SELECTION, CHARACTERIZATION AND EVALUATION OF *STREPTOMYCES* FOR THE CONTROL OF ANTHRACNOSE IN CHILLI

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ABSTRACT

The actinomycetes, mainly *Streptomyces* spp., have been widely studied as potential biocontrol agents against plant pathogenic fungi. This project was undertaken to isolate and select *Streptomyces* strains showing potential *in vitro* and *in vivo* antifungal activity. Isolation was done from rhizosphere-derived soil of healthy and *Colletotrichum*-infested chilli plants in Ulu Chechuh in Kuala Selangor and Sungai Burung in the state of Selangor, Malaysia. The selected strains were evaluated for their ability to control chilli anthracnose pathogen, *Colletotrichum* spp. *in vitro*, and the best strain among them was chosen for green house experimental trials on chilli *in vivo*. A total of 130 isolates of actinomycetes were isolated from healthy and *Colletotrichum*-infested chilli rhizospheric soil on starch-casein, humic acid-vitamin and raffinose-histidine agar. 81.5% of the isolates produced abundant aerial mycelia and were categorized as *Streptomyces*-like. Twenty-four colonies that lacked aerial mycelia and formed compact orange or brown coloured colonies belonged to the genus *Micromonospora*. A high proportion (67.9%) of the *Streptomyces*-like isolates was isolated from the Sungai Burung's *Colletotrichum*-infested and healthy rhizospheric soil samples. Moreover, 50% of the *Micromonospora*-like isolates were obtained from the Sungai Burung's *Colletotrichum*-infested rhizospheric soil samples. Four groups of aerial mycelia colour observed in the *Streptomyces*-like isolates were grey (84.9%), white (8.5%), red (1.9%) and pink (4.7%). 106 *Streptomyces*-like isolates were assayed for *in vitro* antagonistic activity against three dominant species of *Colletotrichum* namely *C. acutatum*, *C. capsici* and *C. gloeosporioides*. The majority of the isolates having pathogen-inhibitory capabilities were from the grey colour group. Twelve isolates inhibited the growth of all test fungi at varying degrees and among them four isolates, P8, P39, P42 and P115,

showed the best inhibitory action. Isolate P42 was selected for further biocontrol studies because of its strong antifungal activity against all tested *Colletotrichum* species with the highest degree of antagonism. The antifungal compounds produced by isolate P42 were responsible for 81-94% reduction in mycelial growth of *C. acutatum*, *C. capsici* and *C. gloeosporioides*. Whole cell hydrolysates showed the presence of LL-DAP, classifying selected isolate P42 as a species of *Streptomyces*. Its aerial spore mass colour was grey with spiral spore chain and smooth-ornamented spores, and the isolate did not produce diffusible pigments. Good growth was observed on ISP2, ISP3, ISP4 and ISP6 agars with optimum growth at pH 7.0 and 28 ± 2°C. It degraded L-tyrosine, xanthine, chitin and starch. Isolate P42 did not require NaCl for growth yet was tolerant to 8% NaCl. It was susceptible to Neomycin, Nanamycin, Novobiocin, Gentamycin, Tetracycline and Streptomycin with inhibition zones ranging from 10-50 mm. It grew well on a variety of carbon sources including D-glucose, galactose, I-inositol, D-xylose, L-arabinose, D-mannitol and rhamnose, but not on sucrose and raffinose. Polyphasic taxonomic studies assigned the isolate under the *Streptomyces rochei* in the phylogenetic tree. Genotypically it was similar with marker strains *S. vinaceusdrappus* NRRL 2363T, *S. rochei* NBRC 12908T, *S. plicatus* NBRC 13071T and *S. enissocaesilis* NRRL B-16365T, but differed in some phenotypic characteristics. The strain was designated as *Streptomyces rochei*. Phytotoxicity effects of isolate P42 were assessed by using low dosage (1.16×10^5 cfu/ml) and high dosage (1.16×10^7 cfu/ml) of strain P42 spore suspension on the growth of maize seeds. Isolate P42 in low dosage did not reveal any adverse effect on the seeds and promoted maize seed growth significantly ($p<0.05$) compared to a high dosage of the isolate. The treatment of the chilli seeds prior to planting and 45 days after planting with 1.16×10^5 cfu/ml isolate P42 suspension, significantly ($p<0.05$) reduced the Fruit Symptom Index (FSI) when matured fruits were

challenged with 10^5 spores/ml of three different *Colletotrichum* species. Disease Severity Index (DSI) was reduced by 52-54% when plants were treated with low dosage of isolate P42. Isolate P42-treated and untreated control plantlets which were not challenged with *Colletotrichum* species were healthy.

ABSTRAK

Aktinomiset, terutamanya *Streptomyces* spp. telah dikaji secara meluas sebagai agen kawalan biologi yang berpotensi terhadap kulat patogen tumbuhan. Projek ini dijalankan untuk mengasing dan memilih strain *Streptomyces* yang menunjukkan potensi aktiviti antikulat *in vitro* dan *in vivo*. Pemencilan dilakukaw dari tanah rizosfera pokok cili yang sihat dan yang ditular *Colletotrichum* di Ulu Chechuh di Kuala Selangor dan Sungai Burung di negeri Selangor, Malaysia. Strain yang dipilih telah dinilai bagi keupayaan mereka untuk mengawal secara *in vitro* cili patogen bintik berpusar, *Colletotrichum* spp. dan strain yang terbaik di antara mereka telah dipilih untuk eksperimen *in vivo* percubaan rumah hijau ke atas cili. Sebanyak 130 pencilan aktinomiset dari tanah rizosfera pokok cili yang sihat dan yang ditular *Colletotrichum* dipencilkan pada agar kanji-kasein, asid humik-vitamin dan raffinose-histidine. 81.5% daripada pencilan menghasilkan miselium udara yang banyak dan dikategorikan sebagai menyerupai *Streptomyces*. Dua puluh empat koloni yang kurang miselium udara dan membentuk koloni berwarna jingga atau coklat adalah dalam genus *Micromonospora*. Sebahagian besar (67.9%) daripada strain yang menyerupai *Streptomyces* telah diasingkan daripada sampel tanah rizosfera Sungai Burung yang sihat dan yang ditular *Colletotrichum*. Selain daripada itu, 50% daripada pencilan yang menyerupai *Micromonospora* telah diperolehi daripada sampel tanah rizosfera Sungai Burung yang ditular *Colletotrichum*. Empat kumpulan warna miselium udara yang diperhatikan dalam pencilan yang menyerupai *Streptomyces*, iaitu kelabu (84.9%), putih (8.5%), merah (1.9%) dan merah jambu (4.7%). 106 yang menyerupai *Streptomyces* telah dinilai bagi aktiviti antagonisme *in vitro* terhadap tiga spesies dominan *Colletotrichum*, iaitu *C. acutatum*, *C. capsici* dan *C. gloeosporioides*. Majoriti pencilan yang mempunyai

keupayaan perencatan patogen adalah dari kumpulan warna kelabu. Dua belas pencilan merencat pertumbuhan semua kulat ujian pada tahap yang berbeza dan di autara mereka empat pencilan, P8, P39, P42 dan P115, menunjukkan tindakan perencatan yang terbaik. Pencilan P42 telah dipilih untuk kajian kawalan biologi lanjut an kerana aktiviti antikulat yang kuat terhadap semua spesis *Colletotrichum* yang diuji, dengan tahap antagonisme tertinggi terhadap tiga spesies *Colletotrichum*. Sebatian antikulat yang dihasilkan oleh pencilan P42 bertanggungjawab mengurangkan 81-94% pertumbuhan miselia *C. acutatum*, *C. capsici* dan *C. gloeosporioides*. Hidrolisat keseluruhan sel menunjukkan kehadiran LL DAP, dan mengkelaskan dipilih isolate P42 yang sebagai spesies *Streptomyces*. Warna spora udara adalah kelabu dengan rantaian lingkaran spora dan spora licin atau dangan ornamentasi tidak menghasilkan pigmen terlarat. Pertumbuhan yang baik telah diperhatikan pada agar ISP2, ISP3, ISP4 dan ISP6 dengan pertumbuhan optimum pada pH 7.0 dan 28 ± 2°C. Ia mendegradasi L-tirosina, xanthine, kitin dan kanji. Pencilan P42 tidak memerlukan NaCl untuk pertumbuhan tatapi toleran terhadap 8% NaCl. Ia rentan terhadap Neomycin, Nanamycin, Novobiocin, Gentamycin, tetracycline dan Streptomycin dengan zon perencatan antara 10-50 mm. Ia tumbuh dengan baik pada pelbagai sumber karbon termasuk D-glukosa, galaktosa, I-inositol, D-xilosa, L-arabinosa, D-manitol dan rhamnos, tetapi tidak pada sukrosa dan rafinosa. Kajian taksonomi "polyphasic" meletakkam pencilan ini di bawah *Streptomyces rochei* dalam pokok filogen. Secara genotip, ia adalah serupa dengan strain *S. vinaceusdrappus* NRRL 2363T, *S. rochei* NBRC 12908T, 13071T NBRC *S. plicatus* dan *S. enissocaesilis* NRRL B-16365T, tetapi berbeza pada beberapa ciri fenotip. Strain ini telah ditetapkan sebagai *Streptomyces rochei*. Kesan fitotoksik pencilan P42 dinilai menggunakan dos rendah (1.16×10^5 cfu / ml) dan dos tinggi (1.16×10^7 cfu / ml) ampaian spora P42 ke atas pertumbuhan benih jagung. Strain P42 dalam

dos rendah tidak memberi kesan buruk kepada biji benih, dan menggalakkan pertumbuhan benih jagung ($p < 0.05$) berbanding dengan penggunaan dos yang tinggi. Perlakuan ke atas biji cili sebelum penanaman dan 45 hari selepas penanaman dengan 1.16×10^5 cfu / ml strain P42 mengurangkan ($p < 0.05$) Indeks Gejala Buah (FSI) apabila buah matang diinokulat dengan 10^5 spora / ml tiga spesies *Colletotrichum* yang berbeza. Indeks Keporahan Penyakit (DSI) dikurangkan sebanyak 52-54% apabila pokok dirawat dengan dos rendah strain P42. Anak pokok yang diperlaku dengan strain P42 dan anak pokok kawalan tanpa perlakuan yang tidak diinokulat dengan spesies *Colletotrichum* tumbuh dengan sihat.

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CHAPTER 5. DISCUSSION

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
bp	Base pair
ºC	Degree Celsius (centigrade)
$CaCO_3$	Calcium carbonate
$CuSO_4$	Copper(II) sulfate
cfu	Colony forming unit
cm	Centimeter
DDBJ	DNA Data Bank of Japan
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
EtBr	Ethydium bromide
$FeSO_4$	Iron (II) sulfate
g	Gram
h	Hour
HCl	Hydrochloric acid
ISP	International *Streptomyces* Project
kb	Kilo-base pair
KCl	Potassium chloride
KH_2PO_4	Monopotassium Phosphate
K_2HPO_4	Dipotassium phosphate
KNO_3	Potassium Nitrate
L	Litre
LL-DAP	LL-diaminopimelic acid
M	Molar

meso-DAP	*meso*- diaminopimelic acid
mg	Milligram
ml	Milliliter
mm	Millimeter
min	Minute
µ	Micron
µl	Microlitre
µM	Micromolar
µm	Micrometer
µg	Microgram
ηg	Nanogram
$MgSO_4$	Magnesium sulfate
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese(II) chloride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2HPO_4	Disodium hydrogen phosphate
$(NH_4)_2SO_4$	Ammonium Sulfate
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
sp.	species
TBE	Tris-borate-EDTA
TLC	Thin Layer Chromatography

UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
$ZnSO_4$	Zinc Sulfate
%	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Chilli

Pepper, chilli, chile, aji, paprika, and *Capsicum*, are some of the names use to describe the plants and fruits of the genus *Capsicum* in *Solanaceae* family (Ochoa-Alejo & Ramirez-Malagon, 2001) which is closely related to eggplant, tomato and potato. Central and South America were the first places to discover this fruit (Berke & Shieh, 2000) and it is now widely grown in the Asian region. The five domesticated species of *Capsicum* that are widely cultivated are *C. annuum, C. pubescens, C. baccatum, C. Chinese* and *C. frutescens* (Idris *et al*., 2001). Amongst the mentioned species, one of the commonly cultivated and local species is *C. annuum* (Idris *et al.*, 2001). Among Malaysians, the plant and fruit of *C. annuum* is simply known as chilli. Varieties such as Kulai, Cili Puteh, MC11, Langkap, Tanjung Minyak and MC12 have been cultivated more among Malaysian local farmers (Idris *et al.*, 2001).

Multi-alkaloid compounds including carotenoids and capsaicinoids can be found in *Capsicum*. Morphological characteristics such as white flowers, blue to purple anthers, simple leaves with 5 lobed, a toothed calyx and typically single-fruited nodes distinguish *Capsicum annuum* (Hyam *et al.*, 1995).

Capsaicin is responsible for the producing of chilli's pungency and alkaloid compounds that are unique to the genus of *Capsicum.* In Medicine, capsaicin has been known as one of the beneficial medicines to relieve pain, and is highly suggested for arthritis (Bosland & Votava, 2000). Another usage of capsaicin has been as repellent sprays for dogs and would-be thieves. Preparation of industrial hot sauce is another widely global demand

that needs capsaicinoids (Ochoa-Alejo & Ramirez-Malagon, 2001). Moreover, attractiveness of chilli fruit colour, has led to plant chilli as an ornamental plant (Husain *et al*., 1999).

Different types of soil can help the chilli to grow and develop well. Providing well drained soil including appropriate soil enrichments will help the crop to achieve its highest yield. The best pH value to grow chilli plant is 5.5-6.8. Areas with day temperatures between 24 - 32°C and night temperatures between 15.5 - 20.5°C are best for chilli crops (Boswell, 1964; Somos, 1984). Setting fruit above 35°C has been limited to only few varieties (Boswell, 1964). Pollen fertility decreased dramatically when the day temperature reached to 38°C (Takagaki *et al*., 1993). Areas with annual rainfall of 600-1250 mm can be the best places for developing chillies. Moisture stress at flowering and during fruit development would adversely affect yield and fruit quality of chilli (Gracza, 1978; Robson & Johnson, 1985). Low moisture level of soil would cause flower abortion (Wien *et al*., 1989) and consequently reduces yield. Likewise, low humidity and high temperature caused abscission of fruit buds, flowers and small fruits because of excessive transpiration and water deficits in the plant (Cochran, 1936). As a result of water deficit, the wall of the collenchymas cell layer becomes thickened due to the starch content being used up and more oil drops were secreted, while epicarp was highly cutinized. Shading also indicated harmful effect on flower formation chillies (Quagliotti *et al*., 1974; Bare & Smeets, 1987). Research done by Quagliotti *et al*., (1974) revealed that even at 30% shading, a 30% decrease was observed in fruit weight and fruit number's development.

1.2 The Importance of Chilli

Chilli has an economic and nutritive importance (Table 1.1). Chilli is an indispensable culinary ingredient throughout the world and is available fresh, dried, powdered, or processed into sauce. According to Sanatombi & Sharma (2006), chilli is also beneficial in the pharmaceutical industries as it has nutritional values (Markus & Kapitany, 2000) and medicinal values (Stewart *et al.*, 2005). A rich source of Vitamins A, B, C and E has been reported in chilli (Anu & Peter, 2000; Garcia-Pineda *et al.*, 2004). It is also beneficial in aiding digestion and general well-being (Markus & Kapitany, 2000). In chilli, oleoresins contain the sensory qualities of fresh chilli: aroma, colour, flavour and pungency, whereas capsanthin and capsorbin are the coloring pigments in *Capsicum.*

1.3 Chilli Cultivation in Malaysia

Chilli pepper is an important and high demand crops in Malaysia, Indonesia and Thailand. There are many types of chilli grown such as the medium long, smooth and cayenne, sweet peppers and hot short chilli. Anthracnose of peppers occurs in each country that produces chilli. Chilli anthracnose is a major problem for chilli growers in Malaysia as well as other chilli production countries such as Thailand. In Malaysia and Thailand anthracnose of peppers has been responsible for 60% and 50% losses respectively. *Colletotrichum* species are known to be seed borne and is prevalent in Indonesia, Malaysia and Thailand.

As of 2009, Malaysia has been in seventh rank among 20 chilli producing countries (Figure 1.1, Table 1.2) with production of 23,210 (MT) according to FAO (Food and Agriculture Organisation).

Table 1.1: Nutritional composition of red chilli

Composition	Quantity		
	Fresh red Chilli	Dry Red Chilli	Processed Red Chilli
Energy	116 Calorie	288 Calorie	35 Calorie
Moisture	65.4%	15.3%	87%
Protein	6.3 g	11.7 g	0.4 g
Fat	1.4 g	12.4 g	0.3 g
Carbohydrate	24.8 g	43.7 g	8.9 g
Fibre	15.8 g	13.4 g	0.6 g
Ash	2.1 g	16.9 g	3.4 g
Calcium	86.0 g	223 mg	28 mg
Phosphorus	120 mg	853 mg	24 mg
Ferum	3.6 mg	45 mg	7.5 mg
Sodium	23 mg	60 mg	-
Potassium	1286 mg	1500 mg	260 mg
Thiamine	0.37 mg	1.14 mg	0.01 mg
Riboflavin	0.51 mg	1.53 mg	0.01 mg
Niacin	2.5 mg	19.8 mg	0.1 mg
Ascorbic Acid	296 mg	184 mg	18 mg

Source: Ministry of Agriculture, http://agrolink.moa.my

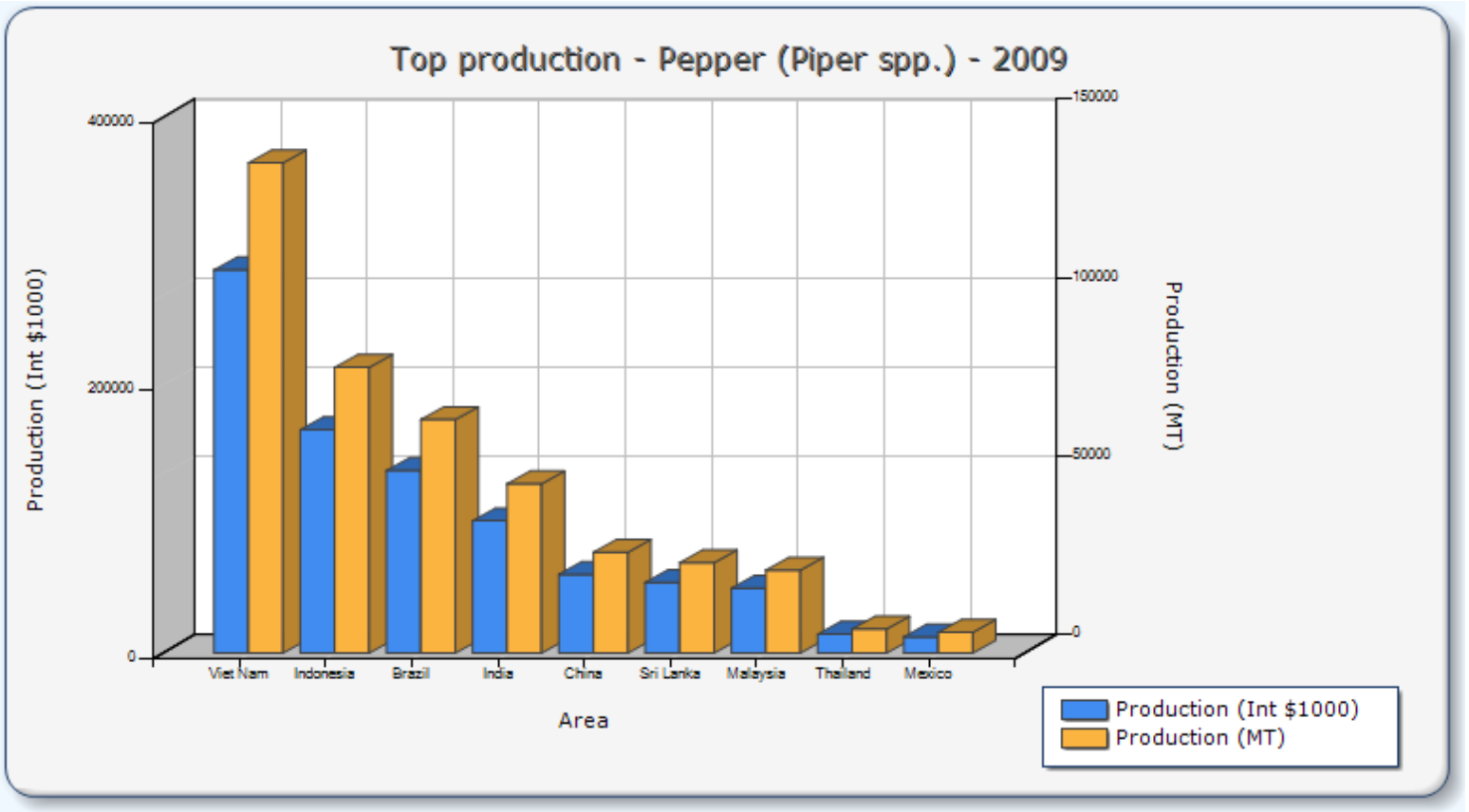

Figure 1.1: Top 20 chilli producer countries by FAO.

1.4 Anthracnose Disease of Chilli

Species of *Colletotrichum* have been shown to be one of the most important plant pathogens. *Colletotrichum* species have caused severe damages to the large range of plants such as fruit trees, legumes, cereals, vegetables and perennial crops in a form of anthracnose (Bailey & Jeger, 1992). Chilli (*Capsicum* spp.) has been one of the targets of anthracnose disease that is responsible for losses of chilli up to 50% (Pakdeevaraporn *et al.*, 2005). Infected seed, alternate hosts and crop debris, can be the places to keep and transfer the disease fungus. Periods of excess irrigation or rain can be responsible for the occurring of infection on immature pods; however, the symptoms will be appeared when the pod completes its final color change and becomes mature. Sunken necrotic tissues, with concentric rings of acervuli are the typical anthracnose symptoms that can be seen on chilli fruits. Marketability will be reduced notably when the fruits showing blemishes (Manandhar *et al*., 1995).

Different *Colletotrichum* species can be associated with anthracnose of the same host in the *Colletotrichum* patho-system (Simmonds, 1965; Freeman *et al.*, 1998; Cannon *et al.*, 2000). *Colletotrichum* species that are responsible for causing anthracnose contain of more than one species that include *C. acutatum* (Simmonds), *C. capsici* (Syd.) Butler and Bisby, *C. gloeosporioides* (Penz.) Penz. and Sacc. (Simmonds, 1965; Johnston & Jones, 1997; Kim *et al.*, 1999; Nirenberg *et al.*, 2002; Voorrips *et al.*, 2004; Sharma *et al.*, 2005; Pakdeevaraporn *et al.*, 2005; Than *et al.*, 2008). Method ofcontrol of the disease by chemical methods include the fungicides Manganese ethylenebisdithiocarbomate (Maneb) (Smith, 2000); azoxystrobin (Quadris), trifloxystrobin (Flint), and pyraclostrobin (Cabrio) that are commonly used for the control of anthracnose of chilli; however no effective chemical control measures for this disease (Alexander & Waldenmaier, 2002; Lewis & Miller, 2003). Other control

methods including use of biofungicides, and cultural practices do not provide an effective disease control. Recently, the selection of resistant cultivars has decreased losses from diseases; moreover chemical and mechanical expenses of disease control has decreased but could not be stopped completely (Agrios, 2005).

1.5 Biological Control of Plant Pathogen

In recent years, research and development efforts have increased on the use of biological alternatives for harmful chemical pesticides for the control of plant diseases (Raaijmakers *et al*., 2002).This may be due to increasing concerns about the environmental risks of chemical agents and their effects on sensitive non-target microorganisms (Lange & Lopez, 1996; Thirup *et al*., 2003). The other commercial reason for the current interest in biological agents can be explained as the development of resistance in fungal pathogens to fungicides constantly requires new chemicals. Consequently, it is becoming more difficult and more expensive to find new antifungal compounds (Campbell, 1986). A further stimulus is the prospect that development and registration will cost less for biological agents than for chemical fungicides (Pusey, 1996). For many plant diseases, it is doubtful that biocontrol agents by themselves will provide the efficacy associated with synthetic fungicides. However, the use is predicted to increase as a significant part of integrated pest management systems that can provide adequate disease control (Pusey, 1996).

1.6 Actinomycetes as Biocontrol Agents

Actinomycetes consist of a very broad phylogenetic group of Gram-positive bacteria (Thirup *el al.,* 2001). In the present study, attention has been centered on species of *Streptomyces* as potential fungal antagonists. This choice was made because actinomycetes, mainly *Streptomyces*, are ubiquitous and abundant in soil (Broadbent

et al., 1971). They also tend to be well distributed through the surface-soil mass, rather than persisting in pockets, as many fungi do (Singh & Mehrotra, 1980). Furthermore, actinomycetes are efficient producers of antifungal compounds (Doumbou *et al.*, 2001). They have the ability to germinate from spores and grow as filamentous masses along soil particles. When environmental conditions become less optimal, actinomycetes will sporulate and lie dormant until conditions become conducive for growth (Korn-Wendisch & Kutzner, 1992).

The actinomycetes have been, by far, the richest source of secondary metabolites (45%), followed by fungi, *Bacillus* and *Pseudomonas* (Berdy, 2005). They have an unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities (Lechevalier, 1992; Wang *et al.*, 1999; Ndonde & Semu, 2000). Actinomycetes, especially those belonging to the genus *Streptomyces*, have been studied as potential biocontrol agents against fungal plant pathogens (Sabaratnam & Traquair, 2002).

According to Lang & Lopez (1996), secondary metabolites produced by microorganisms are responsible for interactions within populations and between the organism and the environment. Rice *et al*. (1998) reported that microbes, which demonstrated potential biocontrol abilities, could act as a rich source of bioactive compounds. Therefore, it has been speculated that the actinomycetes may produce antifungal compounds in soil and play an active role in fungal antagonism. Antifungal produced on or near the root surfaces (rhizosphere) decrease the competition for scarce food reserves by killing or inhibiting fungal growth (Gottlieb, 1976; Korn-Wendisch & Kutzner, 1992).

In order to introduce the plant to an antagonistic organism for control or prevention of root disease, first and foremost, the antagonist should be able to live as a symbiont within the respective plant rhizosphere. It is also very important to choose an antagonist which is antagonistic to the disease causal agent and at the same time does not harm the plant. Actinomycetes of the genus *Streptomyces* are well known for their ability to suppress growth of a wide variety of fungal pathogens due to their ability to produce fungal cell wall-degrading enzymes such as cellulose, hemicellulase, chitinase, amylase and glucanse (Yuan & Crawford, 1995).

So far, studies have been done to characterize antagonistic microbial populations from the rhizosphere of crop plants and use them as biological control agents of fungal plant pathogens (Sabaratnam & Traquair, 2002). Others who have actively pursued in biocontrol research have also achieved some notable success (Paulitz & Belanger, 2001). Malaysia has been identified as one of the 12-mega biodiversity hotspots in the world (Amilita, 2002). This country has a wider diversity of vegetation with tropical rain forests, mangrove coastlines, and high altitude mountains. Since photosynthetic activity or productivity of plants is quite high in the tropics compared to the temperate regions, a wider variety of rhizospheric microorganisms can be expected (Bull *et al.*, 1992). Due to the lack of fundamental information on the available microbial diversity in Malaysia, the vast possibilities of using microbes as sources for new bioactive compounds and other applied research have not been adequately tapped. Therefore, if the right approaches are taken, there will be plenty of scope for successful use of Malaysian rich microbial resources for the development of biological disease control strategies.

Previous studies done in Malaysia on actinomycetes isolated from coastal mangrove mud, shown that mentioned isolated actinomycetes had antifungal activity against selected plant pathogenic fungi (Vikineswary *et al.*, 1997 and 1998). Ismet (2004) showed that a strain belonging to the genus *Micromonospora* isolated from coastal mangrove rhizosphere soil, showed strong antifungal activity against *P. oryzae* and *Ganoderma boninense* (basal stem rot pathogen of oil palm). Biological control of *Colletotrichum* sp. had been recommended by Lenne & Parbery in the early 1976. Antagonistic bacteria strains such as DGg13 and BB133 were found to be effective in controlling *C. capsici* in Thailand that has been the major anthracnose pathogen in that country (Intanoo & Chamswarng, 2007). These studies showed that actinomycetes derived from natural and unexploited ecological niches can be a rich and useful source of antifungal properties. While, none of the above workers have evaluated the biocontrol properties of indigenous strains of actinomycetes derived from chilli pepper farms in Malaysia.

In this study, attention has been focused on the species of actinomycete, which inhabit the rhizosphere soil of chilli plants. Therefore, this study was undertaken to explore the potential of indigenous actinomycetes which inhabit the rhizosphere or colonize inside the root of chilli plant as possible biological control agent of anthracnose disease of chilli caused by *Colletotrichum* spp.

1.7 Objectives of Research

a. To isolate rhizosphere actinomycetes from healthy and *Colletotrichum*-infected chilli plants and to screen against *Colletotrichum* spp.

b. To characterize and identify actinomycetes derived from healthy and diseased (*Colletotrichum*-infected) chilli plants.

c. To evaluate selected bioactive actinomycetes to control anthracnose disease on chilli.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Actinomycetes

Actinomycetes are Gram-positive bacteria with DNA rich in guanine plus cytosine (G+C) content of about 57%-75% (Ensign, 1992). Although prokaryotic by nature, the actinomycetes are considered as transition microorganisms between bacteria and fungi because they may have fungal morphology during some stage in their life cycle (Goodfellow & Cross, 1984). Actinomycetes have a distinctive and diverse macroscopic and microscopic morphology (Dietz, 1994).

Actinomycetes are mainly mesophiles and grow optimally at 25°-35°C (Williams *et al*., 1984). Most actinomycetes are neutrophiles with a growth range from about pH 5.0 to 9.0 and an optimum around pH 7.0 (Williams *et al*., 1984). However, acidophilic actinomycetes that grow at pH 3.5 to 6.5 are widespread in acidic soils (Goodfellow & Williams, 1983). Most actinomycetes are strictly aerobic (Goodfellow & Williams, 1983).

Chemotaxonomy or the analysis of chemical composition of actinomycete cell wall, such as the type of diaminopimelic acid (DAP) isomers, whole cell sugars, mycolic acids and isoprenoid quinones composition, are important for the classification and identification of different groups of actinomycetes (Stackebrandt *et al*., 1983). A combination of morphological, spore and chemical characters can be used to identify actinomycetes to the genus level, but not at the species level (Goodfellow & Williams, 1983).

Phylogenetic analysis has been used to classify actinomycetes into a number of discrete suprageneric groups (Wang *et al*., 1999). The 16S rRNA sequence-based phylogenetic analysis has been widely used to determine taxonomic positions of many organisms (Embley & Stackebrandt, 1994). Assisted by the polymerase chain reaction (PCR) technique and the ability to directly sequence PCR products, the 16S rRNA gene sequence of an unknown organism can be quickly isolated and immediately compared with thousands of sequences in public databases.

2.2 Distribution of Actinomycetes in Nature

Actinomycetes are primarily saprophytes that live in a wide range of natural and man-made environments (Goodfellow & Williams, 1983). They are isolated from environmental samples by applying appropriate selective pressures at various stages of the dilution plate procedure (Williams *et al*., 1984). The population and types of actinomycetes in an ecosystem are determined by numerous physical, chemical and biological factors (Wang *et al*., 1999). These include various environmental factors such as temperature, pH, moisture, season and climate (Saadoun & Gharaibeh, 2002). The main natural reservoir of actinomycetes is soil where they play an important role in the decomposition of organic matter (Labeda & Shearer, 1990). Localized organic substrates such as root fragments and dead fungal hyphae are rapidly colonized by actinomycetes mycelium. From there, their hyphae grow toward soil particles and produce spores when nutrients are exhausted (Williams *et al*., 1984). Spores can be dispersed above the soil when soil aggregates are disturbed by wind or rain. Dispersal within soil is assisted by water movement and by migration of arthropods that carry the hydrophobic spores at their exocuticles (Korn-Wendisch & Kutzner, 1992).

2.3 Economic Importance of Actinomycetes

Actinomycetes play a major role in the degradation of naturally occurring polymers in soil by producing various enzymes as shown in Table 2.1 (Kim, 1992). They have the capacity to degrade lignocellulose, a major, naturally occurring component of recalcitrant plant residues (Antai & Crawford, 1981).

Extracellular amylase complexes produced by many strains of actinomycetes are commercially used for hydrolyzing starch to concentrated maltose syrups (Korn-Wendisch & Kutzner, 1992). Chitinolytic actinomycetes are important degraders of chitin (Mahadevan & Crawford, 1997). Chitin is found in the cuticle of insects and the shell of crustaceans and molluscs, as well as, in the cell walls of most taxonomic groups of fungi (Doumbou *et al*., 2001).

With the ability to degrade various complex organic compounds, actinomycetes play a major role in composting where organic waste materials are decomposed under controlled conditions. Composts are beneficial when used as fertilizers in agriculture (Hoitink & Fahy, 1986). Obligate thermophiles such as *Thermomonospora curvata* produces thermostable cellulases and is active in decomposition of municipal wastes and animal manures (Williams *et al*., 1984). Previously, Ohta & Ikeda (1978) have reported that *Streptomyces griseus* and *Streptomyces antibioticus* were used in deodorization of pig faeces. Furthermore, Kurtboke (2000) reported that some strains of *Streptomyces*, *Actinomadura* and *Nocardia* isolated from composted pig manure produced ligninolytic enzymes and were active in decolourising polymeric dye Poly R478 when grown on agar plates. Moreover, keratinolytic actinomycetes isolated from poultry manure samples are used in a poultry waste composting system to break down

recalcitrant material, and to produce odourless and pathogen-free end-product (Pettett & Kurtboke, 2004).

Table 2.1: Polymer-degrading enzymes produced by actinomycetes

Producer	Enzyme	Polymer degraded	Reference
Streptomyces viridosporus	aromatic aldehyde oxidase, aromatic acid lignin esterase, peroxidase	lignin	Crawford, 1988
Actinomycetes	peroxidase	polyaromatic hydrocarbons, polychlorinated aromatic, hydrocarbons dioxins	Kurtboke, 2000
S. thermodiastaticus *S.antibioticus*	endoglucanase exoglucanase	cellulose	Korn-Wendisch & Kutzner, 1992
Actinomycetes	xylanase	xylan	Deobald & Crawford, 1987
S. fradiae	endopectate lyase	pectin	Korn-Wendisch & Kutzner, 1992
S. hygroscopicus *S. preacox*	amylases	starch	Korn-Wendisch & Kutzner, 1992
S. lydicus	chitinases	chitin	Mahadevan & Crawford, 1997
S. albidoflavus *S. pactum*	keratinolytic enzyme	keratin	Bressollier *et al*., 1999

2.4 Secondary Metabolites from Actinomycetes

Secondary metabolites from microbes are defined as naturally produced substances that are not required for growth in culture, although they probably have survival value in nature (Lee, 1992; Maplestone *et al*., 1992). These low molecular weight compounds are structurally diverse and have various physiological activities such as metal transporting agents, as sexual hormones, as differentiation effectors, and as agents of symbiosis between microbes and plants, nematodes, insects, and higher animals.

During the main nutritive phase of colonial development in *Streptomyces* spp., the substrate mycelium develops. Later it gives rise to an aerial mycelium that is eventually converted in part into chains of spores. The streptomycetes are particularly vulnerable to competition from other soil microorganisms when the substrate mycelium lyses to yield nutrients used to build the aerial mycelium and spores (Korn-Wendisch & Kutzner, 1992). Therefore, secondary metabolites that are mainly produced during the idiophase of cultures play a major role as competitive weapons against other organisms (Walker & Colwell, 1975).

The practical importance of microbial secondary metabolites is tremendous (Demain & Fang, 2000). Table 2.2 summarized the main types of bioactivities and the numbers of discovered and published bioactivities of microbial secondary metabolites. A large number of these metabolites showed antibiotic activities. Table 2.3 summarized the numbers of antibiotics produced by microbial sources according to the main producer types. Actinomycetes represent the largest group (45%) of bioactive microbial metabolites producer. Almost 76% of its metabolites were derived from the genus *Streptomyces*, while the rare actinomycetes represent 24% of the total (Table 2.3).

The genus *Streptomyces* has been the most potential microbial source for all types of bioactive metabolites (Tanaka & Omura, 1993). They produce compounds that exhibit great diversity in chemical structure and bioactivity (Sanglier *et al*., 1993). For example, lactacystin isolated from *Streptomyces* sp. OM-6519 has a new type of neurotrophic factor-like activity that helps in the investigation of nerve cells differentiation. These studies may become a useful tool in finding a cure for nerve diseases associated with dysfunction of the nervous system (Omura, 1992).

Table 2.2: Bioactivity types and the approximate number of discovered bioactivities of microbial secondary metabolites (Berdy, 2005)

Type of Activity		Numbers of Discovered Bioactivities
ANTIBIOTIC ACTIVITIES:		
Antimicrobial activity:		
Antibacterial:	Gram-Positive	~11000-12000
	Gram-Negative	~5000-5500
	Mycobacteria	~800-1000
Antifungal:	Yeasts	~3000-3500
	Phytopathogenic Fungi	~1600-1800
	Other Fungi	~3800-4000
Antiprotozoal:		~1000
Chemotherapeutic Activity:		
Antitumor (Cytotoxic)		~50000-5500
Antiviral		~1500-1600
OTHER BIOACTIVITIES:		
Pharmacological Activity:		
Enzyme Inhibitor		~3000-3200
Immunological Activity		~800
Biochemical Activity		~1000
Other Activities		~2000-2500
Agricultural Activity:		
Pesticide		~900-1000
Herbicide		~1800-1900
Insecticide/Miticide/Larvicide/Detterent		~1100-1200
Feed Additive, Preservative		~300-400
Other activities:		
Microbial Regulators		~500
Biophysical Effects		~300

Table 2.3: Number of bioactive microbial secondary metabolites according to their producer (Berdy, 2005)

Source	Antibiotics	Total Bioactive Secondary Metabolites
Bacteria	**2900**	**3800**
Eubacteriales	2180	2750
Bacillus sp.	795	*860*
Pseudomonas sp.	610	795
Mycobacter	410	410
Cyanobacter	310	640
Actinomycetales	**8700**	**10100**
Streptomyces sp.	6500	7630
Rare Actinomycetes	2200	2470
Fungi	**4900**	**8600**
Microscopic Fungi	3750	6400
Penicilium / Aspergillus	1000	1950
Basidiomycetes	1030	2000
Yeasts	90	140
Slime Mould	30	60
Total Microbial	**16500**	**22500**

Isolation of a group of antiviral antibiotics known as fattiviracins from *Streptomyces microflavus* gives us hope for the treatment of viral-related diseases. Fattiviracins exhibited potent activity against enveloped DNA viruses such as the herpes virus, and enveloped RNA viruses such as influenza A and B viruses, and strains of human immunodeficiency virus-1 (Uyeda, 2003). Recent discovery of a novel cyclic peptide with anticancer properties, YM-216391, from *Streptomyces nobilis* further demonstrated the potentials of streptomycetes in human disease therapy (Sohda *et al*., 2005).

In the rare actinomycetes group, strains from the genera *Micromonospora, Actinomadura, Streptoverticillium, Actirtoplanes, Nocardia, Saccharopolyspora* and *Streptosporangium* are the most frequent producers. Isolation of these non-streptomycetes from the environment is difficult and their preservation and cultivation methods are complicated (Berdy, 2005). However, application of more efficient isolation procedures can demonstrate that these rare actinomycetes are quite widespread and numerous in soil (Athalye *et al*., 1981). Antibiotics produced by this group include gentamicin from *Micromonospora* spp. and vancomycin-type glycopeptides from *Actinomadura* spp. The antitumor enediyne antibiotics were produced exclusively by rare actinomycetes. Members of new groups of macrolactam and naphtacene-quinone antibiotics were isolated from *Actinomadura*. While *Micromonospora* and *Saccharopolyspora* strains were relatively rich sources of macrolides (Moncheva *et al*., 2002). Numerous chemotherapeutic and agricultural agents such as ziracin, dalbavacin and spynosin were also isolated from the rare actinomycetes (Berdy, 2005).

2.5 Antifungal Compounds against Plant Pathogenic Fungi

Chemical fungicides that control fungal diseases of crop plants are limited due to the narrow host-pathogen specificity and emergence of resistance pathogenic strains (Tanaka & Omura, 1993). Problems with high chemical fungicide use include fungicide-enhanced disease resurgence, which refers to the return of diseases with increased severity after treatment. Further, many fungicides such as benomyl, chlorothalonil, anailazine, mareb, chloroneb, and iprodione are detrimental to the advantageous association between mycorrhizal fungi and plants (Boulter *et al*., 2000).

On the other hand, microbial metabolites are varied in structure and activity, and they are biodegradable. Thus, they are expected to overcome the resistance and pollution likely caused by synthetic fungicides (Misato *et al*., 1977). Actinomycetes, especially the genus *Streptomyces*, have been and remain the most potential source of microorganisms for agroactive metabolites (Doumbou *et al*., 2001). Antifungal compounds from actinomycetes are active against a wide range of plant pathogenic fungi and they act against a number of different targets in fungal cell (Table 2.4).

2.6 Targets for Antifungal Agents

A brief review of antifungal compounds produced by microbes and their various modes of action are outlined in Figure 2.1.

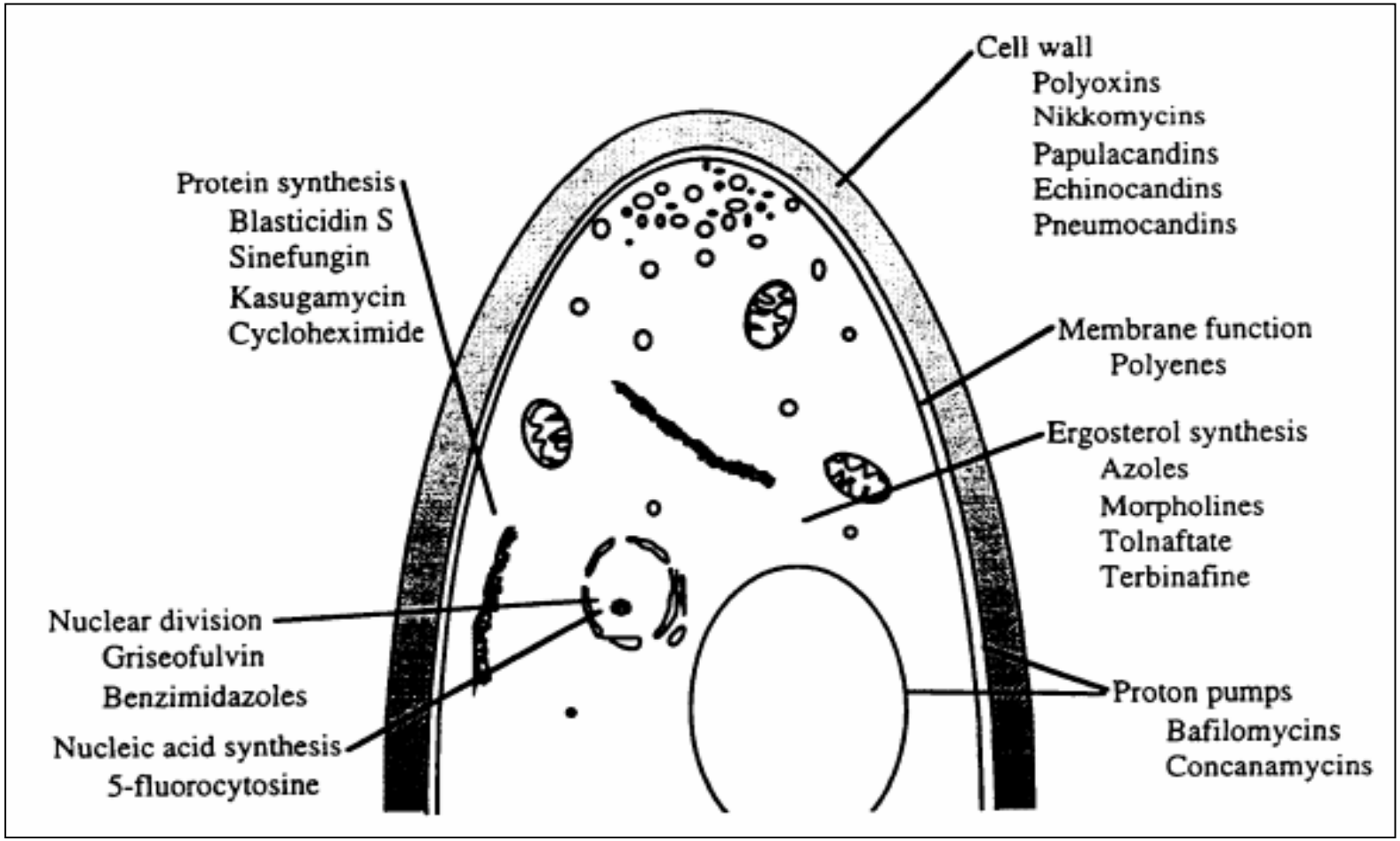

Fig. 2.1: Site of action of some antifungal agents (Debono & Gorde, 1994; Misato *et al*., 1977).

2.6.1 Antifungal Agents Targeting Fungal Cell Wall

Fungal cell wall is composed of a complex of proteins and polycarbohydrates such as chitin (Figure 2.2). Its complex structure serves many functions including osmotic protection, transport of macromolecules, growth, conjugation, and spore formation. Major disruption in cell wall organization or metabolism could deleteriously affect fungal growth (Debono & Gordee, 1994).

2.6.1.1 Chitin Synthase Inhibitors

Chitin, the structural component of fungal cell wall, is synthesized by a group of enzymes known as chitin synthases (Gupte *et al*., 2002). Chitin synthases catalyse the formation of glycosidic bonds using uridine diphospho-*N*-acetylglucosamine (Figure 2.3) as substrate (Debono & Gordee, 1994). Chitin synthases are specifically inhibited by the nikkomycins and polyoxins (Figure 2.3) produced by the actinomyetes (Tanaka & Omura, 1993). Nikkomycins and polyoxins are nucleoside peptide antibiotics that act as competitive inhibitors of chitin synthases by mimicking UDP-*N*-acetylglucosamine (Gupte *et al*., 2002). Fungi susceptible to these inhibitors show characteristic morphological features, notably bulging hyphae and swelling tips (Debono & Gordee, 1994).

