IMPROVEMENT OF PEANUT RHIZOBIAL INOCULANT BY INCORPORATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AS BIOCONTROL AGAINST SEED BORNE FUNGI, *ASPERGILLUS FLAVUS* AND *A. NIGER*

Watcharin Yuttavanichakul

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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การปรับปรุงหัวเชื้อไรโซเบียมโดยเพิ่มคุณสมบัติการควบคุมเชื้อราที่ติดมากับเมล็ดถั่วลิสง *Aspergillus flavus* และ *A. niger*

นายวัชรินทร์ ยุทธวานิชกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IMPROVEMENT OF PEANUT RHIZOBIAL INOCULANT BY INCORPORATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AS BIOCONTROL AGAINST SEED BORNE FUNGI, *ASPERGILLUS FLAVUS* AND *A. NIGER*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master s Degree.

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วัชรินทร์ ยุทธวานิชกุล : การปรับปรุงหัวเชื้อไรโซเบียมโดยเพิ่มคุณสมบัติการควบคุมเชื้อโรคที่ติดมากับเมล็ดถั่วลิสง *ASPERGILLUS FLAVUS* และ *A. NIGER* (IMPROVEMENT OF PEANUT RHIZOBIAL INOCULANT BY INCORPORATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AS BIOCONTROL AGAINST SEED BORNE FUNGI, *ASPERGILLUS FLAVUS* AND *A. NIGER*) อาจารย์ที่ปรึกษา : อ. ดร.พรรณลดา ติตตะบุตร, 75 หน้า

เชื้อจุลินทรีย์ก่อโรคในพืช นับเป็นปัญหาสำคัญต่อการเพาะปลูก และการเจริญเติบโตของพืช มีผลทำให้ผลผลิตทางการเกษตรลดลงและส่งผลต่ออุตสาหกรรมอาหาร ดังนั้นจึงมีการใช้สารเคมีในปริมาณมากเพื่อควบคุมโรคในพืช ทำให้เป็นอันตรายต่อผู้ใช้และผู้บริโภค นอกจากนี้ยังส่งผลกระทบต่อสิ่งมีชีวิตและสิ่งแวดล้อม ดังนั้นการควบคุมในทางชีวภาพหรือชีววิธีจึงถูกนำมาใช้ในการเกษตร ในงานวิจัยนี้มีวัตถุประสงค์เพื่อคัดเลือกเชื้อไรโซเบียมและเชื้อจุลินทรีย์ในกลุ่ม PGPR ที่มีความสามารถในการควบคุมการเจริญของเชื้อราก่อโรครากเน่าในถั่วลิสง ซึ่งมีสาเหตุมากจากเชื้อ *Aspergillus niger* และ *A. flavus* จากการศึกษาพบว่า เชื้อในกลุ่มไรโซเบียมจำนวน 265 ไอโซเลต ที่ได้รับความอนุเคราะห์จากกรมวิชาการเกษตร และ 500 ไอโซเลต จากการคัดแยกจากปมรากถั่วลิสง ไม่มีเชื้อใดที่สามารถควบคุมเชื้อราก่อโรครากเน่าในถั่วลิสง แต่เมื่อนำเชื้อ PGPR จำนวน 350 ไอโซเลต ที่ได้รับความอนุเคราะห์จากสาขาวิชาเทคโนโลยีชีวภาพ มหาวิทยาลัยเทคโนโลยีสุรนารี มาทดสอบ พบเชื้อ PGPR จำนวน 11 ไอโซเลต สามารถควบคุมเชื้อรา *A. niger* ได้ และจากการทดสอบพบเชื้อที่มีความสามารถสูงสุด 4 ไอโซเลต คือ A20, A45, A62, และ A106 โดยพบว่า ไอโซเลต A20 และ A62 ยังสามารถยับยั้งการเจริญของ *A. flavus* ได้อีกด้วย จากผลการระบุเชื้อด้วยการอ่านลำดับนิวคลีโอไทด์ของยีน 16S rDNA พบว่าไอโซเลต A20, A45, A62, และ A106 มีความใกล้เคียงกับ *Bacillus megaterium* strain AM1C7 (99%), *B. subtilis* strain Setapak 8 (99%), *B. subtilis* subsp. *subtilis* strain SB 3130 (99%) และ *Pseudomonas* sp. NJ-61 (95%) ตามลำดับ จากนั้นได้นำไปทดสอบการยับยั้งการเจริญของเชื้อ *A. niger* พบว่า ไอโซเลต A20, A45, A62, และ A106 สามารถควบคุมการเจริญของเชื้อ *A. niger* ได้ 42.5%, 51.42%, 67.81%, และ 44.53% ตามลำดับ เมื่อนำเชื้อ PGPR ไปทดสอบการผลิต lytic enzyme พบว่าไอโซเลต A20, A45, และ A62 สามารถผลิต เอนไซม์โปรติเอสได้ และเพื่อทำการทดสอบสารที่จุลินทรีย์หลั่งออกมาเพื่อยับยั้งการเจริญของเชื้อรา ได้นำอาหารเหลวที่ได้จากการเลี้ยงเชื้อ PGPR ในแต่ละไอโซเลตที่ทำการแยกเซลล์ออกแล้วมาทดสอบ พบว่าอาหารเหลวที่ได้จากการเลี้ยงเชื้อไอโซเลต A20 และ A62 สามารถยับยั้งเชื้อรา *A. niger* ได้อย่างสมบูรณ์ และเมื่อนำอาหารเหลวดังกล่าวไปบ่มกับเอนไซม์ proteinase k ก่อนนำไปทดสอบกับเชื้อรา พบว่าอาหารจากไอโซเลต A45 และ A62 ไม่สูญเสียความสามารถในการยับยั้งเชื้อรา *A. niger* แสดงว่าความสามารถในการยับยั้งเชื้อรา *A. niger* ของเชื้อดังกล่าวไม่เกี่ยวข้องกับเอนไซม์ที่เชื้อผลิตได้ เชื้อ PGPR ทุกไอโซเลต สามารถผลิตฮอร์โมนพืชออกซิน (Indole-3-acetic acid, IAA) โดยเชื้อ PGPR ไอโซเลต A62 ผลิตฮอร์โมน IAA สูงสุดที่ 65.50 ppm ต่อ 10^8 เซลล์ ฮอร์โมน IAA ที่เชื้อผลิตได้มีผลสนับสนุนต่อการเจริญและการเปลี่ยนแปลงทางกายภาพของรากถั่วลิสง และเมื่อทดสอบการป้องกันการเกิดโรครากเน่าในถั่วลิสงโดยใช้ไอโซเลต A20 หรือ A45

ที่ความเข้มข้น 10^8 เซลล์ต่อมิลลิลิตร ร่วมกับการใช้เชื้อ *Bradyrhizobium* sp. TAL 173 ที่ความเข้มข้นเดียวกัน พบว่าเชื้อไอโซเลต A20 หรือ A45 สามารถควบคุมการเกิดโรครากเน่าจากเชื้อ *A. niger* ได้ ดังนั้น การพัฒนาหัวเชื้อไรโซเบียมให้มีความสามารถทั้งในการสนับสนุนการเจริญเติบโตของพืชโดยการตรึงไนโตรเจน จากอากาศและสามารถควบคุมเชื้อราก่อโรค *A. niger* ซึ่งเป็นสาเหตุสำคัญของการเกิดรากเน่าในถั่วลิสงสามารถทำได้โดยการใช้เชื้อไรโซเบียมร่วมกับเชื้อในกลุ่ม PGPR

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

WATCHARIN YUTTAVANICHAKUL : IMPROVEMENT OF PEANUT RHIZOBIAL INOCULANT BY INCORPORATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AS BIOCONTROL AGAINST SEED BORNE FUNGI, *ASPERGILLUS FLAVUS* AND *A. NIGER*. THESIS ADVISOR : PANLADA TITTABUTR, Ph.D., 75PP.

BRADYRHIZOBIA/PEANUT/PGPR/RHIZOBIAL INOCULANTS/BIOCONTROL/ *ASPERGILLUS NIGER*

Pathogenic microorganisms are one of the most important problems for plant growth, which eventually affect the food production system whenever the chronic threat of pathogens has occurred. Biological control is considered as an alternative or supplemental way for reducing the use of chemical agents in agricultural system. In this study, the inhibition of seed borne pathogenic fungus *Aspergillus niger* that causes root rot diseases in peanut (*Arachis hypogaea* L.) was investigated by using root nodulating *Bradyrhizobium* and soil-isolated Plant Growth Promoting Rhizobacteria (PGPR) as biological controllers. A total of 265 peanut bradyrhizobial strains were obtained from the Department of Agriculture (DOA), Thailand, and 500 isolates were isolated from peanut nodules, and then their antagonistic activities to *A. niger* were determined. However, none of them could inhibit *A. niger* growth. Thus, 350 PGPR isolates obtained from School of Biotechnology, Suranaree University of Technology were further screened to achieve this purpose. The total of 11 isolates were found to be able to inhibit *A. niger* growth. Based on their ability to inhibit *A. niger* growth and root colonization, the best 4 PGPR isolates were selected which were A20, A45, A62, and A106. Among these isolates, it was found that isolates A20 and A62 could also inhibit *A. flavus*. The sequence of 16S rDNA genes of these selected strains indicated that A20, A45, A62, and A106 were highly homology to *Bacillus megaterium* strain

AM1C7 (99%), *B. subtilis* strain Setapak 8 (99%), *B. subtilis* subsp. *subtilis* strain SB 3130 (99%), and *Pseudomonas* sp. NJ-61 (95%), respectively. These 4 PGPR, A20, A45, A62, and A106, were able to inhibit *A. niger* growth at 42.5%, 51.42%, 67.81%, and 44.53%, respectively. The production of lytic enzyme protease was detected in A20, A45, and A62, but not found in A106. Some antifungal activities were found clearly in cell-free supernatants of A20 and A62. Interestingly, the antifungal activity of isolates A45 and A62 was proteinase k resistant. This implied that the mode of action against fungus from these isolates was not from protease enzyme. All of PGPR isolates could produce an auxin (Indole-3-acetic acid, IAA) hormone. Isolate A62 produced a significantly high amount of IAA hormone at 65.5 ppm per 10^8 cells. IAA hormone produced from PGPR isolates could promote peanut root growth. When either isolate A20 or A45 (10^8 cells per ml) was co-inoculated with *Bradyrhizobium* sp. TAL 173 (10^8 cells per ml), the peanut root rot disease caused by *A. niger* (10^5 and 10^6 spores per seed) could be inhibited. Therefore, improvement of rhizobial inoculant for peanut to increase nitrogen fixation and reduce fungicide usage by incorporating of rhizobia with selected PGPR might be an appropriate approach.

School of Biotechnology

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Watcharin Yuttavanichakul

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CHAPTER I

INTRODUCTION

1.1 Significance of this study

Peanut or groundnut (*Arachis hypogaea* L.) is an important legume crop. It takes about 80% of the crop growing areas in developing countries. It is unknown when peanuts became a part of the Thai food system, but it is believed that foreigners brought peanuts into Thailand about 400 years ago. Today, peanut is a commercial crop and growing spread throughout the country; the Northern, North-Eastern, Central, and Southern regions of Thailand. Normally, the peanut seed was coated with rhizobial inoculants before sowing in order to fix nitrogen from the air to plant and soil. However, the yield is low due to diseases caused by different microorganisms. The crown rot disease of peanut caused by *Aspergillus niger* and *A. flavus* are the most important disease in both temperate and tropical countries. This disease is caused by seed borne pathogen that can survive in infected peanut seeds (Magnoli et al., 2006). Both of them can produce the harmful mycotoxin. The *A. niger* can produce Ochratoxin and *A. flavus* can produce Aflatoxins. Those mycotoxins are considered as a human carcinogen and it can be accumulated in the meat of animals (Hussein and Brasel, 2001). Thus, seed treatment with chemical fungicides, such as Carbendazim (methyl-2-benzimidazol carbamate) was used to protect the seed from pathogenic organisms before sowing. Carbendazim is a systemic benzimidazole fungicide that plays a very important role in plant disease control. It is a derivative of other fungicides, such as benomyl and is applied world-wide on several crops (tobacco, fruit, vegetables, cereals, etc.,) to control fungi that cause plant diseases.

