Biophys**. **Biochem**. **Cytol**. 10(4): 555–565.

Paczkowski, M.W. and D.L. Berryhill. 1979. Survival of *Rhizobium phaseoli* in coal-based legume inoculants. **Appl**. **Environ**. **Microbiol**. p. 612-615.

Phiromtan, M.,T. Mala and P. Srinives. 2009. Effect of various habitats in Thailand on the abundance of Azotobacte*r* in Rhizospheric Soils, pp. 89. *In* **Proceedings of The ISSAAS International Congress 2009 "Agriculture for better living and global economy"**. January 11-15, 2010, Nong Nooch Tropical Botanical Garden & Resort, Pattaya, Thailand.

Phuangsaeng, W., J. Phumpetch and T. Mala. 1991. The use of Azotobater as biofertilizer. **Annual Report of Research and Development Institute of Kasetsart University**. 20 p.

Pinton, R., Z. Varnini and P. Nannipieri. 2007. **The Rhizosphere: Biochemistry and Organic Sustances at the Soil-Plant Interface**. CRC Press.

Prabhjeet, S. and S.C. Bhargava. 1994. Change in growth and yield components of *Brassica napus* in response to Azotobacter inoculation and different rates of nitrogen application. **J. Agric. Sci.** 122: 241-247.

Quispel, A. 1974. **The Biology of Nitrogen Fixation**. North-Holland Publishing Company, Amsterdam.

Raja, S.K. and N. Karmegam. 2010. Earthworm casts as an alternate carrier material for biofertilizers: Assessment of endurance and viability of *Azotobacter chroococcum*, *Bacillus megaterium* and *Rhizobium leguminosarum*. **Sci. Hort**. 124: 286-289.

Copyright by Kasetsart University All rights reserved

Rajaramamohan-Rao, V. 1976. Nitrogen fixation as influenced by moisture content, ammonium sulphate and organic sources in a paddy soil. **Soil Biol**. **Biochem**. 8(5): 445-448.

Rao, D.L.N. 1975. Azotobacter inoculants. **Soil Microorganisms and Plant Growth**. Oxford & IBH Publisher Co., New Delhi.

Ravikumar, S., K. Kathiresan, S. Thadedus Maria Ignatiammal, M. Babu Selvam and S. Shanthy. 2004. Nitrogen-fixing azotobacters from mangrove habitat and their utility as marine biofertilizers. **J. Ext. Marine Biol. Ecol**. 312(1): 5-17.

Robson, R.L. and J.R. Postgate. 1980. Oxygen and hydrogen in biological nitrogen fixation. **Ann**. **Rew**. **Microbiol**. 34: 183-207.

Rovira, A.D. 1956. Plant root excretions in relation to the rhizosphere effect: I. The nature of root exudate from oats and peas. **Plant Soil** 7: 178-194.

Rovira, A.D. 1959. Root excretions in relation to the rhizosphere effect: IV. Influence of plant species, age of plant, light, temperature, and calcium nutrition on exudation. **Plant Soil** 9: 53-64.

Rudnick, P. and C. Kennedy. 2002. Chapter 6 - Regulation of Mo Nitrogenases, pp. 155-166. *In* G.J. Leigh, ed. **Nitrogen Fixation at the Millennium**. Elsevier Science, Amsterdam.

Saleh, E.A., F.R. Nassar and A.M. Yassen. 2001. Survival of microorganisms of multi-strains inoculant on different carriers. **Anna**. **Agric**. **Sci**. 39: 2163-2169.

Satrulee, V. 1979. **Efficiency of Nitrogen Fixation by Azotobacter in Sakaerat Forest Soils**. M.E. Thesis, Kasetsart University, Bangkok.

Copyright by Kasetsart University All rights reserved

Scher, F.M., and R. Baker. 1982. Effect of *Pseudomonas putida* and a synthetic iron chealator on induction of soil suppressiveness to Fusarium wilt pathogens. **Phytopathology** 72: 1567–1573.

Shanahan, P., D.J. O'Sullivan, P. Simpson, J.D. Glennon and F. O'Gara. 1992. Isolation of 2,4-diacetylphlorogucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. **Appl**. **Environ**. **Microbiol**. 58: 353–358.

Slonnezewski, J.L. and J.W. Foster. 2009. **Microbiology An Evolving Science**. W. W. Norton & Company Inc., New York, USA.

Smaly, V.T., and O.I. Bershova. 1975. Heteroauxin formation in Azotobacter cultures. **Microbiologia** 26: 520-526.

Soil Science Society of America. 1996. **Methods of Soil Analysis**. Madison, Wisconsin, USA.

Sokolova, M., G. Akimova and O. Vaishlya. 2011. Effect of phytohormones synthesized by rhizosphere bacteria on plants. **Appl**. **Biochem**. **Microbiol**. 47(3): 274-278.