Table 2.4: Antifungal compounds produced by actinomycetes and their mode of action against plant pathogenic fungi

Antifungal compound	Producing strain	Target pathogen (Plant disease)	Mode of action	Reference
Blasticidin S	*Streptomyces griseochromogenes*	*Pyricularia oryzae* (rice blast)	Inhibition of protein synthesis	Okuda & Tanaka, 1992
kasigamycin	*S. kasugaensis*	*P. oryzae* (rice blast)	Inhibition of protein synthesis	Pachlatko, 1998
Polyoxin D	*S. cacaoi* var. *asoensis*	*Rhizoctonia solani* (rice sheath blight)	Inhibition of cell wall chitin synthesis	Isono, 1998
Validamycin A	*S. hygroscopicus var.limoneus*	*R. solani* (rice sheath blight)	Inhibition of trehalase	Kameda *et al*., 1987
Hygrolidin	*S. hygroscopicus D-1166*	*Valsa ceratosperma* (apple canker)	-	Worthington 1998
Bafilomycins	*S. griseus* subsp. *sulphurus*	*Botrytis cinerea*	-	Worthington 1998
Venturicidins	*S. aureofaciens*	*Venturia inaequalis*	-	Worthington 1998
Irumamycin	*S. subflavus sunsp. Irumaensis* AM-3603	*P. oryzae* *B. cinerea* *Sclerotinia cinerea*	-	Worthington 1998
Capsimycin	*Streptomyces* strain *C 49-87*	*Phytophthora capsici* (cucumber leaf blight) *Pythium debaryanum* (cucumber damping off)	-	Worthington 1998
Mildiomycin	*Streptoverticillium rimofaciens*	*Ascomycetes* (powdery mildews)	Inhibition of protein synthesis	Pachlatko, 1998
Rustmicin (galbonolide A)	*Micromonospora chalcea*	*Puccinia graminis* (Wheat stem rust)	Inhibition of cell wall (germ tube elongation)	Tanaka & Omura, 1993
Dapiramicin A	*Micromonospora* SF-1917	*R. solani* (rice sheath blight)	-	Worthington 1998

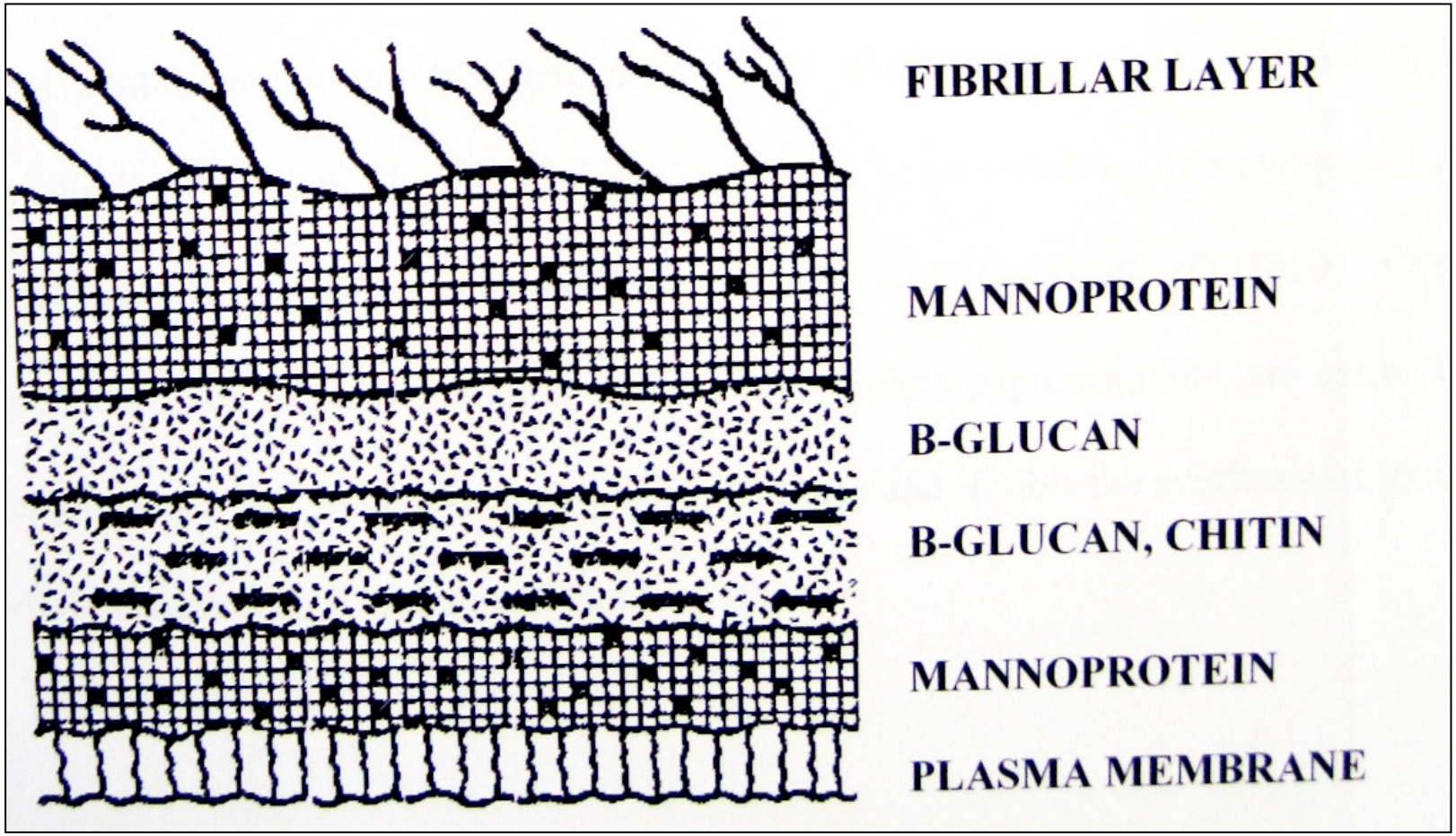

Fig 2.2: Schematic representation of the cell wall of Candida albicans (Debobo & Gorde, 1994).

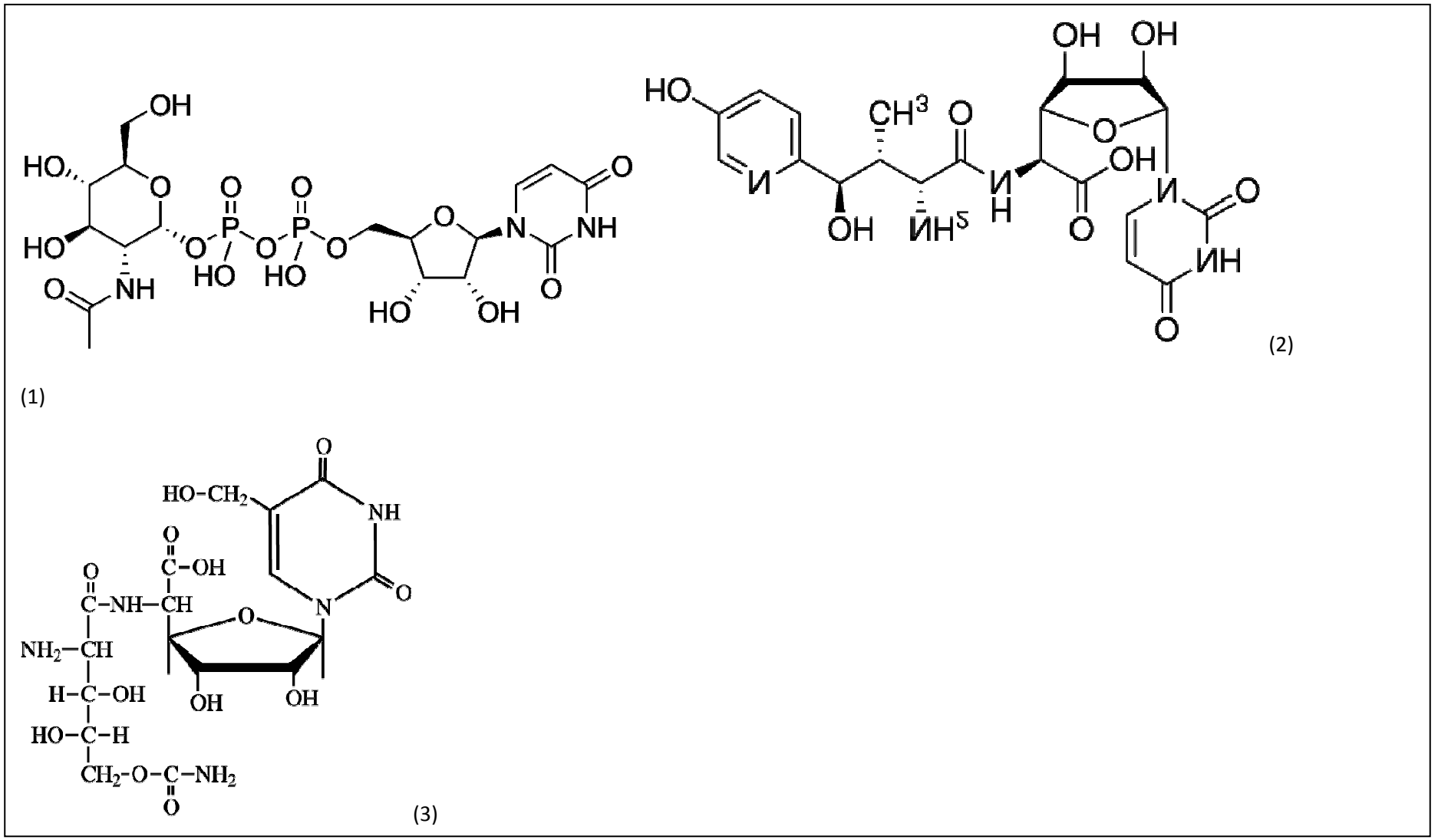

Figure 2.3: Structures of UDP-*N*-Acetylglucosamine (1), Nikkomycin Z (2) and Polyoxins (3).

2.7 Biological Control of Plant Pathogenic Fungi

Biological control is defined as the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than human (Cook & Baker, 1983). Some soils are naturally suppressive to soilborne plant pathogenic fungi such as *Fusarium oxysporum* (Alabouvette *et al*., 1998) and *Gaeumannomyces graminis* var. *tritici* (Raaijmakers et al., 1997). The observation that the suppressive factor could be eliminated through soil pasteurization affirmed the role of soil microorganisms in disease suppression (Mazzola, 2002).

Plant diseases caused by soil-borne fungal pathogens such as wilt, root rot, collar rot, seedling blight and damping-off are major problems that have no direct control measures so far. Soil-borne pathogens are mostly associated with the roots of plants. Therefore, the use of chemicals is restricted because the roots are embedded in soil and hence protected from most chemicals (Turhan, 1981a). Public concern about using chemical fungicides has fostered an interest in using microbes as biological control agents to protect agricultural crops against soil-borne fungal pathogens (Thirup *et al*., 2001). Some of the commercially available bacteria for biocontrol of fungal diseases in plants are shown in Table 2.5.

Generally, biocontrol of fungal pathogens is a direct result of the action of antagonists through one or more of the following mechanisms. These mechanisms reduce the infection level and bring about the desired results (Benyagoub *et al*., 1998).

2.7.1 Antibiosis

Antibiosis is defined as inhibition or destruction of the pathogen by the metabolic product of the antagonist. These products include volatile compounds, toxic substances

and antibiotics that result in the destruction, disintegration and decomposition of the pathogen (Doumbou *et al*., 2001).

According to Demain & Fang (2000), microorganisms produce various types of antibiotics in nature. In many cases, the efficacy of potential strains of microbes to control fungal disease in plants was correlated to the formation of antifungal metabolites. Mutant strains that did not produce antibiotics were ineffective in controlling the plant disease (Fischer *et al*., 1992). For example, mutants of *Gliocladium virens* showed a strong correlation between the ability to produce the antibiotic gliovirin and the ability to protect cotton seedlings from disease caused by the fungal pathogen, *Pythium ultimum* (Howell & Stipanovic, 1983).

Table 2.5: Commercially available bacterial strains for biological control of fungal diseases in plants (Glick *et al*., 1999)

Bacterium	**Commercial product**	**Pathogen**	**Crop(s)**	**Application method**
Bacillus subtilis	Epic	*Rhizoctonia solani, Fusarium* spp., *Alternaria* spp., *Aspergillus* spp. that attack roots	Cotton, legumes	Dry powder added to a slurry and mixed with commercial fungicide for seed treatment
Bacillus subtilis	Kodiak	*Rhizoctonia solani, Fusarium* spp., *Alternaria* spp., *Aspergillus* spp. that attack roots	Cotton, legumes	Dry powder, mixed with chemical fungicides
Burkholderia cepacica	Blue Circle	*Fusarium* spp., *Pythium* spp.	Vegetables	Seed treatment mixed with peat or drip irrigation
Burkholderia cepacica	Deny	*Rhizoctonia* spp., *Fusarium* spp., *Pythium* spp.	Alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetables, wheat	Seed treatment mixed with peat and a sticking agent or drip irrigation
Pseudomonas syringae	Bio-save10	*Botrytis cinerera, Penicillium* spp., *Mucor pyroformis, Geotrichum candidum*	Citrus and pome fruit	Wettable powder applied postharvest to fruit as drench, dip or spray
Streptomyces griseoviridis	Mycostop	*Fusarium* spp., *Alternaria brassicola, Phomopsis* spp., *Botrytis* spp., *Pythium* spp., *Phytophtora* spp.	Field, ornamental and vegetable crops	Drench, spray or through irrigation system

2.7.2 Direct Competition

Competition occurs when the antagonist directly competes with the fungal pathogens for resources such as nutrients available in root exudates, oxygen and space (Schroth & Hancock, 1982). Under iron-limiting conditions, bacteria produce a range of iron chelating compounds or siderophores that bind strongly to iron, making it unavailable to other soil microorganisms that cannot grow for lack of it (Whipps, 2001). Through continual removal of nutrients, fungal pathogens are prevented from germinating and remain inactive. Dormant propagules such as sclerotia, chlamydospores and oospores may be stimulated to germinate but are unable to compete with the active antagonists (Paulitz *et al.*, 1990). Thus, the pathogens are subjected to nutrient deprivation, leading to autolysis of hyphae (Boulter *et al*., 2000).

2.7.3 Mycoparasitism

Mycoparasitism involves hyphal interference between the biocontrol agent and fungal pathogen (Cook & Baker, 1983). The biocontrol agent penetrates fungal cell walls by a combination of physical pressure and the excretion of extra cellular enzymes, phenols, chitinases, cellulases and other lytic enzymes (Widden & Scattolin, 1988).

Production of digestive enzymes was seen in the degradation and penetration of the pathogen *Botrytis cinerea* by a strain of *Trichoderma harzianum* (Belanger *et al*., 1995).

2.7.4 Induced systemic resistance (ISR)

Induced systemic resistance (ISR) is the structural or chemical changes induced by bacterial control agents that delay infection in the host plant by pathogens (Whipps,

2001). Some of the changes observed in plant roots that exhibited ISR are strengthening of epidermal and cortical cell walls, and deposition of newly formed barriers beyond infection sites such as callose, lignin and phenolics (Boulter *et al*., 2000). Other changes include increased levels of pathogenesis-related proteins such as chitinase, peroxidase and polyphenol oxidase in the host plant (Sequeira, 1990).

2.8 Plant Pathogenic Fungi of Economic Importance

2.8.1 Anthracnose Disease Caused by *Colletotrichum* spp.

Anthracnose, essentially from a Greek word meaning 'coal', is the common name for plant diseases characterized by very dark, sunken lesions, containing spores (Isaac,1992). In general, anthracnose disease is caused by *Colletotrichum* species which belongs to the Kingdom Fungi; Phylum Ascomycota, Class Sordariomycetes; Order Phyllachorales; and Family *Phyllachoraceae*. The anamorphs are *Glomerella* species. In 1890 and for first time, anthracnose of chilli was reported from New Jersey, USA, by Halsted (1890), who could describe the causal agents as *Gloeopsorium piperatum* and *Colletotrichum nigrum*. Then Von Arx (1957) considered these taxa were as synonyms of *C. gloeosporioides*.

Anthracnose caused by *C. capsici* is considered to be a dry fruit rot (Pearson *et al*., 1984). *Colletotrichum capsici* and *C. gloeosporioides* are the two main causal agents of pepper anthracnose in the tropical countries of Asia. *Colletotrichum capsici* and *C. gloeosporioides* are the most important *Colletotrichum* spp. in reducing marketable fruit yields of pepper (Manandhar *et al*., 1995) (Table 2.6). Anthracnose has been found not only on mature fruit but also on seedlings, leaves and immature green fruits (Lee & Chung, 1995). According to Park and Kim (1992), five anthracnose fungi, *C.*

gloeosporioides (Penz.) Penz. and Saac., *C. dematium* (Persoon:Fries) Grove, *C. coccodes* (Wallr.) S. Hughes, *C. acutatum* Simmonds, and *Glomerella cingulata* (Stoneman) Spaulding & v. Schrenk, were pathogenic to different tissues of pepper plants. Of these anthracnose fungi, *C. gloeosporioides* attacks the fruit at all stages of development, but not the leaves and stems of plants. *Colletotrichum coccodes* that is responsible for leaf anthracnose of pepper was first found in pepper-growing fields in Chungnam province of Korea in 1988 (Hong & Hwang, 1998). Even though pepper fruits of all ages were susceptible to infection by *C. gloeosporioides*, purple and ripe red fruits developed more anthracnose than the immature stages (Oh *et al*., 1999).

Among pepper cultivars from Korea, the United States, India, and Thailand or accessions tested, irrespective of genetic or country origin, there were not significant differences in susceptibility to anthracnose (Hong & Hwang, 1998). Isolates of *C. gloeosporioides* from almond, apple, avocado, and mango, and also *C. acutatum* from anemone, apple, and peach, colonized in fruits including apple, avocado, almond, mango, and nectarines (Freeman *et al*., 1998).

Table 2.6: Reported causal agents of chilli anthracnose

Countries and regions	Causal agent	Reference
Australia	*Colletotrichum acutatum*, *C. atramentarium*, *C. dematium, C. gloeosporioides* var. *minor*, *C. gloeosporioides* var. *gloeosporioides*	Simmonds, 1965
India	*C. capsici*	Maiti & Sen. 1979
Indonesia	*C. acutatum, C. capsici, C. gloeosporioides*	Paul & Behl, 1993
Korea	*C. acutatum, C. gloeosporioides, C. coccodes, C. dematium*	Voorrips *et al*., 2004
Myanmar (Burma)	*Gloeosporium piperatum* E. and E., *C. nigrum* E. and Hals	Dastur, 1920
Papua New Guinea	*C. capsici, C. gloeosporioides*	Pearson *et al*., 1984
New Zealand	*C. cocodes*	Johnston & Jones, 1997
Taiwan	*C. acutatum, C. capsici, C. gloeosporioides*	Manandhar *et al*., 1995
Thailand	*C. acutatum, C. capsici, C. gloeosporioides*	Than *et al*., 2008
UK	*C. acutatum, Glomerella cingulata*	Adikaram *et al*., 1983
USA	*C. acutatum*	Roberts , 2001
Vietnam	*C. acutatum, C. capsici, C. gloeosporioides, C. nigrum*	Don *et al*., 2007

2.8.1.1. Anthracnose and *Colletotrichum* Disease Epidemiology

Among *Colletotrichum* spp., five species namely *C. acutatum, C. coccodes, C. dematium, C. gloeosporioides*, and *G. cingulata* have been identified as fungal species responsible for anthracnose of pepper in Korea, and *C. capsici, C. gloeosporioides*, and *G. cingulata* in Taiwan. According to Oh et al., (1999) among these species, *C. gloeosporioides* was the dominant species causing anthracnose on pepper fruits. *Colletotrichum coccodes* is responsible for contamination of pepper seeds, seedling leaves and stems, mature leaves, and sometimes green but not red fruits.

Conidia do not function as survival structures as their viability declines rapidly. Mycelium, however, may remain viable for long periods in/on colonized seeds, plant debris, or as latent infections in plants not showing any disease symptoms. Microsclerotia, formed thinly by species such as *C. gloeosporioides* and *C. coccodes*, play an important role in survival (Baxter *et al*., 1985). *Colletotrichum gloeosporioides* was recovered from leaf spots on sicklepod (*Senna obtusifolia*), and *Colletotrichum* spp. has been reported on sicklepod.

Different researchers have observed that some growers left infected fruit on the plant at the time of harvesting thus providing an inoculum source for further infection (Pearson *et al*., 1984). *Colletotrichum* spp. can be seed borne in crop plants. *Colletotrichum capsici* and *C. gloeosporioides* occur either externally or internally in pepper seeds. Manandhar *et al*., (1995) have reported that mycelia and stromata can survive in colonized pepper seeds. It was shown that the pathogen readily colonizes the seed coat and peripheral layers of endosperm even in moderately colonized seeds. Seeds that had been colonized heavily, had abundant inter- and intra-cellular mycelium and acervuli in seed coat endosperm and embryo, showing separation of parenchymatous layers of the

seed coat and reduction of food material in endosperm and embryo (Chitkara *et al*., 1990). Another study done by Lee & Chung in 1995, clarified that seed-borne *C. gloeosporioides* was transmitted from endosperm tissue to hypocotyls and radicals in red pepper.

Conidia germinate on fruit and produce germ tubes with adhesive appressoria (Manandhar *et al*., 1995). Germination and development of appressoria occurred at 95 to 100% RH and at 20 to 30 degrees C; however, abundant surface moisture was only visible on leaf and fruit surfaces at 100% RH (Dodd *et al*., 1991). The conidia of *C. gloeosporioides* germinated on both green and red fruits within 2 hr after inoculation. Contamination of green fruits by the fungus may lead to anthracnose development on immature fruit (Oh *et al*., 1998). On green fruits of pepper, only one isolate caused dark, brown to black lesions 6 days after inoculation. Later, these lesions slowly increased in size and became sunken. Yu *et al*. (1987) declared that on red fruit of pepper, all isolates produced more severe symptoms. The colonizing hypha grows both intra- and intercellular as a lesion improves. It is during the initial phase of colonization that the resistance responses of the plant may be expressed (Jeffries *et al*., 1990).

Many post-harvest diseases of fruit exhibit the phenomenon of dormancy in which symptoms do not develop until the crop ripens. *Colletotrichum* and *Glomerella* species are by far the most important pathogens that cause this type of infection. Although these genera have been the subject of numerous investigations, there remain many gaps in the knowledge of the disease process and understanding of the complex relationships between the various fungi involved (Jeffries *et al*., 1990). Appressoria are known to form adhesive disks for adhering to plant surfaces and remain latent until physiological changes occur in fruits. Appressoria that formed on immature fruits may remain

dormant until changes in life cycle occur in the fruits. The symptoms observed on different age fruits may be due to differences in the formation of infection hyphae (penetration pegs) and not to differences in conidial germination and appressoria formation (Manandhar *et al*., 1995).

Conidia often do not germinate in its natural place or position because of the presence of germination-inhibitors in the spore matrix, but will germinate after being washed or rain-splash disseminated (Manandhar *et al*., 1995). Estrada *et al*. (1993) believed that under normal conditions, conidia dispersed by rainfall may remain on a plant surface and retain potential to cause disease for periods of over 7 days. According to Waller, 1972 and Hong & Hwang, 1998, spread of pathogen is mainly through splash dispersal of spores during the raining season, and able to attack all stages of the chilli pepper plant. Typical fruit symptoms are circular or angular sunken lesions, with concentric rings of acervuli that are often wet and produce pink to orange conidial masses (Figure 2.4, 2.5). During wet periods, appressoria have been reported to produce secondary conidia, which may be involved in secondary spread to pepper fruits (Manandhar *et al*., 1995).

Figure 2.4: Anthracnose symptoms on chilli fruits and plants.
(http://agropedia.iitk.ac.in/?q=content/chilli-anthracnose)

Figure 2.5: Anthracnose symptoms on chilli fruits and seeds. (http://agropedia.iitk.ac.in/?q=content/chilli-anthracnose)

2.8.1.2 Variability in *Colletotrichum*

The reproduction mode in many *Colletotrichum* populations is mainly or exclusively vegetative. In the absence of a sexual stage, the only means of exchanging genetic material between two strains would be anastomosis and heterokaryosis. Microscope examination reveals that anastomosis occurs between side branches, which grow out of neighbouring hyphae and form cross-connection bridges connecting two hyphae. The resultant fused cells are binucleate and appear not to proliferate, but support neighbouring uninucleate cells with genes of either nuclei (Katan, 2000). Heterokaryosis and parasexuality are the crucial factors determining the phenotypic heterogeneity within the group and a thorough analysis at the molecular genetic level is needed. Around 98% of conidia from a single agar culture of *Colletotrichum* have one nucleus, but a small number is always multinucleate. Nuclear heterogeneity can be increased under different environmental conditions and growth in liquid culture can increase the quantity of binucleate conidia to 17% and tri-nucleate conidia to 35% in some species (Jeffries *et al*., 1990).

2.8.1.3. Disease Management of Chilli Anthracnose

Bailey (1987) and Agrios (2005) recommended integrated management techniques, as no single specific management program could eliminate chilli anthracnose. Effective control of *Colletotrichum* diseases usually involves the use of a combination of cultural control, biological control, chemical control and intrinsic resistance (Wharton & Dieguez-Uribeondo, 2004).

2.8.1.3.1 Cultural Practices

In cultural practices pathogen-free chilli seed should be planted and weeds eradicated. Crop rotation should be used every 2 to 3 years with crops that are not alternative hosts of *Colletotrichum*. Keeping transplants clean by controlling weeds and solanaceous volunteers around the transplant houses. The field should have the ability of good drainage and be free from infected plant debris. If disease was previously present, crops should be rotated away from solanaceous plants for at least 2 years (Roberts, 2001). Sanitation practices must be used in the field including control of weeds and volunteer chilli plants. Choosing cultivars that bear fruit with a shorter ripening period may allow the fruit to escape infection by the fungus. Reducing wounds in fruit from insects or other means seems necessary because wounds provide entry points for *Colletotrichum* spp. and other pathogens such as bacteria that cause soft rot. At the end of the season, infected plant debris from the field must be removed completely or deep ploughed to entirely cover crop diseases (Agrios, 2005).

2.8.1.3.2 Use of Biofungicides

The control of chilli anthracnose fruit rot has, for many years, relied on chemicals and resulted in many undesirable problems. There is a need to incorporate alternative control components that are effective in the field. Biological control of fruit rot and dieback of chilli with plant products tested in many laboratories and field trials showed that the crude extract from rhizome, leaves and creeping branches of sweetflag (*Acorus calamus* L.), palmorosa (*Cymbopogon martinii*) oil, *Ocimum sanctum* leaf extract, and neem (*Azadirachia indica*) oil could restrict growth of the anthracnose fungus (Jeyalakshmi & Seetharaman, 1998; Korpraditskul *et al*., 1999). Among the biofungicides used against the fungus *Colletotrichum* spp. on chilli fruit, Charigkapakorn (2000) found that the most effective control was sweet flag crude

extract when applied in two intervals when the majority of the plants were at the first bloom stage and at the mature bloom stage.

2.8.1.3.3 Biological Control

So far, biological control methods for chilli anthracnose disease have not been much focused. The potential for biological control of *Colletotrichum* species had been suggested in 1976 by Lenne & Parbery (1976). Jeger & Jeffries (1988) also stressed the possibilities of biological control of post-harvest fruit diseases by using *Pseudomonas fluorescens*. Some antagonistic bacterial strains such as DGg13 and BB133 have been found to be effectively inhibiting *C. capsici*, the major anthracnose pathogen in Thailand (Intanoo & Chamswarng, 2007). Some researchers also believed that *Trichoderma* species have ability to compete effectively for surface area, thereby reducing pathogen infection success (Jeffries & Koomen, 1992; Maymon *et al*., 2004). *Trichoderma* species have been applied to control *Colletotrichum* species in chilli (Boonratkwang *et al*., 2007), strawberries (Freeman *et al*., 2001), and citrus in Belize (Moretto *et al*., 2001) with concomitant disease reduction. Other biological control agents that have been tested effectively against *C. acutatum* include *Bacillus subtilis* and *Candida oleophila* (Wharton & Dieguez-Uribeondo, 2004).

2.8.2 Pathogens of Rice

Sheath blight of rice caused by an aerial form of *Rhizoctonia solani*, is a major concern of rice production in many countries. The pathogen is not host-specific but is capable of causing serious damages if the environment becomes conducive (Gnanamanickam *et al*., 1992). Mew & Rosales (1986) have reported the use of selected strains of fluorescent and non-flourescent bacterial antagonists isolated from the rice rhizosphere in seed bacterization studies for reducing sheath blight severity in rice.

Rice blast is another important rice disease caused by *Pyricularia oryzae*. Krishnamurthy & Gnanamanickam (1998) evaluated the potential of *P. flourescens* strain Pf7-14 for biological control of rice blast in field experiments. When applied as seed treatment followed by foliar applications, the strain provided 68.5% suppression of rice blast in seedbed experiment and 59% suppression in the field experiment.

2.8.3 Pathogen of Durian

Phytophthora palmivora, the causal pathogen of fruit rot in durian, is a major constrain to the future expansion of the industry in all durian-growing regions. The fungus also causes leaf blight in wet conditions. The significance of the threat to production resulting from *Phytophthora* is reflected by the priority given by the Thai and Vietnamese governments into research on the crop and its disease (Sangchote *et al.*, 2004).

2.9 Actinomycetes as Biocontrol Agents of Plant Pathogenic Fungi

Over the past one hundred years, research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). Screening is a critical step in the development of biocontrol agents. It is important to screen for the one exhibiting the best properties to suppress the pathogen and to reduce disease incidence (Doumbou *et al*., 2001). The first step is finding the potential antagonist *in vitro*, and the second is determining their success *in vivo* (Turhan, 1981b). Agar tests of challenging cultures of the pathogen with those of the candidate microorganisms are usually used to identify antagonistic properties of microorganisms (Merriman *et al.*, 1993).

Actinomycetes have demonstrated *in vitro* antagonistic effects towards a wide range of plant pathogenic fungi by producing zones of inhibition in Petri plates (Figure 2.6). Among the fungal pathogens tested were *R. solani, Macrophomina phaseolina* and *Colletotrichum* spp. (Reddi & Rao, 1971), *F. oxysporum* fsp. *lycopersici*, *F. oxysporum* fsp. *melonis*, *F. oxysporum* fsp. *niveum*, *F. oxysporum* fsp. *cucumerinum* and *Verticillium dahliae* (Turhan, 1981a), *Alternaria brassicicola*, *Fusarium culmorum* and *Pythium debaryanum* (Tahvonen, 1982); *Phytophthora fragariae* var. *rubi* (Valois *et al*., 1996), and *Pythium ultimum*, *Sclerotinia homeocarpa*, *Gaeumannomyces graminis* and *Microdochium nivale* (Chamberlain & Crawford, 1999).

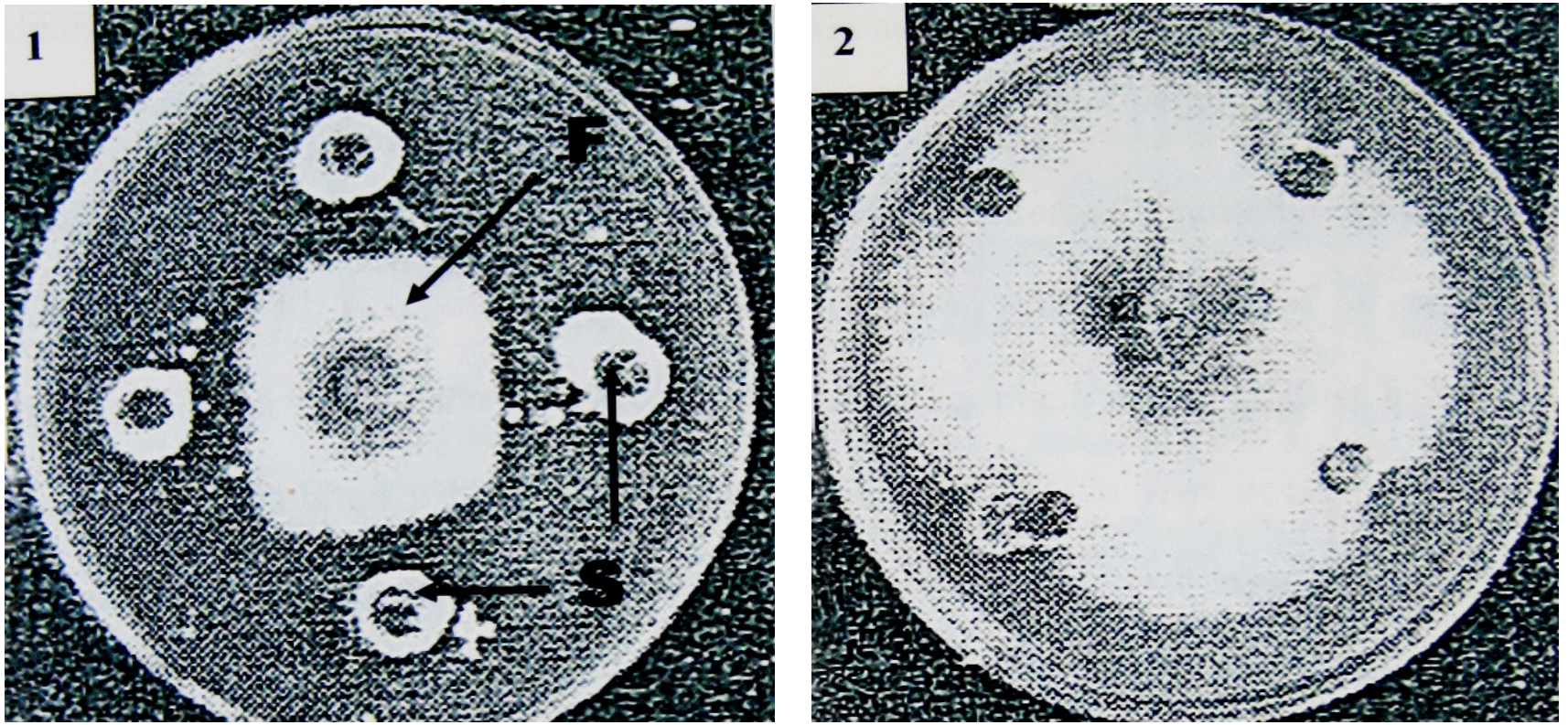

Figure 2.6: *in vitro* assay showing the antagonistic effect of various *Streptomyces* isolates (S) placed at the edge of petri dish on the growth of *Fusarium culmorum* (F) in the centre of PDA medium (Tahvonen, 1982).

1: Agar plugs of four *Streptomyces* isolates are highly inhibitory to *F. culmorum*
2: Agar plugs of four *Streptomyces* isolates are not inhibitory to *F. culmorum*

Intensive screening programmes have yielded numerous candidate strains of actinomycetes with potential disease control effects *in vivo*. A strain of *Streptomyces ochraceiscleroticus* that showed strong *in vitro* antagonistic effect against many soil-borne fungi, exhibited a high percentage of protection *in vivo* as pot assays against *Verticillium* wilt of cotton, pepper, and eggplant, *Fusarium* wilt of tomato, watermelon, muskmelon and cucumber, *Phytophthora* blight of pepper and tomato, and *Colletotrichum* wilt of eggplant (Turhan, 1981a) (Figure 2.7). Actinomycetes have also been used commercially to control plant diseases. Mycostop^{TM}, a powdery biofungicidal product that contains spores and mycelium *of Streptomyces griseoviridis* strain K61, is effective in controlling various soil-borne fungi such as *Fusarium* wilt in ornamentals, damping-off in vegetables, root rot in cucumber and seedling blight in wheat (Table 2.5). The product is used as root dipping or growth nutrient treatment of cut flowers and greenhouse-grown vegetables (Mohammadi, 1992).

Some of the properties associated with actinomycetes that make them attractive as biocontrol agents include the ability to colonize plant surfaces, and synthesis of particular extracellular proteins and antibiotics against plant pathogens (Sivasithamparam & Parker, 1978, Doumbou *et al*., 2001).

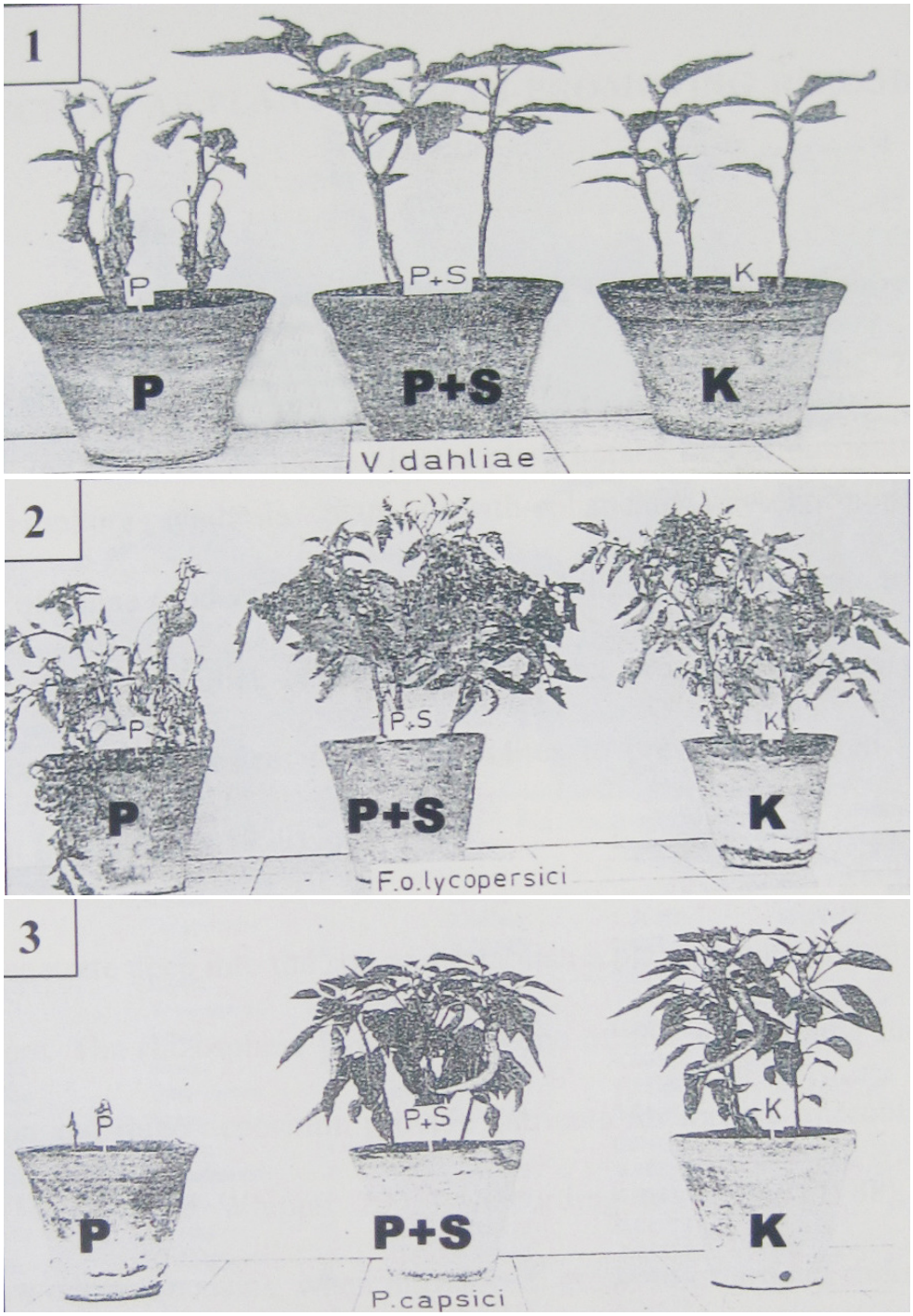

Figure 2.7: *in vivo* pot assays showing the effective disease controlling ability of *Streptomyces Ochraceiscleroticus.* Growth and yield of test plants treated with the pathogen and antagonist (P+S) were compared with plants treated with only the pathogen (P) and control plants not treated with either (K) (Turhan, 1981a).

1: The effect of *S. ochraceiscleroticus* application on *Verticillium* wilt of eggplant.
2: The effect of *S. ochraceiscleroticus* application on *Fusarium* wilt of tomato
3: The effect of *S. ochraceiscleroticus* application on *Phtophthora* blight of pepper

2.10 Actinomycetes as Plant Growth-Promoting Rhizobacteria (PGPR)

Plant growth-promoting rhizobacteria (PGPR) are bacteria that colonized roots and caused plant growth promotion, either directly or indirectly (Whipps, 2001). Direct promotion occurs when the PGPR facilitates plant uptake of soil nutrients by nitrogen fixation, siderophore synthesis, plant growth-enhancing phytohormone synthesis, regulation of ethylene production in roots and solubilization of minerals to make them available for plant use (Glick *et al*., 1999). Indirect promotion occurs when PGPR colonizes roots and synthesizes toxic metabolites to prevent pathogen invasion and establishment (Glick *et al*., 1999).

Plant roots penetrate deep into the soil and establish a plant root environment known as the rhizosphere. The rhizosphere is a narrow zone of soil surrounding the root where root exudation and rhizo-deposition provide nutrients for intense microbial activity to take place (Morgan & Whipps, 2001). According to Weller (1988), rhizosphere colonizers are microorganisms, when introduced, grow on or around roots and survive for several weeks in the presence of competitive indigenous rhizosphere microflora. Nutrients are the limiting factor in competition among bacteria in the rhizosphere. Since bacteria congregate in grooves between root cells where nutrients are most abundant, an introduced strain must preempt the establishment of indigenous bacteria if it is to become established (Weller, 1988). Root colonization is affected by different plant species that produce various types and quantities of root exudates (Weller, 1988).

This was observed when the biocontrol agent, *Streptomyces griseoviridis*, colonized turnip rape seedling roots better than carrot roots. The root exudates of carrot may lack some characteristics necessary for the proliferation of *S. griseoviridis* (Doumbou *et al*., 2001).

The growing roots and root tips are susceptible to invasion by soil-borne fungal pathogens (Sneh, 1990). Actinomycetes that actively colonize new infection sites along the growing roots and protect roots against invasion by soil-borne pathogens are promising biocontrol agents (Barakate *et al*., 2002). Actinomycetes belonging to the genus *Streptomyces* are promising sources of biocontrol-PGPR. They are abundant in soil and are effective colonizers of plant root systems. They also have the ability to endure unfavorable growth conditions by forming spores (Doumbou *et al*., 2001). Various beneficial effects shown by actinomycetes on plant growth are given in Table 2.7. Direct and indirect interactions between actinomycetes and other non-pathogenic soil microorganisms also influence plant growth. For example, Becker *et al*. (1999) reported that actinomycetes stimulated the intensity of mycorrhizal formation and that resulted in improved plant growth.

Manulis *et al*. (1994) described the production of plant growth hormone indole-3-acetic acid (IAA) by various *Streplomyces* spp. such as *S violaceus*, *S. scabies*, *S. griseus*, *S exfoliatus, S. coelicolor* and *S. lividans*. Likewise, Aldesuquy *et al*. (1998) showed that the culture filtrates of *S. olivaceoviridis* enhanced the growth and yield of wheat plants. This activity may be due to an increase in available phytohormones since PGPR strains produced substantial amounts of exogenous auxins (IAA) as well as gibberellins and cytokinins (Doumbou *et al*., 2001).

Some rhizobacteria exhibited both external and internal root colonizing ability. Isolation of the genus *Actinosynnema* from grass blade was the first report that described the isolation of endophytic actinomycetes (Hasegawa *et al*. 1978). Endophytic actinomycetes colonize an ecological niche similar to that of pathogens such as the

vascular wilt pathogen. This may favor them as candidates for biocontrol agents (Doumbou *et al*., 2001). Streptomycetes present inside root tissues play an important role in plant development and health by nutrient assimilation or by producing plant growth regulators (Sardi et al., 1992).

Table 2.7: Application method and plant growth-promoting effects of Streptomycetes

***Streptomyces* sp.**	**Plant species (Application method)**	**Effects on plant growth**	**Reference**
S. griseus	Wheat, oat (seed treatment with cells)	Increased average grain yield, dry foliage weight, tiller number & advanced head emergence	Merriman *et al*., 1974
S. griseus	Carrot (seed treatment with cells)	Increased carrot yields in field trials	Merriman *et al*., 1974
S. canescens, *S. pulcher,* *S. citreofluorescens*	Tomato (seed treatment with cells)	Improved plant growth	El Abyad *et al*., 1993
S. olivaceoviridis *S.rochei*	Wheat (seed treatment with cells)	Increased length and wet weight of shoot	Aldesuquy *et al*., 1998
S. hygroscopicus	Radish, carrot (seed treatment with cells)	Increased plant wet weight in greenhouse trials	Doumbou *et al*., 2001
S. violaceusniger YCED9	Radish (seed treatment with cells)	Increased plant wet weight in greenhouse trials (in non-sterile soil)	Doumbou *et al*., 2001
S. lydicus WYEC108	Radish (seed treatment with cells)	Increased plant wet weight in greenhouse trials	Crawford *et al*., 1993

This effect was observed when pteridic acids A and B produced by *Streptomyces hygroscopicus* TP-A0451 isolated from the stem of bracken fern, induces the adventitious root formation in kidney bean hypocotyls (Igarashi, 2004). These studies suggest the widespread ability of *Streptomyces* spp. to promote plant growth, independent of its characteristics as antagonist of pathogens.

2.11 Role of Enzymes in Biocontrol

Chitin and β-1,3-glucans are major constituents of many fungal cell walls. Various workers have demonstrated *in vitro* lysis of fungal cell walls either by bacterial chitinases or glucanases, or by a combination of both enzymes. Thus, chitinase-producing strains of actinomycetes could be used directly in biocontrol of fungi or indirectly by using the purified proteins (Doumbou *et al*., 2001).

Streptomyces lydicus WYEC108, an antifungal biocontrol agent isolated from the rhizosphere of linseed plants, produced both antifungal antibiotics and extracellular chitinase (Mahadevan & Crawford, 1997). Results showed that WYEC108 not only destroyed germinating oospores of *Pythium ultimum* but also damaged the cell walls of the fungal hyphae. These results showed that *S. lydicus* WYEC108 is a potential biocontrol agent for use in controlling *Pythium* seed and root rot (Yuan & Crawford, 1995).

Valois *et al*. (1996) screened various actinomycete isolates for their ability to grow on fungal cell wall of pathogenic strains of *Phytophthora* spp. Out of these, thirteen isolates showed the ability to produce extracellular glucanases. These enzymes hydrolysed glucan from *Phytophthora* cell walls and caused lysis of living

Phytophthora cells. Further, these strains protected raspberry plants from the infection of *Phytophthora fragariae* var. *rubi*.

Besides these hydrolases, other proteins from actinomycetes such as alkaline protease inhibitor (API) also act against plant pathogenic fungi. The activity of API against *Fusarium* and *Rhizoctonia* is associated with its ability to inhibit fungal serine alkaline protease that is necessary for their growth (Vernekar *et al*., 1999).

2.12 Role of Antifungal Compounds in Biocontrol

The role played by antifungal compounds from actinomycetes in biological control of plant disease has been studied in several systems. Rothrock & Gottlieb (1984) presented evidence that the antifungal compound geldanamycin is produced in sterile soil by *Streptomyces hygroscopicus* subsp. *geldanus*. They further demonstrated the role of geldanamycin in the antagonism of the strain towards soil-borne root rot pathogen, *R. solani*, in soil. The strain has the ability to control *Rhizoctonia* root rot of pea in sterilized soil.

Liu *et al*. (1995) demonstrated control of potato scab disease by using two strains of streptomycetes, *S. diastatochromogenes* PonSSII and *S. scabies* PonR, in a 4-year field plot experiment. Both strains produced antifungal compounds against the pathogenic strain of *S. scabies* in antibiosis assays. Seed tubers treated with the two strains showed significant decrease in the appearance of scab on potato tubers as compared to the non-amended control treatment. Treatment with strain PonSSII showed fewer scab lesions than strain PonR. The *in vivo* results are correlated with the more vigorous growth and larger inhibition zones of PonSSII against pathogenic strains than those of PonF in *in vitro* tests.

Streptomyces violaceusniger YCED9, isolated from rhizosphere soil, suppressed damping-off in lettuce caused by *Pythium ultimum* (Crawford *et al*., 1993). The strain also inhibited the *in vitro* and *in vivo* growth of turfgrass pathogens such as *Microdochum nivale*, *Sclerotinia homeocarpa*, *Gaeumannomyces graminis* and *R.solani* (Chamberlain & Crawford, 1999). Subsequent studies showed that the strain produced polyene-like antifungal compounds that included guanidylfungin A (Trejo-Estrada *et al*., 1998).

Endophytic actinomycetes also play a major role in protecting plants against soil-borne fungal pathogens by producing antifungal compounds (Kunoh, 2002). Fistupyrone, produced by an endophytic *Streptomyces* sp. TP-A0569 isolated from *Allium frstulosum*, inhibited the infection of *Alternaria brassicicola* in pretreated *Brassica* seedlings at 100 ppm (Aremu *et al*., 2003). An endophytic actinomycete strain isolated from a field-grown rhododendron plant, *Streptomyces* sp. R-5, showed broad antimicrobial spectrum against bacteria, yeast and filamentous fungi. The strain exhibited promising biocontrol effects against the pathogen *Pestalotiopsis sydowiana* when applied to tissue-cultured seedlings of rhododendron (Kunoh, 2002).

These studies presented evidences to indicate that antibiotics produced by actinomycetes may function in the biological control of plant pathogenic fungi in nature. However, fundamental studies are needed to characterize the different mechanisms by which a selected biocontrol strain reduces plant disease and the means to utilize the potential strain most effectively to produce disease control.