It is also used in postharvest food storage, in seed pre-planting treatment, and used as a fungicide in paint, paper, and wood (Medina et al., 2007). However, the non-target soil microorganisms, such as symbiotic nitrogen-fixing bacteria or the beneficial bacteria as well as the coated rhizobial inoculants were affected by these chemical substances (Castro et al., 1997). Moreover, there is considerable interest in finding alternatives to chemical pesticides due to environmental concerns for suppression of soil borne plant pathogens (Haggag, 2007). Therefore, the use of microorganisms to control plant diseases offers an attractive alternative way instead of using synthetic chemicals (Roberts et al., 2005).

Rhizosphere bacteria that exhibit root colonization and exert beneficial effects on plants are termed plant growth promoting rhizobacteria (PGPR) (Karthikeyan et al., 2010). PGPR, in combination with efficient rhizobia, could improve the growth and nitrogen fixation by inducing the occupancy of introduced rhizobia in the nodules of legumes (Tilak et al., 2006). Co-inoculation with *Pseudomonas* spp. (PGPR) and *Rhizobium* spp. has been shown to increase the degree of colonization of the legume rhizosphere by rhizobia resulting in enhanced plant nodulation (Cook and Baker, 1983).It has been reported that *Rhizobium trifolii* had potential as biological control agents against the root rot of *Trifolium subterraneum* seedlings caused by *Phytophthora clandestine* (Simpfendorfer et al., 1988). *In vitro* tests of *Rhizobium meliloti* inhibited growth of *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani* while *Bradyrhizobium japonicum* inhibited growth of *M. phaseolina* and *R. solani* (Ehteshamul-Haque and Ghaffar, 1993). PGPR can also elicit plant defenses (Van Loon and Glock, 2004) and antagonize or prevent phytopathogens or deleterious microorganisms (Kloepper et al., 2004). The biocontrol agents of *Pseudomonas fluorescens*, *Trichoderma virens* and *Bacillus subtilis* showed complete inhibition of *A. flavus* growth (Reddy et al., 2009) and *Paenibacillus polymyxa, P. aeruginosa* strains GPS 21, GSE 18, GSE 19, and GSE 30 can control crown root rot disease in peanut (Haggag and Timmusk, 2008; Kishore et al., 2007).

Since, PGPR has potential to promote plant growth, enhance legume plant nodulation with *Rhizobium* spp., and inhibit the growth of plant pathogens, it is possible to use rhizobial inoculant for controlling diseases in peanut caused by *A. flavus* or *A. niger* (Ahmad et al., 2008; Moretti et al., 2008). However, it has not been studied to use PGPR co-inoculated with *Rhizobium* spp. for enhancement of peanut growth and inhibition the growth of plant pathogenic fungi *A. niger* and *A. flavus* on peanut. Therefore, the aims of this study were to obtain the antagonistic peanut rhizobia or PGPR against *A. niger* and *A. flavus,* and to investigate the symbiosis efficiency and biocontrol activity of selected isolates on peanut infected with these fungi. The developed rhizobial inoculant containing rhizobia and PGPR would be able to use instead of fungicide coated on peanut seed surface before sowing.

1.2 Research objectives

1. To determine the antagonistic activity of peanut rhizobial strains and Plant Growth Promoting Bacteria (PGPB) strains on seed borne pathogenic fungi
2. To identify and characterize the selected peanut PGPB strains
3. To determine the appropriate dose of fungal, rhizobial, and PGPB inoculum size to be used in the experiment
4. To evaluate the symbiosis efficiency and biocontrol activity of the selected peanut rhizobial strain and PGPB strains when using as rhizobial inoculant

CHAPTER II

REVIEW OF THE LITERATURES

2.1 Peanut in Thailand

The cultivated peanut (*Arachis hypogaea* L.) is one of the most widespread and important food crops in Thailand (Lampang et al., 1980). However, it is unknown that when peanuts became a part of the Thai food system, but it is believed that foreigners (Spanish or Portuguese) brought peanuts into Thailand about 400 years ago. Butaratanu, (1997) reported that a French missionary record of 1854 indicated that peanuts were planted in Chantaburi Province, a province in the Eastern part of Thailand. In 1929, Thailand imported peanuts for domestic consumption (Kritsadakorn, 1929). In 1932, the Department of Commerce published a document in Thai language describing how to grow peanuts based on the Indian data. In 1947, commercial plantation of peanuts occurred in Chachengsao and Prachineburi provinces (Sawaddecha, 1948). Chutip, 2000 reported that the total peanut planted area was 623,000 rais (99,680 hectares), and the yield was 220-250 kilogram per rai (1375-1562 kilogram per hectare) in Thailand. Nowadays, peanut growing area is spread throughout the Kingdom; the Northern, North-Eastern, Central and Southern regions of Thailand.

2.2 Peanut lines and varieties

Type and accession of peanuts genetically determine yield, the resistance to insects, diseases and aflatoxin, and the percentage of threshed seeds. Spanish type is the raditional accession that has been popular among Thai farmers since it requires less fertile land as compared to the Valencia type (Banterng et al., 2006). Traditional accession of peanuts has

been gathered by agricultural stations to select the best line since 1933. In 1962, two lines of peanuts, named Sukhothai 38 and Lampang, were recommended by the Department of Agriculture (DOA). The dry pod yield of the two recommended lines, Sukhothai 38 and Lampang is 279 and 247 kilogram per rai, respectively. Since then, those two lines of peanuts have been officially recommended to farmers for the different regions of Thailand. However, farmers still use local lines since the recommended seeds are not accessible to all farmers on the planting period due to limited supply and the distance from the agricultural extension station to a main source of recommended seed supply. Starting in 1958, other different types and accessions of peanuts were imported for research and experimentation to select lines that fit local conditions. The new accessions of peanuts were certified by the Department of Agriculture in 1972 under the name of Tainan 9. In 1987, fifteen years later, the new lines were certified under the name of Khonkaen 60-2 and Khonkaen-3; in 1994 named Khonkaen 60-4 and under the name of Khonkaen 5. In 1994, a survey conducted by the Department of Agricultural Extension showed that in the Northern region, peanut farmers use both the recommended seeds of Lampang and Tainan 9, and traditional seeds. However, the percentage of traditional and officially recommended seed use is not known (Loywanitch et al., 1994). A study in 1986 has shown that in the North-Eastern region, 94 percentages of farmers use Sukhothai 38, Lampang and Tainan 9. Only 6 percentages of farmers use traditional seeds. In the Eastern region, 60 percentages of farmers use Sukhothai 38, Lampang and Tainan 9. In the Western part of the Central region, 100 percentages of farmers used Sukhothai 38 or Tainan 9. It should be noted that the percentage of farmers using the recommended seeds reported in this survey were fairly high. Other reports, however, generally indicate that most farmers use traditional and mixed seeds with unknown lines.

2.3 Peanut production in Thailand

Peanut production can be viewed as a system structure of the relationship of inputs required for peanut production. The inputs include natural environmental factors where peanuts are grown, farming entrepreneurs, power and technology. These kinds of inputs are used in 90 to 120 days of timeline activities starting from land preparation, planting, caring and harvesting. Types and amounts of these inputs used in peanut production in Thailand vary regionally (Sukharomana and Dobkuntod, 2003).

2.4 Production system

There are three systems of growing peanut: (1) inter-cropping of peanuts in Para rubber plantations, fruit trees, and field crops (especially cassava and corn), (2) growing of peanuts as a major crop in the wet season, and (3) growing of peanuts after harvesting rice, corn and other field crops. In 1981, about 93.32 percentages of farmers in the major peanut production areas of the Northern, North-Eastern and Eastern regions grow peanuts as a single crop either in the wet season or after harvesting other crops; inter-cropping was practiced by 6.67 percentages of peanut growers. In the Southern region, 68.8 percentages of peanut planting is as an inter-crop of para rubber trees (Sukharomana and Dobkuntod, 2003).

2.5 Farm technology used in peanut production

Farm technology currently used in producing peanuts includes biological, chemical and labor saving technology. Biological technology is seed inoculation with rhizobia which is practiced by a small group of farmers participating in the peanut technology extension program of the Department of Agricultural Extension of MOAC. Peanut growers mainly use traditional seeds. Recommended and certified seeds officially produced under the seed

multiplication program are of good quality. However, good seeds are not widely spread because the Department of Agricultural Extension cannot produce enough to meet the entire country's demand. No private farm commercially produces peanuts for seed. Chemical technology includes the use of insecticides, herbicides, fungicides, and gypsum and calcium. All of these chemicals are used by a small group of farmers. Pesticides are used when necessary by farmers who have enough cash to afford them. The information on the usefulness of gypsum and marl is known by farmers, but the number of farmers and the amount of actual use is not known (Sukharomana and Dobkuntod, 2003).

2.6 Importance of crown rot disease of peanut

Peanut seeds and seedlings are highly susceptible to several pathogens (El-Wakil et al., 2001). One of the most important significant seed and seedling diseases is *Aspergillus* crown rot. Crown rot disease of peanut causes poor standing of plant after planting and/or death of entire plants soon after emergence of seedlings. The succulent and elongating hypocotyls of seedlings are highly susceptible to this disease. Diseased tissues will appear sunken and tan to dark brown in color. Masses of black sooty spores of the fungus usually cover the decayed tissues (http://ipm.ncsu.edu/ peanuts/ diseases/ guide/ aspergillus_ crown_ rot. html). Crown rot disease of peanut is caused by *A.niger* or *A. flavus*. These fungi can survive in peanut seed as well as in the soil. Seed may be attacked by these fungi immediately after placement in a moist condition. These fungi are easily identified by the masses of spores produced on seed. *A. niger* produces sooty black masses of spores, whereas *A. flavus* produces masses of yellow green spores. *A. flavus* is well known for its production of "aflatoxin" which results in placement of molded peanuts in Segregation III at the buying point (http://ipm.ncsu.edu/peanuts diseases /guide/ aspergillus_seed_ decay.html). The crown rot diseases reduce the peanut yields more than 50% if farmers grow peanut in the same area (http://contact.doae.go.th/cts/resultDtl.jsp?

id=273). A comprehensive study was carried out on the fungi occurring in commodities normally traded in Thailand, obtained from farmer's stocks and middlemen in major producing areas throughout the country. From this study, the major fungi of the peanut were *A. flavus* and *A. niger* which could be detected 95% and 86% of the samples, respectively (Pitt et al., 1993).

2.7 Rhizobia and legume symbiosis

Rhizobia are gram-negative soil bacteria that can live in the soil as free-living organism or live in the plant nodule when symbiosis with host leguminous plant. The free-living rhizobia in the soil can enter the root hairs of the susceptible host legume and form nodule through the complex series of interactions between plant and rhizobia. Rhizobia after entering to host plant will divide and differentiate the cell structure inside the nodule, this form of rhizobia inside of nodule are called bacteroids (Alexander, 1962; Sahgal and Johri, 2003). Bacteroids inside nodule are able to converse atmospheric nitrogen (N_2) into ammonia (NH_3), which can be used as nitrogen fertilizer for plant directly. This reaction is occurred by the activity of nitrogenase enzyme, which is a complex of two proteins, a Fe-containing protein and a Fe-Mo protein (Moat et al., 2002). This complex biochemical reaction is called biological nitrogen fixation (BNF) (Somasegaran and Hoben, 1994), while the reaction is shown below:

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

Beside the usefulness of nitrogen fixation ability, rhizobia also produce other chemical molecules that can influence plant development, including lipochito-oligosaccharide Nod factors, phytohormones, lumichrome, riboflavin and H_2 which is

produced through nitrogen fixation activity. Nod factors can stimulate seed germination, promote plant growth and increase yield of both legume and non-legume crops, as well as stimulate the photosynthetic rates after plant leaf spraying (Dakora and Phillips, 2002). Lumichrome and riboflavin can increase root respiration and promoted plant growth when applied to seed or seedling roots (Dakora et al., 2005). Moreover, most of rhizobial strains are able to produce siderophores, indole-3-acetic acid (IAA), and organic acids in culture media (Antoun et al., 1998). Rhizobia can exude these compounds to enhance the availability of nutrition for plant growing under low nutrient environment (Dakora and Phillips, 2002). Due to the benefits of nitrogen fixation and plant growth stimulation activities, rhizobia are used for biofertilizer production in a form of rhizobial inoculant, which can reduce the utilization of chemical compounds as well as the cost of legume production in agriculture.