Somasegaran, P. 1985. Inoculant production with diluted liquid cultures of Rhizobium spp. and autoclaved peat: Evaluation of diluents, *Rhizobium* spp., peats, sterility requirements, storage, and plant effectiveness. **Appl**. **Environ**. **Microbiol**. 50: 398.

Staff of Soil Science Department. 1998. **Introduction of Soil Science**. Kasetsart University Press, Bangkok, Thailand.

Subba Roa, N.S. 1984. **Biofertilizers in Agriculture**. Oxford & IBH Publishing Co., New Delhi.

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Sukthong, M. 2008. Influence of inflorescence thinning on seed quality and seed yield of broccoli-Chinese kale hybrid in Loei Province, Thailand. **Acta Hortic**. 771: 83-88.

Tangchum, B. 1985. Free-living microorganisms, pp. 99-129. *In* Department of Agriculture, Research groups of soil microorganisms. **Documentation Training of Biofertilizer**. Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

Tate, R. L. 2000. **Soil Microbiology**. John Wiley and Son, Inc., USA.

Tchan, Y.T. 1984. **Azotobacteriaceae in Bergey's Manual of Systematic Bacteriology**. Williams and Wilkins, London.

Thananusont, V. 1993. Efficiency of rhizobium in peat carrier and modified carrier, pp. 13. *In* **Workshop on Research Activities of ADRC Contributed to Agricultural Development in Northeast Thailand**. Sep 1-3, 1993, **Khon Kaen**, **Thailand**.

Thompson, J.P. and V.B.D. Skerman. 1979. **Azotobacteraceae: The Taxonomy and Ecology of Aerobic Nitrogen-Fixing Bacteria.** Academic Press, London.

Thompson, J.P and V.B.D. Skerman. 1981. Classification, identification and isolation of Azotobacteriaceae. *In* A.H. Gibson and W.E. Newton, ed. **Current Perspectives in Nitrogen Fixation**. Elsvier: North Holland Biomedical Press, Amsterdam.

Thungtrakul, M. 1987. **Factors Affecting Survival of Rhizobia in Carriers**. M.E. Thesis, Kasetsart University, Thailand.

Copyright by Kasetsart University All rights reserved

Tippannawar, C.M. and T.K. Ramachandra Reddy. 1989. Azotobacter in root system and root tissue of cell of *T. aestivum* and *T. duram* L **Curr**. **Sci**. 58: 1342-1343.

Trevors, J.T., J.D. van Elsas, H. Lee and van L.S. Overbeek. 1992. Use of alginate and other carriers for encapsulation of microbial cells for use in soil. **Microbiol**. **Releases** 1: 61-69.

Van Schreven, D.A., D.J. Lindenbergh and A. Koridon. 1970. Effect of several herbicides on bacterial peopulations and activity and the persistence of these herbicides in soil. **Plant Soil** 33: 513-532.

Vegetable Research and Extension. 2013. **Broccolini**. Available Source: http://agsyst.wsu.edu/Broccolini.html, May 12, 2013.

Wangaruro, S., N. Karanja, B. Makatiani, D. Odee and P.Woomer. 2000. Physico-chemical properties, initial microbial population and survival of rhizobia in peat, vermiculite and filtermud, pp. 404. *In* **Proceeding of the 15th annual general meeting: Soil technologies for sustainable small holder farming systems in East Africa**. August 19-23, 2000, Soil Science Society of East Africa, Sportman's Arms Hotel, Nyayuki, Kenya.

Wardle, D.A., G.W. Yeates, K.S. Nicholson, K. I. Bonner, R. N. Watson. 1999. Response of soil microbial biomass dynamics, activity and plant litter decomposition to agricultural intensification over a seven-year period. **Soil Biol. Biochem.** 31: 1707-1720.

Weaver, R.W., S. Angle, P. Botomley, D. Bezdicek, S. Smith, A. Tabatabai and A. Wollum. 1994. **Method of Soil Analysis Part 2: Microbiological and Biochemical Properties**. Soil Science Society of America, Inc.

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Yadav, K.S., S. Suneja, and H.R. Sharma. 1994. Effect of dual inoculation of Rhizobium and Azotobacter in chickpea (*Cicer arietinum*). **Envir**. **Ecol**. 12(4): 865-868.

Yasmin, S., M. Abdul Rahman Bakar, A. M. Kausar and Y. Hafeez Fauzia. 2004. Isolation, characterization and beneficial effects of rice associated plant growth promoting bacteria from Zanzibar soils. **J**. **Basic Microb**. 44(3): 241-252.

Youssef, H., M.Z. Sedik, M. Fayez and N.A. Hegazi. 1997. Response of some Graminae to peat-based inoculum of associative diazotrophs. **Bulletin of Faculty of Agriculture, Cairo University** 48(1):159-174.

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