CHAPTER 3

MATERIALS AND METHODS

3.1 Isolation and Screening of Rhizosphere-Derived Actinomycetes for Fungal Antagonistic Properties

3.1.1 Sample Collection

Chilli rhizosphere soil samples were collected from diseased (*Colletotrichum*-infested) and healthy chilli farms in Ulu Chechuh in Kuala Selangor and Sungai Burung in the state of Selangor, Malaysia (Plates 3.1a, 3.1b).

Soil samples were taken from 5-15cm depth with sterile cork borer from the root area of plants without eradicating them. Soil samples were then put in sterile universal containers and transferred to the Laboratory.

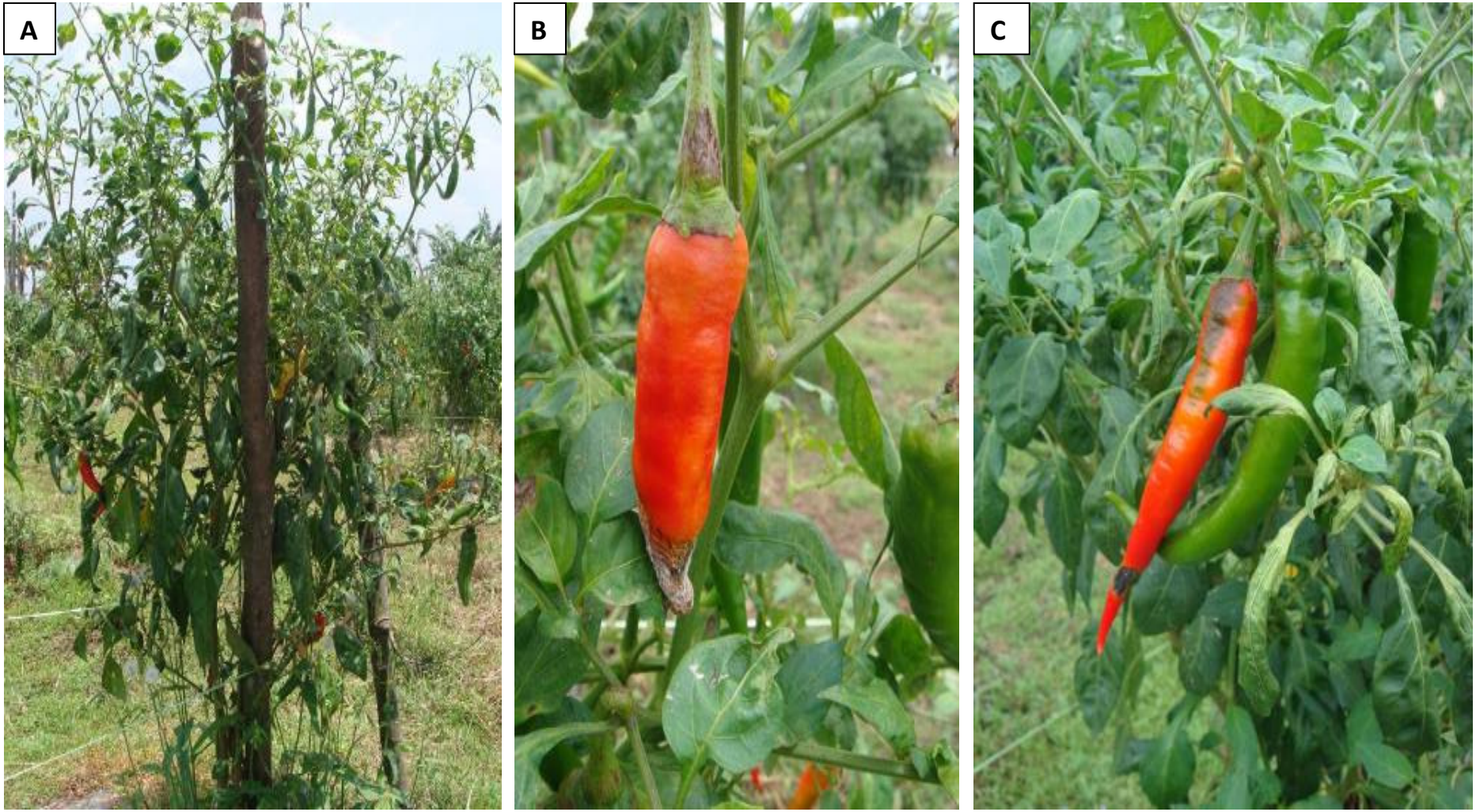

Plate 3.1(a): A - C: Diseased chilli plants for rhizosphere-soil sampling.

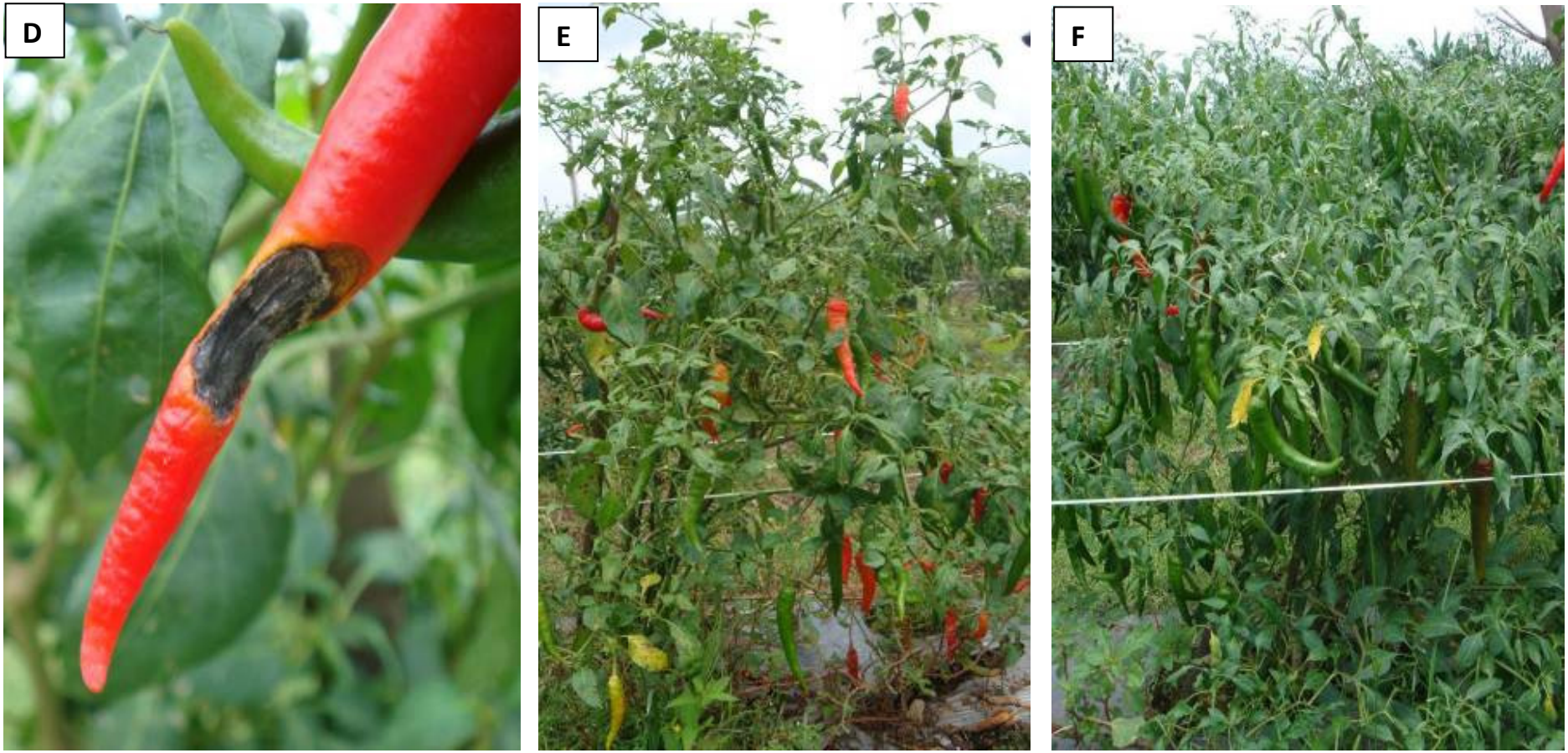

Plate 3.1(b): Diseased and healthy chilli plants for rhizosphere-soil sampling. D: Diseased chilli; E,F: Healthy chilli

3.1.2 Isolation of Rhizosphere-Derived Actinomycetes from Soil Samples

Soil samples were air dried in sterile Petri dishes at room temperature for three days. This was done to eliminate most of the unwanted non-spore forming bacterial contaminants (Labeda & Shearer, 1990). The soil samples were then either treated with dry-heat or moist-heat prior to isolation of actinomycetes.

For dry-heat treatment, 1g of the soil sample was oven dried at 60°C for 40 min (Getha *et al*. 2005). The soil sample was then suspended in 9ml of sterile saline (0.9% NaCl, w/v) solution and vortexed for one min followed by sonication for 30 min. The serial dilution spread plate technique was used, where 0.1ml of suspension at dilutions of 10^{-2} and 10^{-3} were plated onto several different agar media plates. While for moist-heat treatment, 1g of the soil sample was suspended in 9ml sterile yeast extract-SDS solution (Appendix A1.1) and was sonicated for 30 min followed by incubation in a water bath at 45°C for 30 minutes (Getha *et al*. 2005).

Both the dry-heat and moist-heat treatments eliminated all unnecessary non-sporing contaminant organisms. Three isolation media were used to isolate soil actinomycetes: starch-casein agar (SC; Appendix A1.2), humic acid-vitamin agar (HV; Appendix A1.3) and raffinose-histidine agar (RH; Appendix A1.4). To minimize the growth of fungal and bacterial contaminants, 50µg/ml cyclohexamide, 50µg/ml nystatin and 20µg/ml nalidixic acid were added into the isolation medium. Inoculated plates were incubated at 28 ± 2°C for 2-4 weeks. Representatives of isolates putatively assigned as actinomycetes, were picked randomly from the plates using sterile toothpicks and streaked onto yeast extract-malt extract agar (ISP2; Appendix A1.5), inorganic salts-starch agar (ISP4; Appendix A1.6) and sporulating agar (Appendix A1.7) plates. The isolates were incubated at 28 + 2°C for 10 to 14 days. Pure cultures of the isolates were maintained as suspension of

spores and mycelial fragments in 30% (w/w) glycerol at -20°C (Labeda & Shearer, 1990), and on ISP2 and ISP4 agar slant at 4°C.

3.1.3 Macroscopic Determination, Characteristics and Colour Groups of Actinomycetes

Actinomycetes representing all colony morphologies observed were isolated and classified into groups based on macroscopic features such as the presence of aerial mycelium, colony morphology and pigmentation characteristics on ISP2 and ISP4. Isolates producing aerial mycelium were streak-inoculated on ISP4 and incubated at 28 ± 2°C for 14 days. The colours of mature sporulating aerial surface growth and/or substrate mycelium as viewed from the reverse side of the cultures were observed (Kornerup & Wanscher, 1967). The colour group chosen was closest to the colour of the aerial mycelium of a particular isolate (Ndonde & Semu, 2000). The colour of soluble pigment was determined by observing the colour changes in the medium due to the diffusing pigments as described by Shirling & Gottlieb (1966).

3.1.4 Screening of Antagonistic Actinomycetes against *Colletotrichum* spp.

3.1.4.1 Fungal Pathogen

Three virulent and dominant fungal strains used in the *in vitro* antagonism assay were: *Colletotrichum acutatum*, *C. capsici and C. gloeosporioides* provided by the Institute of Biological Sciences, University of Malaya. The working cultures were grown on potato dextrose agar (PDA; Appendix A1.8) plates at 27 ± 2°C. The cultures were maintained in sterile distilled water at 4ºC.

3.1.4.2 *In vitro* Antagonism Assay against *Colletotrichum* spp.

A modified "cross-plug" assay method (Nadaraj, 1996) was used to test the ability of these putative actinomycete isolates to inhibit the growth of three species of *Colletotrichum*. A 5-mm-diameter agar plug fully covered with a grown lawn of a seven-day-old actinomycete isolate on ISP4 agar was placed 2 cm away from the edge of a PDA plate and incubated at 28 ± 2°C for five days. This was done to allow the culture to establish on the agar surface, to sporulate and to produce antifungal metabolites, prior to inoculation of the plates with *Colletotrichum* (Getha & Vikineswary, 2002). For each test fungi, 5-mm-diameter agar plugs of a seven-day-old culture of *Colletotrichum* (*C. acutatum*, *C. capsici and C. gloeosporioides*) were placed 3 cm away from the actinomycete colony (Figure 3.1). The control plates were cultures of test fungi on PDA agar without any actinomycete isolates. The plates were then incubated at 28 ± 2°C and the inhibition of fungal growth, if any, was observed after 48 and 96h (Getha & Vikineswary, 2002). The test was done in three replicates.

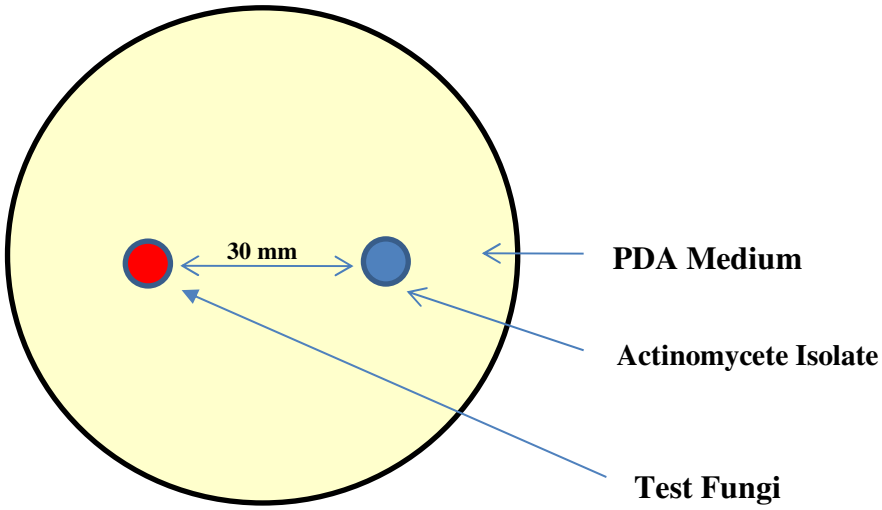

Figure 3.1: Cross plug test plate with actinomycete isolate and test fungi

3.1.4.3 Inhibition of Linear Growth of Three Different Species of *Colletotrichum* spp. By Isolate P42

The best antagonist strain known as P42 showed the best and strong inhibition against the *in vitro* growth of 3 species of *Colletotrichum.* For this test, an agar-streak assay (Crawford *et al.,* 1993) was carried out using 3 different species of *Colletotrichum* to quantitavely measure the ability of isolate P42 to inhibit fungal linear growth.

Fresh culture of strain P42 was streak-inoculated in a 2 cm wide band from the edge of one side of PDA plates and incubated at 28 ± 2ºC for seven days. Then a 5-mm-diameter PDA plug from the growing mycelium edges of *Colletotrichum* cultures (*C. acutatum*, *C. capsici* and *C. gloeosporioides*), was placed in the test plate and further incubated at 27 ± 2ºC. Control plates without isolate P42 were also prepared. All control and test plates run in three replicates. Plates were scored after two, four, six and eight days by measuring fungal colony radius on the control plate (γ_o) and the distance of fungal colony growth perpendicular to the strain P42 colonies on test plate (γ). Percentage inhibition of fungal linear growth was calculated, as shown below, from each test plate and recorded as an average.

$$\text{Percentage inhibition of fungal linear growth} = [1-(\gamma / \gamma_o)] \times 100$$

3.1.4.4 Statistical Analysis

The statistical software program SPSS version 17 was used for all statistical analysis. Means of percentage inhibition of fungal linear growth of 3 different *Colletotrichum* species were subjected to a double factor analysis of variance (two-way ANOVA) to test for significant interaction between *Colletotrichum* species and incubation period on the inhibition. A single factor analysis of variance (one-way ANOVA) was used to test for significant differences in means of percentage inhibition of different species of

Colletotrichum at individual time-periods. The mean values were then compared by Duncan's multiple range analysis and least significant difference (LSD) was calculated at p=0.05.

3.1.4.5 Observation of Morphological Changes by Scanning Electron Microscopy (SEM)

For the morphological changes of the fungal mycelia occurring at the margin of the inhibition zone, mycelia plugs were cut from the assay plates at different points of the inhibition zones within each plate, to obtain a representative sampling of the antagonistic effects. Samples were then fixed overnight in 2% aqueous osmium tetraoxide at 4°C. Samples were washed three times with distilled water and dehydrated in a graded ethanol series (15 minutes for each step) to absolute ethanol, followed by two changes of absolute ethanol. The alcohol was replaced gradually by different mixtures of acetone and ethanol (30:70, 50:50 and 70:30, v/v). Samples were then washed twice in absolute acetone. The dehydrated samples in acetone were critically point dried using liquid carbon dioxide. The dried samples were mounted on aluminium stubs using double sided adhesive carbon tape, sputter-coated with gold and examined in a JEOL JSM-6400 scanning electron microscope.

3.2 Characterization of Potential Actinomycete Antagonist of *Colletotrichum* spp.

3.2.1 Cell Wall (Chemotaxonomic) Analysis of Antagonistic Chilli Rhizosphere-Derived Actinomycetes

Isomers of 2,6-diaminopimelic acid (DAP) in cell walls of the eight potential antagonistic actinomycetes were analysed by thin-layer chromatography (TLC) (Hasegawa *et al*., 1983). Isolates P8, P11, P39, P42, P43, P44, P45, P77 and P115 were grown on non-sporulation agar (Appendix A1.9) at 28 ± 2ºC for seven days. Three loopful colonies were scraped from agar plates, placed in a screw-capped test tube containing 0.1 ml of 6 N HCl, wrapped with aluminium foil and autoclaved for 15 min at 121ºC. Following the autoclave, 1µl of the hydrolysate was spotted on a thin cellulose sheet (Art 5577, Merck). 1µl of 0.01M DL-DAP (Sigma chemicals) that contained isomers LL-DAP and *meso*-DAP was spotted on the same sheet as a standard. The TLC sheet was developed in a solvent system containing methanol : distilled water : 6N HCl : pyridine (80:26:4:10, v/v) for three hours. The developed sheet was then sprayed with ninhydrin reagent (Appendix A2.1) and heated at 100ºC for 2 min to reveal the spots.

3.2.2 Micromorphological Characteristics of Antagonistic Chilli Rhizosphere-Derived Actinomycetes

The selected isolates were streaked on inorganic salts-starch agar (ISP4; Appendix A1.6) and sterile 12 mm diameter coverslips were inserted at an angle of about 45º into the lawned cultures (Williams & Davies, 1967). Triplicate plates were set up for each of the four selected strains. The plates were incubated at 28 ± 2°C for 14 days.

Coverslips with attached growth of each isolate were carefully removed from the plates and fixed overnight at 4°C in vapor of 2 % (w/v) aqueous osmium tetroxide (Sigma

Chemicals). The coverslips were then gold coated using a Cool E5100 diode sputter coater (Biorad, England) and mounted onto aluminium stubs using carbon adhesive cement. The materials were stored in a desiccator prior to viewing in a JEOL JSM-6400 scanning electron microscope. The SEM preparations were examined for sporophore-spore chain type, spore-surface ornamentation, presence of sclerotia and substrate spores, and fragmentation of the substrate mycelium.

3.2.3 Phenotypic Characteristics of Antagonistic Actinomycetes

Strains P8, P39, P42 and P115 showing higher degree of inhibition and antagonism towards fungal plant pathogens were characterized further. Strain P42 showed the highest inhibition activity. The taxonomic keys of Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989), numerical classification of Williams *et al.,* and International *Streptomyces* projects (Shirling & Gottlieb, 1966 and 1972) were used to compare morphological, cultural and some physiological characteristics of these four strains with those of known species or strains of *Streptomyces*.

3.2.3.1 Morphological Studies

Morphological characteristics such as the presence of aerial mycelium and spore chain morphology and ornamentation were observed on selected strains grown on ISP4 agar. These characteristics were compared with those of *Streptomyces* species assigned to different clusters in the numerical phonetic survey of Williams *et al.* (1983).

3.2.3.2 Cultural Studies

Cultural characteristics and growth of the best strains of actinomycetes were observed on yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3; Appendix A1.10), inorganic salts-starch agar (ISP4), glycerol-asparagine agar (ISP5; Appendix A1.11),

peptone-yeast extract-iron agar (ISP6; Appendix A1.12) and tyrosine agar (ISP7; Appendix A1.13) (Shirling & Gottlieb, 1966). Following 14 days of growth at 28 ± 2°C, the agar plates were examined visually to determine aerial spore-mass colour, substrate mycelial pigmentation and the colour of diffusible pigments, if present. The observations were recorded using the colour charts in Methuen Handbook of Colour (Kornerup & Wnscher, 1967).

3.2.3.3 Physiological Studies

3.2.3.3.1 Melanin Production

Melanin production (greenish-brown to brown or black diffusible pigment or a distinct brown pigment) was determined after four days of incubation in peptone-yeast extract-iron agar (ISP6) and tyrosine agar (ISP7).

3.2.3.3.2 Degradation Activity

The degradation of L-tyrosine (0.5%, w/v), xylan (0.4%, w/v), xanthine (0.4%, w/v) and casein (1.0%, w/v, skimmed milk) was detected using modified Benett's agar (MBA, Appendix A.14) as the basal medium. Seven mm plugs taken from a seven-day old culture of the best strains grown on ISP4 or ISP2 agar were placed on each test plate and incubated at 28 ± 2°C. The plates were examined for clearing zones around the growth areas after 7, 14 and 21 days (Williams *et al*., 1983). Starch (1.0%, w/v) degradation was also detected in MBA as the basal medium (Williams *et al*., 1983). Test plates containing streaked actinomycetes strains were incubated at 28 ± 2°C for seven days and flooded with Lugol's iodine solution (Appendix A2.2). Hydrolysis of starch was shown by the occurrence of clear zones around the growth area.

3.2.3.3.3 Growth Tests

Plates of MBA adjusted to pH values 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with the combinations of 1M sodium hydroxide and 1M HCL, were prepared. Actinomycete strains were streaked on to the test plates. The minimum pH that supported growth of actinomycetes strains was determined after fourteen days of incubation at 28 ± 2°C (Williams *et al*., 1983).

The temperature range of growth of strains was also determined on MBA medium. Growth at 45ºC was read after fourteen days while growth at 10ºC was read after six weeks of incubation (Williams *et al*., 1983).

Tolerance to sodium chloride was determined on NaCl-incorporated ISP4 agar of concentrations ranging from 1 to 10 % (w/v). After fourteen days of incubation at 28 ± 2°C, the growth of strains on the test plates was observed visually and recorded in comparison to the growth on control plates without NaCl (Nadaraj, 1996).

3.2.3.3.4 Susceptibility to Antibiotics

Susceptible actinomycete strains to different antibiotics (kanamycin, gentamicin, neomycin, erythromycin, streptomycin, novobiocin and tetracyline) at the defined concentrations were examined using the method of Rheims *et al.* (1998). Surface spores from a seven-day old culture of strains grown on ISP4 agar medium were scraped with sterile cotton buds and lawned evenly on fresh ISP4 agar plates. Antibiotic sensitivity discs (Difco) were placed centrally in the seeded agar plates, incubated at 28 ± 2°C for two to five days and the resulting inhibition zones measured.

3.2.3.3.5 Utilization of Carbon Sources

The growth of actinomycete strains on nine different carbon sources was determined on Pridham-Gottlieb agar medium (ISP9; Appendix A1.15), as recommended by Shirling & Gottlieb (1966). Carbon sources were filter-sterilized and added aseptically into the ISP9 basal medium to give a concentration of 1% (w/v). The ability of actinomycete strains to use a carbon source was tested after seven to fourteen days of incubation at 28 ± 2°C, by comparing this growth with the negative control (no carbon source) plates.

3.2.3.3.6 Hydrogen Sulphide Production Test

Difco peptone-iron agar was complemented with 0.1% Difco yeast extract. The medium was dissolved and autoclaved at 121°C for 15 minutes. The medium was then poured into the plates and solidified at room temperature. Actinomycete isolates were streaked onto the plates, done in triplicate, prior to incubation at 28 ± 2°C for two days. The production of H_2S was indicated by the presence of a blue-black discoloration of the medium surrounding the colonies (Gottlieb, 1961).

3.2.3.3.7 Nitrate Reduction Test

The purpose of this test was to determine whether actinomycete strains could reduce nitrate to nitrite or free nitrogen gas. Nitrate broth was prepared (Appendix A2.3) and 10 ml was dispensed into 28 ml capacity McCartney bottle. Following autoclaving at 121ºC for 15 minutes, a 5-mm-diameter agar plug of a seven-day old culture of each actinomycete strain was placed in the cooled McCartney bottle and incubated at 28 ± 2°C for seven days. Three replicates were done. The negative control tubes without any strain plugs were used. After incubation, 5 drops of sulphanilic acid (reagent A) and 5 drops of N,N-dimethyl-1-naphthylamine (reagent B) were added to the test bottles and shacken well to mix the reagents with the medium. If the nitrate was reduced to nitrite

by the strain (positive reaction), the colour of the medium would change to red or pink within a few minutes. Colourless suspension following the addition of the reagents could mean a negative or positive reaction. For confirmation, a small amount ("sharp knife point") of Zn powder was added to the medium. The bottles were shaken vigorously and allowed to stand at room temperature for 10-15 min. If the medium remained colourless, this indicated a positive result, but if the medium turned pink, the result was negative.

3.2.3.3.8 Gelatinise Activity (Gelatine Liquefaction) Test

This test was done by dissolving Difco gelatine (12 percent) powder in distilled water and dispensing the medium into 28mL capacity McCartney bottle, followed by autoclaving at 121ºC for 15 minutes. Inoculation of tubes was done by placing 5-mm-diameter agar plugs of a seven-day-old cultures of each actinomycete strain that placed in the McCartney bottles and incubated at 28 ± 2°C for fourteen days. Triplicate tubes were then placed at 4°C for 1 hr, and the degree of liquefaction was determined (Gottlieb, 1961).

3.2.3.3.9 Determination of Chitinase Activity

The isolated actinomycete strains were screened for their chitinase activity. Three loopful of seven-day-old cultures of each isolate was transferred into sterile vials containing 2 ml of semi-solid Starch-Casein Nitrate agar (SCN) (1g agar/l) (Appendix A1.16), and gently shaken for uniformity. 25 µl aliquots from each of the isolate suspensions was placed as a single drop in the centre of colloidal chitin agar (CCA) (Appendix A1.17) plates (Hsu & Lockwood, 1975), with three replicates. Inoculated CCA plates were incubated at 28 ± 2°C for fourteen days. The size of the clear zone around each drop-colony was determined.

3.2.3.4 Molecular Characteristics of Actinomycete Strains

3.2.3.4.1 Biological Material

The biomass for the 16S rRNA sequencing studies was obtained by growing actinomycete strains on a non-sporulating agar medium (Appendix A1.9) for seven days at 28 ± 2°C in darkness (Ishikawa, 2000), as shown in Figure 3.3 and Appendices B1.1, B1.2.

3.2.3.4.2 DNA Extraction, PCR Amplification and Sequencing of 16s rRNA

Genomic DNA was isolated from the rhizosphere-derived actinomycetes using Machery Nagel NucleoSpin DNA extraction kit following the manufacturer's specification. Partial sequences of 16s ribosomal RNA was amplified using two previously reported primers, 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525r (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991). Amplifications were performed using Swift^{TM} Maxi Thermal Cycler (Esco, Singapore) in a final volume of 50 µl containing 20 ηg of genomic DNA, 4 µl of MgCl2 (2.0 mM), 7.5 µl of 10x colorless buffer, 0.2 mM of each dNTP, 0.4 µM of each primer (forward and reverse), and 6.25 units of recombinant *Taq* DNA polymerase (Fermentas). The conditions used for the thermal cycling were as follows: pre-denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 56ºC for 1 min, and extension at 72ºC for 1 min. At the end of the cycles, the reaction mixture was kept at 72ºC for 10 min and stored in -20ºC prior to use. The 1.4 kb amplified 16S rRNA fragment was separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (QIAGEN, Germany). 16s rRNA sequences were determined in both forward and reverse directions using ABI 3730XL automated sequencer.

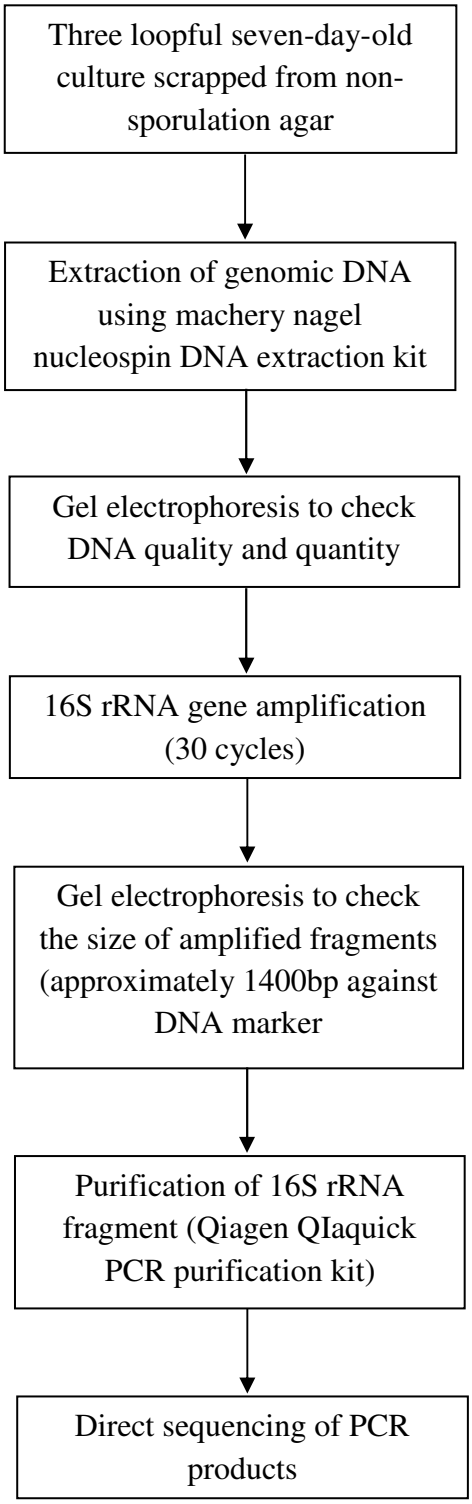

Figure 3.2: Flow-chart of 16S rRNA extraction, amplification and sequencing methods.

3.2.3.4.3 Sequence Alignment and Phylogenetic Analysis

The consequential 16S rRNA sequences of 4 strong strains (P8, P39, P42 and P115) were revised and aligned manually using Chromas version 2.33 (Technelysium Pty Ltd) and Mega 4.0 version 4.0.2, respectively (Tamura *et al*., 2007). The aligned sequences of the four strains were deposited at the National Centre of Biotechnology Information (NCBI) under the accession numbers: JN967800-JN967803. All sequences were compared with 30 similar sequences from the reference organisms, retrieved from the GeneBank/EMBL/DDBJ databases using EzTaxon server (http://www.Eztaxon.org; Kim *et al*., 2012) (Table 3.1).

A phylogenetic tree was constructed based on the neighbour-joining method (Intra, *et al*., 2011) via evolutionary distances calculated with the Jukes-Cantor model (Jukes *et al*., 1969) using Mega 4.0 (Tamura *et al*., 2007) with pairwise deletion option. The tree was statistically tested using bootstrap phylogeny analysis with 1000 replications.

Table 3.1: Representative strains of the family *Streptomycetaceae* producing significant sequence alignment with four strong strains in the 16S rRNA tree

***Streptomyces* Species**	**Source/Strain**	**Accession Number**	**Author**
Streptomyces plicatus	NBRC13071	AB184237	Pridham *et al*., 1958
Streptomyces enissocaesilis	NRRL B-16365	DQ026641	Gause *et al*., 1983
Streptomyces ghanaensis	KCTC 9882	AY999851	Wallhausser *et al*., 1965
Streptomyces geysiriensis	NBRC 15413	AB184661	Wallhausser *et al*., 1965
Streptomyces mutabilis	NBRC 12800T	AB184156	Pridham *et al*., 1958
Streptomyces vinaceusdrappus	NRRL 2363	AY999929	Pridham *et al*., 1958
Streptomyces djakartensis	NBRC 15409	AB184657	Huber *et al*., 1962
Streptomyces tuirus	NBRC 15617	AB184690	Albert and Malaquias de Querioz, 1963
Streptomyces tritolerans	DAS 165	DQ345779	Syed *et al*., 2007
Streptomyces rochei	NBRC12908	AB184237	Berger *et al*., 1953
Streptomyces tendae	ATCC 19812	D63873	Ettlinger *et al*., 1958
Kitasatospora azatica	IFO 13803	U93312	Nakagaito *et al*., 1992
Streptomyces violaceoruber	NBRC 12826	AB184174	Waksman & Curtis, 1916
Streptomyces tricolor	NBRC 15461	AB184687	Waksman, 1961
Streptomyces lienomycini	LMG 20091	AJ781353	Gause *et al*., 1983
Streptomyces purpeofuscus	NBRC 12905	AB184234	Yamaguchi and Saburi, 1995
Streptomyces misakiensis	NBRC 12891	AB184223	Nakamura, 1961
Streptomyces aburaviensis	NRRL B-2218	AY999779	Nishimura *et al*., 1957
Streptomyces rubrogriseus	LMG 20318	AJ781373	Gause *et al*., 1983
Streptomyces violaceorubidus	LMG 20319	AJ781374	Gause *et al*., 1983
Kitasatospora putterlickiae	F18-98	AY189976	Groth *et al*., 2003
Streptomyces xanthocidicus	NBRC 13469	AB184427	Asahi et al., 1966
Kitasatospora kifunensis	IFO 15206	AB022874	Nakagaito *et al*., 1992
Streptomyces chrysomallus subsp. fumigatus	NBRC 15394	AB184645	Frommer, 1959
Streptomyces indigoferus	NBRC 12878	AB184214	Shinobu & Kawato, 1960
Streptomyces coelescens	DSM 40421	AF503496	Pridham, 1970
Streptomyces aurantiogriseus	NRRL B-5416	AY999773	Pridham *et al*., 1958
Streptomyces anthocyanicus	NBRC 14892	AB184631	Pridham *et al*., 1958
Streptomyces humiferus	DSM 43030	AF503491	Goodfellow *et al*., 1986
Streptomyces violaceolatus	DSM 40438	AF503497	Pridham *et al*., 1958

3.3 Evaluation of the *in vivo* Biocontrol Ability of Strain P42 against Three Different Species of *Colletotrichum*

3.3.1 Fungal Inoculum

Three species of *Colletotrichum* (*C. acutatum*, *C. capsici and C. gloeosporioides*) isolated from diseased chilli plants were obtained from Institute of Biological Sciences, University of Malaya, Kuala Lumpur. The pathogens were maintained on sterilized on 2% water agar at 4ºC. The pathogens were cultured on potato dextrose agar (PDA; Appendix A1.18) and incubated at 27 ± 2°C for ten days. The aerial growth from PDA cultures was suspended in 10 ml sterile distilled water and filtered through double-layered cheesecloth to remove mycelial fragments. The inoculum concentration of 10^5 spores/ml (Jetiyanon & Kloepper, 2002) was prepared using a haemocytometer and immediately used for chilli-fruit inoculation in the *in vivo* biocontrol assay.

3.3.2 *Streptomyces* sp. Strain P42 Inoculum

The working culture of strain P42 was obtained by streaking a loopful of spore stock from 30% (v/v) glycerol onto ISP4 agar (Appendix A1.6). Following ten days of incubation at 28 ± 2°C, the aerial growth was suspended in 10ml sterile distilled water. The number of colony forming units (cfu) in the suspension was set at 1.16×10^5 cfu/ml by dilution plate count method.

3.3.3 Dilution Plate Count Technique to Determine cfu Strain P42 Cell Suspension

For dilution purpose, 1 ml of strain P42 cell suspension was added to bottles containing 9ml sterile saline (0.9% NaCl, w/v) solution to make the 10^{-1} dilution. After mixing thoroughly, serial dilution of the suspension was continued until the 10^{-7} dilution.

Aliquots (0.1 ml) from 10^{-5} and 10^{-7} dilutions were spread on ISP4 agar and incubated at 28 ± 2°C for five to seven days.

The numbers of colony forming unit (cfu) were counted from triplicate plates as follows:

$$\text{cfu} = \frac{\text{number of colony} \times \text{final dilution factor}}{\text{ml cell suspension (1ml)}}$$

3.3.4 Evaluation of Phytotoxicity of Selected *Streptomyces* spp. Strain P42 by Seed Germination Assay

3.3.4.1 Preparation of Surface Sterilized Seed of Maize (*Zea mays*)

Maize seeds used in this study were purchased from Giant supermarket, Kuala Lumpur, Malaysia. Seed viability test was first conducted to test the quality of the seeds by the paper towel method. 120 seeds were seeded with three replicates and incubated in a growth chamber at 28°C. After seven days, the number of germinated seeds was determined (Gholami *et al*., 2009). The seeds were then soaked in distilled water overnight. They were then surface-sterilised by soaking in 2% sodium hypochlorite for 5 minutes followed by rinsing with sterile distilled water five times prior to use (Bressan, 2003).

3.3.4.2 Preparation of Inoculum

Ten ml of sterile distilled water was added into a seven day old pure culture of selected antagonistic actinomycete grown on ISP4 agar plate. The spores on the agar surface were scraped and mixed with distilled water to produce a suspension. Serial dilution was done to determine the low dosage and high dosage treatment of actinomycete suspension.

3.3.4.3 Test for Phytotoxicity of Selected Antagonistic Actinomycete

Phytotoxicity assay was conducted to assess the effects of growth of antagonists and their metabolites on the health of the chilli plants. Thirty ml of ISP2 with 2% (w/v) agar was dispensed into plant tissue culture glass jars and autoclaved at 121°C for 15 minutes. Five ml of the spore suspension prepared earlier was then added into the cooled molten agar medium. The content was mixed well by gentle shaking and was left to solidify. Five surface sterilized maize seeds were placed onto the solidified agar surface and left to germinate under light condition for 24 hours per day. The root length, plant height, number of leaves and number of secondary roots were recorded. The experiment was done in triplicates for the selected antagonistic actinomycete strain. Agar medium without actinomycete inoculum served as a positive control.

After ten days of incubation, the plant height, main root length, number of leaves and number of secondary root branches were recorded. The mean and standard deviation of triplicate samples from each set were calculated.

3.3.4.4 Statistical Analysis of Phytotoxicity Results

The statistical software program SPSS version 17.0 was used for the analysis of means of triplicate values.

3.3.5 Planting Materials and Methods

Long red chilli seeds variety Kulai were provided from ACE Hardware Market Kuala Lumpur, and used for the *in vivo* biocontrol assay. Seeds were soaked in a suspension of 1.16×10^5 cfu/ml (low dosage) of P42 strain for 1 hr before planting the seeds into a plastic seedling tray containing sterilized soilless peat-based medium (Florafleur, NEVEMA, Zwolle, Holland). For the control treatment, the seeds were soaked in

sterilized distilled water. Seven days after germination the seedlings were transplanted into 12 cm plastic bags containing sterilized soil (Plate 3.2). A fertilizer (16-16-16; N-P-K) was applied 30 days after planting. All bags were watered normally and placed in a greenhouse that received 12 h of sunlight each day with fluctuating day temperature of 28-35ºC and night temperatures of 20-24ºC.

Plate 3.2: Transferred plantlets from plastic seedling tray to 12cm bags containing sterilized soil.

3.3.6 *In vivo* Biocontrol Assay

P42 treatment (at low concentration 1.16×10^5 cfu/ml) was applied by soil drenching when plants were 45 days old. A fertilizer (13-13-21; N-P-K) was applied after fruit setting, 6 days after the soil drench. The plants were challenged 90 days after planting with 3 species of *Colletotrichum* namely *C. acutatum*, *C. capsici* and *C. gloeosporioides* (10^5spores/ml) by drenching the pepper fruit with a fungal spore suspension or distilled water (control) (Plate 3.3). At this stage, the fruits were fully developed, with at least four fruits per plant. The plants were then maintained in a moist chamber for 24 h and transferred to a greenhouse. Two hundred and forty plants were numbered and grouped for the eight treatments (Table 3.2) in a complete randomized design (CRD). In treatment P, the plants were treated with strain P42 (seeds and soil drenching). In treatment Ca, the plants were inoculated with *C. acutatum* without strain P42 in their soil, while in treatment PCa, the plants were inoculated with the pathogen with strain P42 already in their soil. Similarly treatments Cg and Cc (*C. gloeosporioides* and *C. capsici* respectively) were inoculated with pathogen without P42 strain in their soil, while PCg and PCc inoculated with the pathogen with the presence of strain P42 as the biocontrol antagonist agent.

Plate 3.3: Chilli plants drenched with the fungal spore suspension or sterile distilled water (control).

Table 3.2: Treatment group of chilli plants in *in vivo* biocontrol assay

Treatment group[a]	***Colletotrichum* sp. (10^5spores/ml)**[b]	**Strain P42 (1.16×10^5 cfu/ml)**
C	0 (control)	-
P	0 (control)	+
Ca	+	-
PCa	+	+
Cg	+	-
PCg	+	+
Cc	+	-
PCc	+	+

[a]: Each treatment group contained ten replicates (bags); one plant per bag; complete randomized design. Control plants in treatment group C were treated with sterile distilled water.
[b]: Spore suspension of *Colletotrichum* sp.; +: treated; -: untreated

3.3.7 Symptom Evaluation

Disease severity was rated 9 days after the challenge. Two fruit samples were randomly collected from each plant for the disease rating. The damage was graded in six disease scales (V) (0= all fruits were healthy, 1 = 10% of fruit area was destroyed, 2 = 25% fruit area destroyed, 3 = 50% of fruit area was destroyed, 4 = 75% of fruit area was destroyed, and 5 = 100% of fruit area was destroyed) (Plate 3.4).

Disease severity index (DSI) was rated 9 days after inoculation of chilli fruits and calculated using McKinney's formula (Cegiełko *et al*., 2011):

$$DSI\% = \frac{\Sigma(V \times n)}{N \times Z} \times 100$$

where, V=disease scale; n=number of affected chilli fruits with this disease scale; N=total number of plants; Z=maximum scale number(5).

3.3.8 Statistical Analysis

The experiment was repeated and all data were analyzed using analysis of variance (ANOVA) and the treatment means were separated using Fisher's protected least significant difference (LSD) test at *P*=0.05 using SAS software version 9.1.

Plate 3.4: Scales for chilli fruit symptom evaluation

0: All fruits were healthy,
1: 10% of fruit area was destroyed,
2: 25% fruit area destroyed,
3: 50% of fruit area was destroyed,
4: 75% of fruit area was destroyed,
5: 100% of fruit area destroyed.

3.3.9 Re-isolation of Strain P42 from Experimental Chilli Plant Rhizosphere

Re-isolation of antagonistic P42 strain from experimental chilli plant rhizosphere soil was done to confirm the presence of the strain in the soil. The procedure followed from the same procedure as mentioned in section 3.1.2.

3.3.10 Identification of Strain P42 from Experimental Chilli Plant Rhizosphere

Following the re-isolation and characterization, strain P42 was identified (as mentioned in section 3.2.3.4) to confirm the type of strain P42.

CHAPTER 4

RESULTS

4.1 Isolation and Screening of Rhizosphere-Derived Actinomycetes for Fungus-Antagonistic Properties

4.1.1 Isolation and Macromorphological Characterization of Chilli Rhizosphere-Derived Actinomycetes

A total of 130 colonies of putative actinomycetes from both sampling locations were isolated from the rhizospheric soil of healthy and *Colletotrichum*-infected chilli plants. It was not possible to classify the isolates in the primary isolation plates to the genus level. Hence, the isolates were classified as either *Streptomyces*-like or non-*Streptomyces* based on macromorphological characters.

Isolates presumptively assigned to the *Streptomyces*-like group were distinguished from other bacterial colonies growing on the starch-casein, humic-acid vitamin and raffinose-histidine agar isolation plates by their characteristic colony and pigmentation properties. These isolates formed substrate mycelium and abundant aerial mycelium with powdery spore mass (Plate 4.1). Isolates classified in the non-*Streptomyces* group formed substrate mycelium but lacked aerial mycelium. These bacteria formed small, compact and waxy or slimy colonies. Their substrate mycelium was orange to orange-brown, and spores when present blackened the surface of the colonies (Plate 4.1). These isolates belonged to the suprageneric group actinoplanetes (Goodfellow, 1989). There are six genera within this suprageneric group, and based on macromorphological characteristics, most of the observed isolates appeared to resemble closely to the genus

Micromonospora (Jensen *et al*., 1991). The isolates were classified as *Micromonospora*-like.

The number of isolates picked from total samples of each sampling location studied is given in Table 4.1. A total of 106 colonies with aerial mycelium and 24 colonies with only substrate mycelium were isolated as morphological representatives. There was a higher proportion of *Streptomyces*-like strains isolated from the Sungai Burung *Colletotrichum*-infected soil (47.2% of the total 106 isolates) and healthy soil samples (20.70%), compared to the lower number of isolates from Ulu Chechuh group in *Colletotrichum*-infected (17%), and 15.1% in healthy soils. In *Micromonospora*-like isolates, 50% of them were obtained from the Sungai Burung *Colletotrichum*-infected soil samples compared to the other three sample sources. In fact, 50% of the actinomycetes isolated from Sungai Burung's *Colletotrichum*-infested soil samples corresponded to the *Micromonospora*-like group (Table 4.1).

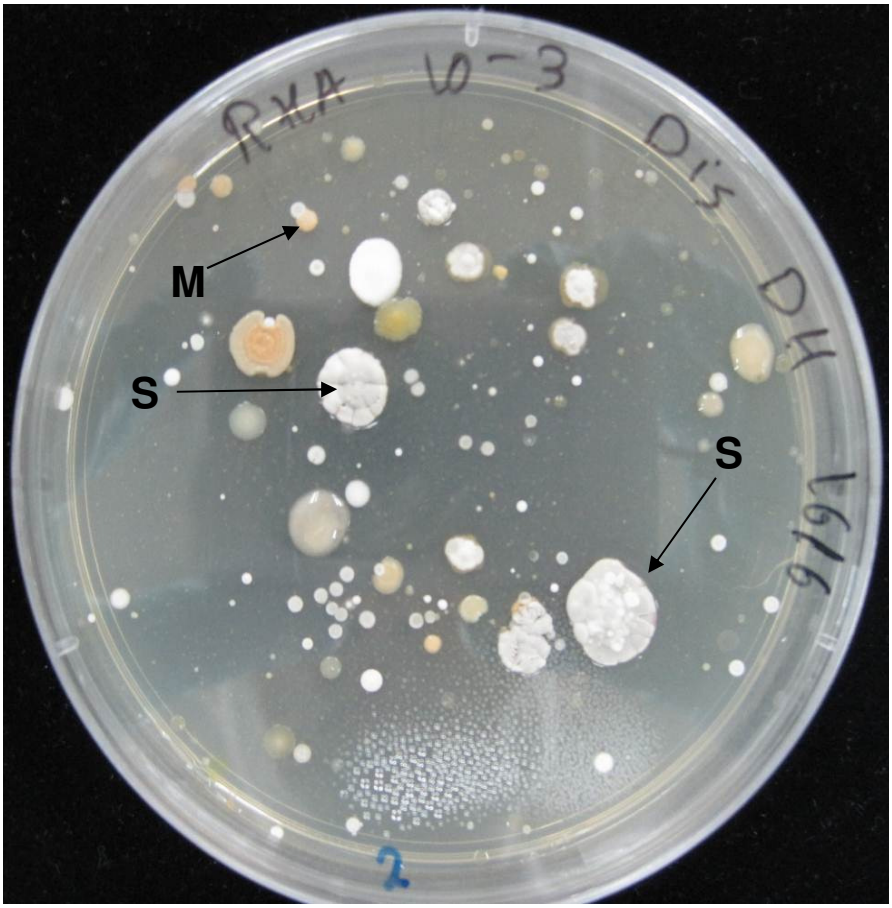

Plate 4.1: Growth of different type of actinomycete colonies on raffinose-histidine agar (RHA) after 2 weeks of incubation at 28 ± 2ºC. (S – *Streptomyces*-like; M – *Micromonospora*-like isolate).