2.8 Rhizobial inoculants

Rhizobial inoculant is the technology on growing rhizobia, preparing inoculant with suitable carrier material for distribution this rhizobial inoculant to farmers (Somasegaran and Hoben, 1994). Rhizobial inoculant can be applied directly to soil or coated on seed surface before sowing (Hynes et al., 1995) to increase the chance of rhizobia entering to root hair and form nodule after seed germination. Peat based inoculant is the most popular type of rhizobial inoculant due to the display of high population and long shelf-life of rhizobia provided by peat material (Stephens and Rask, 2000). However, the main concerns of inoculant production are the quality of culture, adequate cell number in finished product, the processing of carrier, the purity, the efficiency of nodulation and nitrogen fixation efficiency (Lupwayi et al., 2000). Rhizobial inoculants have been applied to many kinds of legumes, such as legumes that are important for economics, animal feeding as well as for

improving the soil fertility by planting legume as rotational crop. Moreover, the mixed legume-cereal intercropping systems or long-term legume-cereal rotations also increase the grain yield (Seneviratne et al., 2000). However, the success of using rhizobial inoculant on legume is depended on many factors, such as the efficiency of rhizobial strain for nodule competition and nitrogen fixation, plant cultivar, soil condition, including environmental factors, such as temperature, moisture or the invasion of plant pathogen. To overcome these difficulties, the rhizobial strain must be well characterized and selected for inoculant production, as well as the attempt of using multiple strains or co-inoculation of rhizobia with other bacteria have been developed to increase the efficiency of rhizobial inoculants (Stephens and Rask, 2000).

2.9 Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly. Many rhizobacteria have the capacity to fix atmospheric nitrogen (Dobbelaere et al., 2003), although it has been reported that in the most cases this amount of nitrogen is negligible for plant demand and they can promote plant growth through the production of plant growth regulators (Dobbelaere et al., 2003; Verma et al., 2001). The plant growth promoting rhizobacteria including *Pseudomonas* spp. have been reported to stimulate the development of healthy root systems (Germida and Walley, 1996), leading to rapid root colonization by beneficial bacteria (Bolton et al., 1990).

The beneficial effects of PGPR are briefly described as followed:

Production as biofertilizers: These groups of bacteria can facilitate plant nutrients uptake via different direct mechanisms, such as nitrogen (N) fixation, solubilization of

phosphate (P), and synthesis of siderophore for iron sequestration making nutrients more available to plants. Though a variety of nitrogen fixing bacteria so called biofertilizers like *Rhizobium, Azotobacter*, *Bacillus*, *Pseudomonas*, *Azospirillum* and *Acetobacter* has been isolated from the rhizosphere of various crops (Steenhoudt and Vanderleyden, 2006), interest in the beneficial nitrogen fixing growth promoting rhizobacteria-plant association has increased recently due to their potential effect for replacing chemical N-fertilizer (Vessey, 2003).

Production of phytostimulators: The promotion of plant growth regulators, such as auxin, cytokinin and gibberellin by PGPR may also aid in growth and development of host plant species. *Azospirillum brasilense*, one of the most studied PGPR has been shown to improve growth development by the production of auxin, cytokinin and gibberellin. Inoculation of plants with this bacterium causes morphological changes, such as an increase in root surface area through the production of more root hairs, which in turn enhance mineral uptake (Steenhoudt and Vanderleyden, 2006). PGPR include the strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Beijeriakia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium*, *Seratia,* etc. were also reported as phytohormone producer (Lucy et al., 2004).

Biological control activity: PGPR also enhance the plant growth via suppression of phytopathogens by a variety of mechanisms such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide (HCN) which suppress the growth of fungal pathogens. Antagonistic microbe-microbe interactions mediated by *Pseudomonas* species are the major drivers in the biological control of phytopathogenic fungi in the rhizosphere and may indirectly benefit plant growth and survival (Winding et al., 2004). The synthesis of molecules involves in antagonistic interactions and disease suppression, such as the antibiotic 2, 4-diacetylphoroglucinal (2, 4-DAPG), pyoluteocin, etc., (Costa et al., 2007).

Biofilm formation: Biofilms are defined as bacterial communities surrounded by a self-produced polymeric matrix, and reversibly attached to an inert or a biotic surface. After attachment to the surface, the bacteria multiply, and the communities acquire a three-dimensional structure. The major components of biofilms are typically water and bacterial cells. The next most component is a polysaccharide matrix composed of exopolysaccharide, which provides a physical barrier against antibiotic, host defense substances and protection against various environmental stresses. In general, cell aggregation involves natural polymers, such as polysaccharides, which are exerted or exposed at cellular surfaces. These polymeric molecules are of sufficient length to form bridges between the microbial cells (Rudrappa and Bais, 2007). The specificity, the high affinity, and the reversibility of microbial aggregation are not due to covalent bonding, but rather to the highly selective affinity of complementary surfaces. The specificity of biological interactions derives from the stereochemical complementarity of molecular structures. The main forces involved in the cell-to-cell adhesion are hydrogen bonding, ionic interactions, Van der Waals forces, and even hydrophobic interactions, depending on the system. *Azospirillum* is one of PGPR which has been studied intensively. The mechanism of attachment of azospirilla to plant roots still remains unclear. A fibrillar material is observed in *Azospirillum*-root association, but its nature is still unknown. *In vitro* binding assays showed that root attachment by *Azospirillum* is a biphasic process (Troch and Vanderleyden, 1996). In a first step, the adhesion of *A. brasilense* to wheat roots is mediated by an adhesion, closely associated with the polar flagellum. This kind of adsorption is relatively rapid (occurring within 2 h), weak, and reversible. Cells lacking the polar flagellum fail to adsorb to wheat roots, whereas purified polar flagella specifically adsorb onto the root surface. A second step of firm and irreversible anchoring, in which bacterial aggregates are also formed, is thought to be mediated by extracellular polysaccharides and establishment of the bacterial-root association. Cell aggregation could increase survival of *Azospirillum* cells under diverse stress

conditions. This phenomenon may also be important during root colonization where cell aggregates are commonly observed. Although much evidence has accumulated during recent years indicating the involvement of extracellular polysaccharides and proteins in both cell aggregation and root attachment process, the precise mechanisms of these phenomena remain unexplained. The difficulty in elucidating these processes derives from their complexity, because it seems that they are mediated by various cell-surface components. Moreover, data from different works are sometimes contradictory. This can be partially explained by the high number of factors that affect these adhesion processes, such as strain variability, culture growth conditions, culture age, bacterium-plant interaction variability in the case of root attachment, physical and chemical conditions of the binding assays, and more (Bianciotto et al., 2009).

2.10 Applications of PGPR in agriculture

Different PGPR including free-living and associative bacteria such as *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas* have been used in agricultural systems as biofertilizer for their beneficial effects on plant growth (Tilak et al., 1982). Researchers in the former Soviet Union and India conducted widespread tests in the early to the mid part of the 20th century studying the effects of PGPR on different crops. Though results from different experiments were not harmonized and were often inconsistent, up to 50 to 70% yield increases were reported. Inconsistency of results was due to a lack of quality in experimental designs and analysis of results (Lucy et al., 2004). Moreover, during this time an understanding of the detailed mechanisms of plant growth promotion by rhizobacteria was largely unknown. Nevertheless, these field experiments provided clues concerning the optimal conditions for bacterial colonization and growth promotion of target crops. Plant growth benefits due to the addition of PGPR include increases in germination rates, root growth, yield including grain, leaf area, chlorophyll content,

magnesium content, nitrogen content, protein content, hydraulic activity, tolerance to drought, shoot and root weights, and delayed leaf senescence. Inoculation with plant growth promoting bacteria (PGPB) can result a significant change in various plants growth parameters, which may affect crop yield (Abeles et al., 1992).

Another major benefit of PGPR use is disease resistance conferred to the plant, sometimes known as 'biocontrol'. The use of PGPR to increase crop yield has been limited due to the variability and inconsistency of results between laboratory, greenhouse and field studies (Lucy et al., 2004). Soil is an unpredictable environment and an intended result is sometimes difficult to obtain (Bashan and Holguin, 1997). For example, in a study by Frommel et al., 1993, poor colonization of the PGPR on plant roots occurred at one site due to adverse conditions, including high *Verticillum* infection of the soil, low soil pH, high mean temperature, and low rainfall during the growing season. These undesirable growing conditions most likely contributed to the low root colonization (Dobbelaere et al., 2003). Climatic variability also has a large impact on the effectiveness of PGPR (Okon and Labandera-Gonzalez, 1994) but sometimes unfavourable growth conditions in the field are to be expected as a normal functioning of agriculture. Increased yields obtained with wheat inoculated by *Pseudomonas* species in the growth chamber have also been observed in the field (Weller and Cook, 1986). Even though there is a possibility of great variability in field results, if a positive effect of a PGPR was seen on a specific crop in greenhouse studies, there is a strong likelihood that those benefits will carry through to field conditions. One research reported that several related PGPR could promote growth of maize (*Zea may* L.) (Marques et al., 2010). PGPR, such as fluorescent pseudomonads, has been used as seed inoculants to promote plant growth and increase yields (Kloepper et al., 2004). Positive effects of PGPR on diverse host such as bean (Anderson and Guerra, 1985), cotton (Raupach and Kloepper, 1998), soybean (Polonenko et al., 1987), peanut (Dey et al., 2004), maize (Saleem et al.,

2007) and sugerbeet (Çakmakçi et al., 2006) are common in literature. In Thailand, PGPR as *Azotobacter* sp. and *Azospirillum* sp. have been produced by Suranaree University of Technology (SUT) and mixed with good quality of organic fertilizer, thus so called bioorganic fertilizer (BOF). The applications of BOF were tested in different areas and plants. In order to compare the plant yields obtained by using BOF and chemical fertilizers in the field experiment, vegetables and rice plants were used. The results indicated that application of BOF could provide almost the same yield of rice as chemical fertilizer amendment. The use of together with chemical fertilizer (half of recommended amount of each) resulted in the highest yield. In the case of vegetable cultivation, results demonstrated that inoculation of the leguminous plants, *Sesbania rostrata* and cowpea (*Vigna unguiculata*) with the appropriate rhizobial strains as green manures followed by plowing before BOF application could enhance the yield of Chinese kale. This demonstrated that application of green manure could enhance the effect of BOF to become more advantageous, thus confirming its replacement of chemical fertilizer application (Teaumroong et al., 2010).

2.11 Effects of chemical fungicides on rhizobial inoculation

Peanut seeds are coated with rhizobial inoculants before sowing. However, fungicides are usually used in agriculture in order to protect seeds from diseases caused by fungi. These fungicides are the problem of using rhizoial inoculants with peanut seeds, since the main known fungicides are toxic to rhizobia (Diatloff, 1970). In most cases, the rhizobial cells remain viable but are not able to nodulate the host plants or their ability to fix nitrogen is reduced (Fisher, 2004). Therefore, using rhizobial inoculants that have biological control activity on these fungi can replace the application of fungicide on seeds, and allow rhizobia to efficiently nodulate and fix nitrogen for plant.

2.12 Seed borne pathogenic fungi, *A. niger*, *A. flavus*, and biological control activity

Seed borne fungi are defined by Ingold (1953) as those which are dispersed in association with some kind of dispersal unit of the host . This description embraces all seed types and all associated microfungi (Maude, 1996). The key control of plant diseases in organic agriculture are crop rotation, mixed cropping and moderate fertilization. A wide range of plant diseases can be controlled or minimized in these ways. However, the seed borne diseases cannot be controlled by these methods due to this diseases are not transmitted through the soil. Therefore, the crop rotation is an insufficient tool for controlling seed borne diseases (Borgen, 2004). "Biological control" or its abbreviated synonym "Biocontrol" have been used in many fields of biology. In plant pathology, the biocontrol means the use of microbial antagonists to suppress diseases as well as the use of host specific pathogens to control weed population. The organism that suppresses the pathogen is referred as biological control agent (BCA) (Pal et al., 2006).

2.12.1 Biological control of fungi by using PGPR

PGPR can enhance the plant competitiveness and responses to external stress factors as well as inhibiting soil borne plant pathogens through antifungal activity (Sharma, 2002) and also siderophore production (Neilands, 1981). Biocontrol of soil borne diseases is known to result from (i) the reduction of saprophytic growth of the pathogens and then of the frequency of root infections through microbial antagonism (Somasegaran et al., 1994), and/or (ii) the stimulation of "induced systemic resistance (ISR)" in the host-plants (Pieterse et al., 1998).

There were several reports that have been shown that the use of various bacteria to control the plant diseases such as *A. niger* considerably decreased in the presence of *Trichoderma harzianum.* Seed dressing with *T. harzianum* resulted in decreasing the crown rot infection by different *Aspergillus* inoculums levels (Podile and Kishore, 2002). Two fluorescent pseudomonad strains, FPC 32 and FPO 4, applied as seed treatment significantly protected peanut against *A. niger* infection and increased the yield (Gupta et al., 2002). Significant control of crown rot was obtained by bacterization of peanut seeds with *B. subtilis* AF 1 in *A. niger* infested soil (Podile and Kishore, 2002). A new strain of *B. pumilus* isolated from Korean soybean sauce showed strong antifungal activity against the aflatoxin-producing fungi *A. flavus* and *A. parasiticus* (Cho et al., 2009). Bottone and Peluso (2003) identified a compound produced by *B. pumilis* which could inhibit *Aspergillus* species. *B. subtilis*, isolated from peanuts, was found to inhibit the growth of *A. flavus* in peanuts. Sommartya et al., (1997) showed that mixing *B. subtilis* with peanut could reduce the damage caused by *A. flavus*. Luna (2000) confirmed the antagonistic ability of *Pseudomonas* on *A. flavus*. Another plant growth promoting microorganism, *Trichoderma* spp. has been accepted as the most potent biological control agents for certain fungal diseases. Inhibitory effect on *A. flavus* by culture filtrate of two isolates of *Trichoderma* and *T. viride* were reported by Calistru et al. (1997). *T. viride* was also found to inhibit the production of aflatoxin B1 (73.5%) and aflatoxin G1 (100%) when cultured together with *A. flavus* (Choudhary, 1992).

There are some cases where PGPR may promote plant growth in non-sterilized soil by controlling fungal diseases. In addition of siderophore-producing *Pseudomonas putida* converted a *Fusarium*-conductive soil into a *Fusarium*-suppressive soil for the growth of three different plants (Scher and Baker, 1982). An isolate of *Pseudomonas cepacia*, positive for β-1,3-glucanase production, decreased the incidence of

disease caused by *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium ultimum* (Fridlender et al., 1993). Similarly, five fluorescent *Pseudomonas* isolates , each positive for antibiotic production, promoted potato (*Solanum tuberosam* L.) growth on non-sterilized soil (Kloepper and Schroth, 1981). Species of *Pseudomonas*, *P. fluorescens*, *P. cepacia* and *P. stuzeri* isolated from plant rhizosphere, were reported to produce chitinases and cellulases (Lim et al., 1991; Fridlender et al., 1993; Niesen et al., 1998). These bacteria inhibited several soil fungi, indicating their potential as biocontrol agents of several fungi pathogens. *Bacillus amyloliquefaciens* BNM 122 strain is a potential microbial biocontrol agent that is able to control the damping-off caused by *Rhizoctonia solani*, both in a plant growth chamber and in the green house conditions. *Bacillus* sp. produce antifungal peptides that inhibit the growth of a large number of fungi, including *Aspergillus*, *Penicillium*, and *Fusarium* species (Munimbazi and Bullerman, 1998).

2.12.2 Biological control of fungi by rhizobia

There is evidence that a strain of *Bradyrhizobium japonicum* can suppress the wide range of pathogens, such as *Phytophthora megasperma, Pythium ultimum, Fusarium oxysporum* and *Ascochyta imperfect* (Omar and Abd-Alla, 1998 ; Simpfendorfer et al., 1988). While *Sinorhizobium meliloti* was found to inhibit the growth of *F. oxysporum*, and rhizobia isolated from root nodule of *Acacia pulchella* could decrease the survival of zoospores of *Phytophthora cinnamoni* in i*n vitro* (Malajczuk et al., 1984). Moreover, it has been reported that some rhizobial strain successfully protected field-grown soybean, mungbean, sunflower, and okra from infection by *Macrophomina phaseolina, Rhizoctonia solani*, and *Fusarium* species (Ehteshamul-Haque and Ghaffar, 1992). The mechanism of bioprotection by these bacteria has not been elucidated. However, it has been proposed that rhizobia achieve this bioprotection by parasitizing the

hyphal tips of the fungal pathogen and decreasing contact with the plant cells (Dakora et al., 1993). Another mechanism has been proposed is the elicitation of isoflavonoid phytoalexins by rhizobial cells or by their Nod factors (Savoure et al., 1994) can indirectly control the pathogens in legumes. Therefore, inoculating plant with rhizobia that have biological control activity can be the cheap and effective method to control seed-borne pathogens in peanut and other plants.

2.13 Co-inoculation effects of PGPR and rhizobia

Although plant growth promoting rhizobacteria occur in soil, usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Therefore, for agronomic utility, inoculation of plants with target microorganisms at a much higher concentration than those normally found in soil is necessary to take advantages of their beneficial properties for plant yield enhancement. An increasing number of PGPR are successfully used as commercial biofertilizers for agricultural improvement (Subba Rao, 1993).

On the basis of beneficial effects of PGPR and rhizobia, studies using inoculants mixtures are very promising (Lugtenberg and Kamilova, 2009). Results of enhance levels of nodulation were recorded when soybean was treated with *B. japonicum* and *P. fluorescens* (Nishijima et al., 1988). Interaction between *Bradyrhizobium* and plant growth-promoting rhizobacteria increased nodulation and nitrogen fixation in soybean and *Lupinus albus* (Dashti et al., 1998). The nodules of chickpea plant obtained by co-inoculation of *P. fluorescens* F113 and *R. leguminosarum* 1112 were much larger and strongly pigmented compared to single inoculation of *R. leguminosaum* (Andrade et al., 1998). Compared to single inoculation, co-inoculation has improved the absorption of nitrogen, phosphorus and mineral nutrients by plants (Andrade et al., 1998 ; Bashan and

Holguin, 1997). However, some *Pseudomonas* strains reduced nodule numbers and their capacity for nitrogen fixation for example on beans (Edwards et al., 1998) and fodder galega (Suominen et al., 2000).

Benefits of plants from co-inoculation have been shown to include increase in seed germination, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to antibiotic stress, shoot and root weights, biocontrol and delay senescence (Yang et al., 2009). Thus, co-inoculation or mix culture inoculant could be an efficient method to enhance plant growth and protect the plant from pathogen.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 *In vitro* antagonistic activity and root colonization

In this study, both peanut bradyrhizobia and PGPR isolates were used to investigate the antagonistic activites on *A. niger*. From a pool of 1,115 bacterial isolates, 265 peanut *Bradyrhizobium* isolates were obtained from Department of Agriculture (DOA) and 500 *Bradyrhizobium* isolates were isolated from 500 peanut nodules, and those were determined for the antagonistic activity on *A. niger*. However, none of them could inhibit *A. niger* growth. Thus, 350 PGPR isolates were further screened regarding to this purpose and the total of 11 isolates were found to have antagonistic activities on *A. niger* (Fig. 1). These 11 PGPR were able to inhibit *A. niger* and colonize on peanut root in the range of 17.98-67.81% and 7.17-9.84% cfu per cm root, respectively (Table 1). However, there were only two isolates, A20 and A62 could inhibit the growth of *A. flavus*, and the percentage of mycelial inhibition was only 26.66% and 32.00%, respectively (Fig. 2). Since the inhibition of *A. niger* by selected PGPR isolates was clearly observed, *A. niger* was continued test in this research for elucidating the biocontrol efficiency of selected PGPR isolates. Isolate A62 gave the significantly highest in fungal inhibition, while isolate A45 performed the highest of root colonization at 5 days after inoculation (DAI). The bacterial cell counts obtained from the roots have increased 1.16, 1.12, 0.84, 0.53, 0.46, 0.46, 0.38, and 0.36 log cfu per cm root for isolates A20, A106, A45, A81, A62, A48, A25, and A67, respectively (Table 1) when compared to the initial inoculum level of 1×10^8 cfu per ml. It has been reported that biocontrol rhizobacteria isolates *B. cereus*, *B. subtilis*, *B. circulans*, *B. licheniformis*, *B. stearothermophilus* and *Chromobacterium violaceum*

were able to colonize the sorghum root (*in vitro*) in a range of 10^4 to 10^9 cfu per cm root, were able to inhibit the *Fusarium oxysporium* both *in vitro* and *in vivo* experiments. The highest root colonization ability of rhizobactria also showed the significant survival ability in the rhizosphere (Idris et al., 2007). The root colonization of *Paenibacillus polymyxa* (10^9 cfu per cm root) was able to suppress the pathogen and the superior biofilm former characteristic of this strain offers significantly better protection against crown rot in peanut (Haggag and Timmusk, 2008b). Thus, root colonization has remained a focus of much research because of the positive relationship between colonization and pathogen suppression in many biocontrol systems (Weller, 2007). Yen et al. (2003) reported that competitive root tip colonization by *Pseudomonas* strains can play an important role in the efficient control of soil borne crop diseases caused by fungi. Inadequate colonization is often the limiting factor in biological control. These results indicated that root colonization ability was one of the factors influences biological control efficiency. Based on the abilities of both fungal inhibition and root colonization, the best 4 PGPR isolates, A20, A45, A62 and A106, were selected for further experiments.

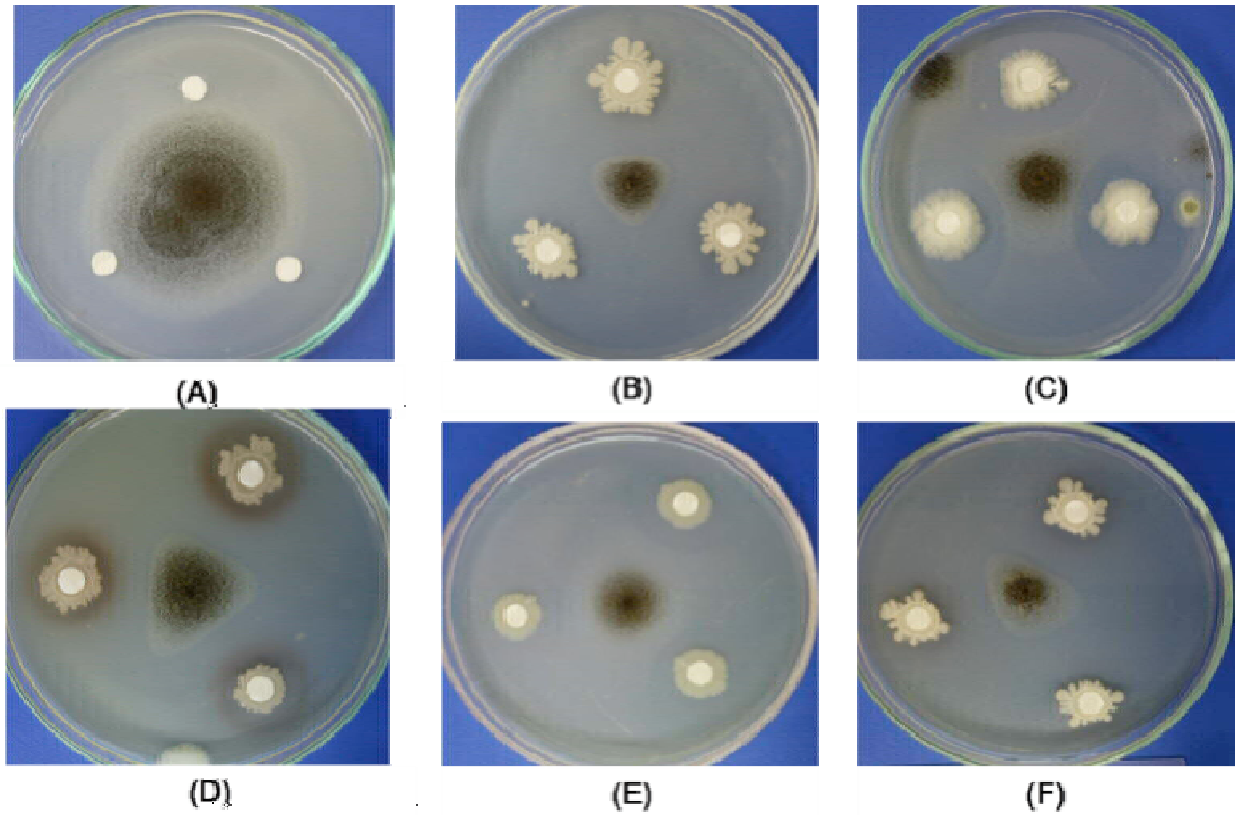

Figure 1 Inhibition of *A. niger* mycelial growth by selected PGPR isolates. A: *A. niger* alone (control), B: *A. niger* with isolate A62, C: *A. niger* with isolate A20, D: *A. niger* with isolate A25, E: *A. niger* with isolate A106 and F: *A. niger* with isolate A45.