Table 4.1: Distribution of representative groups of actinomycetes which were isolated from various chilli rhizosphere-derived sample sources on three different media

Source of sample	*Streptomyces*-like[a]		*Micromonospora*-like[b]		Total isolates
	No.	%	No.	%	
Ulu Chechuh, *Colletotrichum*-Infected Soil (UCD)	18	17	3	12.5	21
Ulu Chechuh, Healthy Soil (UCH)	16	15.1	4	16.6	20
Sungai Burung, *Colletotrichum*-Infected Soil (SBD)	50	47.2	12	50	62
Sungai Burung, Healthy Soil (SBH)	22	20.70	5	20.9	27
Total Isolates	106	81.5	24	18.5	130

[a]: Colonies forming substrate and aerial mycelium with powdery aerial spore mass
[b]: Colonies forming only substrate mycelium, small and compact, waxy or slimy, and orange to orange-brown to black colour.

Representatives of the different types of putative *Streptomyces*-like isolates growing on ISP4 were assigned to four groups of aerial mycelium colour: grey, white, red and pink. Table 4.2 shows the aerial and substrate mycelium colours of the *Streptomyces*-like strains isolated in this study, and their frequency of occurrence. These isolates had colour intergrades within the grey spore-colour and also within the substrate mycelium colour. The largest group of 42 isolates formed grey aerial spores mass and lacked characteristic colours in the vegetative (substrate) mycelium and soluble pigments (Table 4.2).

Generally, the distribution of different colour groups of the *Streptomyces*-like isolates differed according to the sampling locations. Strains isolated from the Sungai Burung diseased (*Colletotrichum*-infested) (SBD) soil samples had a wide diversity or variability in aerial and substrate mycelium colour. This was followed by the Sungai Burung healthy (SBH) soil samples. The Ulu Chechuh (UCD and UCH) soil samples, however, showed less diversity. Many of the isolates with grey aerial mycelium were isolated from Sungai Burung's diseased (*Colletotrichum*-infested) (SBD) and healthy (SBH) soil samples (Table 4.2). According to Tresner and Backus (1963), cultures with spore-colour groups which have shades of red in combination with other colours to give a variety of pinkish values were grouped under the basic red colour group. In the present study, isolates which produce reddish aerial mycelium were isolated only from the Sungai Burung's soil samples. In addition, isolates with pinkish aerial mycelium were obtained only from the same location sampling sites. Based on the distribution factor, isolates with red and pink spore colors were placed in two separate colour groups (Table 4.2).

Table 4.2: Colour grouping for the aerial and substrate mycelium of putative *Streptomyces*-like isolates in each spore-colour, and their frequency of occurrence in the rhizosphere-derived samples

Aerial mycelium and substrate mycelium colour[a]	Sources of sample[b]				Total isolates	
	UCD	UCH	SBD	SBH	No.	%
Grey colour group						
Grey / Grey	5	3	22	12	42	
Grey / Red	1	2	1	2	6	
Grey / Brown (Sp)	3	1	7	1	12	
Grey / Grey-Brown	-	1	5	3	9	
Grey-White / Grey-White	3	2	3	1	9	
Grey-White / Yellow	1	1	-	2	4	
Dark Grey / Dark Grey (Sp)	3	1	4	-	8	
Total	**16**	**11**	**42**	**21**	**90**	**84.9**
White colour group						
White / White	2	5	2	-	9	
Total	**2**	**5**	**2**	**-**	**9**	**8.5**
Red colour group						
Red-Grey / Red	-	-	1	1	2	
Total	-	-	**1**	**1**	**2**	**1.9**
Pink colour group						
Pink-White / Pink	-	-	5	-	5	
Total	**-**	**-**	**5**	**-**	**5**	**4.7**
Total isolates	**18**	**16**	**50**	**22**	**106**	**100**

[a]: Aerial mycelium colour / substrate mycelium colour (sp, presence of soluble pigment) observed on ISP4 (inorganic salts-starch agar) after 14 days of incubation at 28±2ºC (colour groups based on Tresner and Backus, 1963).
[b]: Ulu Chechuh Diseased and Healthy, Sungai Burung Diseased and Healthy soil samples

4.1.2 *In vitro* Antagonism Assay against Fungal Plant Pathogens

Out of the 106 *Streptomyces*-like strains isolated in this study, a total of 26 representative isolates that were positive inhibitor against *Colletotrichum* pathogens from all colour groups were selected for the antagonism assay against plant pathogenic fungi. Colour groups of aerial mycelium of the representative isolates are given in Table 4.3. These isolates were chosen on the basis of their gross morphological differences such as aerial and substrate mycelium colour, and their ability to grow and sporulate well on ISP4 agar. These isolates also represented the sample source from which they were isolated.

The results of the *in vitro* antagonism assay are shown in Table 4.4 and Plates 4.2, 4.3. The ability of isolates to inhibit growth of the test fungi was indicated when mycelial growth near the actinomycete plug was either partially or completely inhibited after 96 h of incubation on PDA plates. When the width of inhibition zone (X) was observed with a distance $\geq$ 23mm from the actinomycete plug, it was taken as very strong inhibition of test fungi (Table 4.4 and Plate 4.2A). In strong inhibition, the width of inhibition zone should be in between 20mm $\leq X <$ 23mm (Table 4.4 and Plate 4.2B). In moderate inhibition, there was an inhibition zone between 15mm $\leq X <$ 20mm from the actinomycete strain (Table 4.4 and Plate 4.2C). In weak inhibition, the hyphal growth was only retarded to a small degree (with a width of inhibition zone, $X <$ 15mm) (Table 4.4 and Plate 4.3D). There was no inhibition when the growth of fungus was comparable to the control (Table 4.4 and Plate 4.3E).

Table 4.3: Colours of aerial mycelium, substrate mycelium and soluble pigment of 26 representatives of *Streptomyces*-like isolates tested for *in vitro* antagonism against chilli plant pathogenic fungi (*Colletotrichum* spp.) after 14 days incubation at 28 ± 2ºC on inorganic salts-starch agar

Isolate codes	Source of samples[a]	Colour group[b]	Aerial spore mass	Substrate mycelium	Soluble pigments[c]
P3	SBD	Grey	Grey	Grey	-
P6	SBD	Grey	Grey	Grey	-
P8	SBD	Grey	Grey	Brown	Light Brown
P10	UCD	White	White	White	-
P11	SBD	Pink	Pink-White	Pink	-
P39	SBH	Grey	Grey	Red	-
P42	SBD	Grey	Grey	Grey	-
P43	UCD	Grey	Grey-White	Yellow	-
P44	SBD	Grey	Grey-White	Grey-White	-
P45	SBD	Pink	Pink-White	Pink	-
P46	UCD	White	White	White	-
P54	SBD	Grey	Grey	Grey	-
P75	SBD	Pink	Pink-White	Pink	-
P77	UCD	White	White	White	-
P78	SBD	Grey	Grey	Grey	-
P79	SBD	Pink	Pink-White	Pink	-
P83	SBD	Grey	Grey	Grey	-
P94	UCD	White	White	White	-
P98	UCD	Grey	Grey-White	Grey-White	-
P101	UCD	White	White	White	-
P103	UCH	Grey	Grey-White	Grey-White	-
P108	UCH	White	White	White	-
P113	UCH	White	White	White	-
P115	SBH	Grey	Grey-White	Grey-White	-
P121	SBD	White	White	White	-
P128	SBD	White	White	White	-

[a]: UCD, Ulu Chechuh *Colletotrichum*-infected soil sample; UCH, Ulu Chechuh healthy soil sample; SBD, Sungai Burung *Colletotrichum*-infested soil sample; SBH, Sungai Burung healthy soil sample.
[b]: Colour group agreed closely with the colour of aerial mycelium of colony (Tresner and Backus, 1963).
[c]: None

Table 4.4: Inhibition of the *in vitro* growth of 3 species of pathogenic chilli plant fungi by rhizosphere-derived actinomycetes using the cross plug assay method after 96h of incubation on PDA (Potato Dextrose Agar) plates

Isolate codes	Spore colour	*In vitro* growth inhibition of[a]		
		C. acutatum	*C. capsici*	*C. gloeosporioides*
P3	Grey	1	1	1
P6	Grey	0	0	2
P8	Grey	3	3	3
P39	Grey	4	3	4
P42	Grey	4	4	4
P43	Grey	3	3	1
P44	Grey	1	2	2
P54	Grey	1	0	2
P78	Grey	0	2	1
P83	Grey	2	2	2
P98	Grey	0	2	1
P103	Grey	0	1	0
P115	Grey	3	3	3
P10	White	0	2	2
P46	White	0	1	0
P77	White	1	4	1
P94	White	1	2	1
P101	White	0	1	0
P108	White	1	0	2
P113	White	0	3	0
P121	White	0	1	1
P128	White	1	0	2
P11	Pink	2	1	2
P45	Pink	0	1	1
P75	Pink	0	0	2
P79	Pink	1	1	2

[a]: *C. acutatum, Colletotrichum acutatum; C. capsici, Colletotrichum capsici; C. gloeosporiodes, Colletotrichum gloeosporioides;* 3 replicates were used

4 – Very strong inhibition, X ≥ 23mm (Plate 4.2A);

3 – Strong inhibition, 20mm ≤ X ≤ 23mm (Plate 4.2C);

2 – Moderate inhibition, 15mm ≤ X ≤ 20mm (Plate 4.2B);

1 – Weak inhibition, X < 15mm (Plate 4.3A);

0 – No inhibition (growth of fungus comparable to control) (Plate 4.3B).

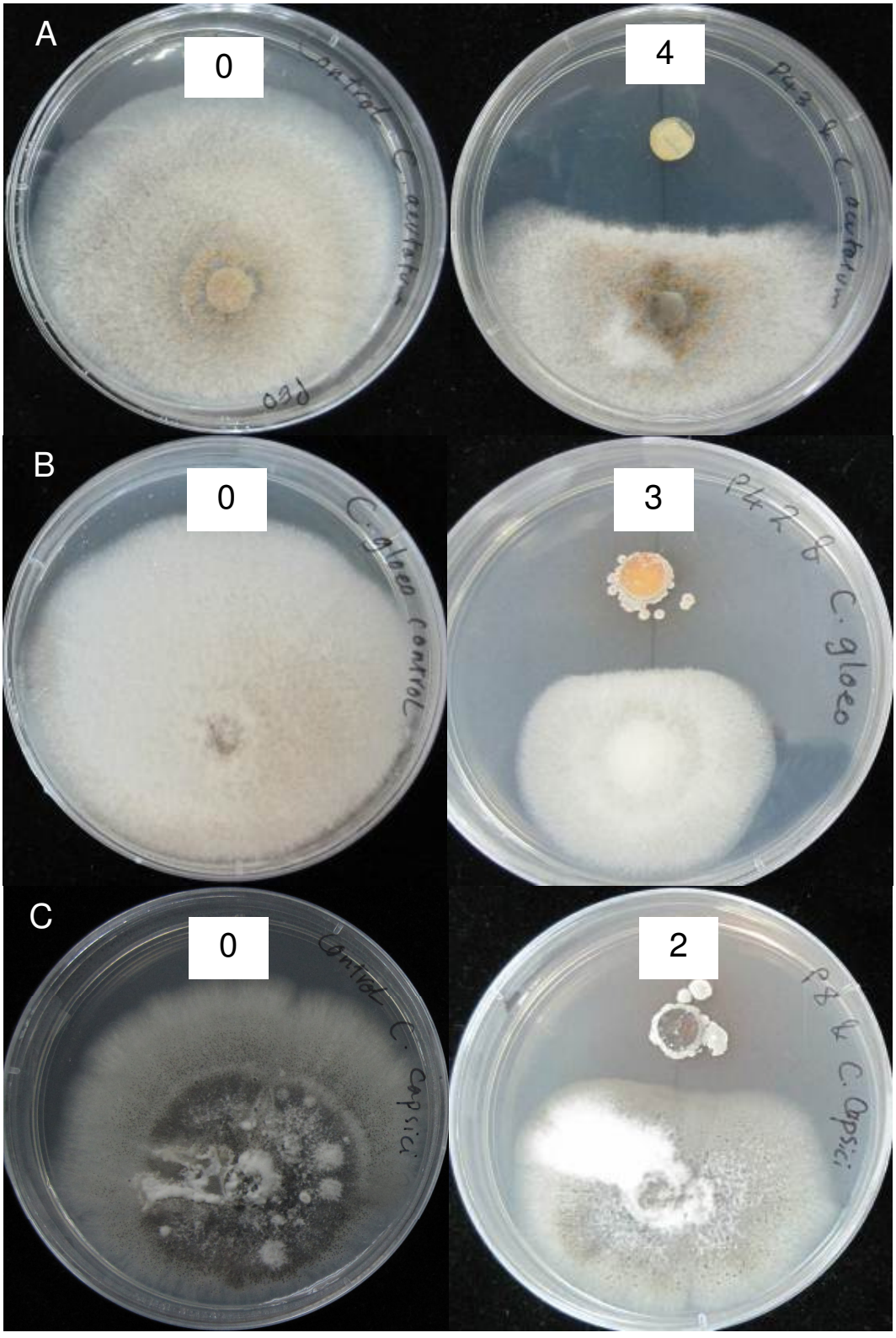

Plate 4.2: Cross-plug assay on Potato Dextrose Agar plate after 96h of incubation at 28 ± 2ºC to test the *in vitro* antagonism of actinomycete isolates against fungi
A: *Colletotrichum acutatum;* B: *Colletotrichum gloeosporioides;* C: *Colletotrichum Capsici*
(4 – Very strong inhibition, 3 – Strong inhibition, 2 – Moderate inhibition, 0 – Control).

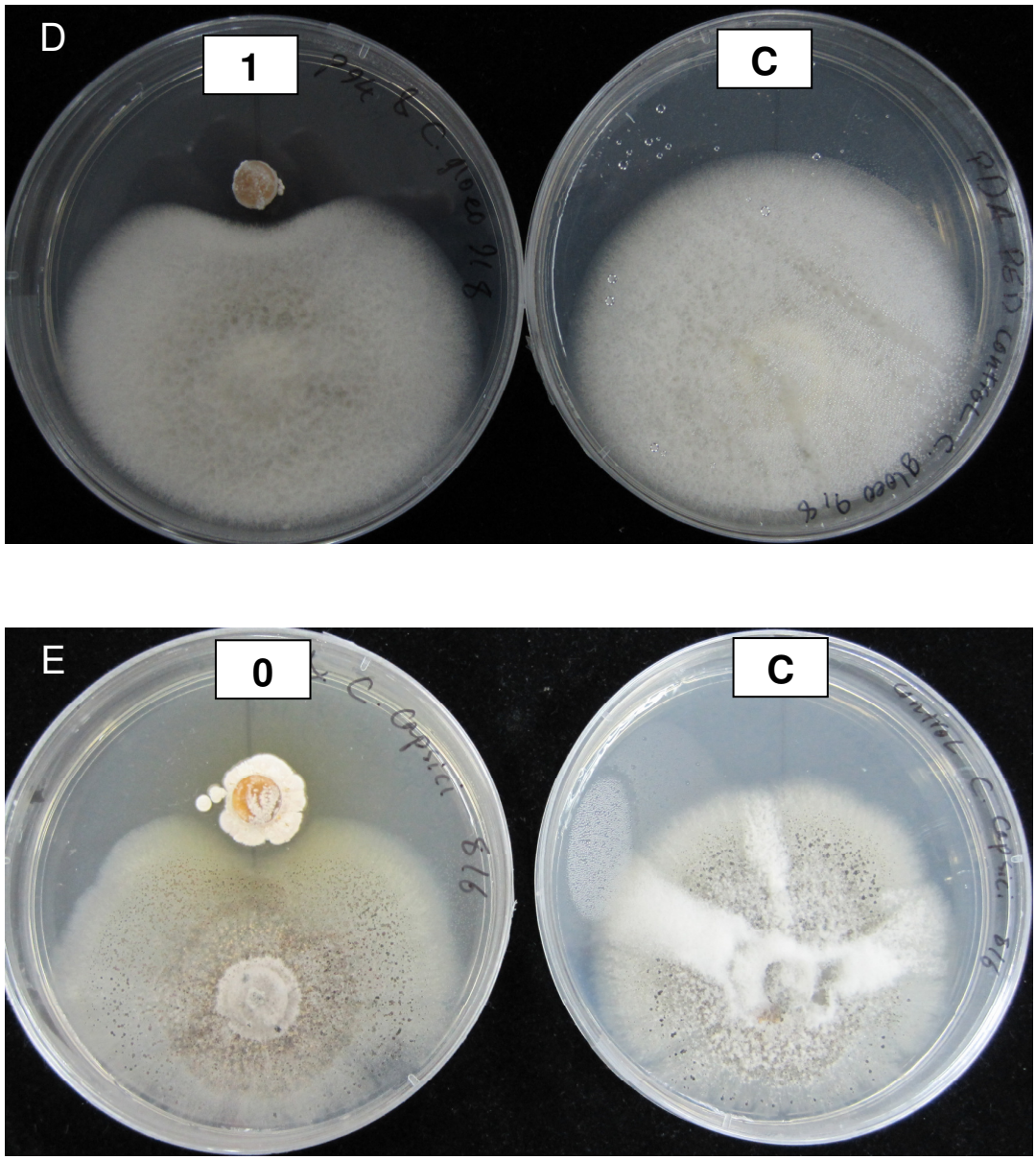

Plate 4.3: Cross-plug assay on Potato Dextrose Agar plate after 96h of incubation at 28 ± 2ºC to test the *in vitro* antagonism of actinomycete isolates against fungi
D: *Colletotrichum gloeosporioides*; E: *Colletotrichum capsici*
(1 – Weak inhibition; 0 – No inhibition; C – Control).

Figure 4.1 summarizes the overall results of the antagonism assay. Out of the 26 isolates screened, 20 (76.9%) showed very strong to moderate inhibition against one or more test fungi. The majority of these isolates were from the grey colour group of aerial mycelium. About 42.3% of the grey colour group and 23.0% of the white colour group and 11.5% of the pink group isolates tested in this study showed antagonistic activity against one or more species of *Colletotrichum*. Out of the 26 antagonistic isolates, 12 isolate or 46.1%, showed inhibitory activity at varying degrees against all three *Colletotrichum* species tested. Eight of the potential fungus-antagonistic isolates belonged to the grey colour group (P3, P8, P39, P42, P43, P44, P83, P115), two isolates belonged to the white colour group (P77, P94) and two isolates belonged to the pink colour group (P11, P79) (Plate 4.4).

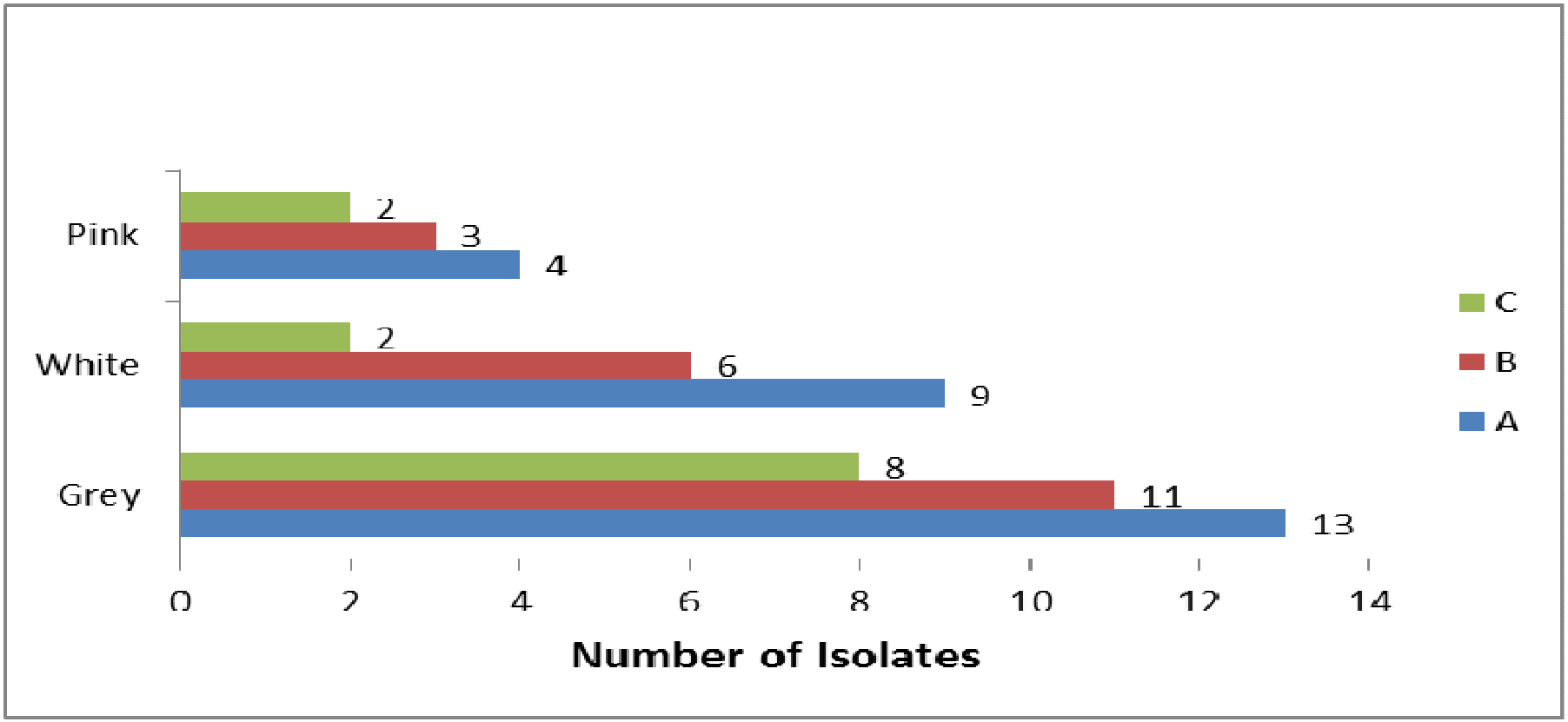

Figure 4.1: Diagram of the distribution of three colour groups of antagonistic rhizosphere-derived actinomycetes

A, Total bioactive isolates tested; B, Isolates showing very strong to moderate inhibition against one or more species of *Colletotrichum* (X<2cm); C, Isolates showing very strong to moderate inhibition against all three species of *Colletotrichum* (X>2cm).

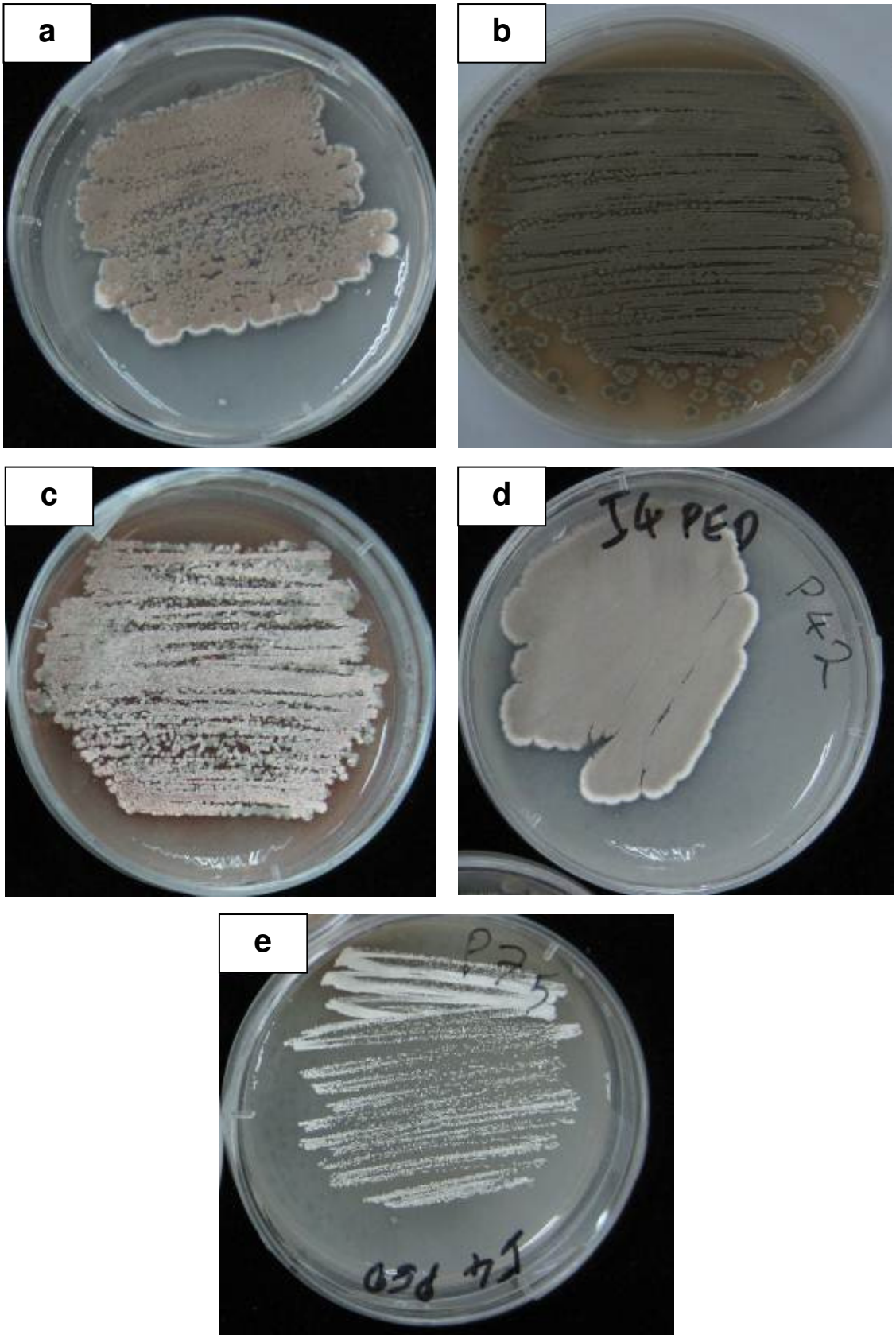

Plate 4.4: 14-day old cultures of antagonistic rhizosphere-derived actinomycete isolates grown at 28 ± 2°C on inorganic salts-starch agar (ISP4)
a - Isolate P3; b - Isolate P8; c - Isolate P39; d - Isolate P42; e - Isolate P75.

Two isolates (P39 and P42) showed very strong antagonism towards *C. gloeosporioides*. Isolates P8 and P115 showed strong antagonism and another ten isolates showed moderate antagonism (Table 4.4). *Colletotrichum gloeosporioides* was inhibited very strongly to strongly by 15.5% of the *Streptomyces*-like isolates; while, 38.5 % of the tested isolates were only moderately inhibitory against *Colletotrichum gloeosporioides*. (Table 4.4). Seven isolates (P42, P77, P39, P8, P43, P113 and P115) or 27% of the isolates were very strong to strong antagonistic while six others were moderately antagonistic towards *C. capsici* (Table 4.4). *Colletotrichum acutatum* was very strongly to strongly inhibited only by isolates P8, P39, P42, P43 and P115. Two other isolates were moderately antagonistic towards *C. acutatum* (Table 4.4). The remaining isolates showed very weak antagonism or none towards the fungal pathogens. Control cultures of all test fungi produced dense mycelial growth on PDA (Plate 4.2, 4.3).

The test fungi used in this study differed greatly from each other in their sensitivity to the antifungal effects of the actinomycetes (Figure 4.2). Three of the isolates (P8, P39, P42) inhibited three pathogenic fungi very strongly or strongly. However, 38% of the isolates tested caused a moderate inhibition. All three species of *Colletotrichum* were extremely sensitive to two of the isolates.

Three isolates (P39, P42 and P77) with potential inhibitory activity were highly inhibitory against the growth of at least one of the *Colletotrichum* sp. (Table 4.4). These isolates, particularly P42 and P39, were highly effective against three different species of *Colletotrichum*.

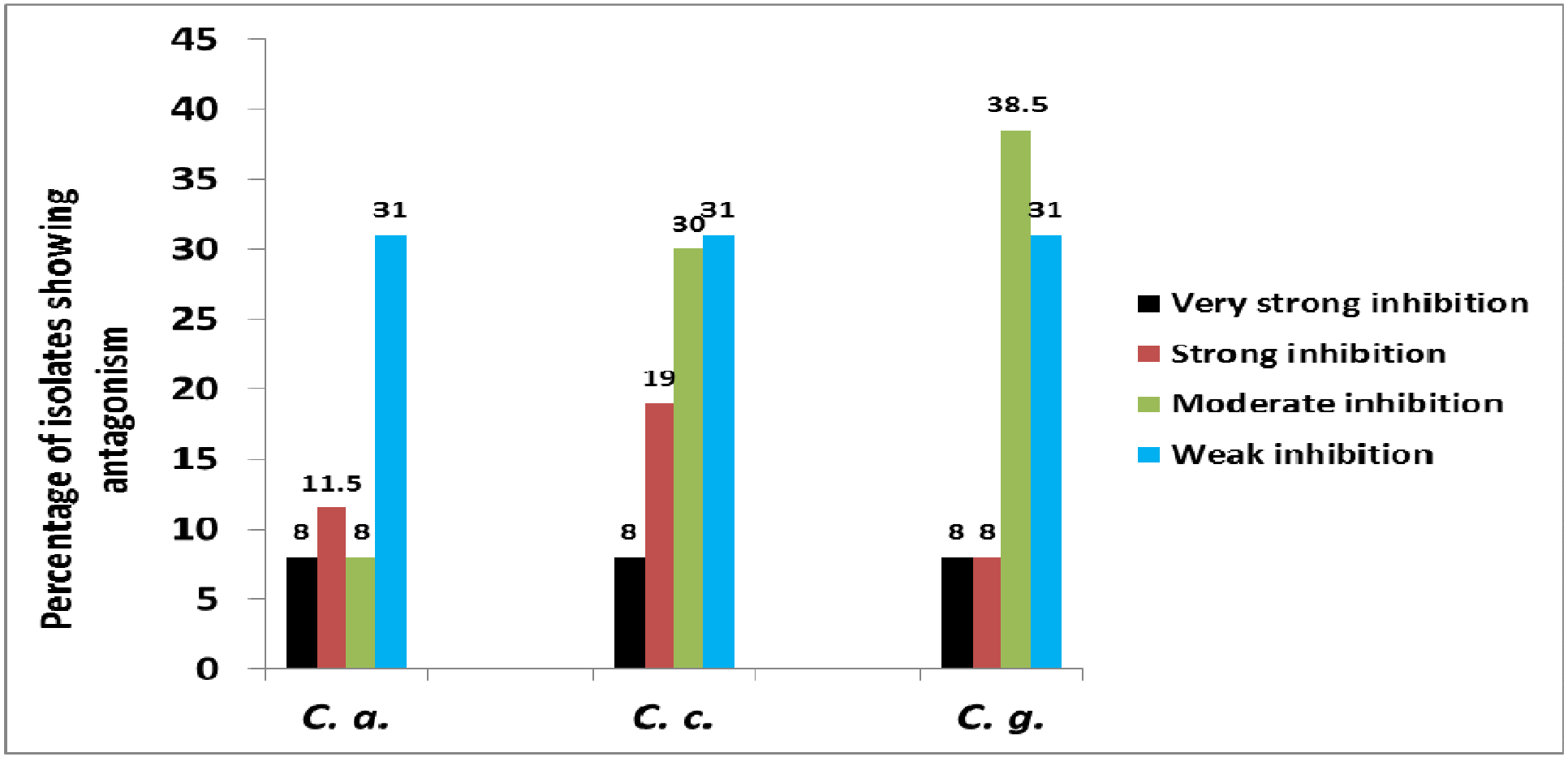

Figure 4.2: Inhibition of three different test fungi by antagonistic rhizosphere-derived actinomycete isolates (*C. a.* : *Colletotrichum acutatum*; *C. c.* : *Colletotrichum capsici; C. g.* : *Colletotrichum gloeosporioides*).

Isolate P42 which very strongly inhibited *Colletotrichum acutatum,* was also very strongly effective against *Colletotrichum capsici* and *Colletotrichum gloeosporioides* with the highest inhibition zone *in vitro*. Turhan and Grossmann (1986) observed similar trend in the antagonistic effects of actinomycetes towards different types of fungi. Their study showed that isolates which completely inhibited resistant fungi such as *Rhizoctonia solani* and *Alternaria alternata*, were also highly effective against the other test fungi studied.

The strategy used in this study was to screen for isolates with the greatest potential as biological control organisms from the standpoint of extracellular phytopathogen-inhibiting metabolites.

4.1.3 Inhibition of Linear Growth of Three Different Species of *Colletotrichum* by Isolate P42

Isolate P42 exhibited a strongest degree of inhibition on linear growth of all three species of *Colletotrichum* tested compared to isolates P8, P39 and P115. The strain showed 89% to 94% inhibition of *C. acutatum*, 84% to 90% of *C. capsici* and 81% to 88% of *C. gloeosporioides* within two to eight days of paired incubation (Figure 4.3). The antifungal activity, expressed as percent inhibition of fungal linear growth, is presented in Appendix C1. Plate 4.5 shows the growth inhibition of different pathogenic species of *Colletotrichum* by isolate P42.

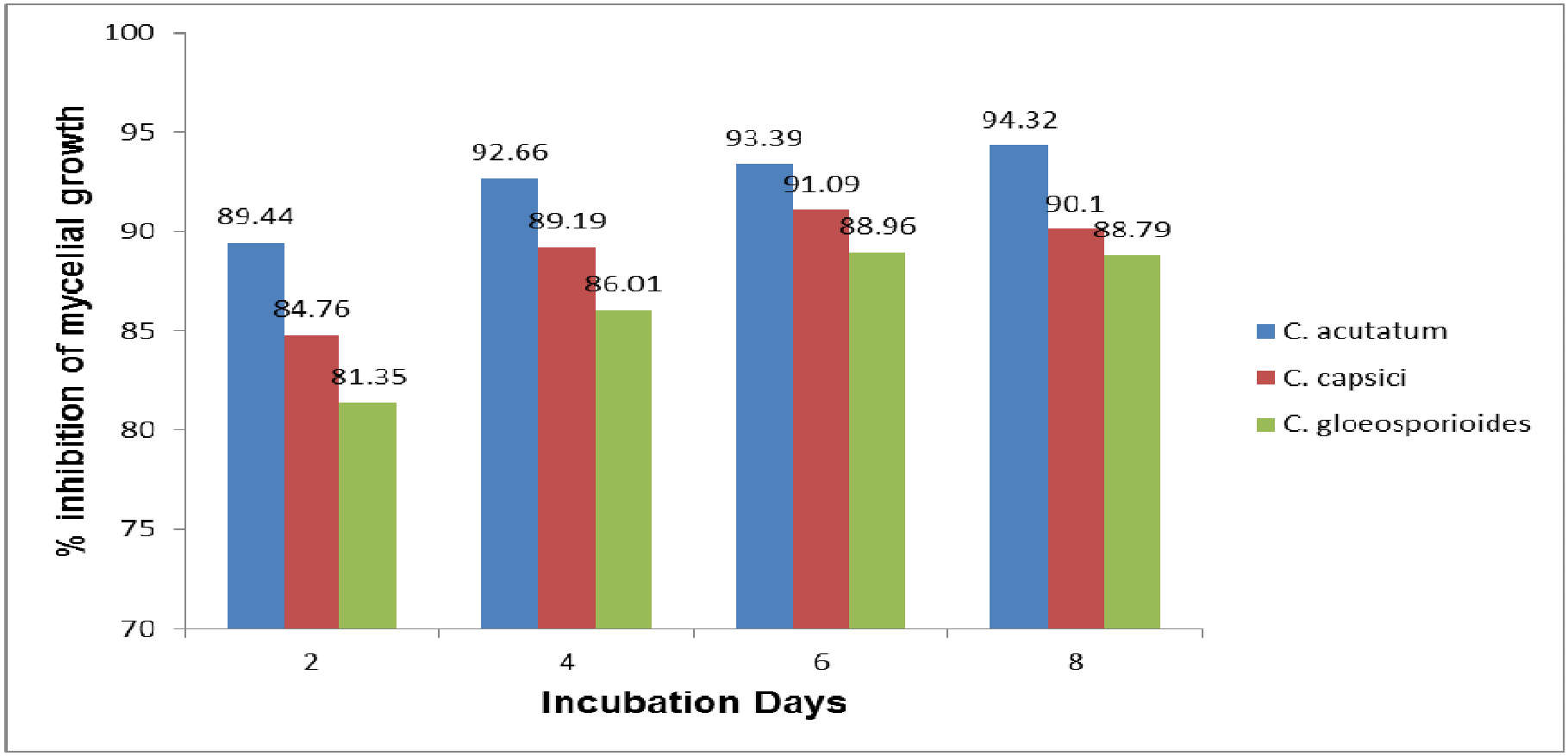

Figure 4.3: Inhibition of linear growth of mycelium of three species of *Colletotrichum* by isolate P42

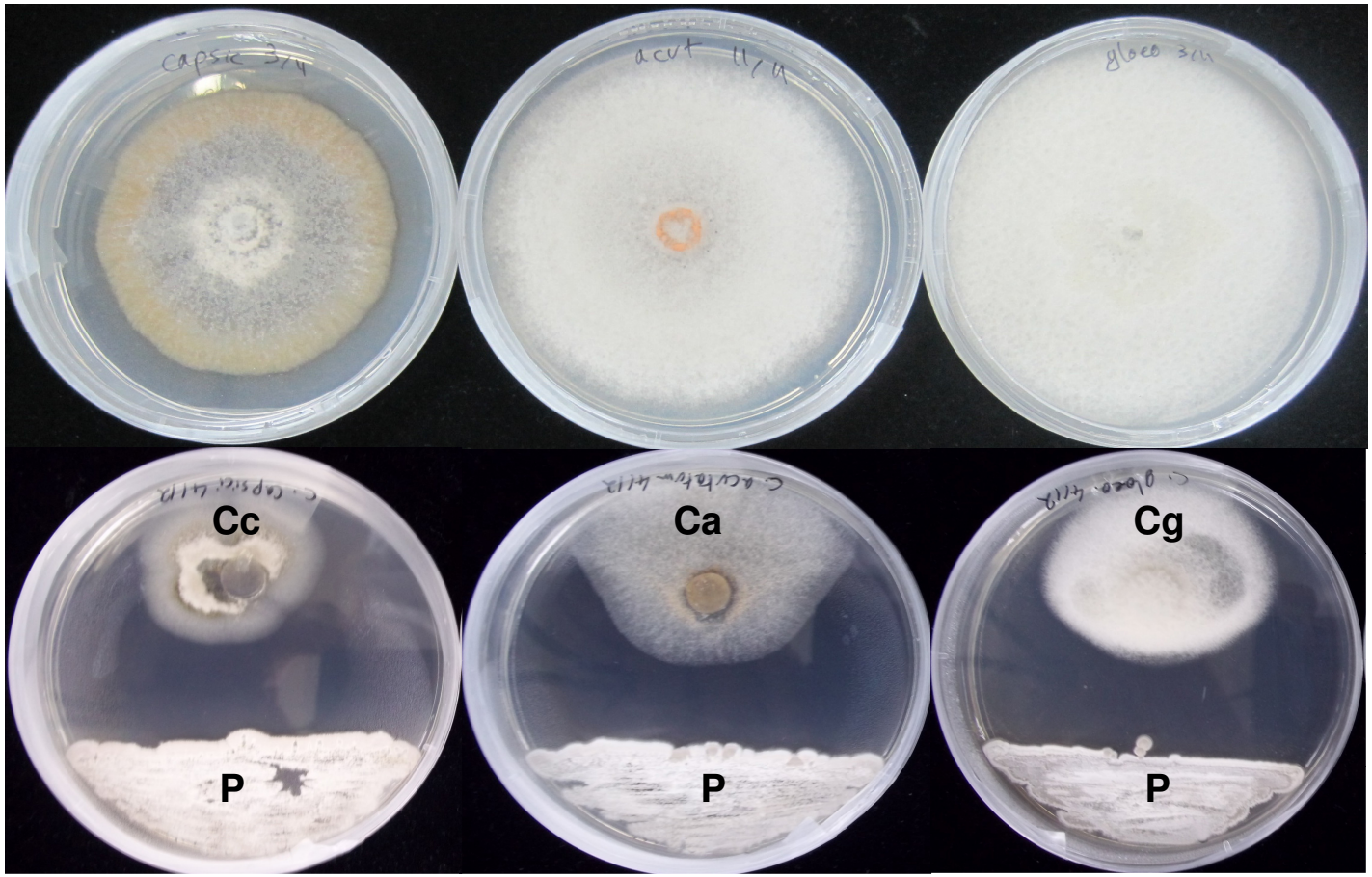

Plate 4.5: Incubation of mycelial growth of different species of *Colletotrichum* by isolate P42 on PDA
P – Isolate P42; Ca – *C. acutatum*; Cc – *C. capsici;* Cg – *C. gloeosporioides*
(Top Row – Control Plates; Bottom Row – Test Plates).

The incubation period and the different pathogenic species of *Colletotrichum* had significant influence (p=0.05) on the inhibition of *Colletotrichum* by isolate P42 (Table 4.5). Moreover, the incubation period and *Colletotrichum* species interaction showed that the percent inhibition of individual species varied significantly (p=0.05) with the incubation period (Table 4.5). Isolate P42 showed greater antagonism against *Colletotrichum acutatum* compared to *Colletotrichum capsici* and *Colletotrichum gloeosporioides*, until the end of eighth day of paired incubation (Figure 4.3). Statistical analysis showed that there was no significant difference (p=0.05) between the percentage inhibition of linear growth of *Colletotrichum capsici* and *Colletotrichum gloeosporioides* within the first two days of incubation (Appendix C2.1). Inhibition of linear growth of all three species of *Colletotrichum* increased significantly (p=0.05) after the second day (Figure 4.3; Appendices C2.2, C2.3, C2.4).

Table 4.5: Analysis of Variance for significant interaction between different species of *Colletotrichum* and incubation period on the inhibition of fungal linear growth by isolate P42

Source	Sum of Squares	DF	Mean Square	F Value	Significance Level
Replicates	34.76	3	11.59	6.32	0.1693
***Colletotrichum* sp.**	308.44	2	154.22	84.18	0.0001*
Incubation Day	280.99	3	93.66	51.12	0.0001 *
***Colletotrichum* sp. × Day**	17.95	6	2.99	1.63	0.0016*

* Significant at the 5% level ($p<0.05$)

4.1.4 Observation of Morphological Changes of Inhibited Fungal Growth by Scanning Electron Microscopy (SEM)

Four rhizosphere strains namely P8, P39, P42 and P115 which exhibited very strong and strong inhibition against three *Colletotrichum* species were chosen for scanning electron microscope studies.

Among these rhizosphere-derived strains, only strain P39 was isolated from *Colletotrichum*-free (healthy) rhizosphere soil; the remaining were isolated from *Colletotrichum*-infested rhizosphere soil. These four strains produced inhibition zones up to 23 mm on tested PDA plates with three different *Colletotrichum* species. All four strains were inhibitory against all fungi tested at varying degrees (Table 4.4). The control plate with no actinomycete strain was also prepared. In the presence of the actinomycete, the inhibition effect on the growing edge of mycelia of *Colletotrichum* was observed to be folding back when they reached the critical margin of inhibitory zone. The growth of mycelium had been stopped by the actinomycete.

Different modes of inhibition were observed against all test fungi when inhibited fungal mycelia were examined under scanning electron microscope, indicating the possibility of diffusible antifungal and bioactive substances which belonged to the actinomycete. Hyphal tip lysis, folding back, stunted mycelium, disintegrated hyphae; curling and/or bulging of hyphae were observed on the affected fungal growth (Plates 4.6 - 4.8). However, not all modes of inhibition were frequently observed for each fungus. Disintegrated hypha was mainly observed in *Colletotrichum capsici* (Plate 4.6C). Curled hyphae without disintegration were observed in all three species of *Colletotrichum* tested (Plates 4.8A,C,D). Folding back and lysed hyphae were observed in *C. gloeosporioides* (Plates 4.6B, 4.7D). Stunted hyphae were observed in *C. acutatum* (Plate 4.7A).

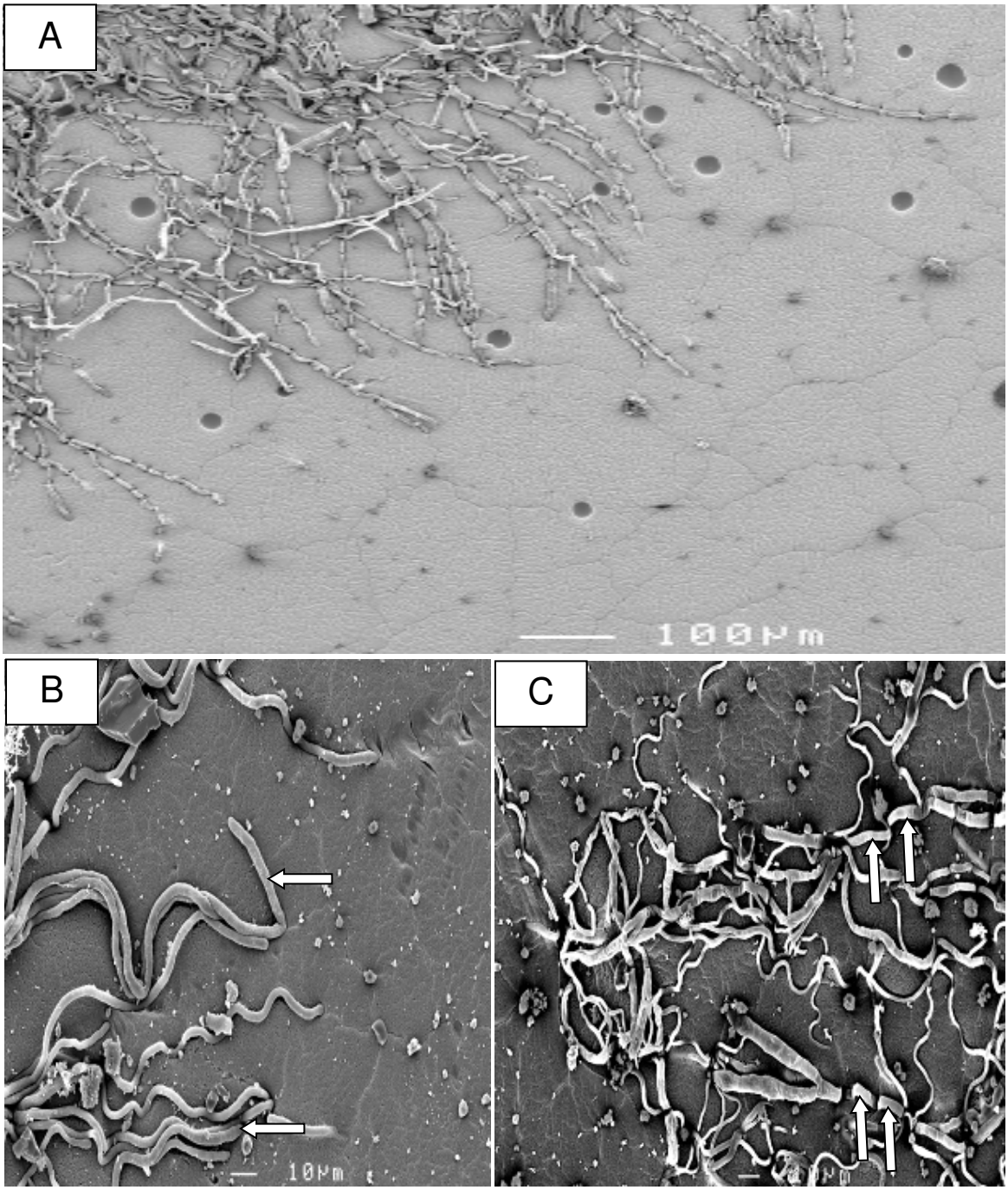

Plate 4.6: Scanning electron micrographs showing modes of fungal inhibition. A: Control plate hyphae with normal radial growth, Bar=100µm; B: Folding back and lysed hyphal tip (Observed in *C. gloeosporioides*) Bar=10µm; C: Fragments of disintegrated hyphae (Observed in *C. capsici*) Bar=10µm.