Table 1 Inhibition of *A. niger* mycelial growth and *in vitro* root colonization by PGPR isolates

PGPR isolates	Dual culture assay % mycelial inhibiton [A]	*In vitro* root colonization log cfu/g roots[B]
A106	44.53 ± 2.40^{bc}	9.12 ± 0.49^{b}
A20	42.52 ± 3.20^{bc}	9.16 ± 0.43^{b}
A25	41.58 ± 2.10^{bc}	7.93 ± 0.15^{d}
A43	36.52 ± 3.01^{bc}	8.38 ± 0.87b^{c}
A44	17.98 ± 4.84^{e}	7.26 ± 0.30^{e}
A45	51.42 ± 2.70^{b}	9.84 ± 0.24^{a}
A48	35.94 ± 3 .07^{bc}	8.46 ± 0.46^{cd}
A62	67.81 ± 3.70^{a}	8.46 ± 0.04^{cd}
A67	33.77 ± 3.48^{d}	8.36 ± 0.28^{cd}
A69	46.00 ± 3.15^{bc}	7.17 ± 0.45^{e}
A81	39.71 ± 4.03^{bc}	8.53 ± 0.11^{cd}

[A] % mycelial inhibition was calculated by (A-B)/A×100, where A is mycelia growth away from the bacterial colony (the maximum growth of the fungal mycelia), B is mycelia growth toward the bacteria.

[B] Root colonization expressed as log cfu/cm root dry weight.

Different letters in the same column indicate a significant different among treatments ($P \leq 0.01$).

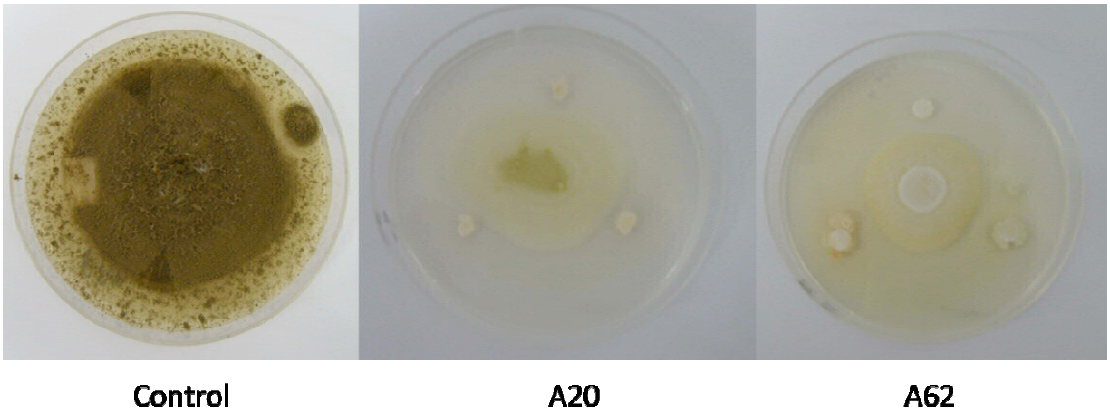

Figure 2. Inhibition of *A. flavus* mycelial growth by selected PGPR isolates.

4.2 Characterization of selected PGPR isolates

Selected PGPR isolates including A20, A45, A62, and A106 were morphologically observed under microscope. The PGPR isolates A20, A45, and A62 were gram-positive, rod-shaped and had the ability to form endospores. The PGPR isolate A106 was a gram-negative and rod-shaped without endospore formation (Fig. 3). The sequence of 16S rDNA genes of these selected isolates were identified, and the results indicated that A20, A45, A62, and A106 were highly homology to *Bacillus megaterium* strain AM1C7 (99%), *B. subtilis* strain Setapak 8 (99%), *B. subtilis* subsp. *subtilis* strain SB 3130 (99%), and *Pseudomonas* sp. NJ-61 (95%), respectively. Generally, *Bacillus* spp. is capable of producing antibiotics as well as a variety of fungal cell wall-degrading enzymes, such as chitinase, proteinase, cellulase, amylase, etc., (Adesina et al., 2007). Most of the antibiotics are the peptides effective against fungi, such as cyclic lipopeptide (CLP), phosphono-oligopeptide, and dipeptide (Ongena and Jacques, 2007). In this study, three *Bacillus* sp. isolates were also found to inhibit the *A. niger* growth as described above. Similar result was reported that peanut seeds bacterized with *B. subtilis* showed a reduced incidence of crown rot in *A. niger* infested soil, suggesting a possible role of *B. subtilis* in biological

control of *A. niger* (Podile and Prakash, 1996). Moreover, it was found that the induction of lipoxygenase LOX (13HPODE and 13HPOTrE) in peanut by *B. subtilis* AF 1 was significantly inhibited the growth of *A. niger* as well as caused extensive lysis of *A. niger* and reduced the incidence of crown rot in peanut (Sailaja et al., 1998). While antifungal activity was also found in *Pseudomonas* sp., it was found that *P. aeruginosa* GSE 18 reduced more than 60% of the pre-emergence rotting and post-emergence wilting of peanut in *A. niger*-infested potting mixture (Kishore et al., 2006). Bacterial seed treatment inoculated with *Pseudomonas* sp. induced the rapid accumulation of defense-related enzymes like chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase (Dey et al., 2004) in peanut seedlings compared with the un-inoculated control seed (Saravanakumar et al., 2007).

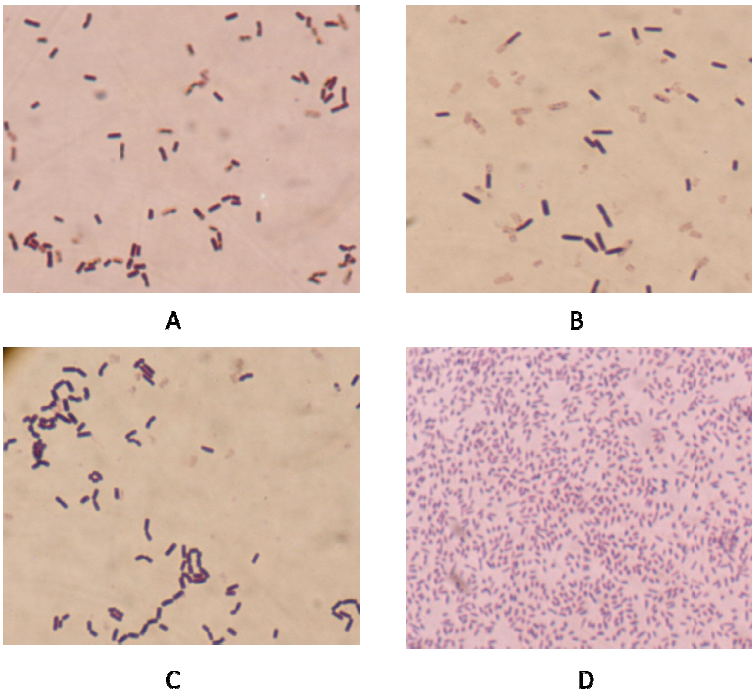

Figure 3. Gram staining and morphological observation of selected PGPR isolates under light-microscope. **A**: Isolate A20, **B**: Isolate A45, **C**: Isolate A62 and **D**: Isolate A106.

The PGPR isolates A20, A45, A62 and A106 were tested for their ability of indole-3-acetic acid (IAA) and biofilms formation, as well as lytic enzyme production including protease, cellulase, and chitinase. All of the tested isolates showed the high production of biofilm formation. Among them, A20 showed the significant highest biofilm formation (Table 2). In the case of IAA production, all of the PGPR isolates were able to produce IAA and isolates A45 and A62 showed the highest amount of IAA among the tested isolates. In addition, isolates A20, A45 and A62 could produce protease enzyme (Table 2 and Fig. 4), however, all of them could not produce cellulase and chitinase enzymes (Table 2).

Table 2. Characterization of biofilm formation, indole-3-acetic (IAA) and antifungal enzymes production of selected PGPR isolates.

Treatment	Biofilm formation	IAA	Protease	Chitinase	Cellulase
A20	2.6631 ± 0.36^{a}	17.75 ± 0.01^{bc}	+	-	-
A45	1.3566 ± 0.14^{b}	28.86 ± 0.04^{b}	+	-	-
A62	1.3090 ± 0.48^{b}	65.50 ± 0.03^{a}	+	-	-
A106	1.3859 ± 0.31^{b}	13.30 ± 0.01^{c}	-	-	-

Biofilm formation (measured by optical density at 595 nm), IAA unit = ppm per 1×10^{8} cfu, + = can produce enzyme, and - = cannot produce enzyme. Different letters in the same column indicate a significant different among treatments ($P \leq 0.01$).

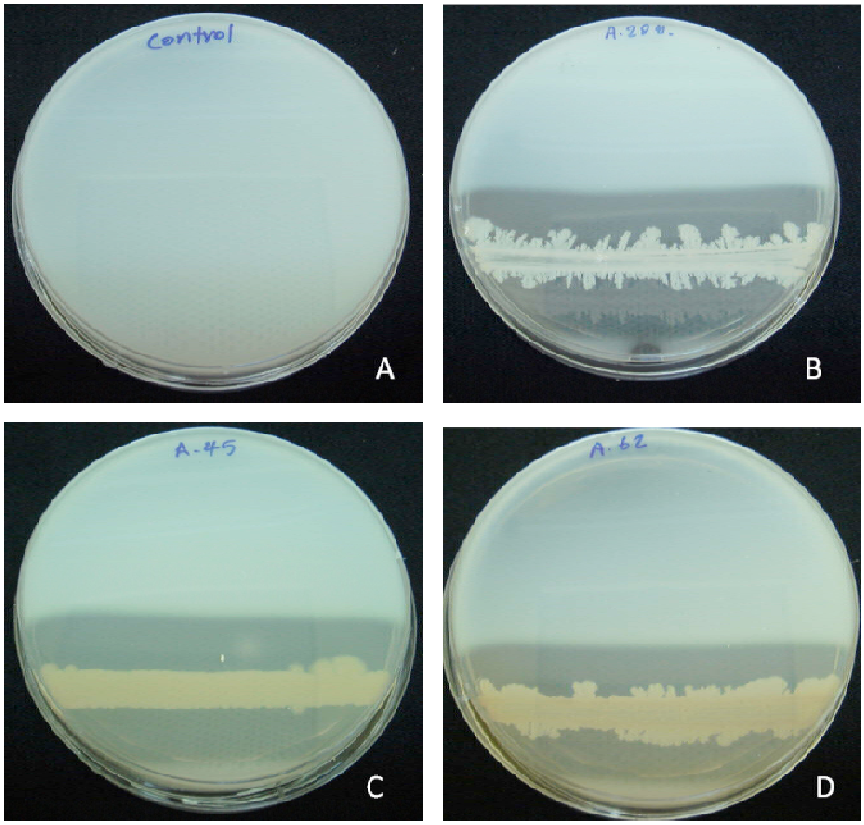

Figure 4. Clear zone of protease enzyme production by the selected PGPR isolates on 0.2% skim milk medium. **A**: PGPR isolate A20, **B**: PGPR isolate A45, **C**: PGPR isolate A62, and **D**: PGPR isolate A106.

Roots and root-cross sections of peanut inoculated with different concentrations of IAA (10-60 µM), or inoculated with each of 4 selected PGPR isolates showed different morphologically changes when compared with control (un-inoculation) (Fig. 5A). Inoculation of peanut with IAA in a range of 10-30 µM was not much effect on root, however, clearly morphological changes were observed in a range of 40-60 µM IAA inoculation that showed the effect on root cortex and epidermis (Fig. 5B). The production of plant hormones by PGPR was reported in numerous studies (Maldonado et al., 2009; Xiao et al., 2009; Cole et al., 2010; Dodd et al., 2010; Shibuya et al., 2010). The important hormones is auxin hormone especially indolacetic acid (IAA) play a significant role in the

root development (Aloni et al., 2006). The effect of exogenous IAA could stimulate or inhibit plant growth and is often a function of hormones concentration available (Figueiredo et al., 2011). Persello-Cartieux et al. (2003) also observed the sensitivity of plant tissue changes according to hormone concentrations. It was reported that isolates of *Pseudomonas* (fluorescent) produced exudates in roots of maize in response to IAA (Figueiredo et al., 2011). Analyzing the sources of IAA with bacterial origin, Loper and Schroth (1986) found that two strains of *Pseudomonas* spp. producing high concentrations of IAA (5-10 mg per ml), which reduced roots elongation and increased shoot/root proportion in sugar beet plants (*Beta vulgaris*) when applied as seed inoculant in this culture. Arau´jo et al. (2005) detected IAA production in two strains of *B. subtilis* which provided benefits in growth of soybean, in addition to be antagonists of phytopathogenic fungi in culture. At higher concentrations of IAA could inhibit the growth of *Saccharomyces* and other fungi (Prusty et al., 2004). Arau´jo and Hungria (1999) found that *B. subtilis* (AP-3) or its metabolites provided increase in nodulation and yield of soybean in the field.