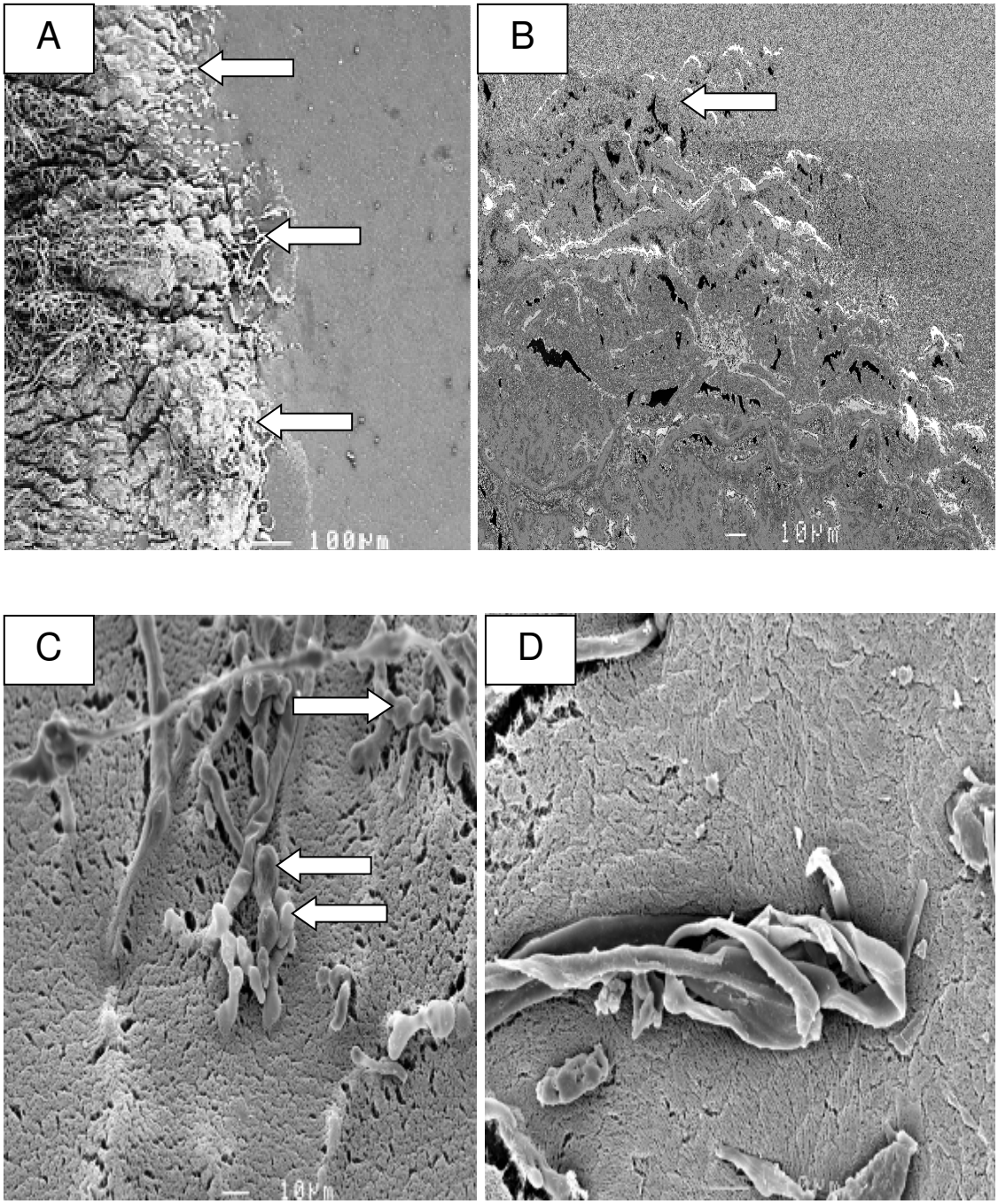

Plate 4.7: Scanning electron micrographs showing modes of fungal inhibition. A: Stunted mycelia (Observed in *C. acutatum*) Bar=100µm; B,C: Bulging hyphae (Observed in *C. capsici*) Bar=10µm; D: Folding back and lysed hyphae (Observed in *C. gloeosporioides*) Bar=10µm.

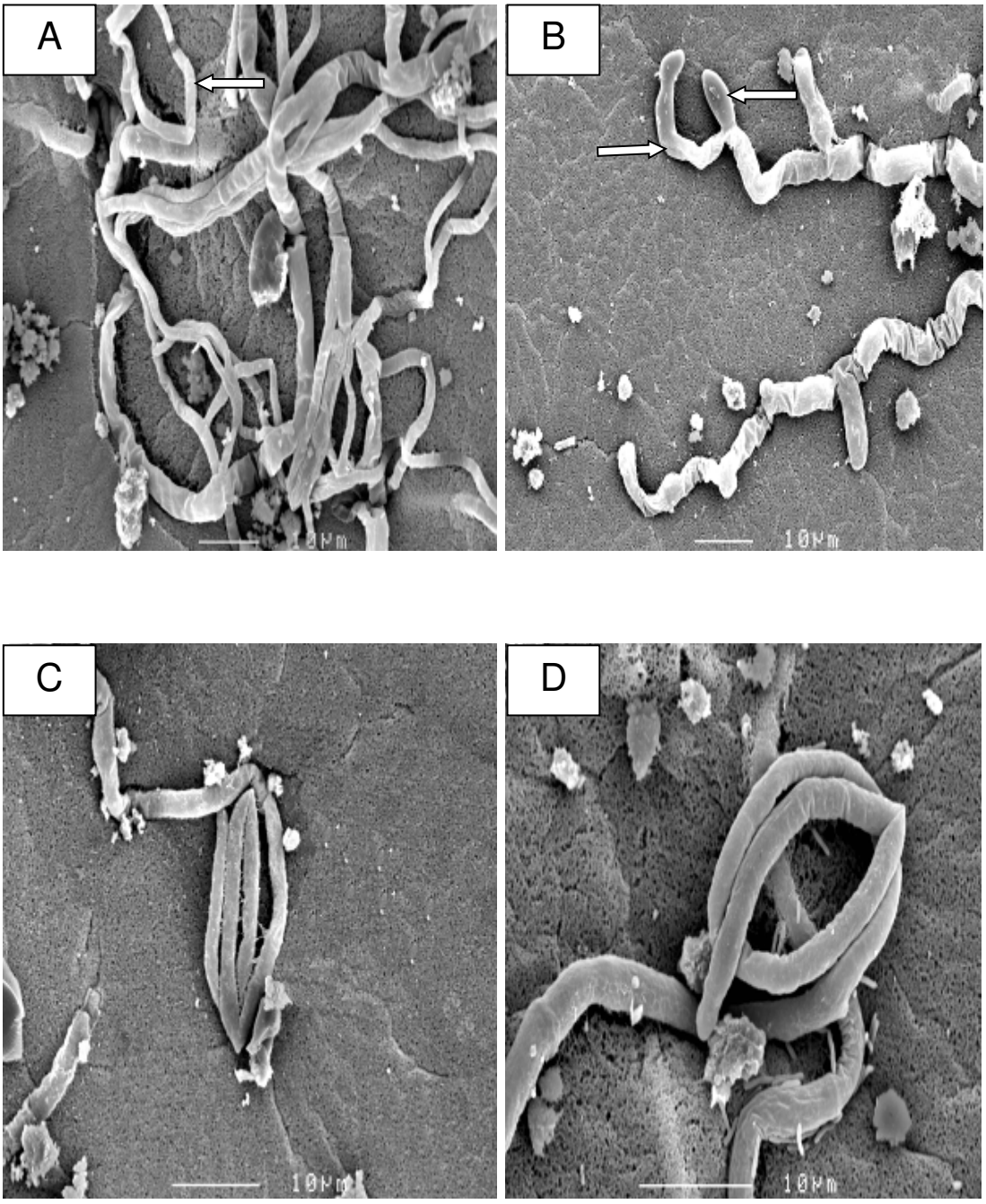

Plate 4.8: Scanning electron micrographs showing modes of fungal inhibition. A: Curling of hyphae (Observed in *C. capsici*) Bar=100µm; B: Folding back and bulging of hyphae (Observed in *C. gloeosporioides*) Bar=100µm; C: Curling of hyphae (Observed in *C. gloeosporioides*) Bar=100µm; D: Curling of hyphae (Observed in *C. acutatum*) Bar=100µm.

4.2 Characterization of Potential Actinomycete Antagonist of *Colletotrichum* spp.

4.2.1 Chemotaxonomic and Micromorphological Characteristics of Antagonistic Chilli Rhizosphere-Derived Actinomycetes

Eight (P8, P11, P39, P42, P43, P45, P115 and P44) out of nine potential inhibitor antagonistic strains tested contained the LL-DAP and thus had cell wall chemotype I (Lechevalier & Lechevalier, 1970). Strain P77 (Plate 4.9) did not contain LL-DAP, so did not have cell wall chemotype I. Based on the cell wall composition, these eight strains could be classified as species of *Streptomyces*. The thin layer chromatogram of DAP isomers in nine of the tested strains is given in Plate 4.9.

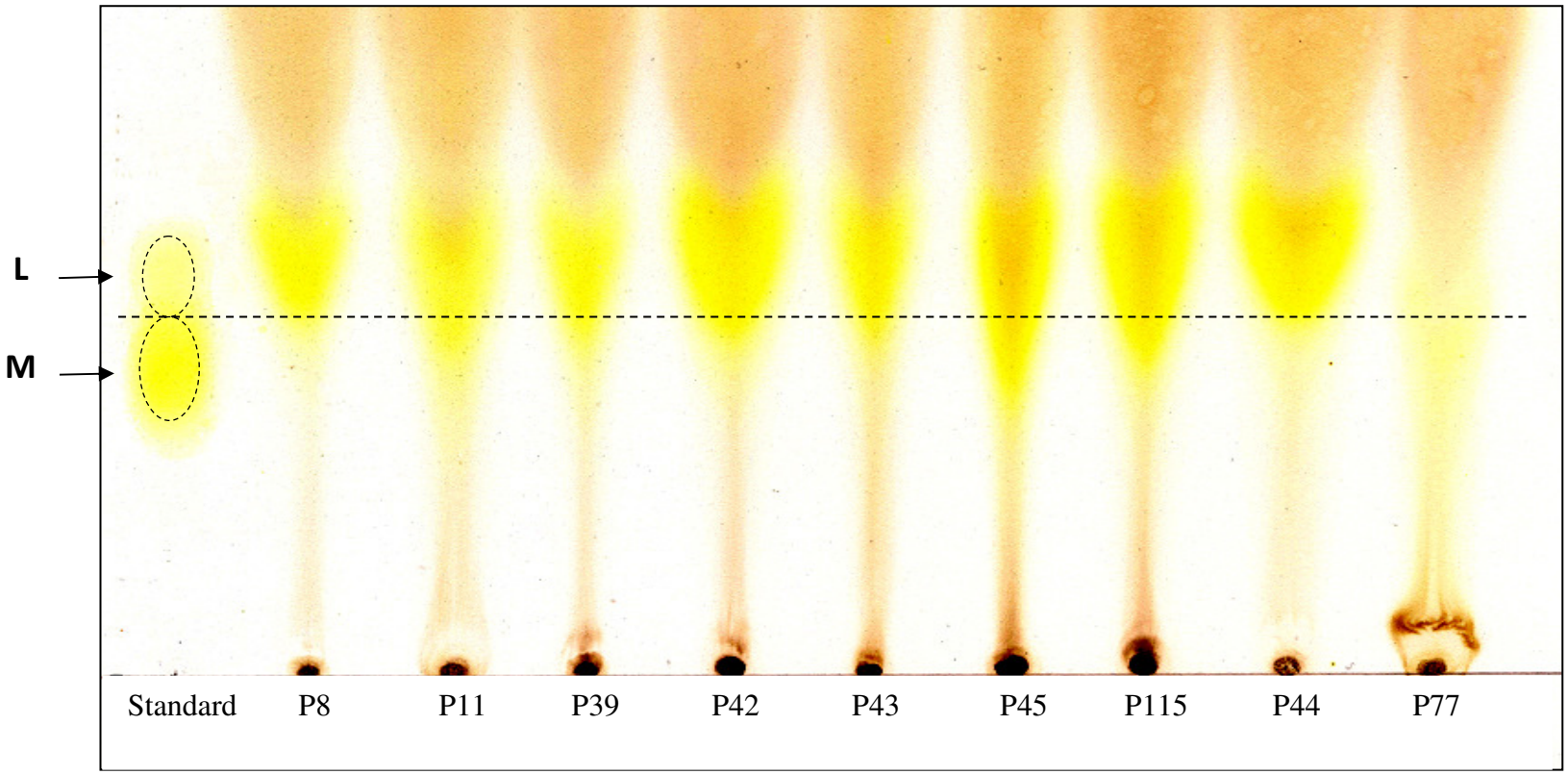

Plate 4.9: Thin layer chromatogram of diaminopimelic acid (DAP) isomers with LL-DAP as the faster moving spot.
L: LL-DAP; M: *Meso*-DAP.

Observation of coverslip cultures by light and scanning electron microscope showed that the four selected isolates were polysporous actinomycetes (e.g. *Streptomyces* spp.) producing long spore chains of varying structures on the aerial hyphae (Plates 4.10, 4.11). The single spores were ovoid-shape and developed on short tips of unbranched sporophores as (Vobis *et al.,* 1997) has described.

Different spore chain structures observed in this study were straight/flexuous (*Rectiflexibiles*), hooked/open looped/with irregular spirals (*Retinaculiaperti*) and closed, compact spirals (*Spirals*), with smooth spore surface as revealed by SEM (Plates 4.10, 4.11).

Light Micrograph SEM Micrograph

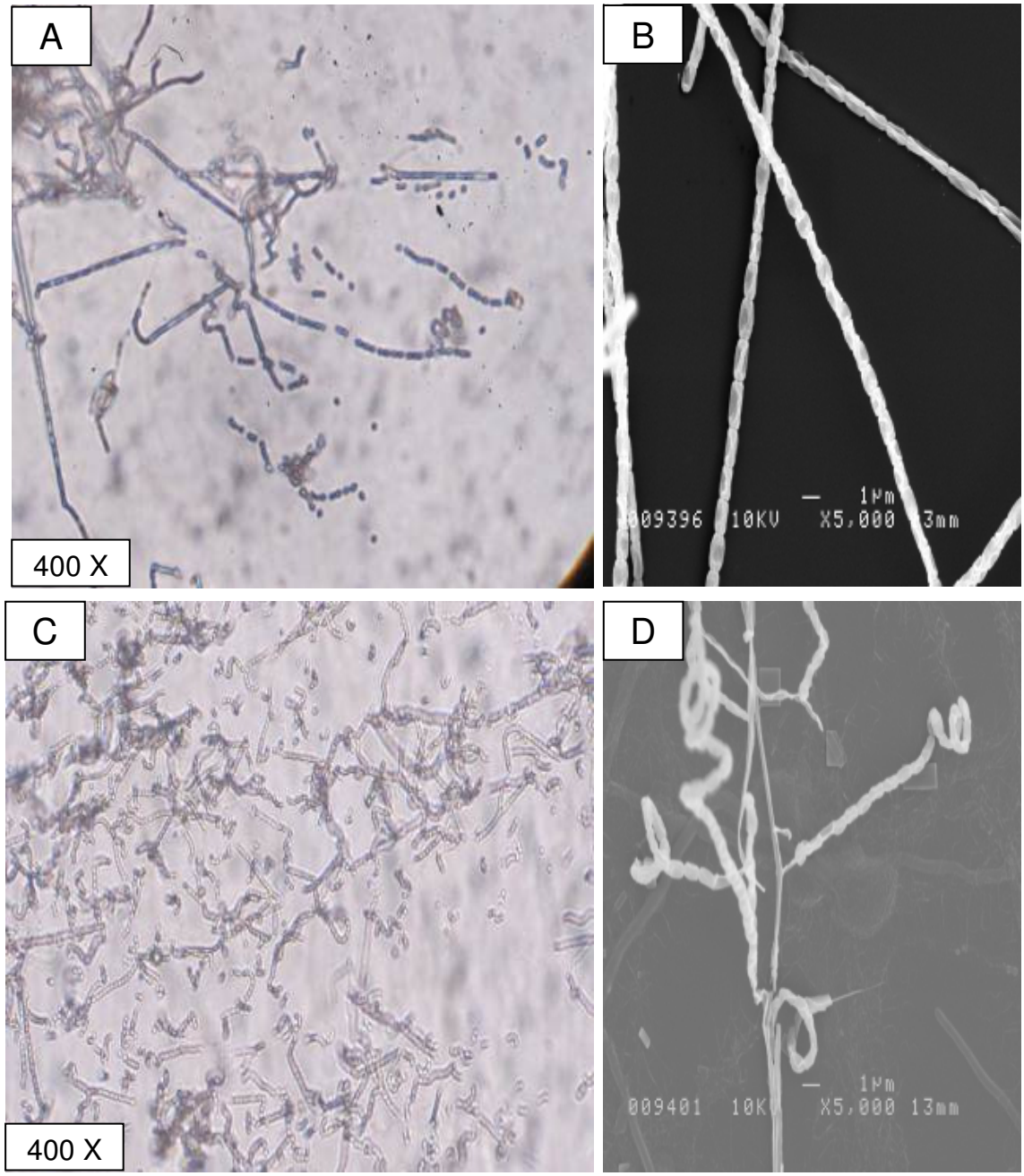

Plate 4.10: Coverslip cultures of streptomycete strains after 14 days of incubation. A: Light micrograph of strain P8 spores (observation at 400x magnification); B: Scanning electron micrograph of strain P8 spores (Bar=1µm); C: Light micrograph of strain P39 spores (observation at 400x magnification); D: Scanning electron micrograph of strain P39 spores (Bar=1µm).

Light Micrograph SEM Micrograph

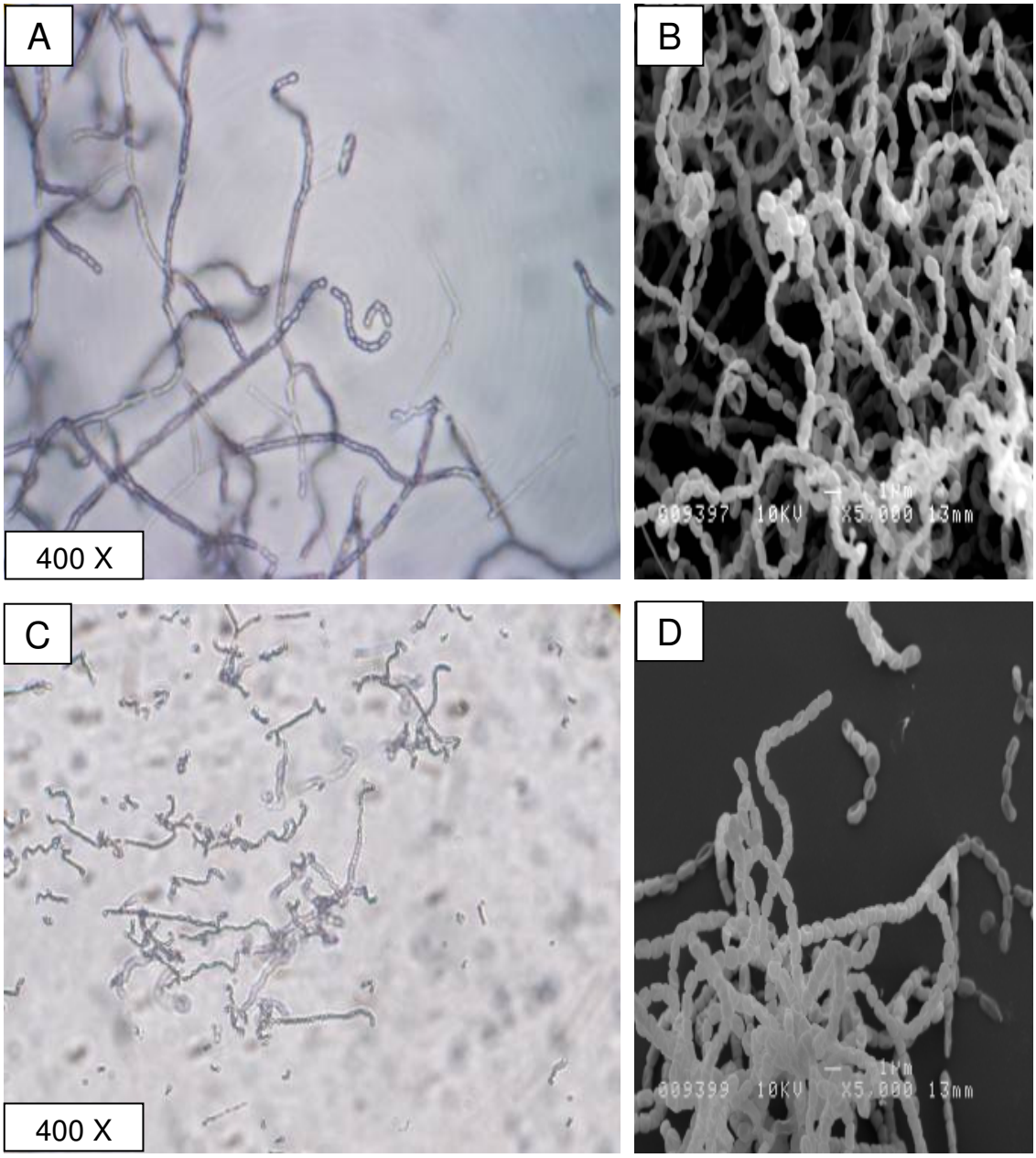

Plate 4.11: Coverslip cultures of streptomycete strains after 14 days of incubation. A: Light micrograph of strain P42 spores (observation at 400x magnification); B: Scanning electron micrograph of strain P42 spores (Bar=1µm); C: Light micrograph of strain P115 spores (observation at 400x magnification); D: Scanning electron micrograph of strain P115 spores (Bar=1µm).

4.2.2 Morphological Characteristics of Antagonistic Actinomycetes

A comparison of morphological characteristics showed that the four selected strains were closely related to family *Streptomycetaceae* showed in Tables 4.6, 4.7, 4.8 and 4.9. The results were compared with those strains assigned to the most relevant cluster.

4.2.3. Cultural and Some Physiological Characteristic of Antagonistic Actinomycetes

Spore mass colour of the four selected strains has shown in Tables 4.10, 4.11, 4.12 and 4.13 Production of distinctive colour in substrate mycelium, diffusible pigment and melanin production of these strains on agar media were observed. The cultural and some physiological characteristics of morphologically related species of *Streptomyces* assigned to each cluster are also given in Tables 4.10 - 4.13. The cultural characteristics of the four strains on various agar media are given in Table 4.15. According the results revealed in Table 4.14, strain P8 had a good growth on ISP2, oatmeal agar (ISP3) and ISP4, but poor on ISP5 and moderate on the other test media. Strain P39 showed a good growth on ISP3, ISP4 and ISP7 but moderate growth on ISP2 and poor growth on ISP5 and ISP6. Strain P42 showed good growth on ISP2, ISP3, ISP4 and ISP6 but moderate growth on ISP5 and ISP7. Strain P115 had moderate growth on ISP2 and ISP3 but poor growth on other media. Soluble pigments were produced in ISP2, ISP3 and ISP6 when strain P8 was streaked on mentioned media and incubated for 14 days.

Aerial mycelium of strains after 14 days of incubation on ISP4 and ISP3 were shown in Plates 4.12, 4.13 and 4.14. On continued incubation, parts of the colony's colour changed. The colour of substrate mycelium of strains was varied (Plate 4.12).

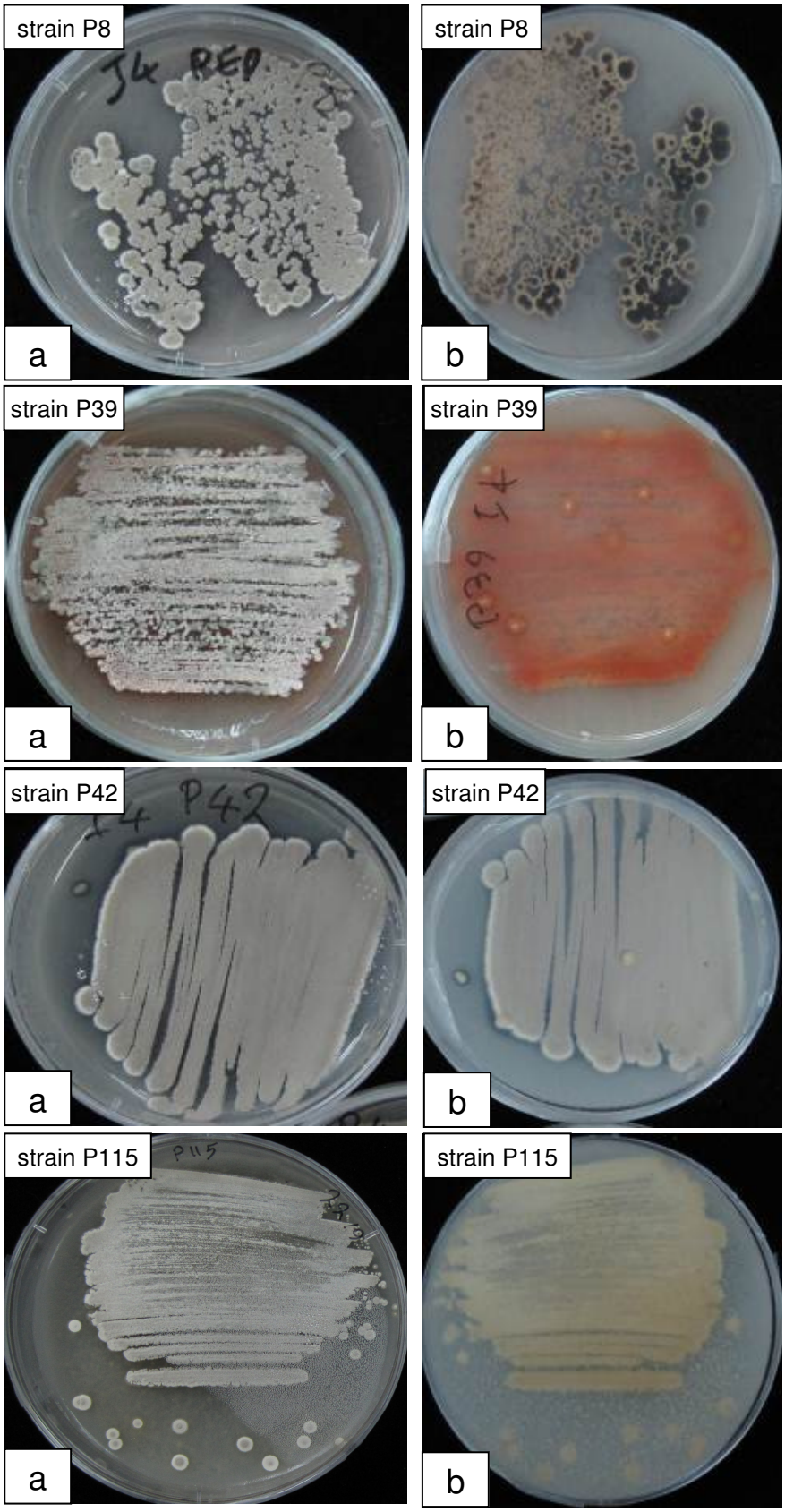

Plate 4.12: 14 day old cultures of 4 selected strains grown on ISP4 and ISP3. a - Aerial mycelium and spore mass; b - Reverse showing substrate mycelium

Table 4.6: Morphological characteristics of strain P8

Characteristics	Strain P8	*S. purpeofuscus*	*K. azatica*	*S. misakiensis*	*K. putterlickiae*
Morphology and Pigmentation:					
Presence of Aerial Mycelium	+	+	+	+	+
Spore Chain Morphology:					
Rectus Flexibilis	+	+	+	+	+
Retinaculum Apertum	-	-	-	-	-
Spirales	-	-	-	-	-
Verticillate	-	-	-	-	-
Spore Chain Surface:					
Smooth	+	+	+	+	+
Warty	-	-	-	-	-
Spiny	-	-	-	-	-
Hairy	-	-	-	-	-
Rugose	-	-	-	-	-
Production of Aerial Spore Mass	+	+	+	+	+
Fragmentation of Substrate Mycelium	-	-	-	-	-
Sclerotia Formation	-	-	-	-	-
Sporulation on Substrate Mycelium	-	-	-	-	-

+, Positive response; -, Negative response

Table 4.7: Morphological characteristics of strain P39

Characteristics	Strain P39	*S. violaceorubidus*	*S. tendae*	*S. tritolerans*
Morphology and Pigmentation:				
Presence of Aerial Mycelium	+	+	+	+
Spore Chain Morphology:				
Rectus Flexibilis	-	-	+	+
Retinaculum Apertum	-	-	-	-
Spirales	+	+	-	-
Verticillate	-	-	-	-
Spore Chain Surface:				
Smooth	+	+	+	+
Warty	-	-	-	-
Spiny	-	-	-	-
Hairy	-	-	-	-
Rugose	-	-	-	-
Production of Aerial Spore Mass	+	+	+	+
Fragmentation of Substrate Mycelium	-	-	-	-
Sclerotia Formation	-	-	-	-
Sporulation on Substrate Mycelium	-	-	-	-

+, Positive response; -, Negative response

Table 4.8: Morphological characteristics of strain P42

Characteristics	Strain P42	*S. rochei*	*S. enissocaesilis*	*S. plicatus*	*S. vinaceusdrappus*
Morphology and Pigmentation:					
Presence of Aerial Mycelium	+	+	+	+	+
Spore Chain Morphology:					
Rectus Flexibilis	-	-	-	-	-
Retinaculum Apertum	-	-	-	-	-
Spirales	+	+	+	+	+
Verticillate	-	-	-	-	-
Spore Chain Surface:					
Smooth	+	+	+	+	+
Warty	-	-	-	-	-
Spiny	-	-	-	-	-
Hairy	-	-	-	-	-
Rugose	-	-	-	-	-
Production of Aerial Spore Mass	+	+	+	+	+
Fragmentation of Substrate Mycelium	-	-	-	-	-
Sclerotia Formation	-	-	-	-	-
Sporulation on Substrate Mycelium	-	-	-	-	-

+, positive response; -, negative response

Table 4.9: Morphological characteristics of strain P115

Characteristics	Strain P115	*S. rubrogriseus*	*S. tricolor*	*S. linomycini*
Morphology and Pigmentation:				
Presence of Aerial Mycelium	+	+	+	+
Spore Chain Morphology:				
Rectus Flexibilis	-	-	-	-
Retinaculum Apertum	-	-	-	+
Spirales	+	+	+	-
Verticillate	-	-	-	-
Spore Chain Surface:				
Smooth	+	+	+	+
Warty	-	-	-	-
Spiny	-	-	-	-
Hairy	-	-	-	-
Rugose	-	-	-	-
Production of Aerial Spore Mass	+	+	+	+
Fragmentation of Substrate Mycelium	-	-	-	-
Sclerotia Formation	-	-	-	-
Sporulation on Substrate Mycelium	-	-	-	-

+, Positive response; -, Negative response

Table 4.10: Cultural and some physiological characteristics of strain P8

Characteristics	Strain P8	*S. purpeofuscus*	*K. azatica*	*S. misakiensis*	*K. putterlickiae*
Colour of Spore Mass:					
Red	-	-	-	-	-
Yellow	-	-	-	-	-
Grey	+	+	-	+	+
Green	-	-	-	-	-
Blue	-	-	-	-	-
Violet	-	-	-	-	-
White	-	-	+	-	-
Distinctive Substrate Mycelial Pigments	-	-	-	-	-
Pigmentation of Substrate Mycelium:					
Red/Orange	-	-	-	-	-
Green	-	-	-	-	-
Blue	-	-	-	-	-
Violet	-	-	-	-	-
Production of Diffusible Pigments	+*	+	-	-	-
Pigmentation of Diffusible Pigments:					
Red/Orange	-	-	-	-	-
Yellow/Brown	+*	+	-	-	-
Green	-	-	-	-	-
Blue	-	-	-	-	-
Violet	-	-	-	-	-
Melanin Production on Peptone-Yeast-Iron Agar and Tyrosine Agar	+	+	-	+	-

+, Positive response; -, Negative response
*, Observed on ISP2, ISP3 and ISP6

Table 4.11: Cultural and some physiological characteristics of strain P39

Characteristics	Strain P39	*S. violaceorubidus*	*S. tendae*	*S. tritolerans*
Colour of Spore Mass:				
Red	-	-	-	-
Yellow	-	-	-	-
Grey	+	+	-	-
Green	-	-	-	-
Blue	-	-	-	-
Violet	-	-	-	-
White	-	-	+	+
Distinctive Substrate Mycelial Pigments	-	-	-	-
Pigmentation of Substrate Mycelium:				
Red/Orange	+	+	-	-
Green	-	-	-	-
Blue	-	-	-	-
Violet	-	-	-	-
Production of Diffusible Pigments	-	-	+	-
Pigmentation of Diffusible Pigments:				
Red/Orange	-	-	-	-
Yellow/Brown	-	-	+	-
Green	-	-	-	-
Blue	-	-	-	-
Violet	-	-	-	-
Melanin Production on Peptone-Yeast-Iron Agar and Tyrosine Agar	-	-	-	-

+, Positive response; -, Negative response

Table 4.12: Cultural and some physiological characteristics of strain P42

Characteristics	Strain P42	*S. rochei*	*S. enissocaesilis*	*S. plicatus*	*S. vinaceusdrappus*
Colour of Spore Mass:					
Red	-	-	-	-	-
Yellow	-	-	-	-	-
Grey	+	+	-	+	+
Green	-	-	-	-	-
Blue	-	-	-	-	-
Violet	-	-	-	-	-
White	-	-	-	-	-
Distinctive Substrate Mycelial Pigments	-	-	-	-	-
Pigmentation of Substrate Mycelium:	-	-	-	-	-
Red/Orange	-	-	-	-	-
Green	-	-	-	-	-
Blue	-	-	-	-	-
Violet	-	-	-	-	-
Production of Diffusible Pigments	-	-	-	-	-
Pigmentation of Diffusible Pigments:					
Red/Orange	-	-	-	-	-
Yellow/Brown	-	-	-	-	-
Green	-	-	-	-	-
Blue	-	-	-	-	-
Violet	-	-	-	-	-
Melanin Production on Peptone-Yeast-Iron Agar and Tyrosine Agar	-	-	-	-	-

+, Positive response; -, Negative response

Table 4.13: Cultural and some physiological characteristics of strain P115

Characteristics	Strain P115	*S. rubrogriseus*	*S. tricolor*	*S. linomycini*
Colour of Spore Mass:				
Red	-	-	-	-
Yellow	-	-	-	-
Grey	-	+	+	+
Green	-	-	-	-
Blue	-	-	-	-
Violet	-	-	-	-
White	+	-	-	-
Distinctive Substrate Mycelial Pigments	-	-	-	-
Pigmentation of Substrate Mycelium:				
Red/Orange	-	-	-	-
Green	-	-	-	-
Blue	-	-	-	-
Violet	-	-	-	-
Production of Diffusible Pigments	-	-	-	-
Pigmentation of Diffusible Pigments:				
Red/Orange	-	-	-	-
Yellow/Brown	-	-	-	-
Green	-	-	-	-
Blue	-	-	-	-
Violet	-	-	-	-
Melanin Production on Peptone-Yeast-Iron Agar and Tyrosine Agar	-	-	-	-

+, Positive response; -, Negative response

Table 4.14: Cultural characteristics of four selected strains after 14 days of growth at 28±2ºC on various agar medium

Sample	Agar Medium	Growth	Aerial Spore Mass and Color	Substrate Mycelium Color	Soluble Pigment
P8	ISP2	Good	Good/Grey	Dark Brown	Brown
	ISP3	Good	Good/Grey	Dark Brown	Brown
	ISP4	Good	Good/Grey	Dark Brown	No
	ISP5	Poor	Poor /Light Brown	Brown	No
	ISP6	Moderate	Moderate/Grey	Brown	Brownish Orange
	ISP7	Moderate	Moderate/Brown	Brown	No
P39	ISP2	Moderate	Moderate/Grey	Dark Red	No
	ISP3	Good	Good/Grey	Reddish Orange	No
	ISP4	Good	Good/Grey	Reddish Brown	No
	ISP5	Poor	Poor /Pastel Red	Orange Red	No
	ISP6	Poor	Poor /Yellowish Grey	Brownish Orange	No
	ISP7	Good	Good/Greyish Orange	Vivid Red	No
P42	ISP2	Good	Good/Grey	White	No
	ISP3	Good	Good/Grey	Green	No
	ISP4	Good	Good/Grey	Grey	No
	ISP5	Moderate	Moderate/White	White	No
	ISP6	Good	Good/White	Pale Yellow	No
	ISP7	Moderate	Moderate/Grey	Reddish Brown	No
P115	ISP2	Moderate	Moderate/White	Pale Yellow	No
	ISP3	Moderate	Moderate/White	Light Green	No
	ISP4	Poor	Poor /White	Light Grey	No
	ISP5	Poor	Poor /Pale Brown	Pale Brown	No
	ISP6	Poor	Poor /Pale Brown	Pale Brown	No
	ISP7	Poor	Poor /Pale Yellow	Pale Brown	No

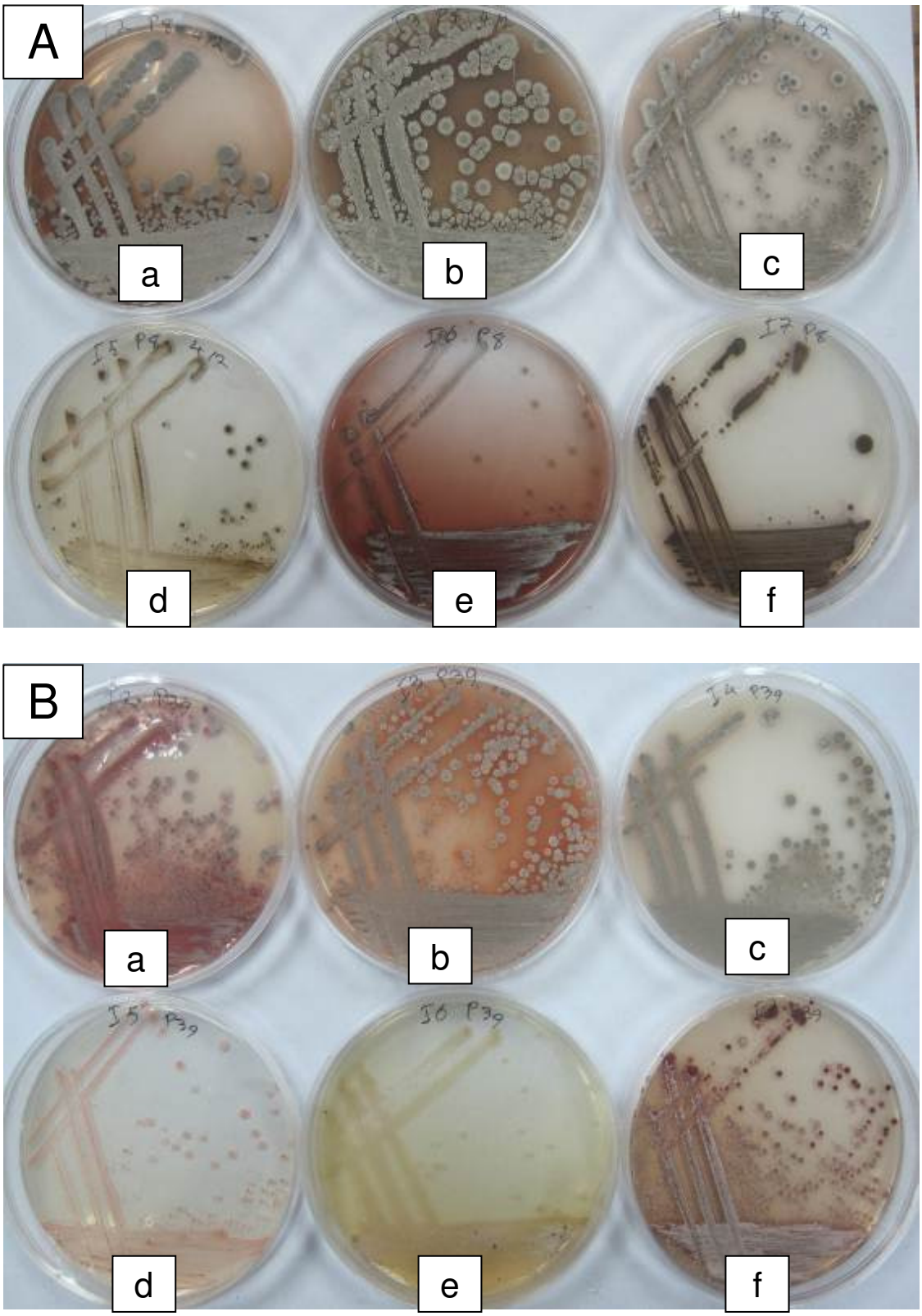

Plate 4.13: Culture characteristics of the selected antagonistic actinomycete strain P8 (A) and strain P39 (B) after 14 days of growth at 28 ± 2ºC on six different ISP medium: a: ISP2; b: ISP3; c: ISP4; d: ISP5; e: ISP6; f: ISP7.

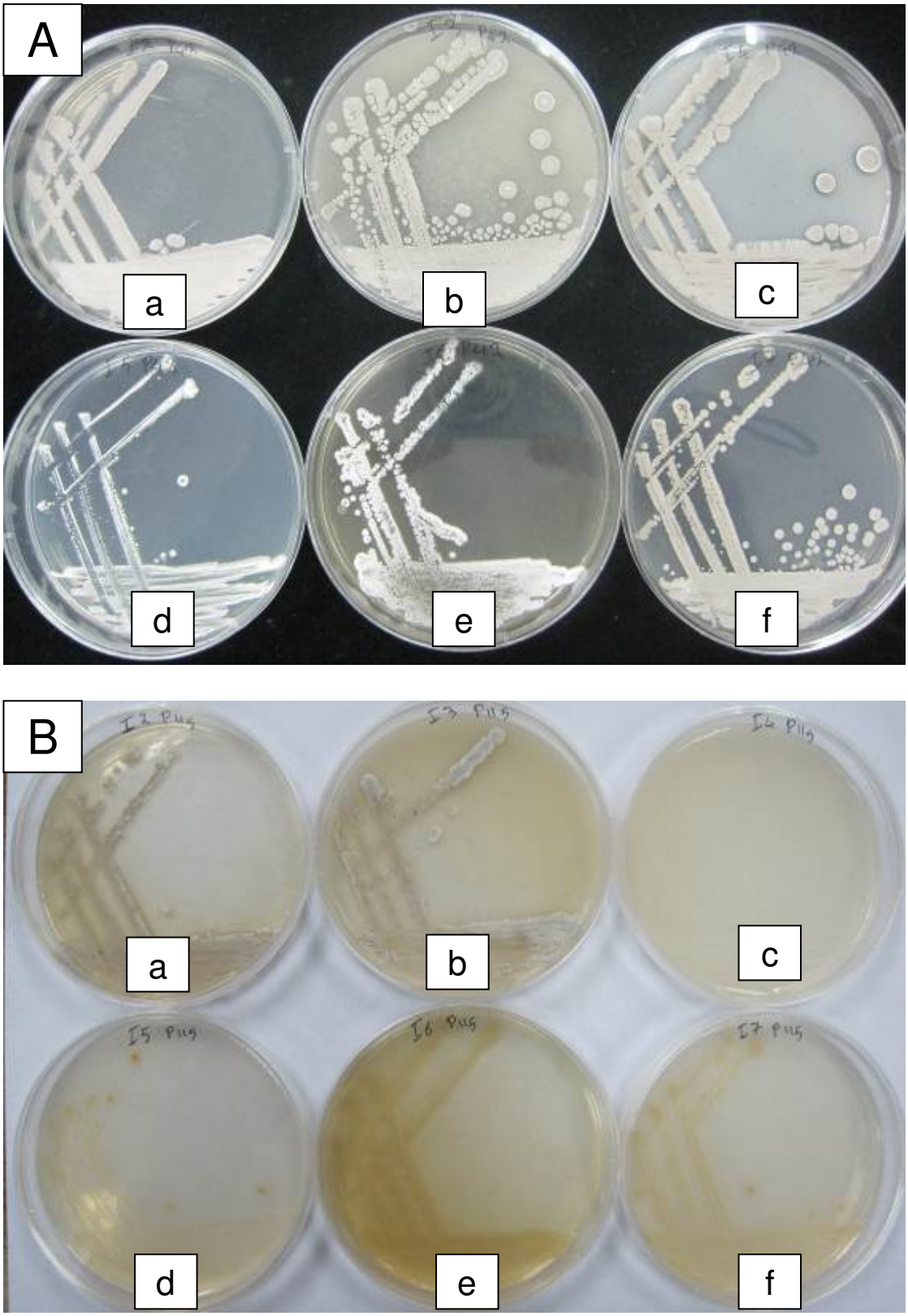

Plate 4.14: Culture characteristics of the selected antagonistic actinomycete strain P42 (A) and strain P115 (B) after 14 days of growth at 28 ± 2ºC on six different ISP medium: a: ISP2; b: ISP3; c: ISP4; d: ISP5; e: ISP6; f: ISP7.

4.2.4 Other Physiological Characteristics of Antagonistic Actinomycetes

4.2.4.1 Degradation Activity

Physiological characteristics of four selected strains are summarized in Table 4.15. Strain P39, P42 and P155 degraded L-tyrosine, xanthine and starch; there was no degradation activity observed for L-tyrosine, xylan, xanthine and casein by strain P8. Strains P8, P39 and P42 could not degrade casein while strain P115 was able to degrade casein (Plate 4.15).

Table 4.15: Degradation activity of four selected strains

Degradation[a] Test	**Results**			
	P8	P39	P42	P115
Starch	+	+	+	+
Casein	-	-	-	+
L-Tyrosine	-	+	+	+
Xylan	-	-	-	-
Xanthine	-	+	+	+

+ Positive action; - Negative action

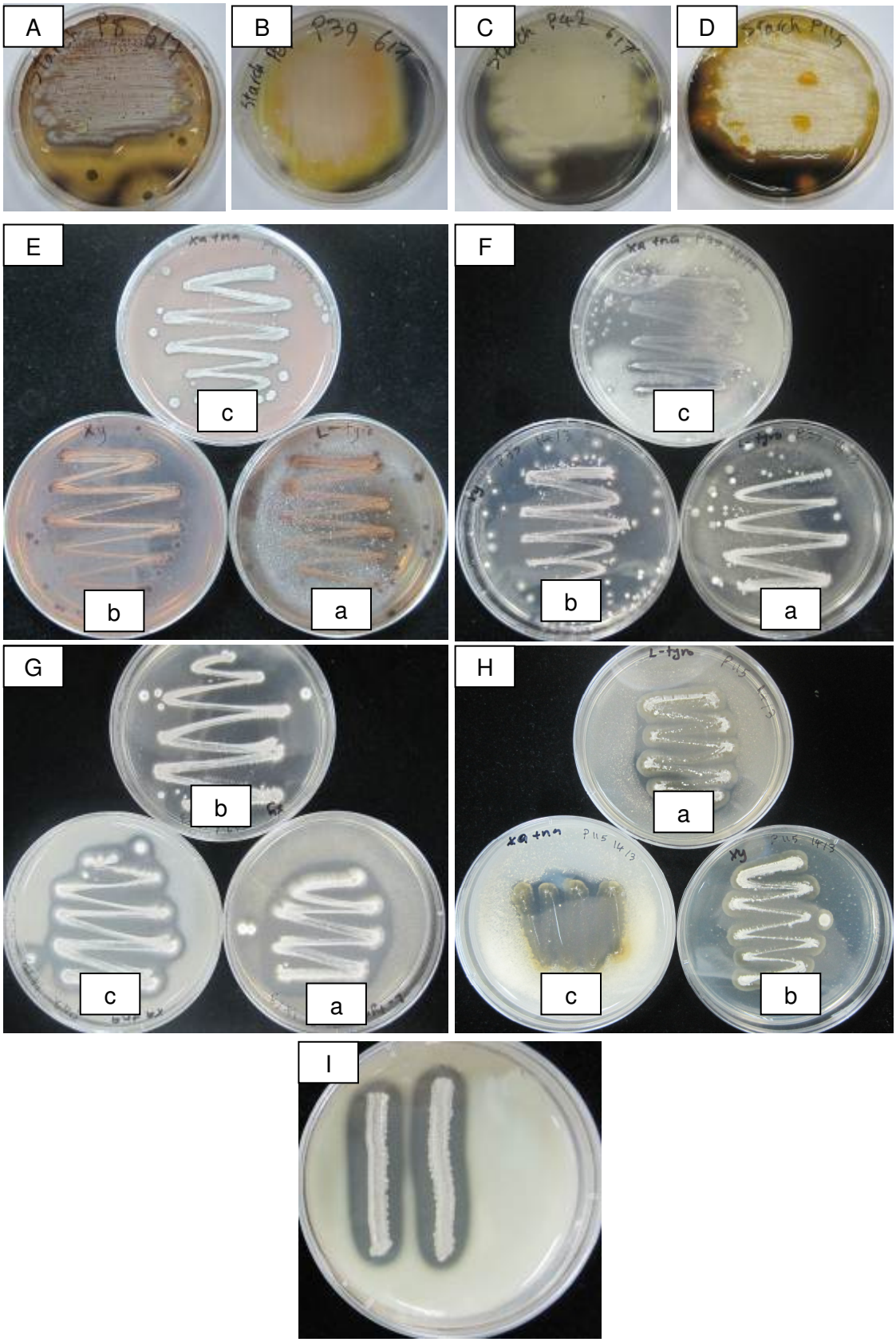

Plate 4.15: Degradation activity in four selected strains (P8, P39, P42 and P115); A, B, C, D degradation of starch; E, F, G, H degradation of L-tyrosine (a), xylan (b) and xanthine (c); I: degradation of casein.

4.2.4.2 Growth at Different pH and Temperature

All strains except P39 could grow well at pH 5 to 10. Strain p39 could grow only from pH 5 to 10. None of the strains could grow at pH 4 or below (Table 4.16; Plates 4.16, 4.17, 4.18, 4.19). All strains except P8 could grow well between 10ºC to 40ºC (Table 4.16; Plate 4.20).

Table 4.16: pH and temperature tests of four selected Strains

	Results			
Growth at	P8	P39	P42	P115
pH 4.0	+	-	+	+
pH 5.0	+	-	+	+
pH 6.0 – 10.0	+	+	+	+
10ºC	-	+	+	+
17ºC	+	+	+	+
28ºC	+	+	+	+
35ºC	+	+	+	+
40ºC	-	+	+	+

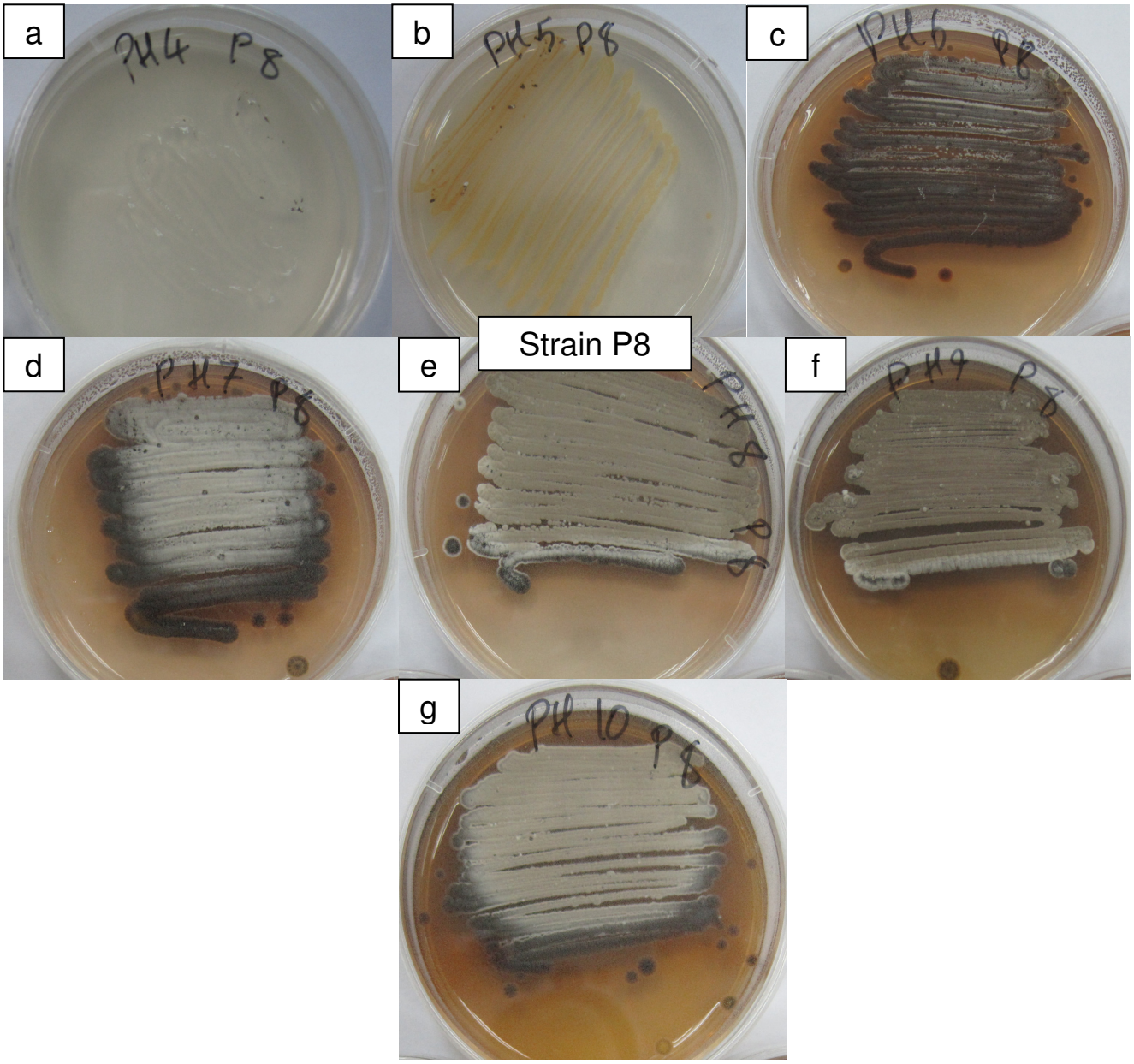

Plate 4.16: pH test of strain P8. a: pH4; b: pH5; c: pH6; d: pH7; e: pH8; f: pH9; g: pH10.

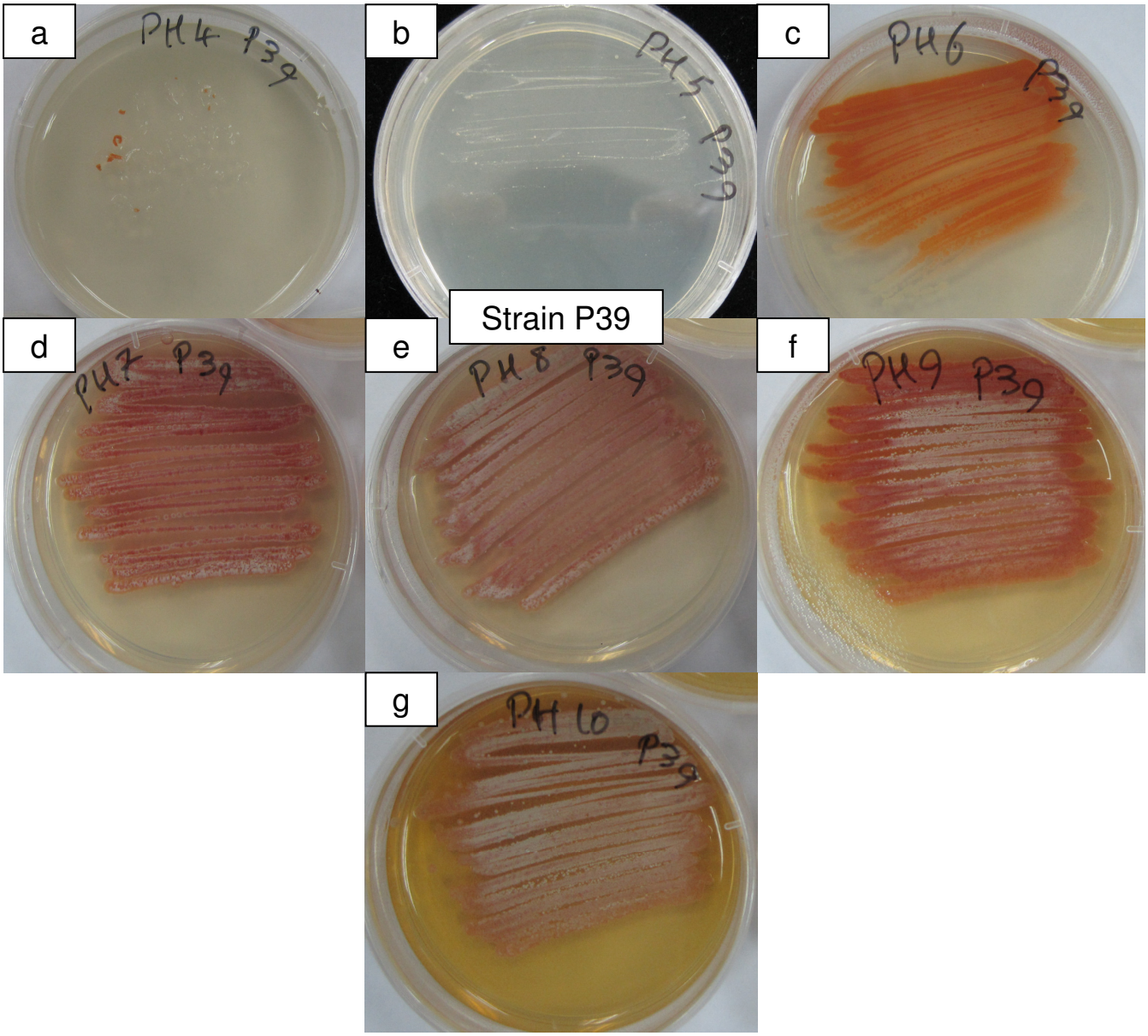

Plate 4.17: pH test of strain P39. a: pH4; b: pH5; c: pH6; d: pH7; e: pH8; f: pH9; g: pH10.

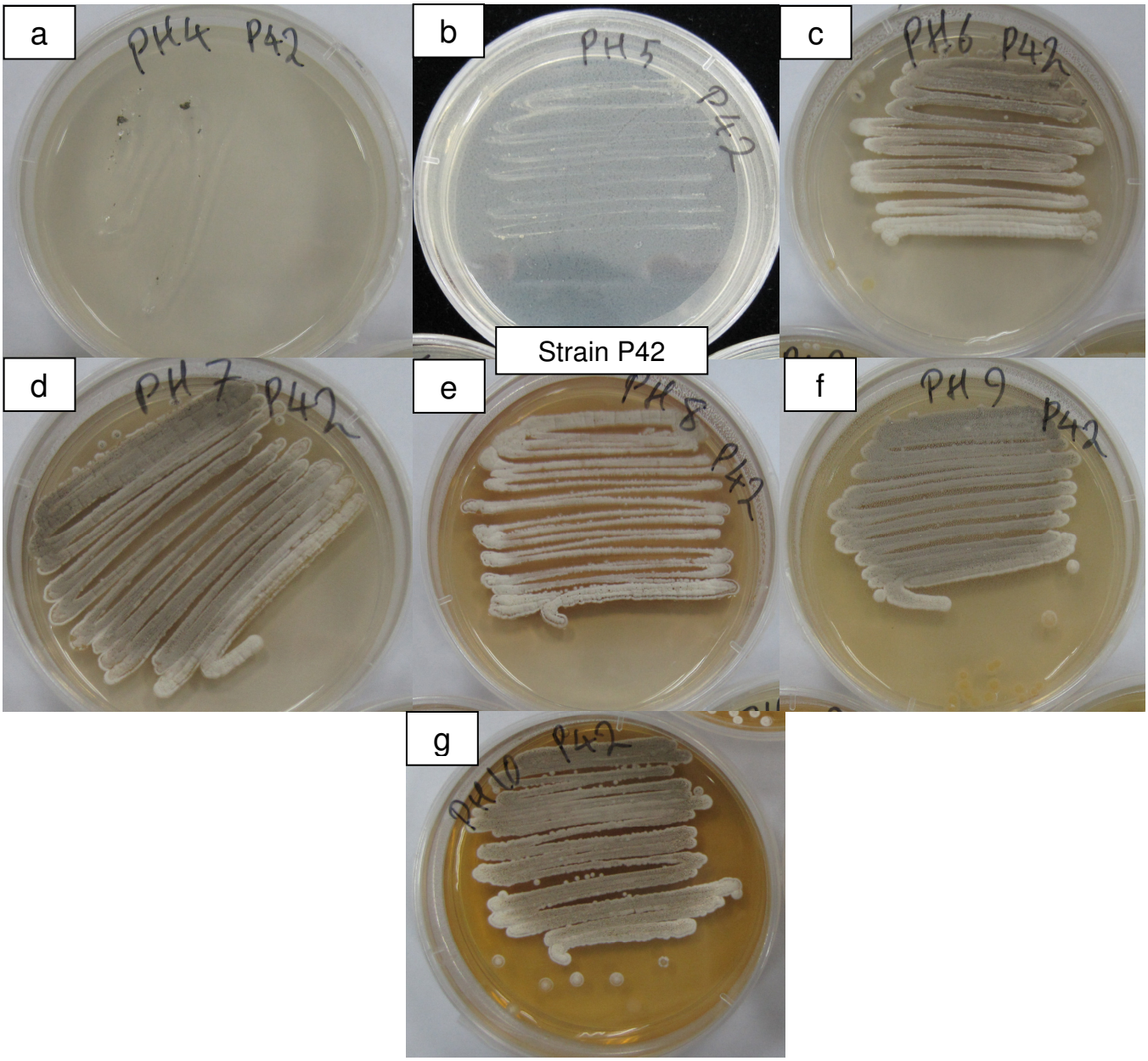

Plate 4.18: pH test of strain P42. a: pH4; b: pH5; c: pH6; d: pH7; e: pH8; f: pH9; g: pH10.

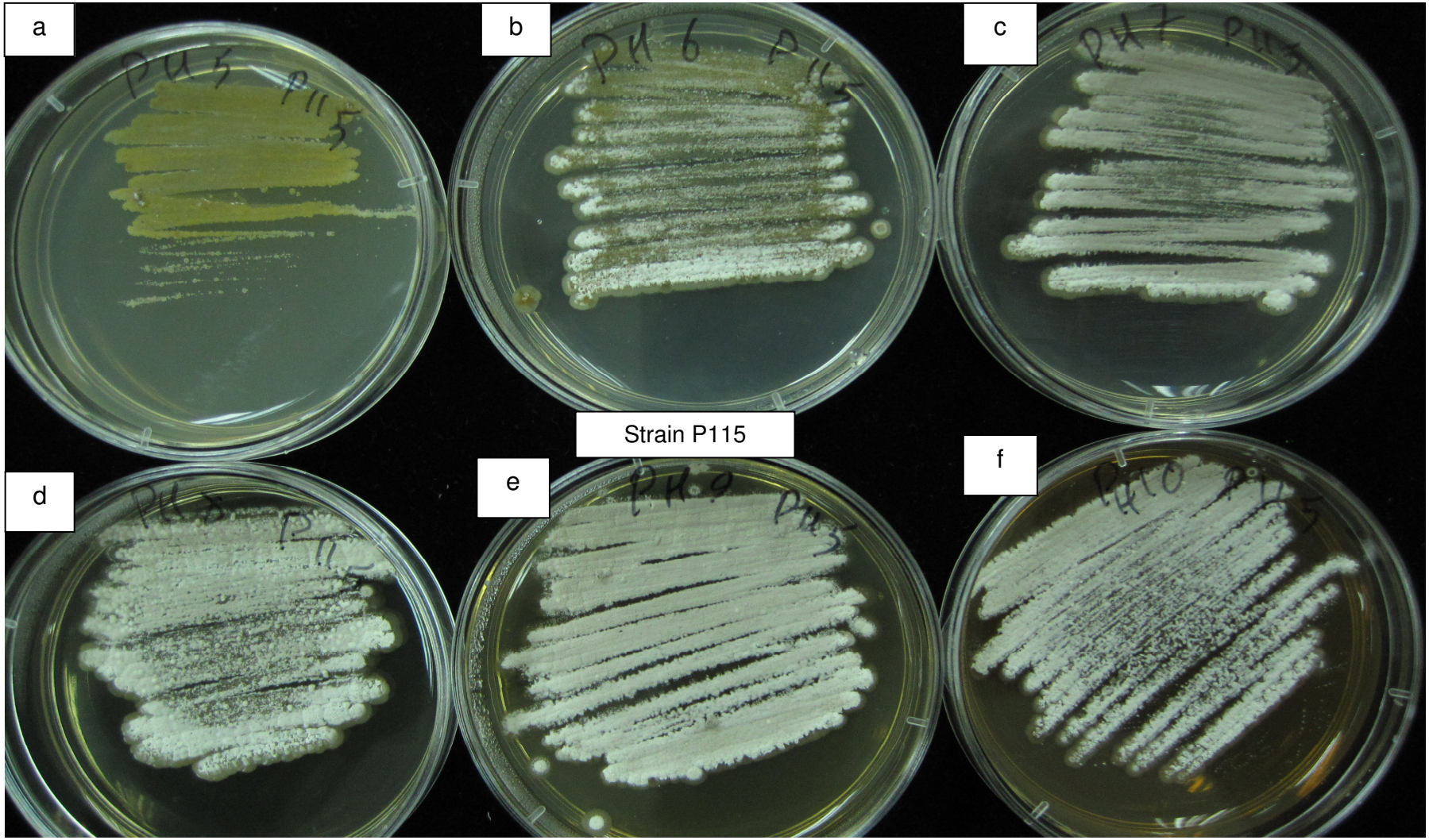

Plate 4.19: pH test of strain P115. a: pH5; b: pH6; c: pH7; d: pH8; e: pH9; f: pH10.

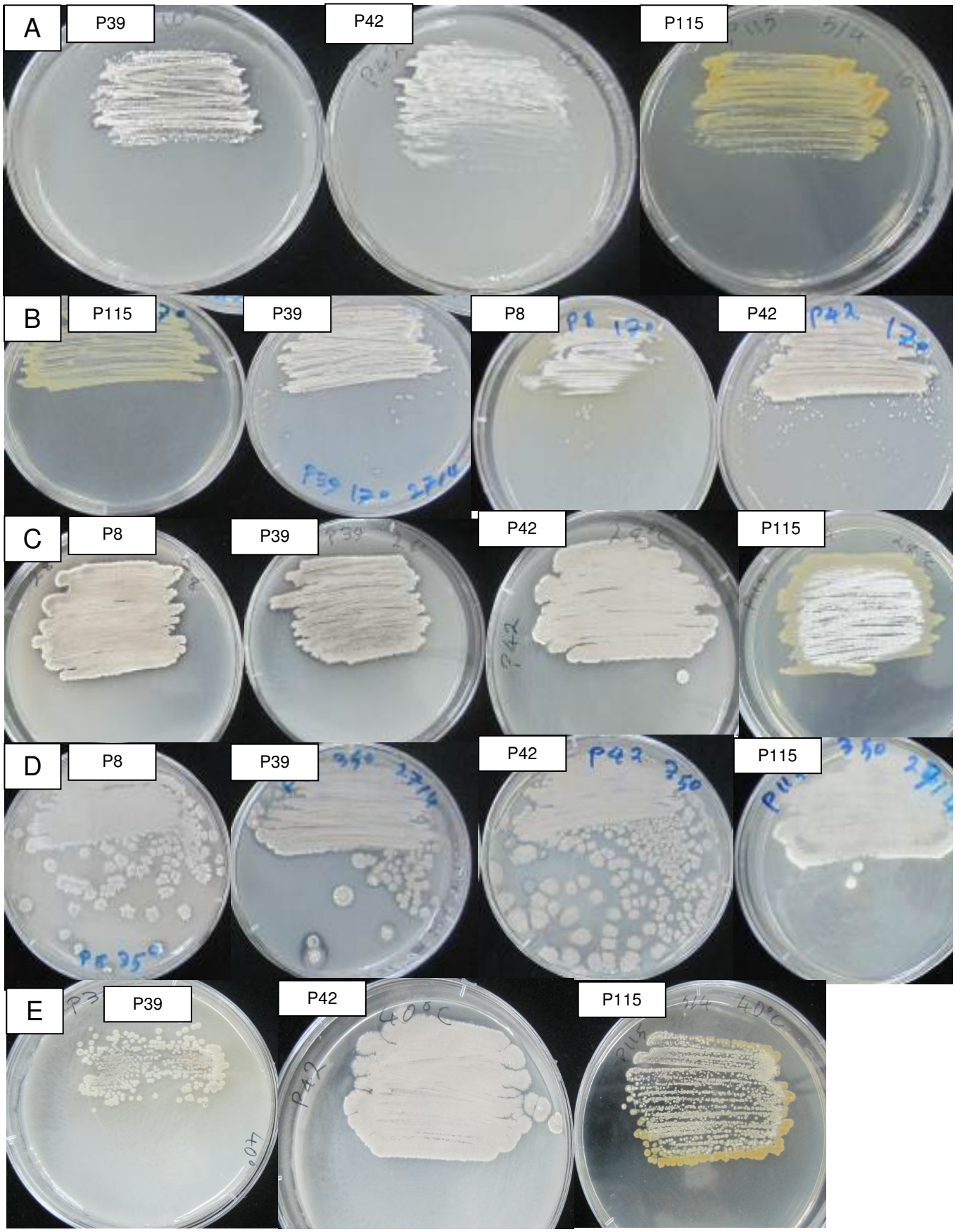

Plate 4.20: Temperature test results of four selected strains. A: 10ºC; B: 17ºC; C: 28ºC; D: 35ºC; E: 40ºC.

4.2.4.3 Sodium Chloride Tolerance Test

Strains P42 and P39 grew well in ISP4 agar medium in the absence of NaCl, as well as in the presence of 1% to 5% for P42 and 1% to 4% for P39. Strain P8 could grow up to 2% salinity level only while strain P115 showed a moderate growth between 0% and 7% and grew well between 8% to 10% salinity level (Table 4.17; Plates 4.21, 4.22, 4.23, 4.24).

Table 4.17: Sodium chloride tolerance test of four selected strains

	Results			
Growth at[a]	P8	P39	P42	P115
NaCl 0% v/w	++	++	++	+
NaCl 1% v/w	+	++	++	+
NaCl 2%. v/w	+	++	++	+
NaCl 3% v/w	-	++	++	+
NaCl 4% v/w	-	++	++	+
NaCl 5% v/w	-	+	++	+
NaCl 6% v/w	-	+	+	+
NaCl 7% v/w	-	+	+	+
NaCl 8% v/w	-	-	+	+
NaCl 9% v/w	-	-	-	+
NaCl 10% v/w	-	-	-	+

[a]: ++, Good growth; +, Moderate growth; -, No growth

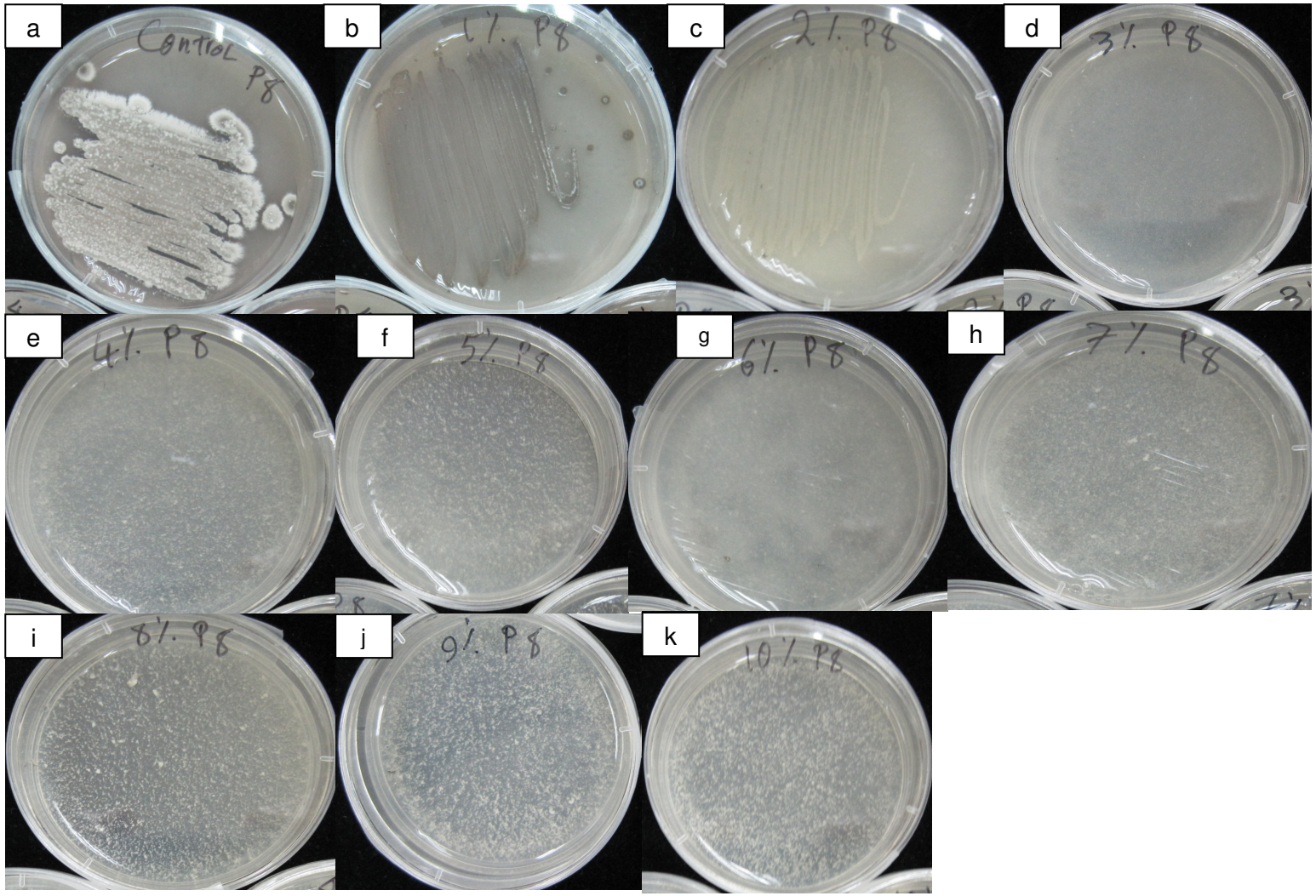

Plate 4.21: NaCl tolerance test results of strain P8.
a: Control; b:1% Salinity; c: 2% Salinity; d: 3% Salinity; e: 4% Salinity; f: 5% Salinity; g: 6% Salinity; h: 7% Salinity; i: 8% Salinity; j: 9% Salinity; k: 10% Salinity.

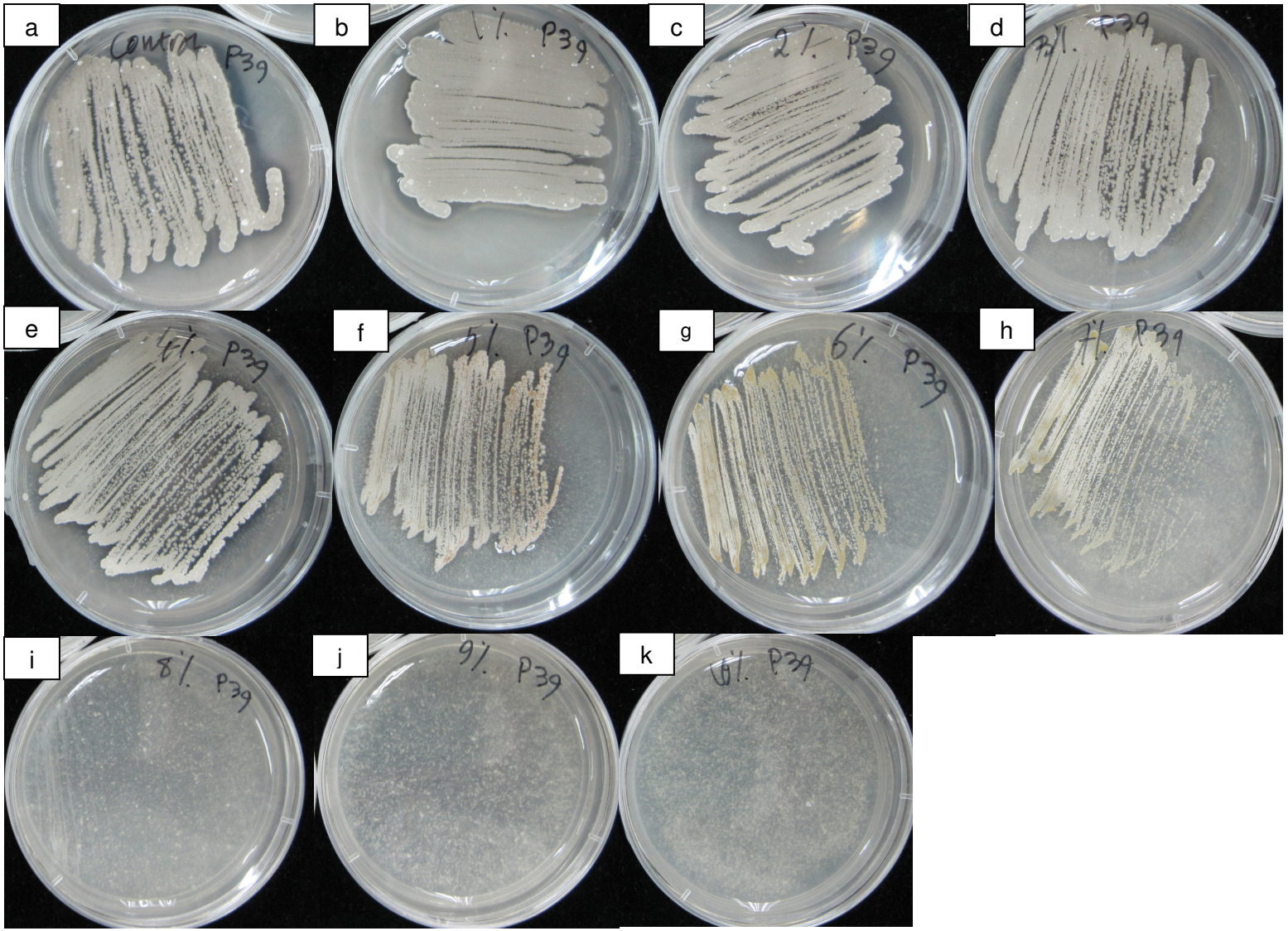

Plate 4.22: NaCl tolerance test results of strain P39.
a: Control; b:1% Salinity; c: 2% Salinity; d: 3% Salinity; e: 4% Salinity; f: 5% Salinity; g: 6% Salinity; h: 7% Salinity; i: 8% Salinity; j: 9% Salinity; k: 10% Salinity.

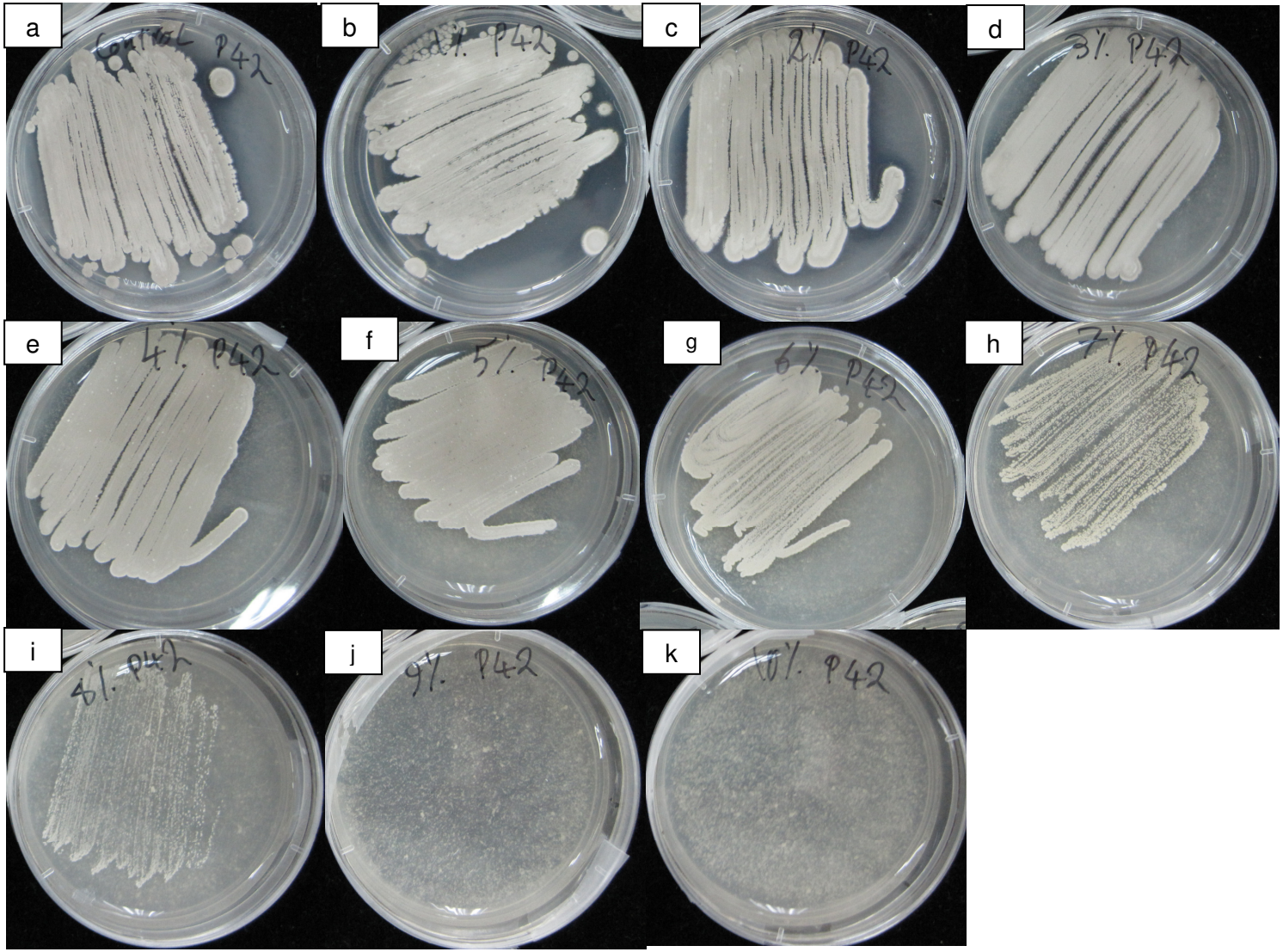

Plate 4.23: NaCl tolerance test results of strain P42.
a: Control; b:1% Salinity; c: 2% Salinity; d: 3% Salinity; e: 4% Salinity; f: 5% Salinity; g: 6% Salinity; h: 7% Salinity; i: 8% Salinity; j: 9% Salinity; k: 10% Salinity.

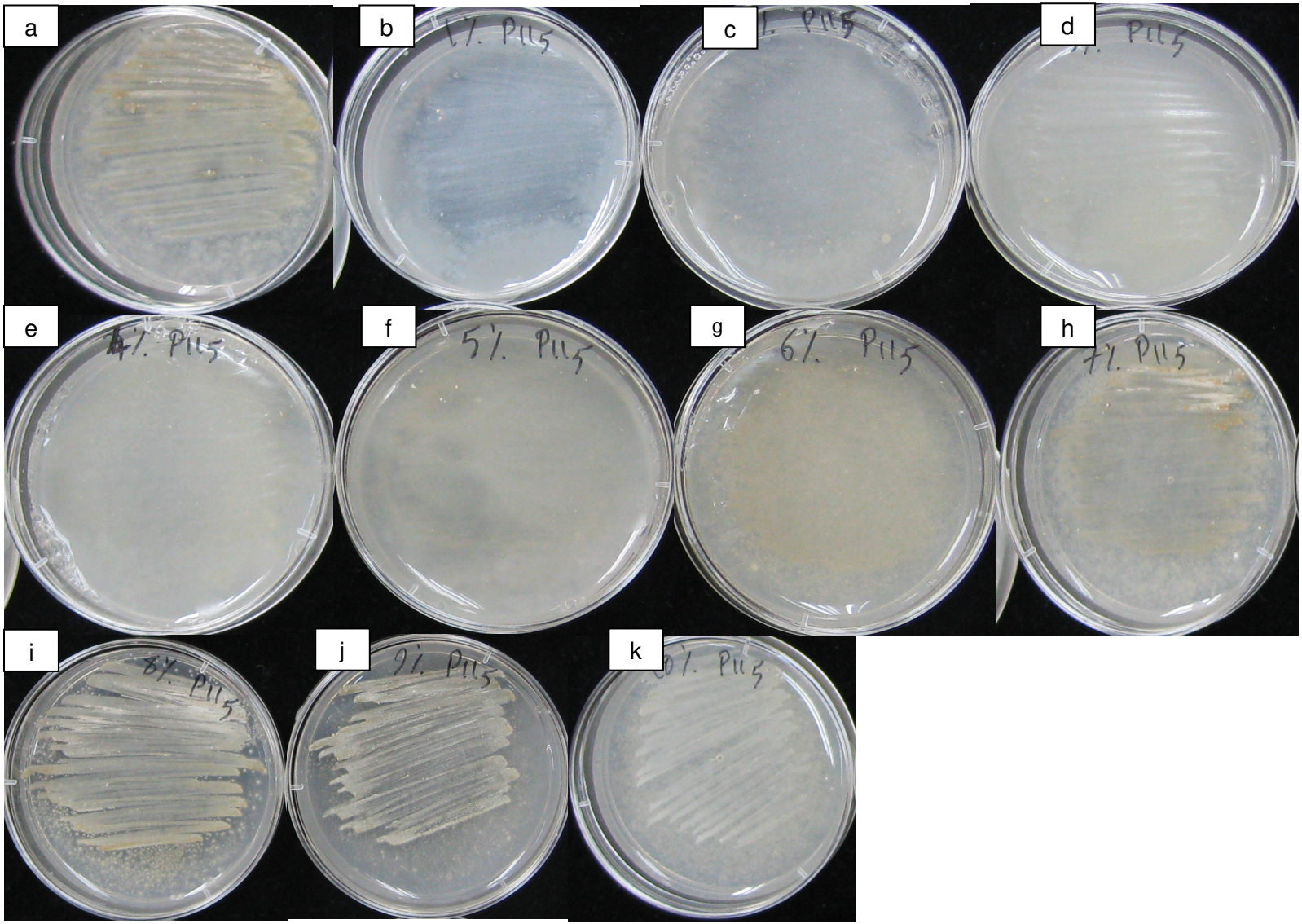

Plate 4.24: NaCl tolerance test results of strain P115.
a: Control; b:1% Salinity; c: 2% Salinity; d: 3% Salinity; e: 4% Salinity; f: 5% Salinity; g: 6% Salinity; h: 7% Salinity; i: 8% Salinity; j: 9% Salinity; k: 10% Salinity.

4.2.4.4 Susceptibility to Antibiotics

Of the seven antibiotics tested, strain P42 demonstrated high susceptibility to Neomycin, Kanamycin, Novobiocin and Gentamycin, moderately susceptible to Streptomycin and Tetracyclin, and low susceptibility to Erythromycin. Strain P39 was highly susceptible to Neomycin, Streptomycin, Kanamycin and Gentamycin, moderate and low susceptibility to Novobiocin and Erythromycin respectively. This strain showed resistance to Tetracycline. Strain P8 demonstrated high susceptibility to Erythromycin, Streptomycin, Gentamycin and Tetracyclin, and moderate susceptibility to Neomycin, Kanamycin and Novobiocin. Strain P115 revealed moderate susceptibility to Streptomycin, Kanamycin, Novobiocin, Gentamycin and Tetracyclin but low susceptibility to Erythromycin and Neomycin at the concentration used (Table 4.18; Plate 4.25).

Table 4.18: Susceptibility to antibiotics (µg per disc)[a]

Susceptibility to:	**Results**			
	P8	P39	P42	P115
Ertythromycin	++	±	±	±
Neomycin	+	++	++	±
Streptomycin	++	++	+	+
Kanamycin	+	++	++	+
Novobiocin	+	+	++	+
Gentamycin	++	++	++	+
Tetracycline	++	-	+	+

[a]: ++, highly susceptible (diameter inhibition zone 31-50 mm); +, moderately susceptible (21-30 mm); ±, low susceptibility (10-20 mm); -, resistant.

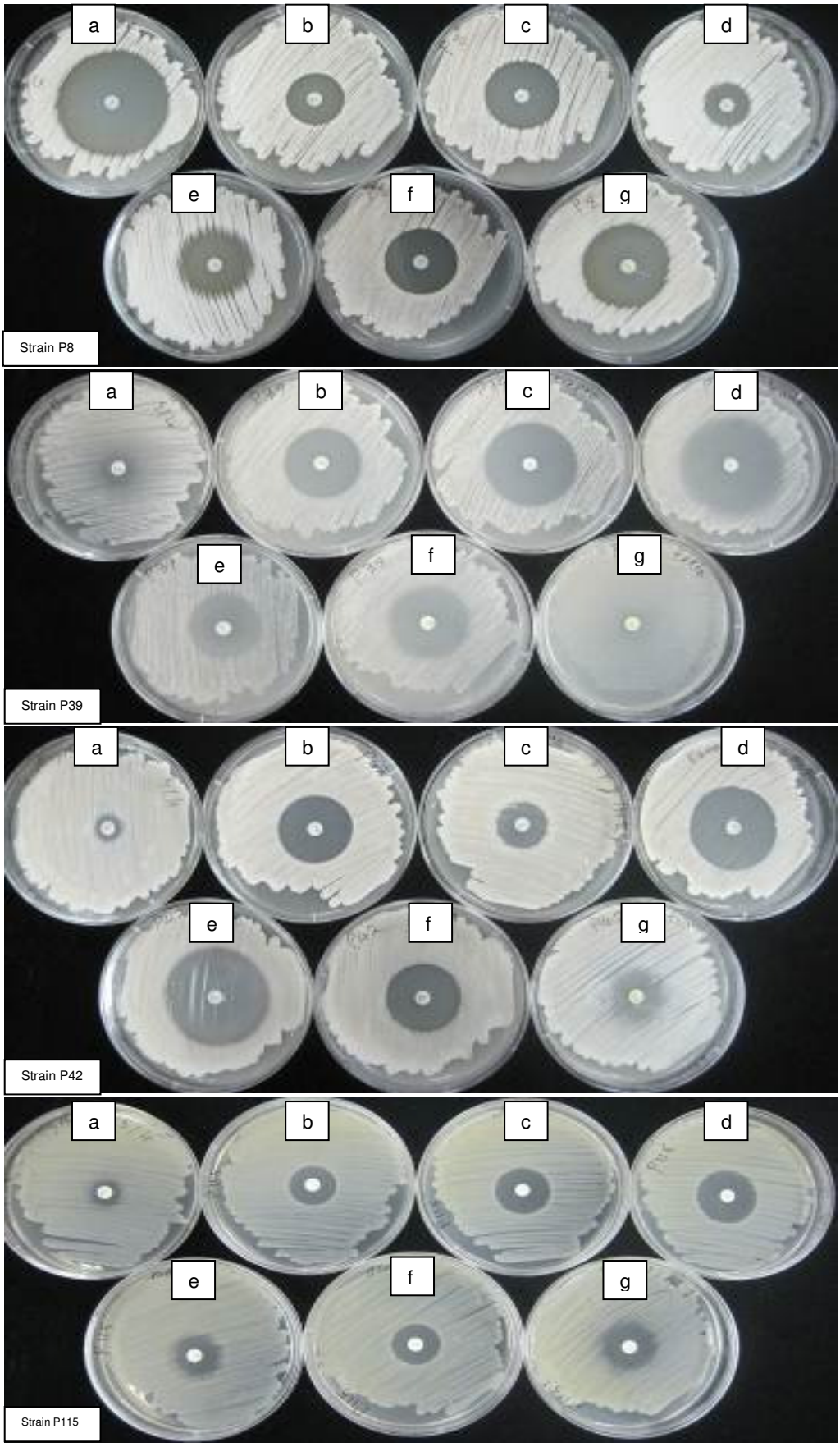

Plate 4.25: Susceptibility to antibiotic discs in four selected strains. a: Ertythromycin; b: Neomycin; c: Streptomycin; d: Kanamycin; e: Novobiocin; f: Gentamycin; g: Tetracycline.

4.2.4.5 Sole Carbon Sources Growth Ability

Utilization of 9 different kinds of sole carbon sources is shown in Table 4.19. All four strains had the ability to grow well on a variety of carbon compounds in the synthetic ISP9 agar medium. Glucose, Xylose and Galactose (all at 1% w/v) were utilized very well as sole sources of carbon for energy and growth. However all strains could not use Sucrose and Raffinose (Table 4.19; Plates 4.26, 4.27, 4.28, 4.29).

Table 4.19: Sole carbon source growth ability

Sole carbon Source	**Growth[a]**			
	P8	P39	P42	P115
Negative Control	-	-	-	-
D-Glucose	+	+	+	+
L-Arabinose	+	+	+	-
Sucrose	-	-	-	-
D-Xylose	+	+	+	+
I-Inositol	-	+	+	+
Rhamnose	-	+	+	+
Raffinose	-	-	-	-
Galactose	+	+	+	+
D-Mannitol	-	+	+	+

[a]: +, Good growth; ±, Weak growth; -, No growth.