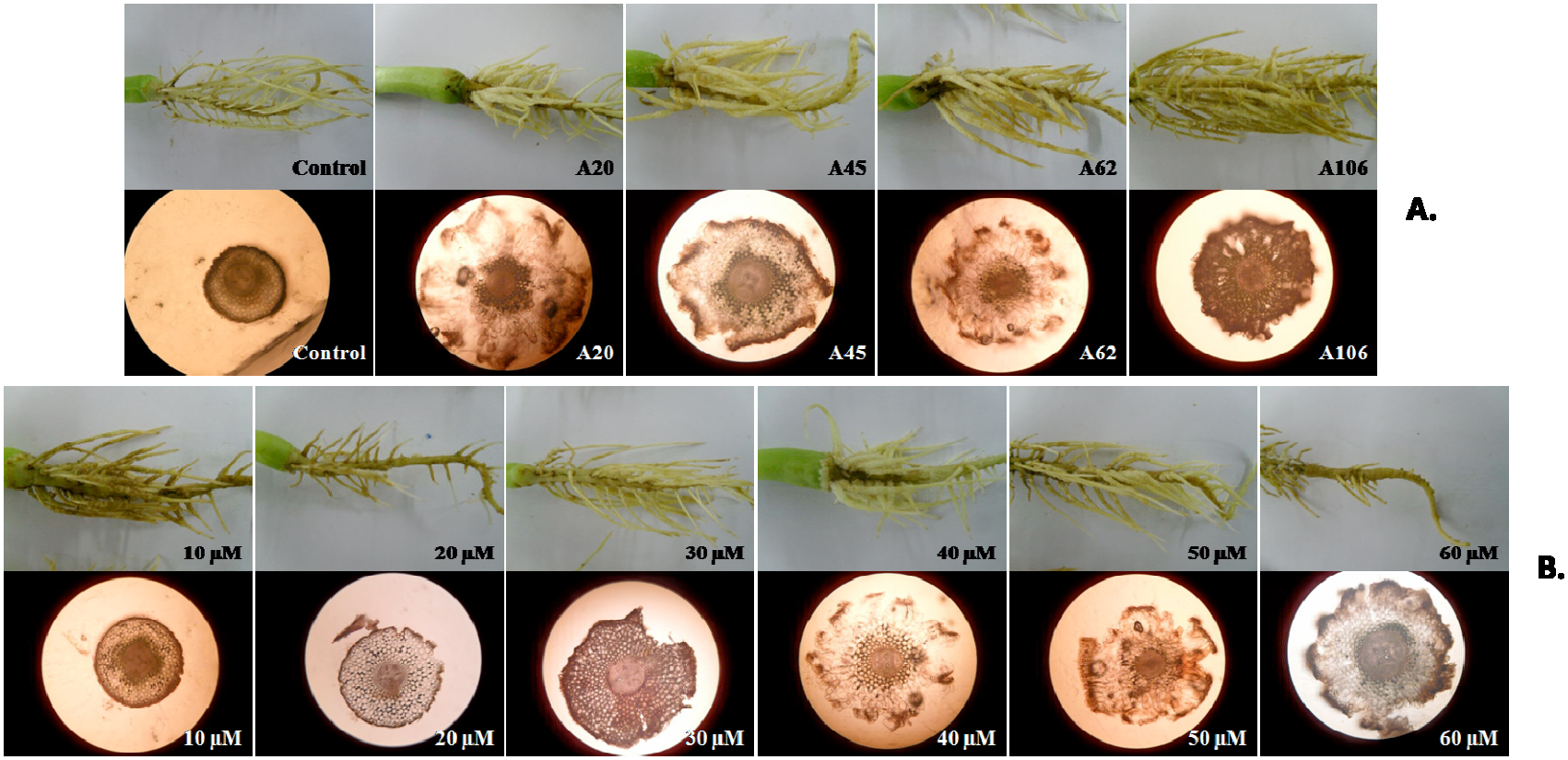

Figure 5 Effects of inoculation different concentrations of IAA, or IAA producing PGPR on peanut root morphology. **A**: Peanut root with different PGPRs inoculation; control (un-inoculation), A20, A45, A62 and A106. **B**: Peanut root with IAA inoculation at different concentrations; 10, 20, 30, 40, 50, and 60 µl.

The biofilm structures of 4 PGPR isolates were observed by a Carl Zeiss Pascal laser scanning microscope. The biofilm structures of A45, A62, and A106 were a dome formed and only A20 was a flat formed (Fig. 6). The long filaments and elongated cell clusters was found in A45 (Fig. 6B).

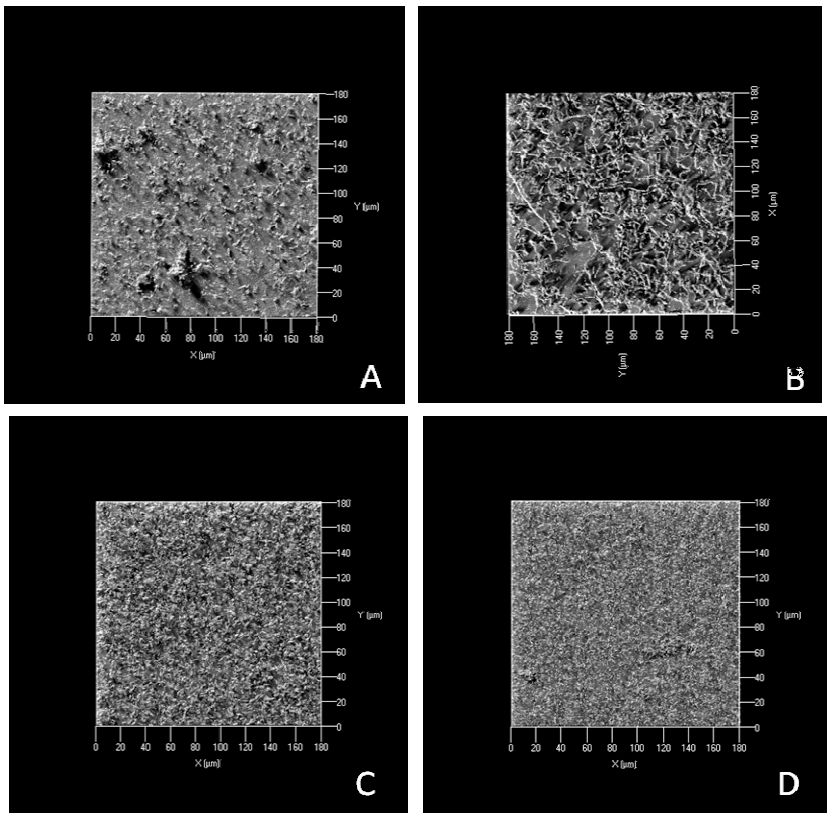

Figure 6. Biofilm structures of selected PGPR cultured in LB medium visualized by the confocal reflection microscopy technique. Images were acquired at 24 h after inoculation and are presented as simulated projections. Each projection shows fields of 140 by 140 µm (x-y), as indicated. **A**: PGPR isolate A20. **B**: PGPR isolate A45. **C**: PGPR isolate A62.and **D**: PGPR isolate A106.

In most natural, clinical and industrial settings, bacteria often grow and attach to surfaces in communities known as biofilms. Biofilm-associated organisms are able to adapt to environmental changes by altering their gene expression (Davies et al., 1993) and altering general physiology including increased resistance to antibiotics (Brooun et al., 2000). One of the ways in which microbial communities adjust to environmental changes is by changing the structural organization of the biofilm (Nielsen et al., 2000). Biofilms has been shown to play a fundamental role in futuristic agricultural approaches, such as biofertilizer, plant growth promoters, and biocontrolling agent. Jayasinghearachchi and Seneviratne (2004) demonstrated that a fungal-rhizobial biofilm (FRB) (*Bradyrhizobium elkanii* SEMIA 5091 and *Penicillium* spp.), a developed biofilmed inoculation, was significantly increasing N_2 fixation compared with single inoculation of rhizobia. *B. subtilis*, another biocontrolling PGPR, protects plant roots from pathogenic by mechanisms which include biofilm formation and antibiotic and surfactin production (Bais et al., 2004). The role of bacterial IAA considers in different microorganism-plant interactions that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms. Moreover, several reports indicated that IAA can also be a signaling molecule in bacteria and therefore can have a direct effect on bacterial physiology (Li et al., 2007, Remans et al., 2006 ; Spaepen et al., 2007). Other targets of indole mediated signaling were found recently indicating a role for indole signaling in biofilm formation (Domka et al., 2006). The bacterial biofilm formation on fungi participates in the synergistic degradation of substrates, antagonism of fungal growth, bacterial utilization of fungi as nutrient sources, and the formation of more complex synergistic associations for the purposes of nutrient acquisition. While bacterial biofilm formation has been described in many systems, the molecular mechanisms that govern these interactions are not yet well understood (Hogan et al., 2004). In the role of antifungal substance or metabolites

antagonistic PGPR have attracted much attention in their role in reducing plant diseases, especially strains of the genus *Bacillus* spp., *Pseudomonas* sp., and *Burkholderia* (Quan et al., 2011). *Bacillus* spp. produces 167 biological compounds active against bacteria, fungi, protozoa, and viruses (Bottone and Peluso, 2003).

4.3 Antifungal activity

In order to predict the mechanism of antifungal activity, the culture filtrates of all isolates were concentrated and the activity test was conducted in different treatments as water, proteinase k, and 70% ethanol. Treated with proteinase k was expected to degrade lytic enzyme activity, and treated with 70% ethanol resulted in the transfer of antifungal activity into the organic phase (Principe et al., 2007). The results showed that the control treatments without cell-free supernatant had no effect on fungal growth (Fig. 7A). In all treatments, cell-free supernatant of A62 showed the greatest (most effective) antagonistic activity among the 4 selected PGPR. Cell-free supernatants of both A20 and A62 showed the antagonistic activity when dissolved in water (Fig. 7B) and were greater when dissolved in 70% ethanol (Fig. 7D). Inhibition on fungal growth was still appeared in the treatments of A62 and A45 when dissolved in proteinase k (Fig. 7C). But antifungal activity of isolated A20 was not resistant to proteinase k activity (Fig. 7C). However, cell-free supernatant of A20 dissolved in 70% ethanol showed the greater inhibition zone than dissolved in water (Fig. 7B). Therefore, it might be thought that antifungal activity of this isolate may have more than one antifungal mechanism. Antifungal activity of A45 and A62 was resistant to proteinase k activity. However, there was confusingly observed that cell-free supernatant of A45 did not showed the antagonistic activity when dissolved in water and 70% ethanol. Even A106 showed the antagonistic ability on *A. niger* growth in *in vitro* test as described before, no clear inhibition was observed from lyophilized cells dissolved

in different treatments. It is possible that there is low concentration of the antifungal substances presence in the cell-free supernatant culture of A45 and A106 or it might be possible that the mechanism of antifungal substances of these isolates involved intra- or inter-cellular substances. Moreover, the biocontrol activities of isolates A20 and A62 were observed in cell-free supernatant, indicating that biocontrol metabolites were secreted into cultured media. The antifungal activity of A62 was found when treated with proteinase k and ethanol. Proteinase k was expected to cut-out of enzyme activity and ethanol was expected to increase the solubility of organic substance, especially in lipid group can dissolve well in ethanol. It is possible that isolate A62 (*Bacillus* sp.) produced antifungal substance in lipopeptides group. It is well known that most of the *Bacillus* strains, such as *B. subtills, B. circulan* and *B. megaterium* produce bioactive compounds belonging to the cyclic lipopeptides group with high stability attributable to their structure (Lugtenberg and Kamilova, 2009).