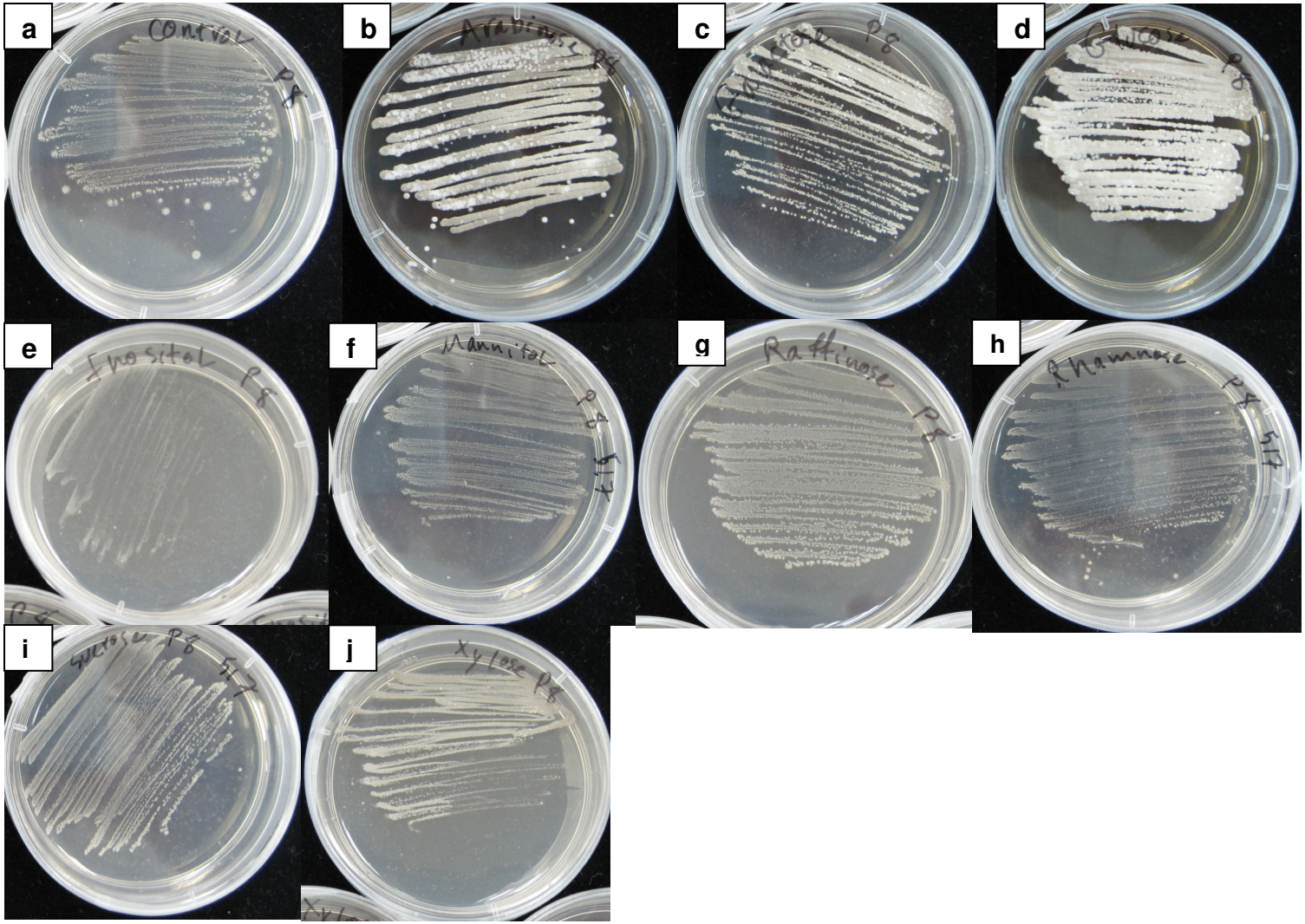

Plate 4.26: Sole carbon source growth ability in strain P8. a: Control; b: L-Arabinose; c: Galactose; d: Glucose; e: I-Inositol; f: D-Mannitol; g: Raffinose; h: Rhamnose; i: Sucrose; j: D-Xylose.

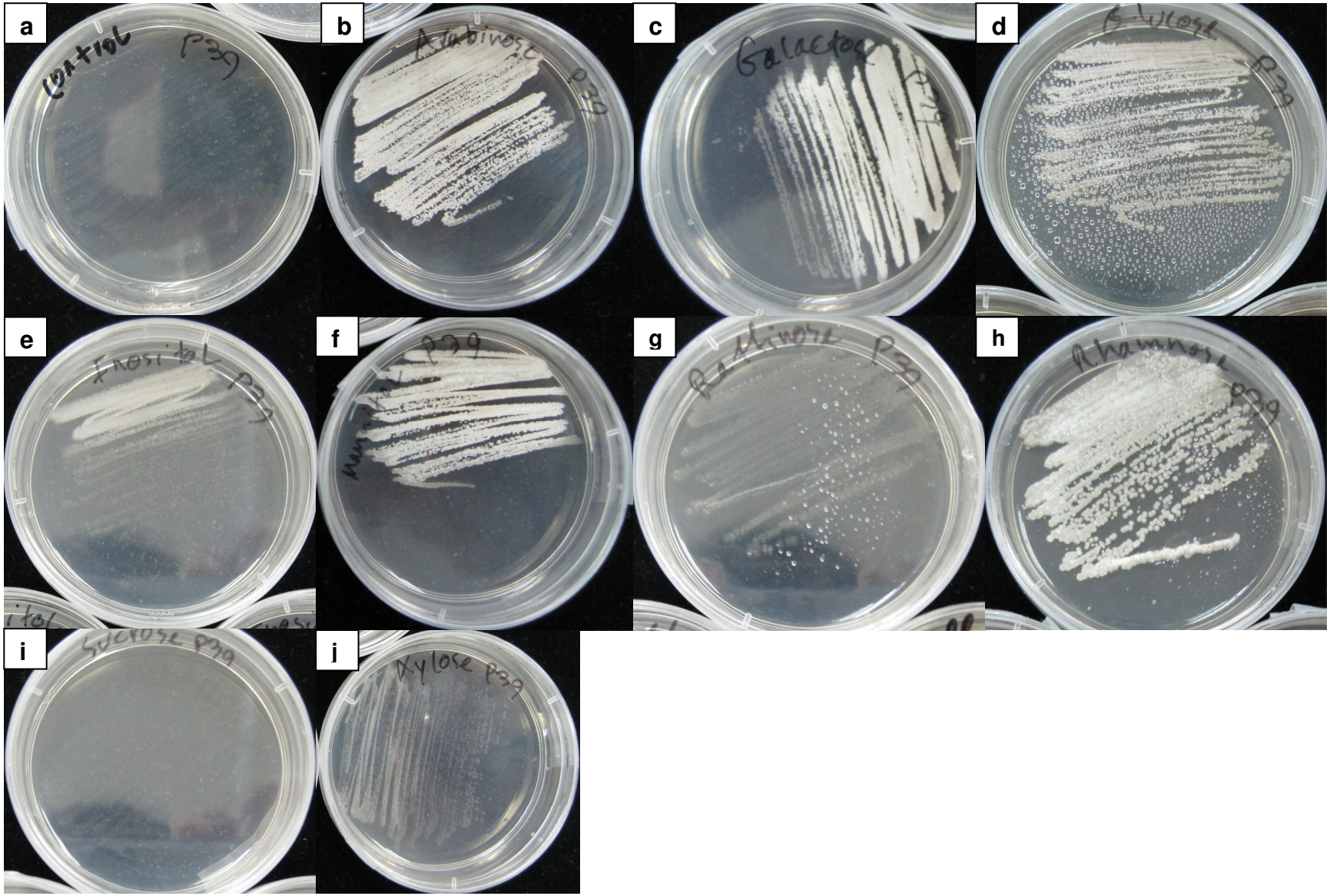

Plate 4.27: Sole carbon source growth ability in strain P39. a: Control; b: L-Arabinose; c: Galactose; d: Glucose; e: I-Inositol; f: D-Mannitol; g: Raffinose; h: Rhamnose; i: Sucrose; j: D-Xylose.

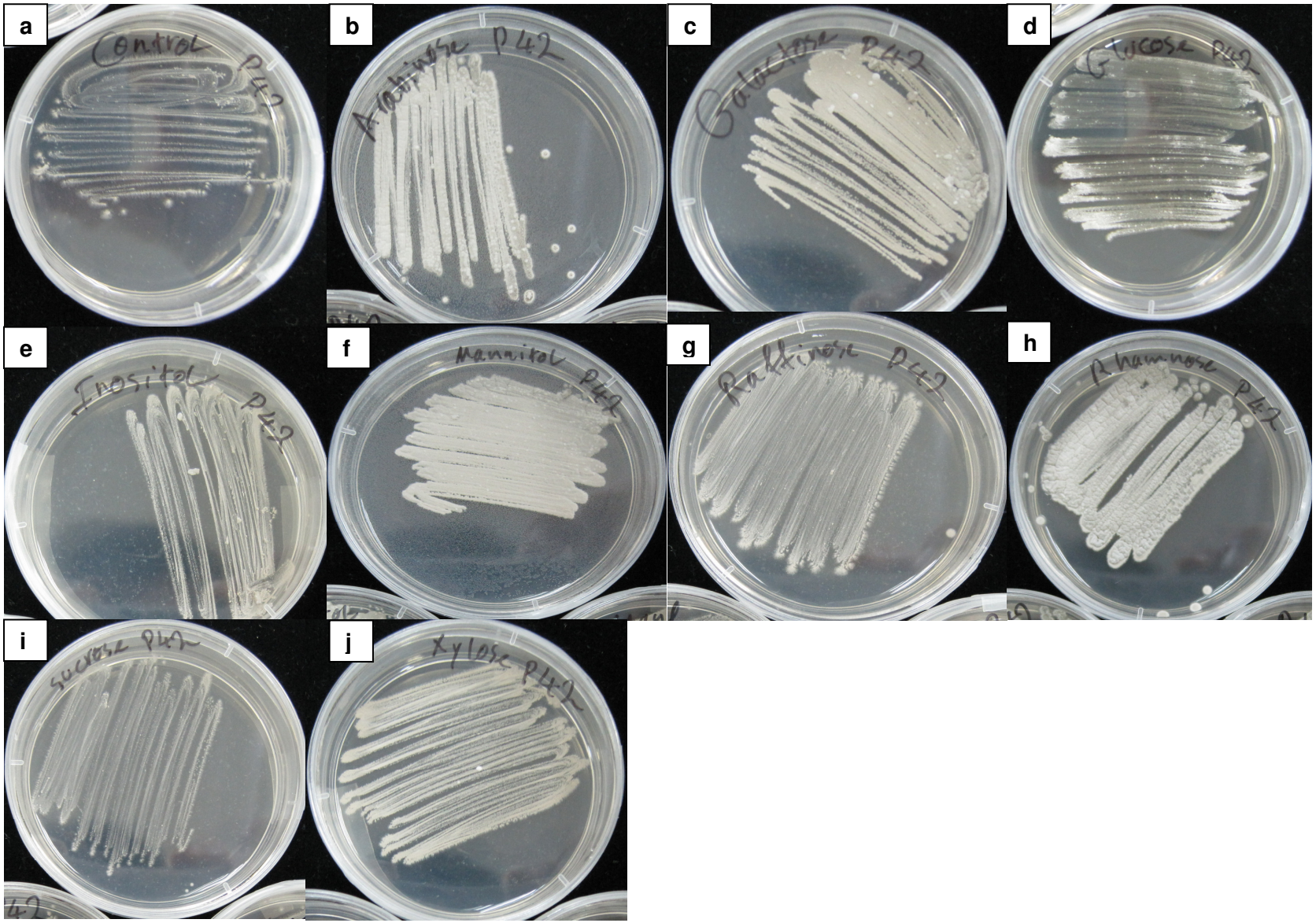

Plate 4.28: Sole carbon source growth ability in strain P42. a: Control; b: L-Arabinose; c: Galactose; d: Glucose; e: I-Inositol; f: D-Mannitol; g: Raffinose; h: Rhamnose; i: Sucrose; j: D-Xylose.

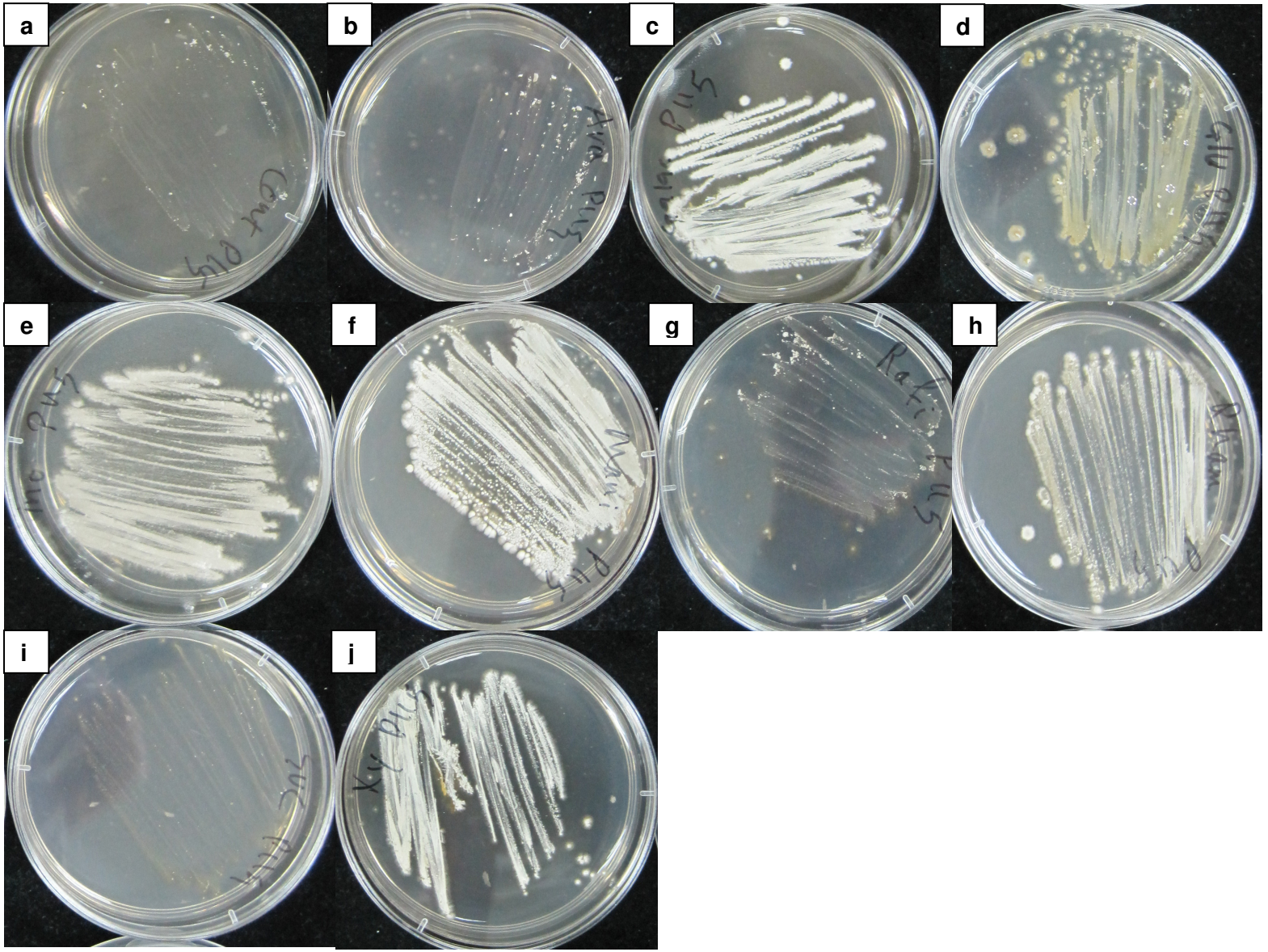

Plate 4.29: Sole carbon source growth ability in strain P115. a: Control; b: L-Arabinose; c: Galactose; d: Glucose; e: I-Inositol; f: D-Mannitol; g: Raffinose; h: Rhamnose; i: Sucrose; j: D-Xylose.

4.2.4.6 Hydrogen Sulphide Production Test

Production of H_2S was observed by the presence of a blue-black discoloration of the medium surrounding the colonies (Gottlieb, 1961) in strain P8 only. The other three strains were not able to produce H_2S (Plate 4.30).

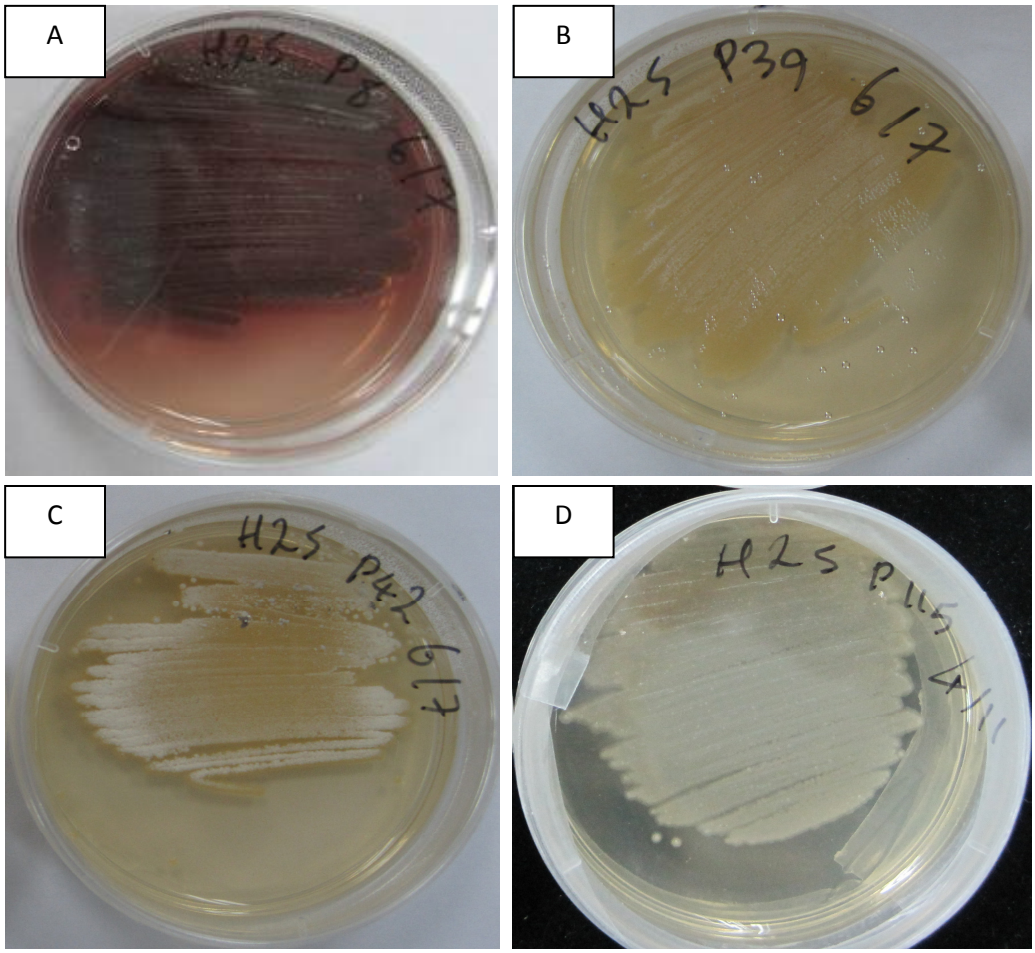

Plate 4.30: Hydrogen Sulphide production test in four selected strains. A: Strain P8; B: Strain P39; C: Strain P42; D: Strain P115.

4.2.4.7 Nitrate Reduction Test

Strains P39, P42 and P115 were able to reduce nitrate to nitrite. However, in strain P8 this ability was not observed (Plate 4.31).

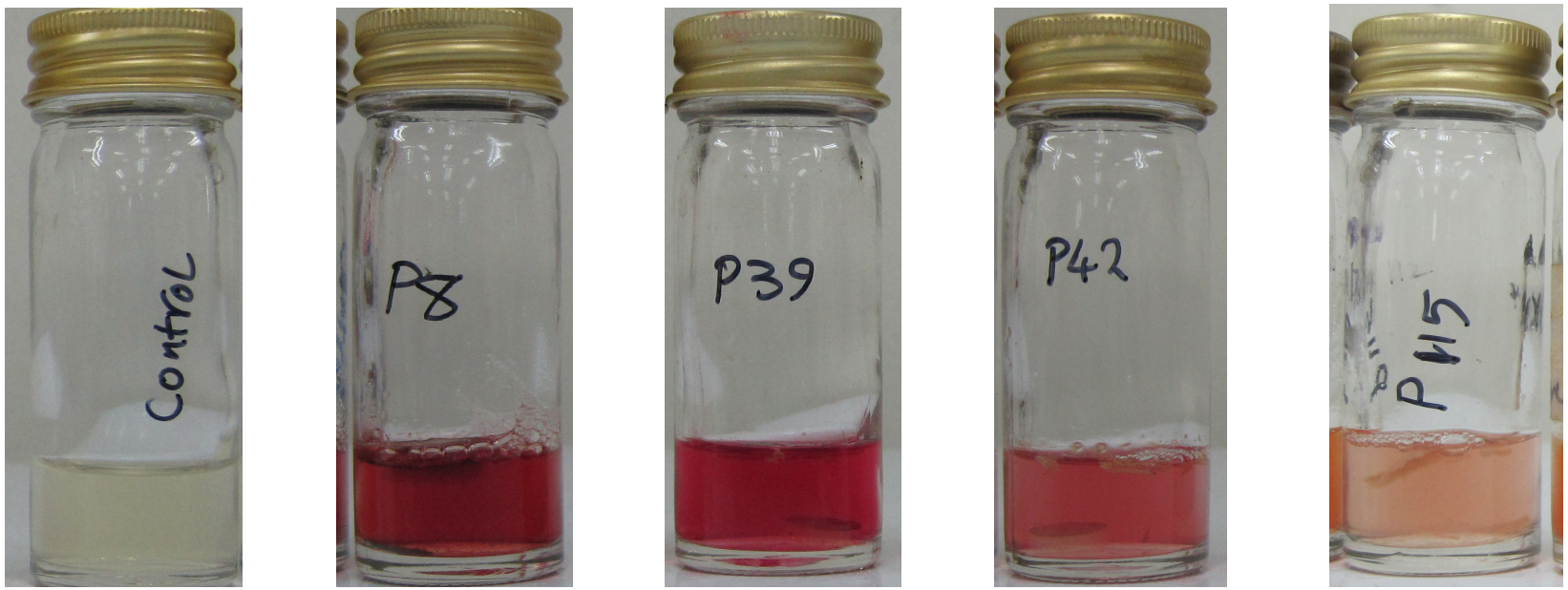

Plate 4.31: Nitrate reduction test in four selected strains. Strain P8 showed negative response to this test since after adding Zn powder the color of medium changed to pink.

4.2.4.8 Gelatinise Activity (Gelatine Liquefaction) Test

The degree of liquefaction of gelatine was determined and observed in strains P8, P42 and P115. This ability was not observed in strain P39 (Plate 4.32).

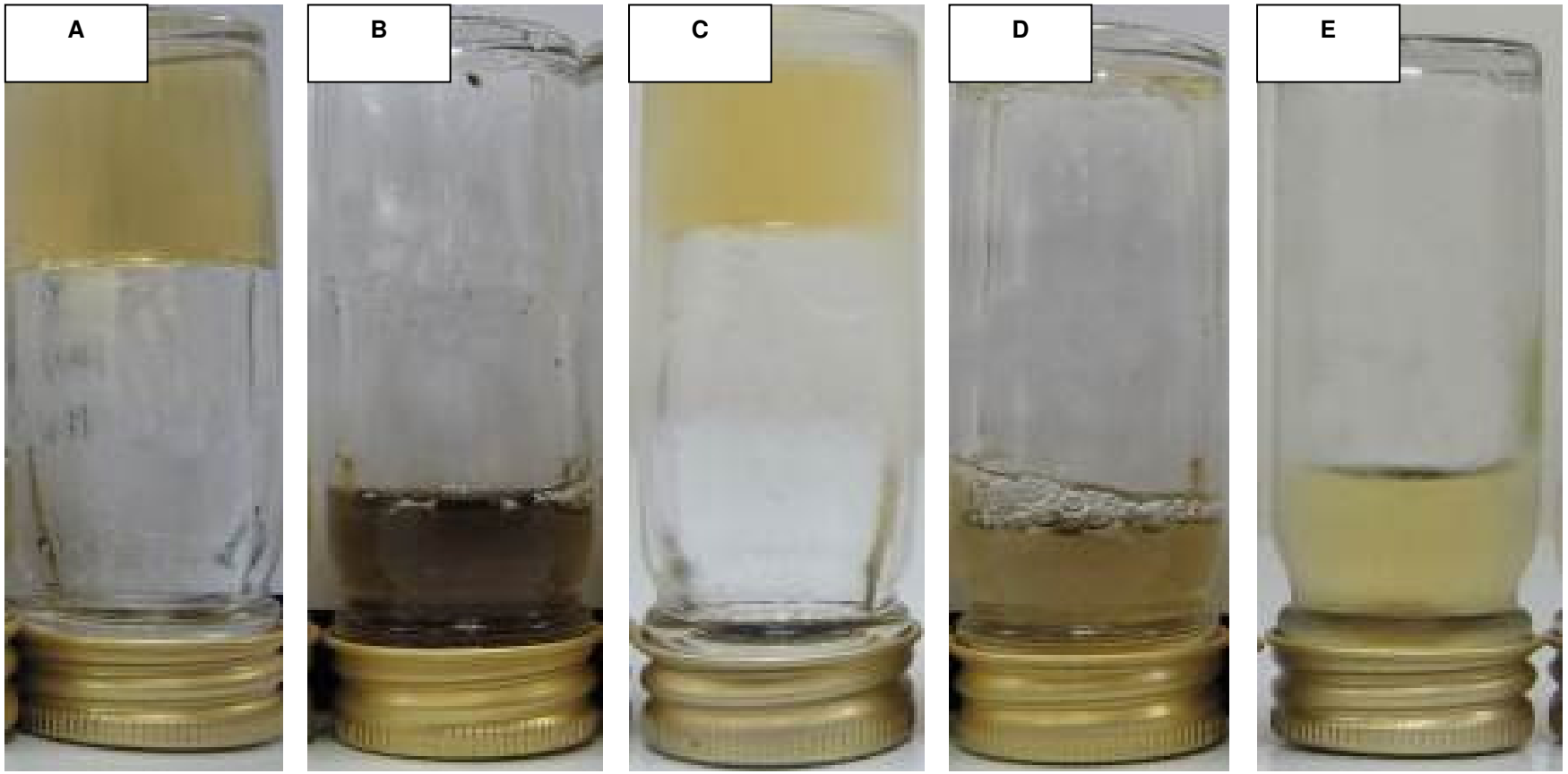

Plate 4.32: Gelatinise activity in four selected strains. A: Control; B: Strain P8; C: Strain P39; D: Strain P42; E: Strain P115.

4.2.4.9 Determination of Chitinase Activity of the Actinomycete Isolates

Determination of chitinase activity was observed in strains P42, P8 and P39 with a clearing zone of 7.5, 7.3 and 6.5 mm, respectively (Plate 4.33). Strain P115 was not able to produce any clear zone around the actinomycete isolate on colloidal chitin media.

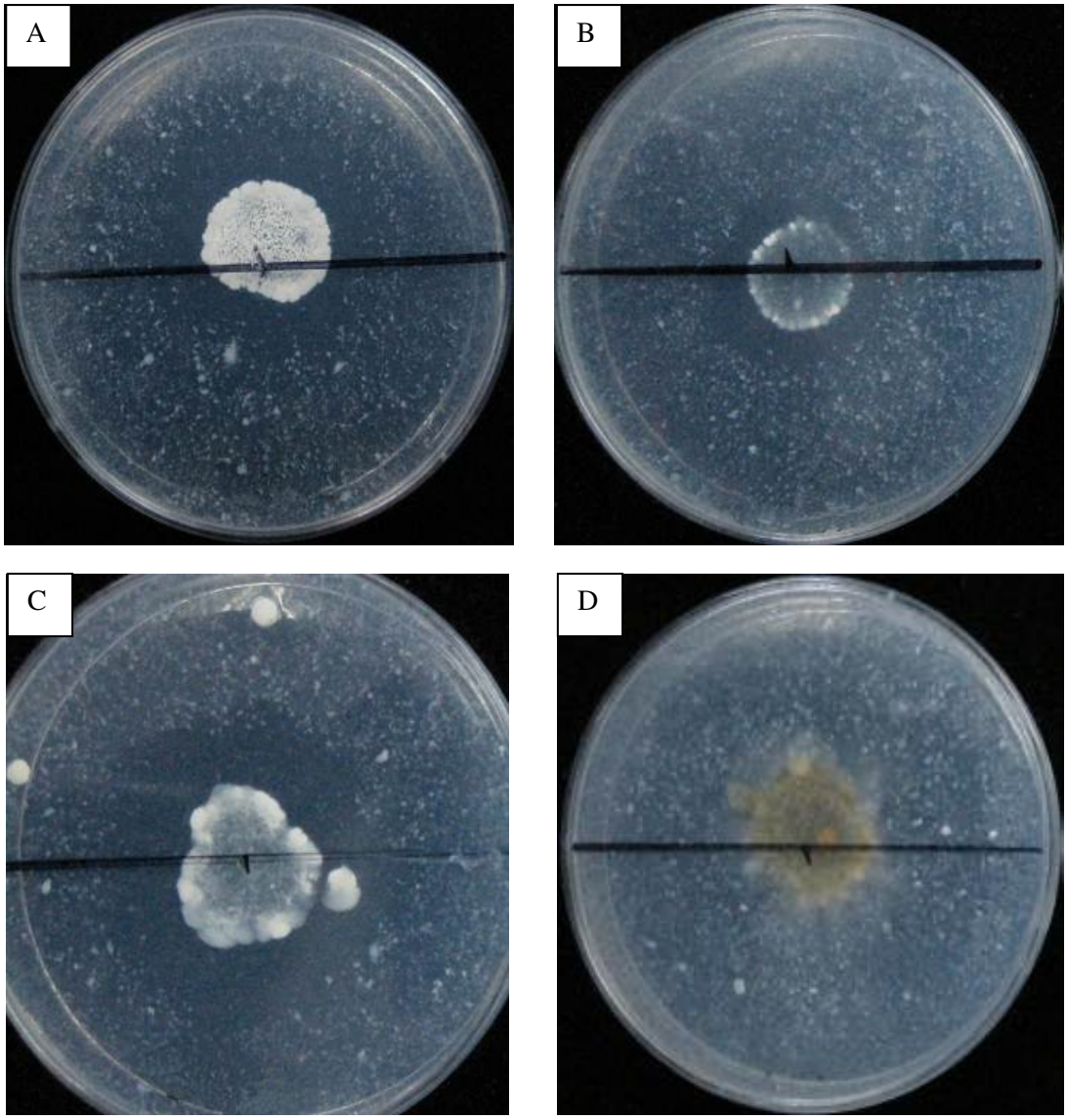

Plate 4.33: Chitinase activity of the four selected strains. A: Strain P8; B: Strain P39; C: Strain P42; D: Strain P115.

4.2.5 Phylogenetic Analysis of Antagonistic Actinomycetes

The nucleotide sequence for a section of the 16S rRNA gene (1420 bp) from the four selected strains (Appendices C3.1, C3.2, C3.3, C3.4) (Gen-Bank Accession numbers JN967800-JN967803) were identified by the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al*., 2012). All four isolates contained different nucleotide sequences for the 16S rRNA gene, indicating that they were different strains. All of four strains belonged to the family *Streptomycetaceae* and contained between 99-100% DNA homology in the 16S rRNA gene. Representative strains of the family *Streptomycetaceae* producing significant sequence alignment with four strong strains in the 16S rRNA tree are shown in Table 3.1.

The strain P8 was closely related to *S. purpeofuscus* (99.47%), *Kitasatospora azatica* (99.39%), *S. misakiensis* (99.32%) and *K. putterlickiae* (99.17%) based on pairwise sequence similarity. Strain P39 showed similarity to *S. violaceorubidus* (100%), *S. tendae* (99.85%) and *S. tritolerans* (99.62) in its neighbour. Strain P42 closely resembled *Streptomyces rochei* (100%), *S. enissocaesilis* (100%), *S. plicatus* (100%) and *S. vinaceusdrappus* (99.84%) in its neighbour. Three strains closely related to strain P115 were *S. rubrogriseus* (99.62%), *S. tricolor* (99.39%) and *S. lienomycini* (99.39%). The results described above were supported by phylogenetic analysis based on the neighbor-joining tree. The four isolates were sorted into clusters with high similarity into the family *Streptomycetaceae* (Figure 4.4).

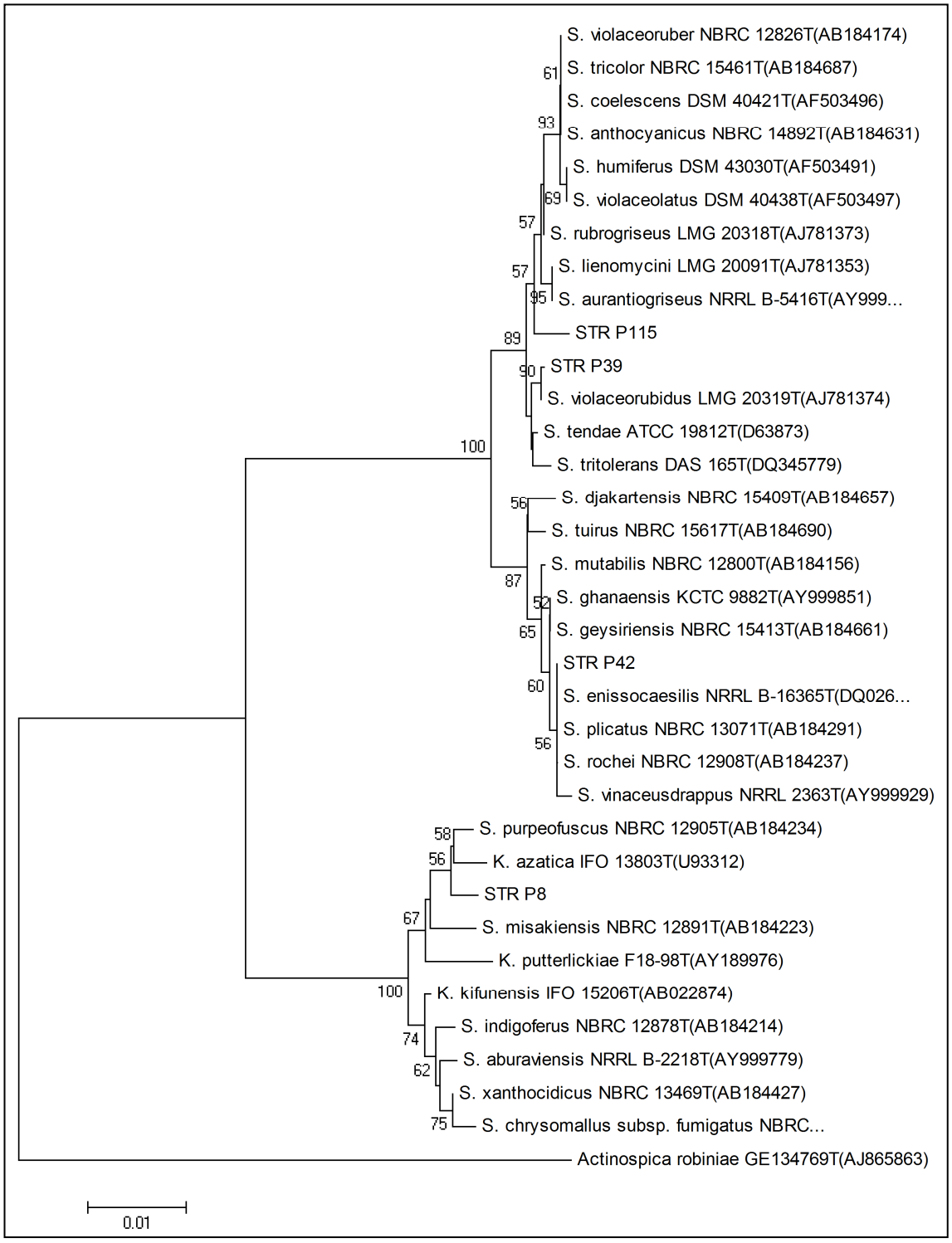

Figure 4.4: Neighbour-Joining tree based on partial 16S rRNA sequences showing relationship between four tested strains and representative streptomycetes.
The numbers at the nodes indicate the levels of bootstrap phylogeny analysis with 1000 replications; only values above 50% are given. *Actinospica robiniae* GE134769^{T} (AJ865863) was used as an outgroup. The scale bar indicates 0.01 substitution per nucleotide position.

Table 4.20: Some differential characteristics of strain P8 and the type strains of the validly described species*

Phenotypic Test	**Strain P8**	***S. purpeofuscus***	***K. azatica***	***S. misakiensis***	***K. putterlickiae***
Growth at:					
10ºC	-	-	-	-	-
28 ºC	+	+	+	+	+
40 ºC	-	-	-	-	-
Growth on Sole Carbon Source:					
D-Glucose	+	+	+	+	+
L-Arabinose	+	+	+		+
Sucrose	-	-	+	+	+
D-Xylose	+	+	+	-	+
I-Inositol	-	-	-	-	-
Rhamnose	-	-	-	-	-
Raffinose	-	-	-	-	-
Galactose	+	ND	ND	ND	+
D-Mannitol	-	-	-	-	+
Spore Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Spore Chain	RF	RF	RF	RF	RF
Colony Color on ISP4:					
Aerial Mass	Grey	Grey	White	Grey	Dark Grey
Reverse Side	Brown	Brown	Beige	Yellow	Pale Grey
Pigmentation	-	-	-	-	-
Enzymes:					
H_2S	+	-	ND	-	+
Gelatine	+	+	+	-	-

+, positive response; -, negative response; ND, Not Determined; RF, *Rectiflexibiles*

*: Data compiled from Shirling E.B (1968); Caruso *et al*., (2000); Wink J.M. (1971); Groth (2003).

Table 4.21: some differential characteristics of strain P39 and the type strains of the validly described species*

Phenotypic Test	**Strain P39**	***S. violaceorubidus***	***S. tendae***	***S. tritolerans***
Growth at:				
10ºC	+	+	-	-
28 ºC	+	+	+	+
40 ºC	+	+	-	+
Growth on Sole Carbon Source:				
D-Glucose	+	+	+	+
L-Arabinose	+	+	+	+
Sucrose	-	+	-	+
D-Xylose	+	+	-	+
I-Inositol	+	+	+	-
Rhamnose	+	+	-	+
Raffinose	-	+	-	+
Galactose	+	+	ND	+
D-Mannitol	+	+	+	+
Spore Surface	Smooth	Smooth	Smooth	Smooth
Spore Chain	SS	SS	RF	RF
Colony Color on ISP4:				
Aerial Mass	Grey	Grey	White	White Grey
Reverse Side	Red	Red Orange	Yellow/Brown	Yellow
Pigmentation	-	-	Brown	-
Enzymes:				
H_2S	-	+	+	+
Gelatine	+	+	+	-

+, positive response; -, negative response; ND, Not Determined; RF, *Rectiflexibiles*; SS, *Spirals*

*: Data compiled from Bensultana *et al*., (2010); Wink J.M. (1971).

Table 4.22: Some differential characteristics of strain P42 and the type strains of the validly described species*

Phenotypic Test	**Strain P42**	***S. rochei***	***S. enissocaesilis***	***S. plicatus***	***S. vinaceusdrappus***
Growth at:					
10ºC	+	+	+	+	+
28 ºC	+	+	+	+	+
40 ºC	+	+	-	+	+
Growth on Sole Carbon Source:					
D-Glucose	+	+	+	+	ND
L-Arabinose	+	+	+	+	+
Sucrose	-	-	-	-	-
D-Xylose	+	+	+	+	+
I-Inositol	+	+	+	+	ND
Rhamnose	+	+	+	+	+
Raffinose	-	-	-	-	ND
Galactose	+	+	-	ND	+
D-Mannitol	+	+	-	+	+
Spore Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Spore Chain	SS	SS	SS	SS	SS
Colony Color on ISP4:					
Aerial Mass	Grey	Grey	Pinkish Lilac	Grey	Grey
Reverse Side	Grey	Grey	Brown	Pale Yellow	Grey
Pigmentation	-	-	-	-	-
Enzymes:					
H_2S	-	-	-	ND	-
Gelatine	+	+	-	ND	-

+, positive response; -, negative response; ND, Not Determined; SS, *Spirals*

*: Data compiled from Shirling E.B (1968); Wink J.M. (1971); Ningthoujam *et al.*, (2009).

Table 4.23: Some differential characteristics of strain P115 and the type strains of the validly described species*

Phenotypic Test	**Strain P115**	***S. rubrogriseus***	***S. tricolor***	***S. linomycini***
Growth at:				
10ºC	+	+	+	+
28 ºC	+	+	+	+
40 ºC	+	+	+	+
Growth on Sole Carbon Source:				
D-Glucose	+	+	+	+
L-Arabinose	-	+	-	+
Sucrose	-	-	-	-
D-Xylose	+	-	-	+
I-Inositol	+	+	-	-
Rhamnose	+	+	-	+
Raffinose	-	-	-	-
Galactose	+	ND	ND	ND
D-Mannitol	+	+	-	+
Spore Surface	Smooth	Smooth	Smooth	Smooth
Spore Chain	SS	SS	SS	RA
Colony Color on ISP4:				
Aerial Mass	White	Grey	Grey	Grey
Reverse side	White	Red	Red	Colorless
Pigmentation	-	-	-	-
Enzymes:				
H_2S	-	-	-	ND
Gelatine	+	+	+	ND

+, positive response; -, negative response; ND, Not Determined; SS, *Spirals*; RA, *Retinaculiaperti.*

*: Data compiled from Wink J.M. (1971).

4.3 Evaluation of the *in vivo* Biocontrol Ability of Strain P42 against Three Different Species of *Colletotrichum*

4.3.1 Phytotoxicity Test of Selected *Streptomyces* spp. Strain P42 by Seed Germination Assay

Seed viability test results indicated that maize seed had 85.83% germination rate, therefore selected for the following stages of phytotoxicity test.

(germinated seeds - non germinated seeds / 120 × 100)

The results of the phytotoxicity assay are shown in Table 4.24. The treated plant heights in low dosage treatments were 20.88 ± 2.05 cm, while in high dosage treatments they were 12.51 ± 3.46 cm. The plant height for control was 18.40 ± 1.82 cm. There was significant difference ($p<0.05$) between the plant height of maize seeds treated with low dosage of P42 compared to plant height of maze seeds treated with high dosage of P42 and non-treated maize seeds (control). The root condition of test plants in comparison with control plants was summarized in Table 4.24 and shown in Plate 4.34. The main root length in treated plants with high dosage and low dosage of P42 were 18.60 ± 6.75 cm and 38.14 ± 6.74 cm respectively, and showed significant difference when compared to non-treated (control) maize seeds (Plate 4.35). The number of secondary roots for low dosage treatments was 6.50 ± 1.59 and for high dosage treatments was 4.07 ± 1.18 (Table 4.24). The results did not reveal any significant difference between the number of secondary roots of low dosage and the control plants. The number of leaves in high and low dosage treated plants with P42 strain was 1.70 ± 0.6 and 2.83 ± 0.37 respectively. Results indicated significant difference between the means of maize plant

leaf numbers in high or low dosage of P42 and the control (non-treated) treatments (Table 4.24).

Table 4.24: Effects of strain P42 spore suspension treatment on the growth of maize

Treatment	Characteristics			
	Plant Height (cm)	Main Root Length (cm)	Number of Leaves	Number of Secondary Roots
Control	18.40 ± 1.82a	34.43 ± 7.79a	2.33 ± 0.54a	6.27 ± 1.81a
P42 (High Dosage)	12.51 ± 3.46b	18.60 ± 6.75b	1.70 ± 0.64b	4.07 ± 1.18b
P42 (Low Dosage)	20.88 ± 2.05c	38.14 ± 6.74c	2.83 ± 0.37c	6.50 ± 1.59a

a,b,c: show significant difference ($P<0.05$) within the same column.

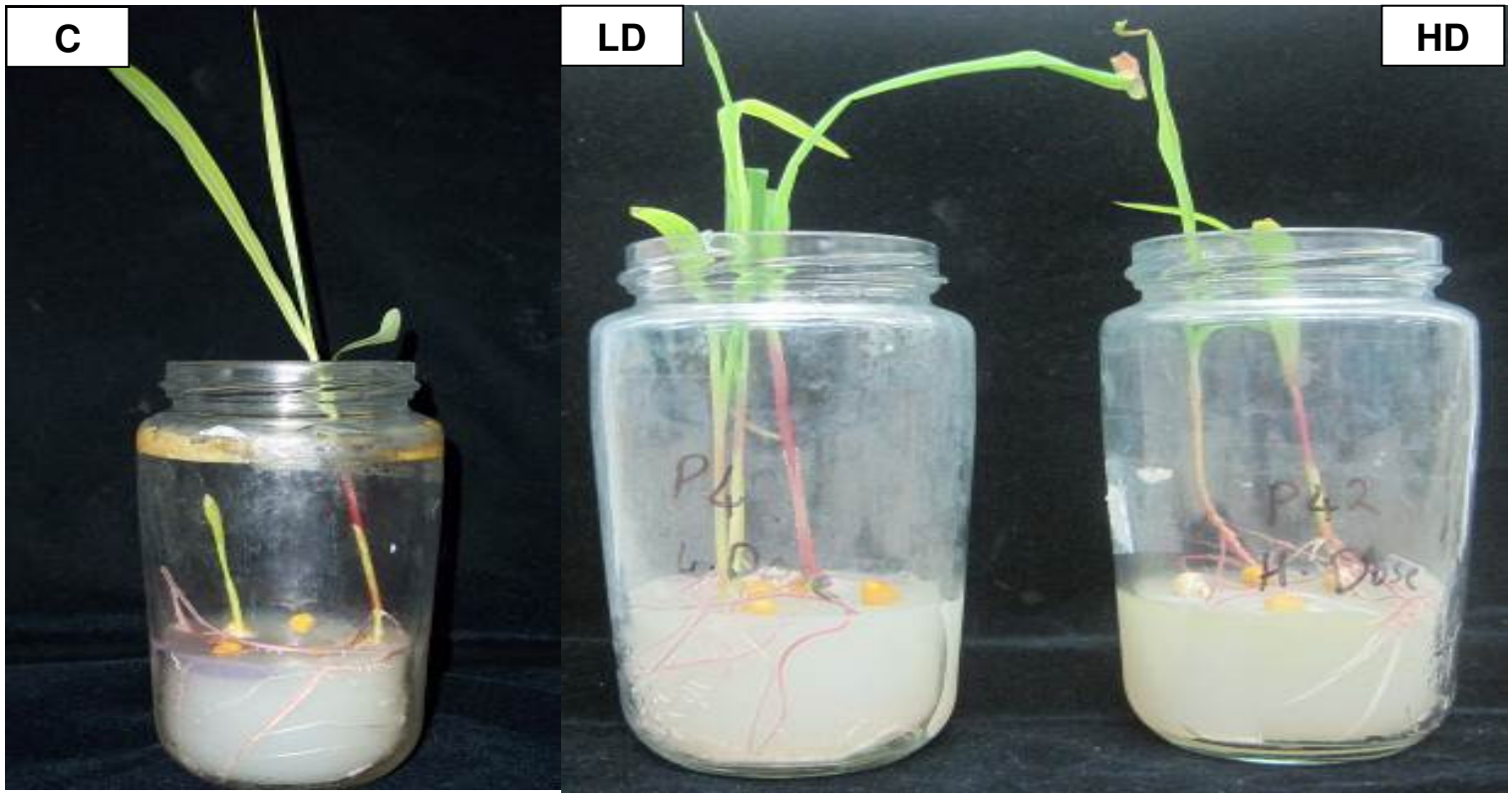

Plate 4.34: Phytotoxicity test. Germination and growth of Maize seeds after 10 days of incubation on ISP2 agar 2% (w/v) at 28 ± 2ºC
C: Control; LD: Low Dosage of P42; HD: High Dosage of P42.