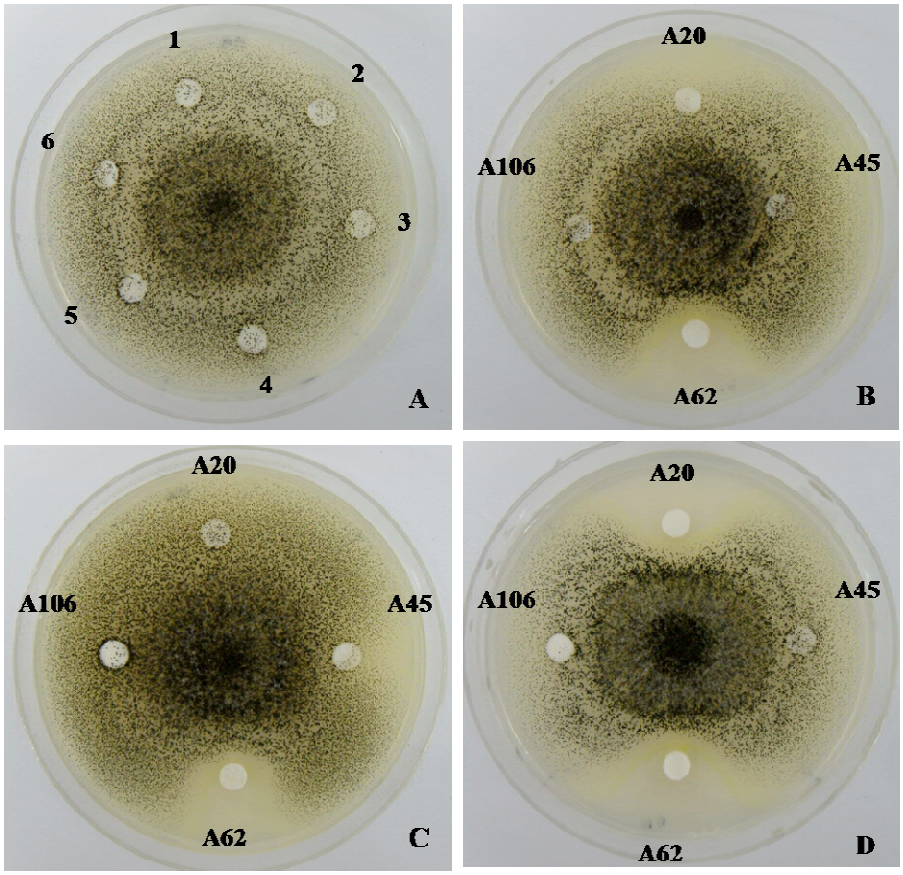

Figure 7. Antagonistic activity of cell-free supernatants of selected PGPR against *A.niger*. **A**: control: 1) Lyophilized LB medium dissolved in sterilized water, 2) Lyophilized LB medium treated with proteinase k, 3) Lyophilized LB medium dissolved in 70% ethanol, 4) LB medium, 5) 3 µl of proteinase k (20 mg per ml or 6 µm AU per ml), 6) 70% ethanol. **B**: cell-free supernatants of 4 selected PGPR dissolved in sterilized water. **C**: cell-free supernatants of 4 selected PGPR treated with proteinase k. **D**: cell-free supernatants of 4 selected PGPRs dissolved in 70% ethanol.

4.4 Effects of different *A. niger* inoculum doses on crown- and root-rot disease of peanut plant

The evaluation of the minimum inoculum dose of *A. niger* on peanut was conducted. The results indicated that at 10 to 10^7 spores per ml of *A. niger* caused 47.66%, 52.66%, 68.33%, 77.33%, 87.33%, 91.33%, and 100% root rot disease severity of peanut plants, respectively (Table 3, Fig. 8A and B). The crown- and root-rot disease of *A. niger* on peanut was shown in Figure. 8C and D. The concentrations of *A. niger* inoculum dose had a major influence on the disease incidence. The result showed that a higher concentration of *A. niger* inoculum dose gave the higher disease incidence. Although it has been reported that the initial population levels of *Aspergillus* sp. in peanut field had a range of approximately 30 to 3,600 spores per g of soil and can cause root rot disease (Horn et al., 1995), in this study, three concentrations of *A. niger* at 10^5, 10^6 and 10^7 spores per ml that caused more than 80% of disease severity were used for further plant fungal infection test.

Table 3. Effect of different doses of *A. niger* on crown rot disease severity in peanut plant.

Concentration of *A. niger* (spores/ ml/ seed)	**Disease Severity (%) ±SD**
Control (no fungal inoculation)	32.00 ± 4.0^{f}
10^1	47.66 ± 7.6^{e}
10^2	52.66 ± 6.4^{e}
10^3	68.33 ± 6.1^{d}
10^4	77.33 ± 6.2^{cd}
10^5	87.33 ± 6.4^{bc}
10^6	91.33 ± 7.6^{ab}
10^7	100.00 ± 0.0^{a}

Different letters in the same column indicate a significant different among treatments ($P \leq 0.01$).

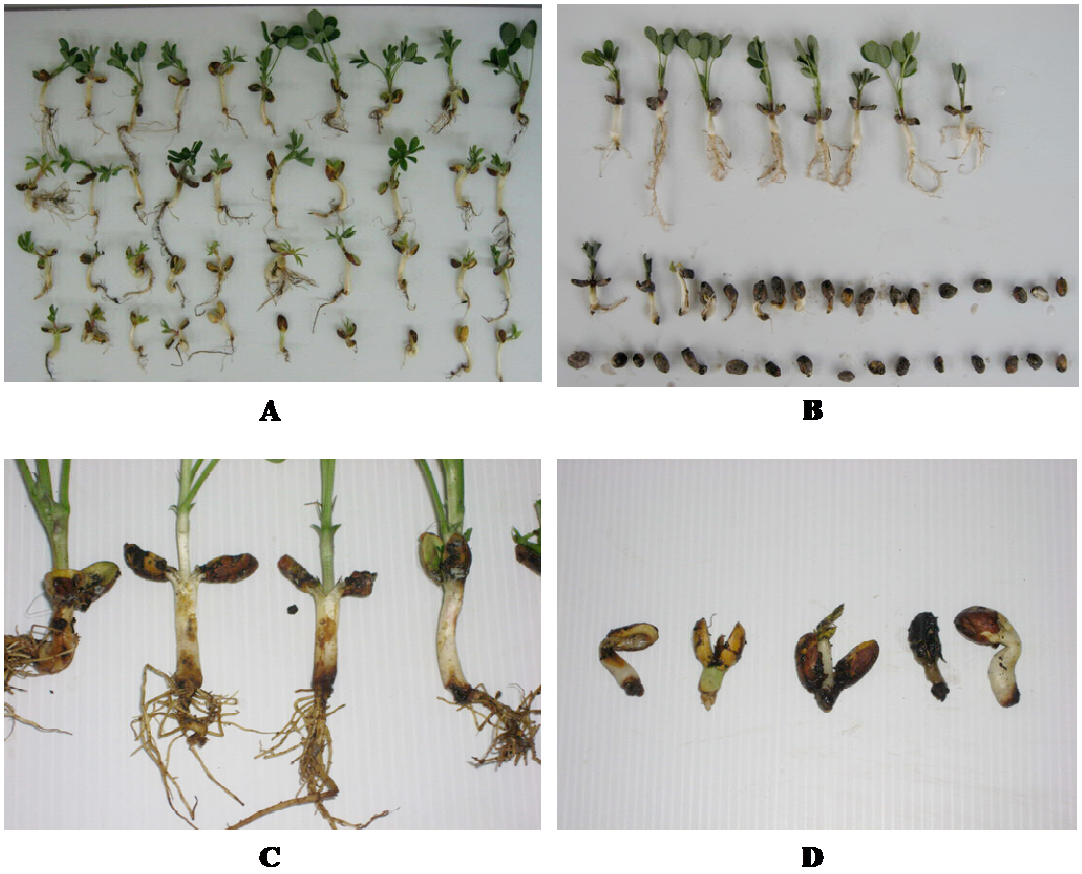

Figure 8. Root rot disease on peanut inoculated with *A. niger* at 10^7 on peanut spores per ml per seed. **A**: Control (no fungal inoculation), **B**: inoculated, **C**: disease symptoms on germinated seedling, and **D**: pre-germinated seed rot.

4.5 Effect of co-inoculation of PGPR isolates with *Bradyrhizobium* sp. TAL 173 on crown- and root-rot disease of peanut plant

The selected PGPR isolates were co-inoculated with *Bradyrhizobium* sp. TAL 173 on the purpose of investigation the biological control activity on *A. niger* infected plant in pot experiments. In this study, the higher concentration of PGPR and *Bradyrhizobium* sp. TAL 173 at 10^8 cells per ml was used for biological control test because there had been reported that higher concentration of the PGPR inoculum size gave better biocontrol activity. The best biocontrol was obtained with the PGPR inoculum size at 10^9 cells per ml (Kong et al., 2010). This experiment was compared to carbendazim as chemical

fungicide treatment. The results in Table 4 and Figure 9 revealed that the application of chemical fungicide or co-inoculation of the selected PGPR treatments could reduce the disease incidence (DI) and disease severity (DS) of crown rot on peanut when compared with plants inoculated only with *A. niger*. No disease symptom was observed in healthy control that was treated with neither fungal nor bacterial inoculants. Carbendazim treated seeds showed significantly decreased of DI and DS from control plant infested only with different concentrations of *A. niger*. However, the DI and DS values of carbendazim treated plants were not significantly different from plants co-inoculated *Bradyrhizobium* sp. TAL 173 with A20 or A45 at concentrations of *A. niger* 10^5 and 10^6 spores per ml. At concentration of *A. niger* 10^7 spores per ml per seed, although the DI and DS of some treatments were not significantly different from only fungal infested control, the incidence and severity were lower than that of control treatment. Of all tested *A. niger* inoculum doses, even co-inoculation treatments could reduce the incidence and severity of crown rot disease, it was not efficient as carbendazim, which showed the highest disease suppression of 100%. The peanut plant dry weight of this experiment was shown in Table 5. Peanut plant dry weight of carbendazim treated plants were not significantly different from those plants co-inoculated *Bradyrhizobium* sp. TAL 173 with A20 or A45 at concentrations of *A. niger* 10^5 and 10^6 spores per ml. At concentration of *A. niger* 10^7 spores per ml per seed, although peanut plant dry weight of some treatments were not significantly different from only fungal infested control, peanut plant dry weight of some treatment was higher than that of control treatment. Of all tested *A. niger* inoculum doses, co-inoculation treatments could increase the peanut dry weight. It is known that *in vitro* assays have certain limitations in which the biocontrol efficiencies may not be equally expressed under natural condition (Jedabi and Awatif, 2009). However, co-inoculation of TAL 173 with A20 or A45 also gave the high disease suppression when compared with the infected control.

Table 4 The effect of selected PGPR co-inoculated with commercial *Bradyrhizobium* sp. TAL 173 on crown rot disease of peanut at different concentrations of *A. niger* under light room condition.

Treatments	Disease incidence (%) *A. niger* (spores/ml/seed)			Disease severity score [C] *A. niger* (spores/ml/seed)		
	10^5	10^6	10^7	10^5	10^6	10^7
Control A[A]	0.00 [d]	0.00 [b]	0.00 [b]	0.00 [d]	0.00 [b]	0.00 [c]
Control B[B]	88.89 [a]	100.00 [a]	100.00 [a]	3.00 [a]	3.67 [a]	4.00 [a]
0.02 % Carbendazim	0.00 [d]	0.00 [b]	0.00 [b]	0.00 [d]	0.00 [b]	0.00 [c]
TAL173 + A20	33.33 [bcd]	55.56 [ab]	77.78 [a]	1.33 [bc]	1.89 [ab]	3.11 [ab]
TAL173 + A45	11.11 [cd]	55.56 [ab]	66.67 [a]	0.44 [cd]	2.22 [a]	2.67 [b]
TAL173 + A62	44.44 [bc]	66.67 [a]	77.78 [a]	1.67 [ab]	2.67 [a]	3.22 [ab]
TAL173 + A106	55.56 [ab]	77.78 [a]	77.08 [a]	2.22 [ab]	3.11 [a]	3.11 [ab]

[A] Un-inoculated plants.

[B] Plants inoculated only with *A. niger* spores at different concentrations.

[C] Crown-rot severity was assessed on a rating scale of 0–4, where 0 = no infection, 1 = 1–25% infection, 2 = 26–50% infection, 3 = 51–75% infection and 4 = 76–100% infection in the root and crown regions

Different letters in the same column indicate a significant different among treatments ($P \leq 0.01$)

Table 5 The effect of selected PGPR co-inoculated with commercial *Bradyrhizobium* sp. TAL 173 on crown rot disease of peanut at different concentrations of *A. niger* under light room condition.

Treatments	**Peanut plant dry weight (g)**			
		***A. niger* (spores/ ml/seed)**		
	No *A. niger* [A]	**10^5**	**10^6**	**10^7**
Control [B]	0.50	0.17 [c]	0.00 [c]	0.00 [b]
0.02 % Carbendazim	0.62	0.58 [a]	0.60 [a]	0.57 [a]
TAL173 + A20	0.91	0.55 [a]	0.37 [ab]	0.21 [b]
TAL173 + A45	1.01	0.51 [ab]	0.44 [ab]	0.20 [b]
TAL173 + A62	0.70	0.46 [ab]	0.33 [ab]	0.21 [b]
TAL173 + A106	0.66	0.37 [ab]	0.29 [ab]	0.22 [b]
F-test	ns	*	*	*

[A] Un-inoculated *A. niger.*

[B] Plants inoculated only with *A. niger* spores in different concentrations.

Mean values within a column followed by different letters were significantly different according to the DUNCAN's test, P≤0.01 (*),

ns = non significant

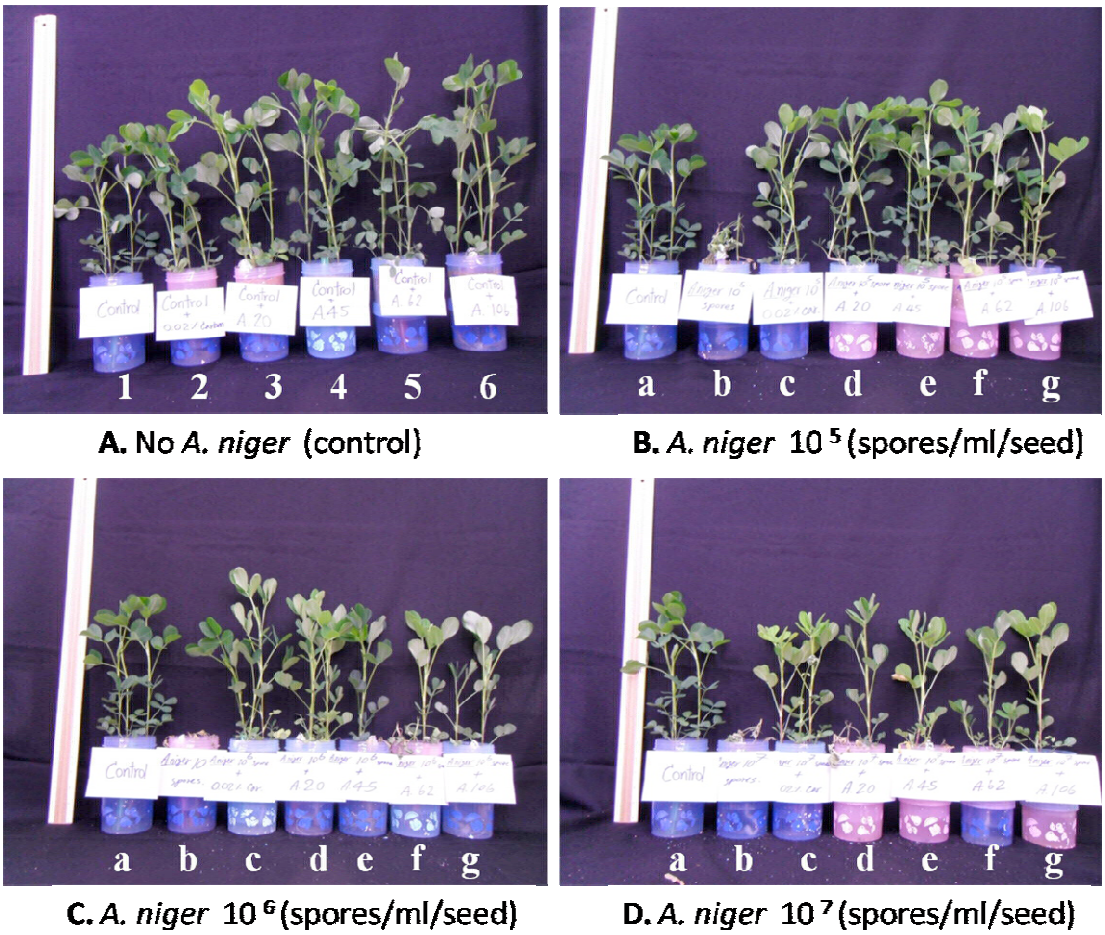

Figure 9. Effect of co-inoculation of PGPR isolates with *Bradyrhizobium* sp. TAL 173 on root rot disease of peanut plant at different inoculum dosage of *A. niger* spores. Pot **1**. Control (un-treated plant), **2**. 0.02% Carbendazim, **3**. inoculated with PGPR A20, **4**. inoculated with PGPR A45, **5**. inoculated with PGPR A62, and **6**. inoculated with PGPR A106. **a**. Control (un-treated plant), **b**. plant inoculated only with *A. niger* inoculum **c**. plant treated with 0.02% Carbendazim, **d**. plant inoculated with PGPR isolate A20, **e**. plant inoculated with PGPR isolate A45, **f**. plant inoculated with PGPR isolate A62, and **g**. plant inoculated with PGPR isolate A106.

4.6 Inoculation effects of PGPR alone and co-inoculation with *Bradyrhizobium* sp. TAL 173 on peanut

The PGPR isolates A20 and A45 were tested for the plant growth promoting effect on peanut in different concentrations (10^4-10^8 cells per ml). Positive response in plant dry weight and root dry weight was observed at inoculation level of 10^6 to 10^8 cells per ml of both isolates (Table 6). Co-inoculation effects of A20 and A45 with commercial *Bradyrhizobium* sp. TAL 173 on peanut was tested in light room conditions. All of the co-inoculation treatments were able to increase the plant-, root-, and nodule-dry weight when compared with single bradyrhizobial inoculation alone (Table 7). The root dry weight of plants co-inoculated by *Bradyrhizobium* sp. TAL 173 with A20 (10^4-10^6 cells per ml) or with A45 (10^4 cells per ml) were significantly different from single *Bradyrhizobium* sp. TAL 173 inoculation. The highest plant dry weight was observed from co-inoculation of *Bradyrhizobium* sp. TAL 173 with A20 (10^7 cells per ml). Up to 10^6 cells per ml of A20 and up to 10^5 cells per ml of A45 gave the significantly higher nodule dry weight than plant inoculated with *Bradyrhizobium* sp. TAL 173 alone. The co-inoculation of each PGPR (A20 and A45) with *Bradyrhizobium* sp. TAL 173 increased plant-, root- and nodule-dry weight significantly different from control, but it was not significantly different from plant inoculated with TAL 173 alone. This effectiveness of microbial inoculation depends on specific combinations of associated PGPR and functional compatibility with the host (Azcón, 1993). However, the phytohormone production is one of the mechanisms described for the rhizobacteria stimulating effect (Probanza et al., 1996). Physiological changes in root or nodule functioning could be involved in the specific

effects obtained from the various microbial combinations. Some combinations of rhizobial and rhizobacterial isolates could not improve growth and nodulation compared to uninoculated control, which might be due to certain compounds (i.e., toxic for plants to some extent) produced by the bacteria. Production of antibiotics and competition for attachment sites on root surfaces could be one of the reasons for negative effects of co-inoculation of PGPR with *Rhizobium* (Chebotar et al., 2001 ; Valverde et al., 2006 ; Mirza et al., 2007). Raverkar and Konde (1988) also reported the adverse effects of co-inoculation with *Rhizobium* and *Azospirillum lipoferum* on nodulation, nitrogen contents and yield of the peanut.

Table 6 The effect of different concentrations of selected PGPR on peanut under light room condition

Treatment	Root length (cm)	Plant height (cm)	Plant dry weight (g)	Root dry weight (g)
Control	9.56 b	33.78 de	0.697 b	0.090 b
A20 (10^4)	10.00 ab	39.33 abc	0.940 ab	0.112 ab
A20 (10^5)	8.80 b	40.67 a	1.027 ab	0.105 ab
A20 (10^6)	14.66 ab	37.61 abcd	1.087 a	0.141 ab
A20 (10^7)	9.54 b	38.28 abcd	1.103 a	0.137 ab
A20 (10^8)	10.06 ab	35.22 bcde	1.087 a	0.102 ab
A45 (10^4)	8.48 b	35.39 bcde	1.030 ab	0.163 a
A45 (10^5)	11.56 ab	34.95 cde	1.043 ab	0.132 ab
A45 (10^6)	11.39 ab	32.50 e	0.837 ab	0.119 ab
A45 (10^7)	11.50 ab	38.11 abcd	1.063 ab	0.133 ab
A45 (10^8)	11.11 ab	37.50 abcd	0.940 ab	0.143 ab
A20+A45 (10^8)	8.78 b	39.83 ab	0.920 ab	0.104 ab

Different letters in the same column indicate a significant different among treatments ($P \leq 0.01$)

Table 7 The effect of different concentrations of selected PGPR co-inoculated with commercial *Bradyrhizobium* sp. TAL 173 on peanut under light room condition

Treatment	Plant dry weight (g)	Root dry weight (g)	Nodule No.	Nodule dry weight (g)
Control (un-inoculate)	0.697 b	0.090 c	0.00 d	0.0000 d
TAL 173 alone	0.803 ab	0.142 bc	7.33 b cd	0.0096 cd
TAL 173+A20 (10^4)	0.883 ab	0.186 ab	8.67 bcd	0.0072 abc
TAL 173+A20 (10^5)	0.890 ab	0.216 a	6.00 bcd	0.0186 abc
TAL 173+A20 (10^6)	0.873 ab	0.215 a	16.67 abc	0.0340 a
TAL 173+A20 (10^7)	1.137 a	0.205 ab	7.00 abcd	0.0219 ab
TAL 173+A20 (10^8)	0.877 ab	0.184 ab	13.67 abc	0.0311 a
TAL173+A45 (10^4)	0.883 ab	0.234 a	16.00 abcd	0.0178 abc
TAL173+A45 (10^5)	0.823 ab	0.185 ab	18.00 a	0.0225 ab
TAL173+A45 (10^6)	0.980 ab	0.206 ab	13.33 abc	0.0306 a
TAL173+A45 (10^7)	0.910 ab	0.208 ab	13.33 abc	0.0276 ab
TAL173+A45 (10^8)	0.810 ab	0.190 ab	17.00 abc	0.0224 ab
TAL173+A20+A45 (10^8)	1.053 ab	0.171 ab	8.67 cd	0.0072 cd

Different letters in the same column indicate a significant different among treatments ($P \leq 0.01$)

CHAPTER V

CONCLUSION

From this study, 765 isolates of peanut bradyrhizobia could not have antagonistic activity on *A. niger*. However, there were 11 of 350 PGPR isolates could inhibit *A. niger*, and A20 and A62 isolates could inhibit *A. flavus*. The best 4 PGPR isolates including A20, A45, A62, and A106 were selected, these isolates had highly homology to *Bacillus megaterium* strain AM1C7 (99%), *B. subtilis* strain Setapak 8 (99%), *B. subtilis* subsp. *subtilis* strain SB 3130 (99%), and *Pseudomonas* sp. NJ-61 (95%), respectively. Isolates A20 and A45 (10^8 cell per ml) significantly reduced the disease incidence and disease severity when *A. niger* 10^5 and 10^6 spores per ml were applied to plant. Isolates A20 and A45 could be co-inoculated with commercial *Bradyrhizobium* sp. TAL 173 (10^8 cell per ml) in order to protect the seed from pathogenic fungus *A. niger* and promote the growth of peanut plant.

This study demonstrated that two PGPR isolates (A20 and A45) could effectively inhibit the growth of *A. niger* and control the crown rot disease of peanut both *in vitro* and *in vivo* under light room condition. Further research should be carried out to confirm the effectiveness of developed inoculants on biocontrol of crown rot disease and the yield of peanut in the field conditions.

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