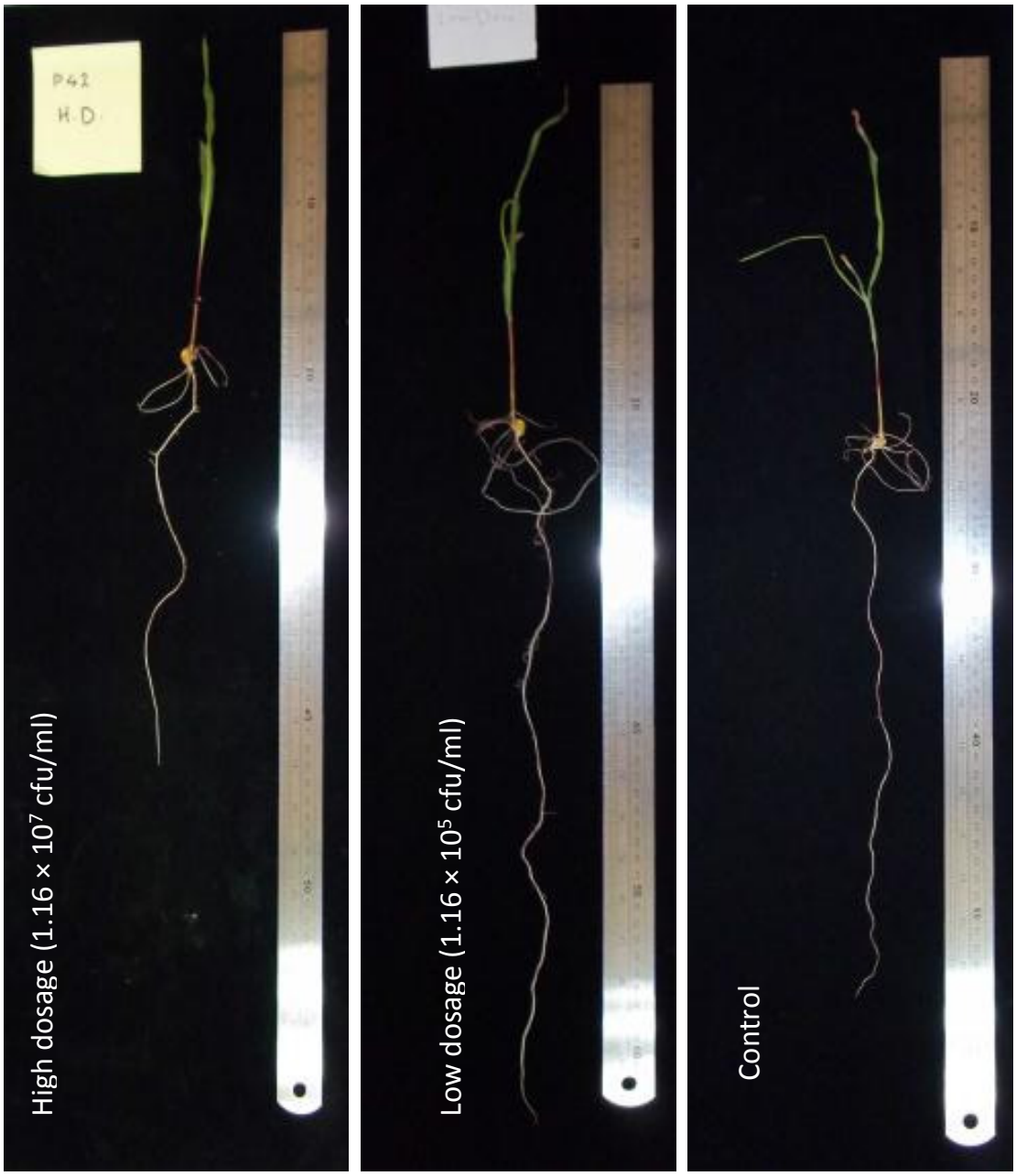

Plate 4.35: Condition and characteristics of Maize plants with different dosage of P42 after 10 days of incubation at 28 ± 2ºC.

4.3.2. *In vivo* Biocontrol Assays

In vivo biocontrol assay was repeated three times. Treating the seeds and roots of chilli plants with strain P42 suspension (1.16×10^5 cfu/ml) significantly ($p<0.05$) reduced anthracnose severity when the chilli fruits were inoculated with 10^5 spores/ml of each *Colletotrichum* species (Table 4.25, Appendices C5, C6).

4.3.2.1 Treatments C and P

The pathogen-free untreated (C) and strain P42-treated (P) control plants remained healthy until the end of the experiment (Plates 4.36 and 4.37; Table 4.25). Plants treated with P42 strain showed bigger size fruits in comparison with P42-free control plants (Plate 4.37).

Table 4.25: The effect of strain P42 on pathogen-free chilli plants (recorded after 70 days)

Treatment Group	Plant #	Fruit Fresh Weight (g)	Damage Scale
Untreated Chilli Plants (C)[a]	1	11.8	0
	2	12.0	0
	3	11.5	0
	4	12.2	0
	5	11.6	0
	6	11.4	0
	7	12.1	0
	8	12.4	0
	9	11.9	0
	10	12.4	0
Strain P42-Treated Chilli Plants (P)[b]	1	13.2	0
	2	13.5	0
	3	12.7	0
	4	13	0
	5	13.4	0
	6	12.9	0
	7	13	0
	8	12.6	0
	9	12.8	0
	10	13.1	0

[a]: C, seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in sterile distilled water.
[b]: P, chilli seeds were treated with *Streptomyces* sp. strain P42 suspension prior to planting; plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in sterile distilled water.

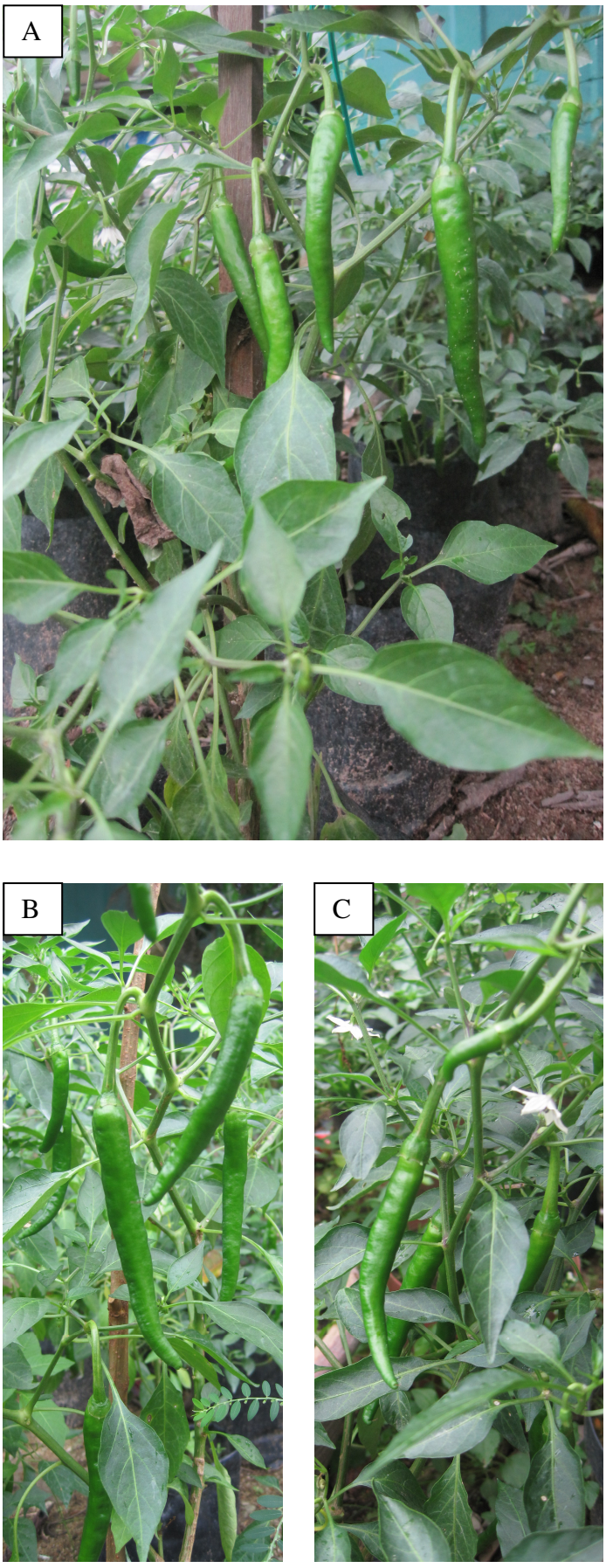

Plate 4.36: A,B,C: Pathogen-free and untreated chilli plants (C) 70 days after planting.

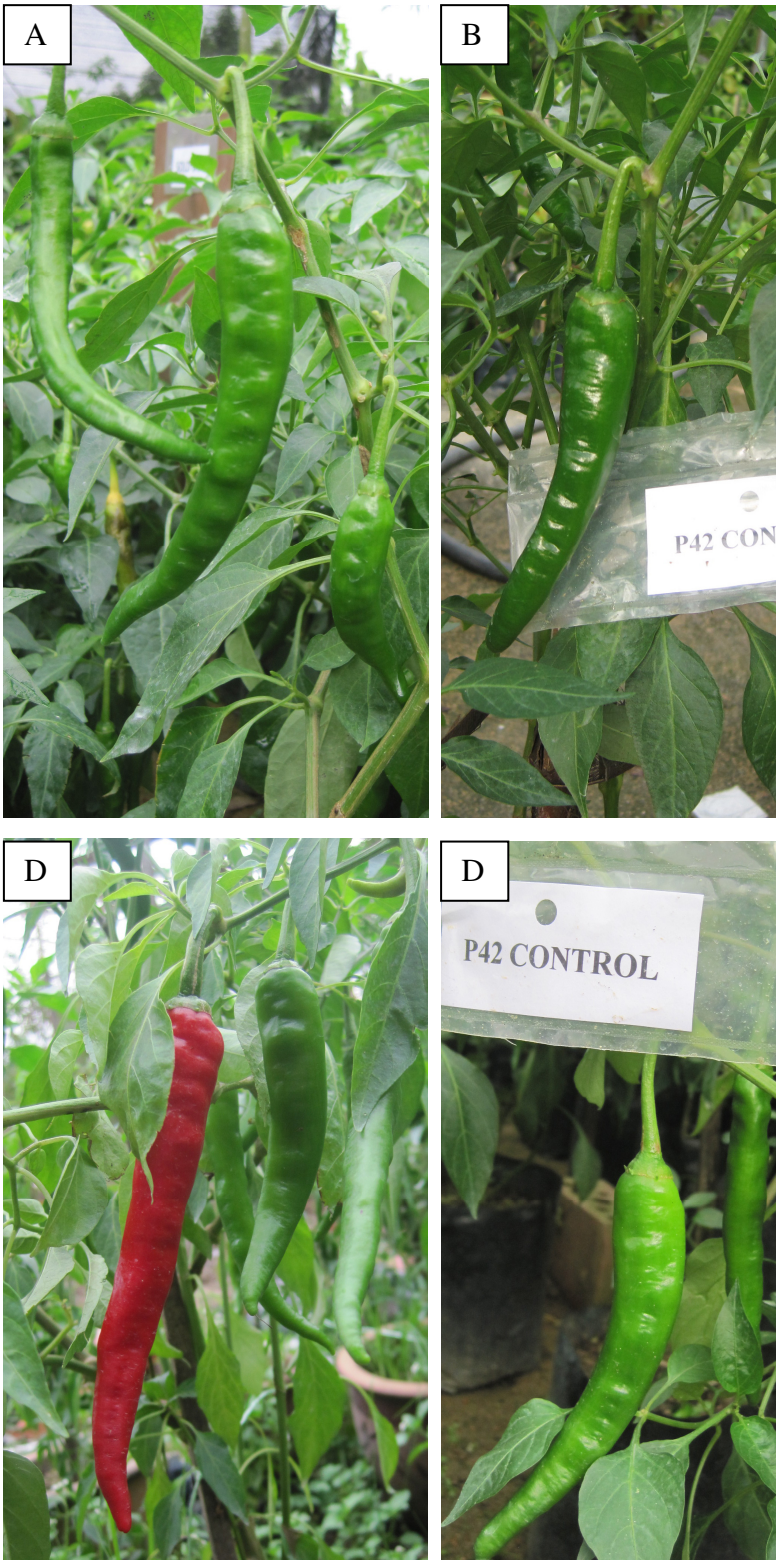

Plate 4.37: A,B,C,D: *Streptomyces* sp. strain P42 treatment in pathogen-free chilli plants (P) 70 days after planting. The bigger size of fruits in the plants is notable.

4.3.2.2 Treatments Ca and PCa

Severe fruit damage symptoms were observed in chilli fruits after 9 days of inoculation with *Colletotrichum acutatum* (Ca) spore suspension. Damage symptoms expanded quickly into a large lesion, leading to the distortion of pepper fruits (Plate 4.38). However, the lesions on fruits treated with strain P42 (PCa) developed more slowly, resulting in smaller lesions than those observed on untreated plants. Chilli plants treated with strain P42 caused significant disease suppression ($P \leq 0.05$) against *C. acutatum* (Plate 4.39; Table 4.26).

Table 4.26: The effect of strain P42 on chilli plants inoculated with *C. acutatum* (recorded after 9 days of inoculation with fungus suspension)

Treatment Group	Plant #	Fruit Fresh Weight (g)	Damage Scale
P42-Free Chilli Plants (Ca)[a]	1	4.1	4
	2	4.3	4
	3	4.0	4
	4	5.1	3
	5	2.5	5
	6	3.5	4
	7	5.8	3
	8	3.9	4
	9	2.8	5
	10	2.6	5
Strain P42-Treated Chilli Plants (PCa)[b]	1	11.2	2
	2	12.0	1
	3	12.4	1
	4	11.5	2
	5	11.6	2
	6	12.3	1
	7	12.1	1
	8	12.2	1
	9	11.0	2
	10	11.9	1

[a]: Ca, seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in 10^5 *C. acutatum* suspension.
[b]: PCa, chilli seeds were treated with *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in 10^5 *C. acutatum* suspension.

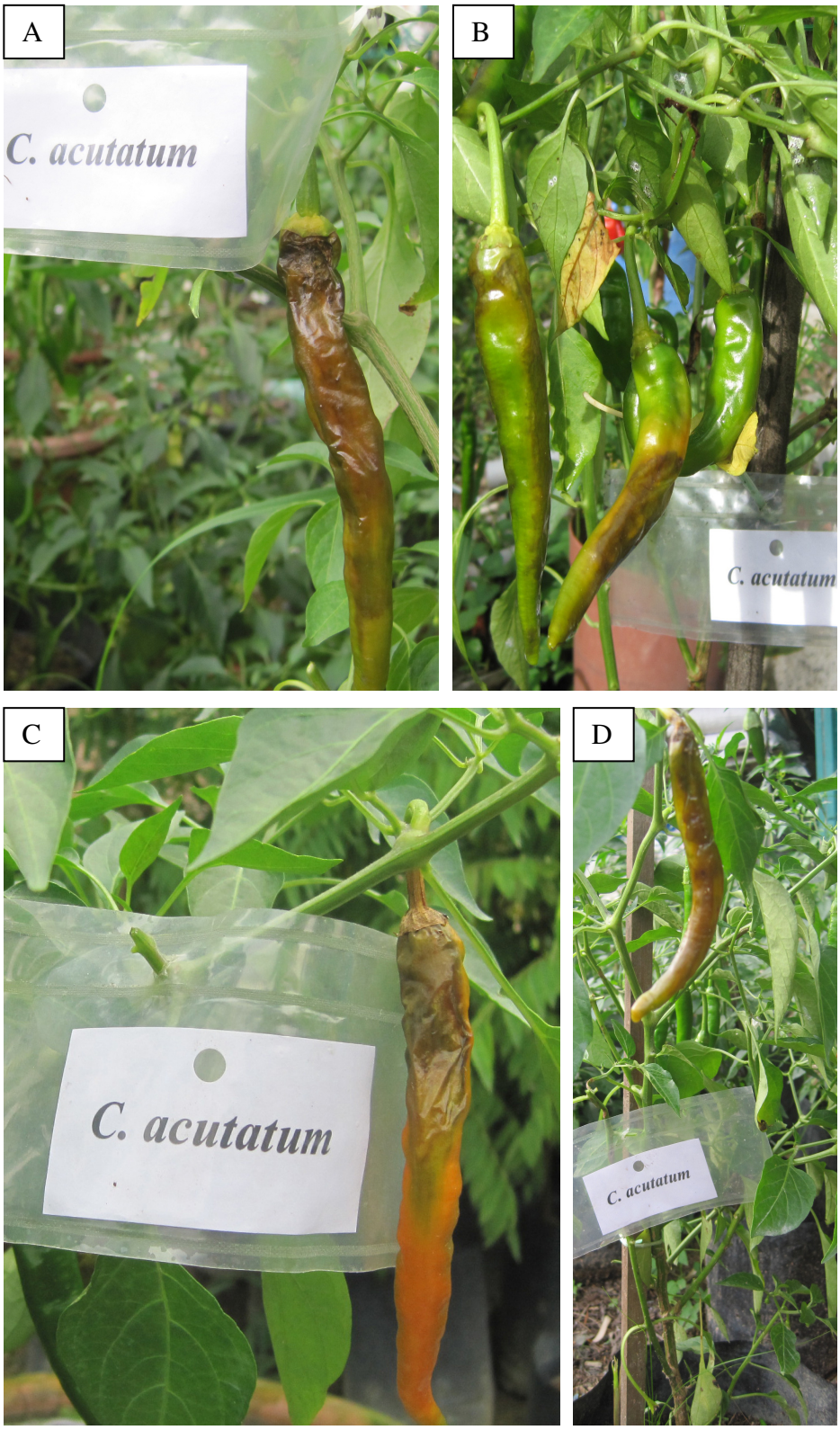

Plate 4.38: A,B,C,D: P42-free chilli plants inoculated with *C. acutatum* 9 Days after inoculation; D: Dead fruit.

Plate 4.39: A,B,C: *Streptomyces* sp. strain P42 treatment in chilli plants inoculated with spores of *C. acutatum* 9 days after inoculation.

4.3.2.3 Treatments Cc and PCc

Severe fruit damage symptoms were detected in chilli fruits after 9 days of inoculation with *Colletotrichum capsici* (Cc) spore suspension. All ten untreated plants failed to resist the disease. Damage symptoms expanded quickly into a large lesion, leading to the death of chilli fruits (Plate 4.40). However, the lesions on fruits treated with strain P42 (PCc) developed more slowly, resulting in smaller lesions than those observed on untreated plants. Chilli plants treated with strain P42 caused significant disease suppression ($P \leq 0.05$) against *C. capsici* (Plate 4.41; Table 4.27).

Table 4.27: The effect of strain P42 on chilli plants inoculated with *C. capsici* (recorded after 9 days of inoculation with fungus suspension)

Treatment Group	Plant #	Fruit Fresh Weight (g)	Damage Scale
P42-Free Chilli Plants (Cc)[a]	1	2.4	5
	2	2.9	5
	3	3.5	4
	4	3.0	4
	5	3.2	4
	6	4.4	3
	7	3.3	4
	8	2.6	5
	9	3.6	4
	10	2.5	5
Strain P42-Treated Chilli Plants (PCc)[b]	1	10.1	1
	2	9.5	2
	3	10.8	1
	4	9.8	2
	5	11	1
	6	8.5	2
	7	7.2	3
	8	8.4	2
	9	5.4	4
	10	9.0	2

[a]: Cc, seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in 10^5 *C. capsici* suspension.
[b]: PCc, chilli seeds were treated with *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in 10^5 *C. capsici* suspension.

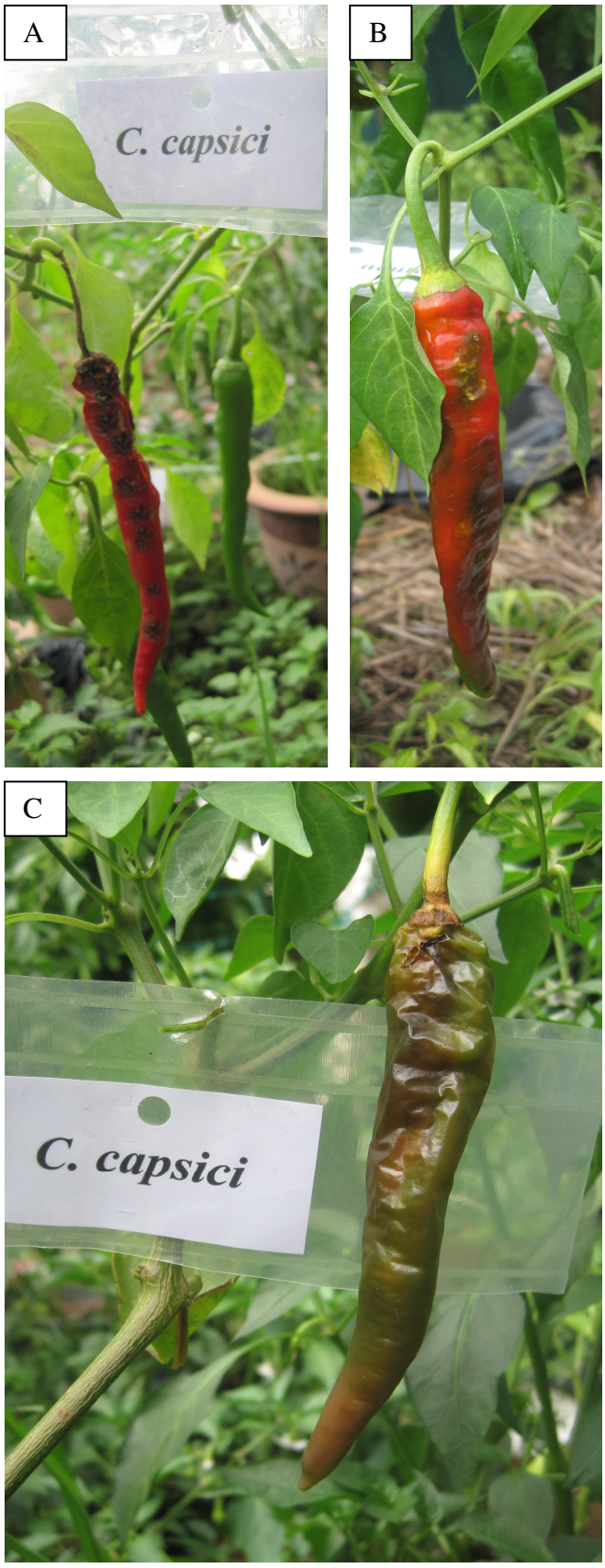

Plate 4.40: A,B,C: P42-Free chilli plants inoculated with *C. capsici* 9 days after inoculation; C: Dead fruit.

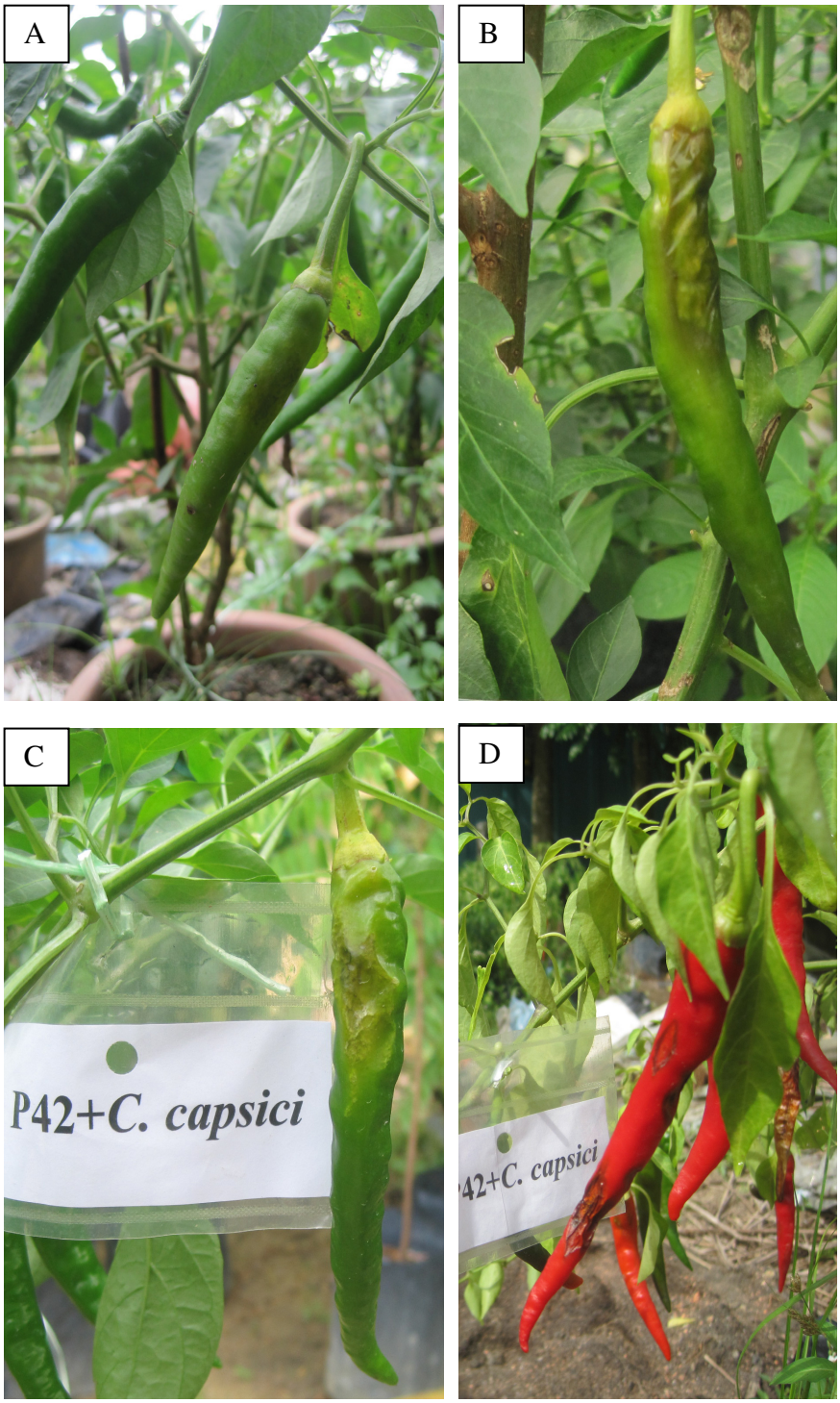

Plate 4.41: A,B,C,D: *Streptomyces* sp. strain P42 treatment in chilli plants inoculated with spores of *C. capsici*, 9 Days after inoculation.

4.3.2.4 Treatments Cg and PCg

High level of fruit damage symptoms were noticed in chilli after 9 days of inoculation with *Colletotrichum gloeosporioides* (Cg) spore suspension. All ten untreated plants failed to resist to the disease at varying degrees. Damage symptoms expanded quickly into a large lesion, leading the death of most of the pepper fruits (Plate 4.42). However, the lesions on fruits treated with strain P42 (PCg) developed more slowly, resulting in smaller lesions than those observed on untreated plants. Chilli plants treated with strain P42 caused significant disease suppression ($P \leq 0.05$) against *C. gloeosporioides* (Plate 4.43; Table 4.28).

Table 4.28: The effect of strain P42 on chilli plants inoculated with *C. gloeosporioides* (recorded after 9 days of inoculation with fungus suspension)

Treatment Group	Plant #	Fruit Fresh Weight (g)	Damage Scale
P42-Free Chilli Plants (Cg)[a]	1	2.5	5
	2	2.1	5
	3	4.5	3
	4	2.9	5
	5	3.6	4
	6	3.8	4
	7	2.0	5
	8	3.3	5
	9	2.6	5
	10	4.2	3
Strain P42-Treated Chilli Plants (PCg)[b]	1	10	1
	2	9.2	2
	3	11.2	1
	4	9.6	2
	5	9.1	2
	6	5.5	4
	7	9.5	1
	8	8.0	3
	9	11.8	1
	10	9.9	2

[a]: Cc, seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in 10^5 *C. gloeosporioides* suspension.
[b]: PCc, chilli seeds were treated with *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in 10^5 *C. gloeosporioides* suspension.

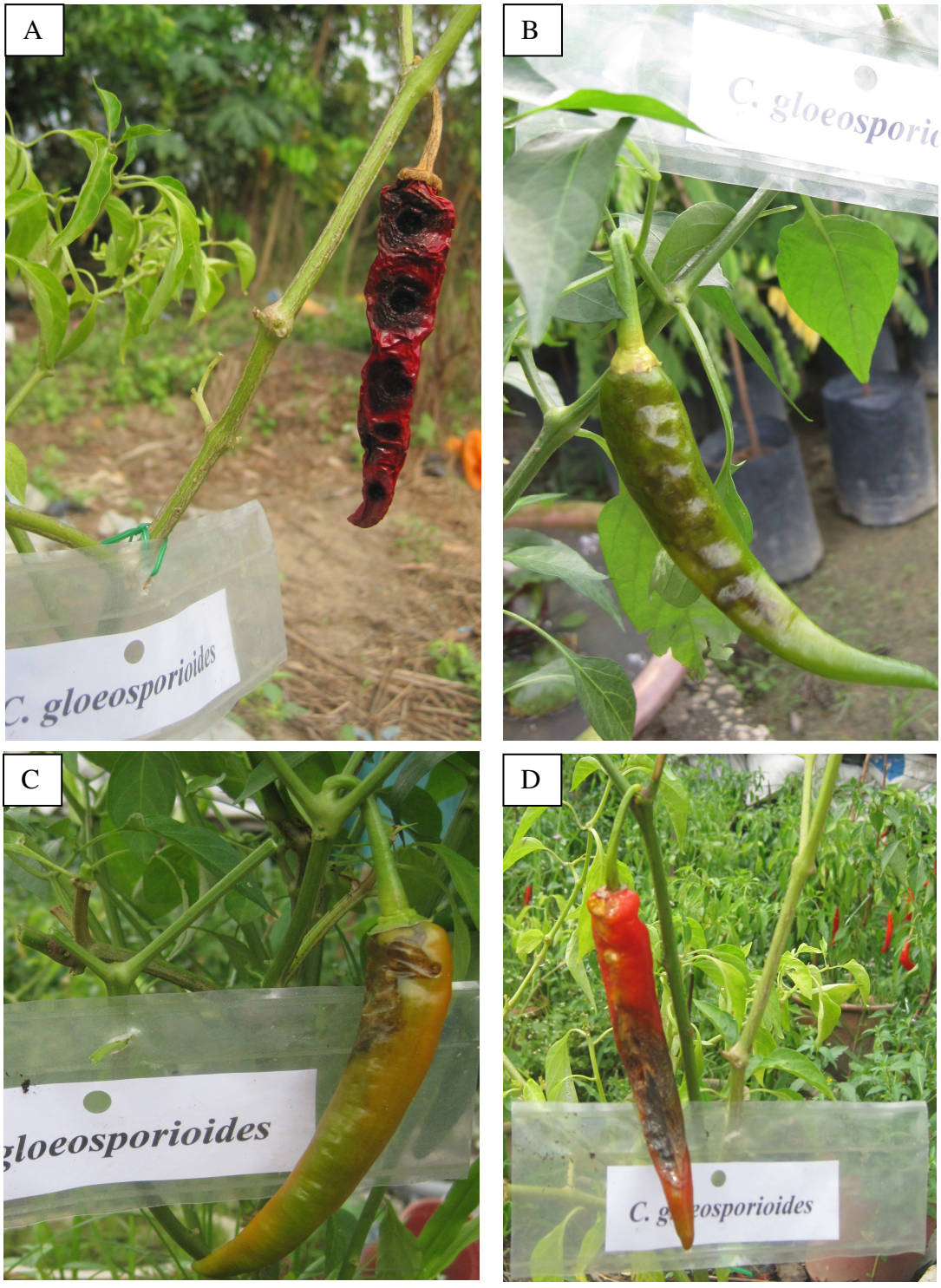

Plate 4.42: A,B,C,D: P42-Free chilli plants inoculated with *C. gloeosporioides*, 9 days after inoculation; A: Dead fruit.

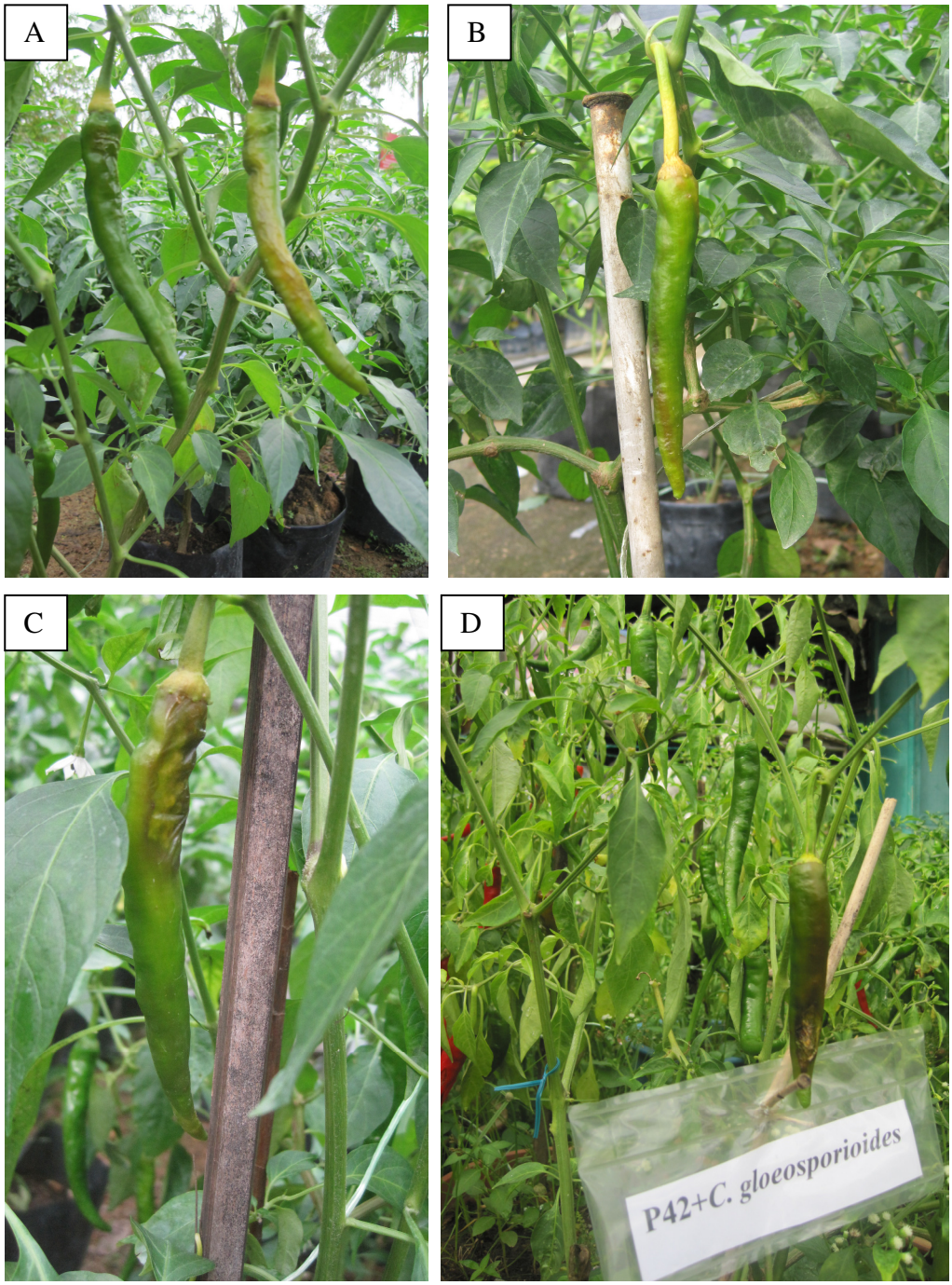

Plate 4.43: A,B,C,D: *Streptomyces* sp. strain P42 treatment in chilli plants inoculated with spores of *C. gloeosporioides*, 9 days after inoculation.

4.3.2.5 Summary of Fruit Symptom Index (FSI) and Disease Severity Index (DSI)

The average of Fruit Symptom Index (FSI) and Disease Severity Index (DSI) for chilli fruit symptoms of each individual treatment group inoculated with different *Colletotrichum* species were calculated (Table 4.29). Results showed that FSI and DSI were reduced in strain P42-treated chilli plants compared to the untreated plants (Appendices C4.3, C5.7, C6.7).

In the presence of strain P42 (PCa, PCc and PCg) FSI was significantly reduced (p=0.05; Appendices C4.1, C5.5, C6.5) compared to untreated chilli plants (Ca, Cc, Cg); moreover treated chilli plants showed 54%, 53% and 56% reduction in DSI values, respectively. Treatment with strain P42 also reduced the damage scale of plant anthracnose symptom and none of the plants died after 9 days inoculation (Table 4.29).

For chilli plants inoculated with any of the *Colletotrichum* species, the mean total fruit fresh weight of strain P42-treated plants was higher than untreated plants (Table 4.29). Pathogen-free strain P42-treated control plants (P) revealed higher fruit fresh weight compared to pathogen-free untreated control chilli plants (C). The mean fruit fresh weights of two control groups were statistically different (p=0.05, Appendices C4.2, C5.6, C6.6). The mean fruit fresh weight of untreated chilli plants inoculated with *C. acutatum*, *C. capsici* and *C. gloeosporioides* (Ca, Cc, Cg) was significantly lower (p=0.05; Appendices C4.2, C5.6, C6.6) than both the pathogen-free control groups (P, C) and P42-treated plants inoculated with *C. acutatum*, *C. capsici* and *C. gloeosporioides* (PCa, PCc, PCg).

The differences in FSI and mean fruit fresh weight of both groups were statistically

significant (p=0.05; Appendices C7-C12).

Table 4.29: Summary of Fruit Symptom Index (FSI) and Disease Severity Index (DSI) and fresh weight of chilli plants with and without strain P42 treatment in the *in vivo* biocontrol assay (recorded after 9 days)

***Colletotrichum* Species**	**Strain P42 treatment[a]**	**Fruit Fresh Weight (g)[b]**	**FSI[c]**	**DSI(%)[d]**	**A**	**B**
-	Untreated (C)	11.92 ± 0.39B[e]	0.0C	0	0	0
-	Treated (P)	13.09 ± 0.29A	0.0C	0	0	0
C. acutatum	Untreated (Ca)	3.78 ± 0.93D	4.1A	83	30	10
C. acutatum	Treated (PCa)	11.60 ± 0.61B	1.4B	29	30	0
C. capsici	Untreated (Cc)	3.15 ± 0.63D	4.3A	87	30	13
C. capsici	Treated (PCc)	9.64 ± 1.45C	1.7B	34	30	0
C. gloeosporioides	Untreated (Cg)	3.05 ± 0.63D	4.5A	90	30	17
C. gloeosporioides	Treated (PCg)	9.87 ± 1.43C	1.8B	36	30	0

[a]: C, seeds and plants were not treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension and fruits immersed in sterile distilled water; P, chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in sterile distilled water; Ca,Cc and Cg seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension; PCa, PCc and PCg chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension.
[b]: Average fruit fresh weight of thirty plants for each treatment (mean ± standard deviation).
[c]: Fruit Symptom Index, FSI (average of thirty plants for each treatments).
[d]: Disease Severity Index, DSI (average of thirty plants for each treatments).
[e]: Means with the same letter within a column are not significantly different ($p<0.05$).
A: Number of plants inoculated with the *Colletotrichum* species; B: Number of dead chilli fruits.

4.3.3 Re-Isolation of Strain P42 from Experimental Chilli Plant Rhizosphere

Strain P42 was re-isolated from chilli plants rhizospheric soils using similar media as in subsection 3.1.2 prior to re-identification. Strain P42 re-isolated from rhizospheric soils of trial chilli plants showed similar morphological characteristics as in section 4.2.2 and Table 4.3.

4.3.4 Identification of Strain P42 from Experimental Chilli Plant Rhizosphere

Strain P42 was re-identified using 16S rRNA method as mentioned in section 3.2.3.4 to confirm the type of strain P42. The nucleotide sequences for a section of the 16S rRNA gene (1477 bp) (Appendix C13) from the strain P42 was subjected to BLASTN analysis using the NCBI database for identification at the genus level. BLASTN results revealed that the strain P42 belonged to the genus *Streptomyces* and contained 99% DNA homology in the 16S rRNA gene. The molecular results confirmed the strain P42's 99% proximity to *Streptomyces rochei*.

CHAPTER 5

DISCUSSION

5.1 Isolation and Screening of Rhizosphere-Derived Actinomycetes for Fungus-Antagonistic Properties

5.1.1 Isolation of Chilli Rhizosphere-Derived Actinomycetes

Actinomycetes are generally saprophytic, soil-dwelling microorganisms that spend the majority of their life cycle as spores (Cao *et. al*., 2005). The cell production rate of actinomycete is generally lower than bacteria, and their rate of radial growth on culture medium is lower than fungi. Actinomycetes may take up to a month incubation time for a visible colony to develop, as was observed in this study.

In the current study, 82% of the strains isolated from rhizosphere-derived samples inoculated on three different agar media (starch-casein agar [SC]; Raffinose-histidine [RH] agar; humic acid-vitamin [HV] agar) fell into the *Streptomyces*-like group. Although the occurrence of distinct aerial mycelium with abundant spore mass formation represents an important macroscopic criterion to identify the genus *Streptomyces*, there are other actinomycete genera that also produce aerial mycelium. Among them are *Streptosporangium, Saccharomonospora* and *Actinomadura* (Goodfellow & Williams, 1983). These organisms can easily be differentiated from streptomycetes by other morphological (Williams & Davies, 1967), biochemical (Hasegawa *et al*., 1983) and physiological (Williams *et al*., 1983) properties. Thus, the possibility that the *Streptomyces*-like strains isolated in this study may consist of

members from other aerial mycelium-producing genera besides *Streptomyces* cannot be ruled out.

Numerous studies have revealed that when SC agar was used to isolate actinomycetes from soil, the majority of colonies developed are *Streptomyces* spp. (Hayakawa & Nonomura, 1987). The abundance of *Streptomyces* spp. on SC agar was also observed during isolation of rhizosphere-derived actinomycetes from Malaysian Langkawi Agro Technology Park (Heng & Hamzah, 2011) and mangrove rhizosphere soil (Kavithambigai *et al*., 2001). The fast growing streptomycetes may have retarded the slower growing actinomycetes, such as *Micromonospora* and other related genera, from growing out in the isolation plates (Vikineswary *et al*., 1997). This could be one of the factors that contributed to a low percentage of *Micromonospora*-like strains (approximately 18%) isolated in the current study. The cycloheximide, nystatin and nalidixic acid in the agar media suppressed the growth of fungi (Williams & Davies, 1965).

In a study done by Maldonado *et al*. (2005), RH was used as a selective agar for the isolation of actinomycetes derived from marine sediments. They successfully isolated numerous and various types of actinomycetes. However, when the isolation was done on a different media such as humic acidvitamin (HV) agar, *Micromonospora*-like strains were mainly obtained from *Colletotrichum*-infested soil. Presumptive rare actinomycetes, including the very slow-growing *Micromonospora*, grew well on the HV agar plates. The activation of spore germination by humic acid was considered as one of the causes for an increase in the number of diverse actinomycete colonies on the HV agar plates (Hayakawa & Nonomura, 1987).

Besides isolation media and incubation conditions, the selectivity of different groups of actinomycete among the isolates was also influenced by the nature of sample pre-treatment regimes. In one of their earlier studies, Nonomura & Ohara (1969) had reported that treating the soil with heat at 120°C was selective for the isolation of the genera *Microbispora* and *Streptosporangium*. Moist heating of soil suspensions at 70°C for 30 minutes selectively isolated members of the genus *Micromonospora* (Labeda & Shearer, 1990).

Numerous procedures has been recommended for the selective isolation of specific actinomycete genera from soil samples (Goodfellow & Haynes, 1984; Pisano *et al*., 1989; Takizawa *et al*., 1993). Heat treatment of samples prior to actinomycete isolation reduced the number of gram-negative bacteria commonly found in marine samples and often over-running isolation plates (Pisano *el al*., 1986). Higher numbers of *Micromonospora* spp. were observed from heat-treated near-shore sediment samples (Jensen *et al*., 1991). This genus is known to be more heat-resistant than other actinomycetes. However, the low number of *Micromonospora*-like isolates obtained in this study suggests that more effective pre-treatment techniques other than air-drying and moist-heat (70°C for 30 minutes) are needed to enhance the isolation of this group and other rare actinomycetes.

The use of antibacterial antibiotics has become another important selective technique for the isolation of different actinomycete genera (Labeda & Shearer, 1990). When nalidixic acid was added to the isolation medium, the number of isolated actinomycetes was increased (Takizawa *el al*., 1993). Hayakawa & Nonomura (1989) have shown that

the sporogenous species, *Bacillus subtilis*, was completely inhibited by nalidixic acid. However, *Micromonospora* strains have been reported to be resistant to nalidixic acid (Wakisaka & Koizumi, 1982). Nalidixic acid had been used to suppress the growth of soil bacteria during the isolation of indigenous actinomycetes from coastal mangrove mud (Kavithambigai *et al*., 2001). Rifampicin had also been used as another selective agent to isolate actinomycetes from soil, hay and straw (Athalye *et al*., 1981). Following this, Pisano *el al*. (1989) developed a technique of adding rifampicin to SC agar for the selective isolation of *Micromonospora* strains. Other selective agents like gentamycin and tunicamycin (Labeda & Shearer, 1990), and novobiocin (Wang *et al*., 1999) had also been described for the isolation of *Micromonospora* spp. from soil. Therefore, selective antibiotics could be used to enhance the isolation of rare actinomycetes. However, since the isolation of specific genera of rare actinomycetes was not targeted in the current study, selective antibiotics were not added to the isolation media.

The relatively low numbers and slow growth of actinomycetes in relation to many common bacteria, were among the main problems encountered during the isolation from rhizosphere soil samples. Rapidly growing bacteria often disguised the presence of actinomycetes on the isolation plates. Therefore, pre-treatment of the sample and/or application of selective pressures were needed to inhibit their growth. According to Matsukuma *et al*. (1994), pre-treatment of pine litter samples with a solution containing 0.05% sodium dodecyl sulphate (SDS) and 6% yeast extract, and the addition of nalidixic acid to isolation agar, were effective in reducing bacterial growth in serial dilution method. These agents were not harmful to the spores of actinomycetes (Hayakawa *et al*., 1988).

Previous researchers had revealed that the relative numbers of different types of actinomycetes isolated were also influenced by the sampling location. Various ecological factors such as water content, organic matter, salinity and climate are interrelated and might influence the actinomycete population (Goodfellow & Haynes, 1984). Findings of previously reported studies suggest that differences in the type of soil and the agriculture practices may influence the population of the actinobacteria. This statement is supported by Athalye *et al*. (1981) and Oskay *et al*. (2004), where they found that the growth rate of microorganisms were influenced by the humidity and pH of the soil. Merckx *et al*. (1987) stated that soil rhizosphere has diverse saprophytic microorganisms due to the organic material derived from the plant roots and its exudates. This statement is further supported by Tewtrakul & Subhadhirasakul (2007), where they obtained a correlation between the diversity of actinobacteria with the type of plants and the soil organic matter content.

In the present study, *Streptomyces*-like isolates were notably dominant in the rhizospheric soil samples collected from different zones in the selected chilli farms. The predominance of streptomycetes in many actinomycete populations had been reported frequently (Xu *et al*., 1996; Wang *el al*., 1999). This is also true in the case of sediments (Okazaki *and* Okami, 1976; Sponga *el al*., 1999), coastal mangrove mud (Vikineswary *et al*., 1997) and seaweeds (Genilloud *et al*., 1994) of the marine environment.

The four main groups of aerial mycelium colours observed in this study had been documented previously (Tresner & Backus, 1963; Shirling & Gottlieb, 1966). Variations in the colours of the aerial and substrate mycelium of the isolates and in the soluble pigments they produced may be an indication of the diversity and variability of

the isolated *Streptomyces*-like strains (Ndonde & Semu, 2000). Besides the grey group, isolates from the white, red and pink colour groups were also observed. Previously, Ndonde & Semu (2000) have also grouped *Streptomyces* strains isolated from Tanzanian soil into separate red and pink aerial mycelium colour groups. In general, the range of diversity observed in the *Streptomyces*-like isolates appeared to be narrow in this study since strains from other colour groups such as yellow and blue (Ndonde & Semu, 2000) were not isolated.

5.1.2 Evaluation of Selected *Streptomyces*-Like Isolates for Antagonism towards Plant Pathogenic Fungi

Streptomycetes are known to have a relatively lower growth rate on agar plates than most of the fungi (Yuan & Crawford, 1995). Therefore, a pre-inoculation (five days) was done in the cross-plug assay to allow the actinomycete colonies to establish and sporulate on the agar surface. According to Crowford *et al.* (1993), if the actinomycetes were allowed to grow and sporulate on the *in vitro* assay prior to inoculation of the fungal pathogen, the antagonism may be due to the production of secondary, as opposed to primary, metabolites. The genetic control mechanisms for the production of secondary metabolites and formation of spores in *Streptomyces* spp. showed cross-correlations (Maplestone *et al.,* 1992). This similarity ensured antibiotic production during sporulation (Demain & Fang, 2000).

The antifungal activity of actinomycetes isolated from rhizosphere soil samples in this study was compared with the activity of isolates obtained from other sources (Table 5.1). In the present study, 24.5% of the selected *Streptomyces*-like strains showed antagonism against one or more test fungi. Previously, 33% of the selected

Streptomyces spp. isolated from a Malaysian coastal mangrove area showed antagonism to fungal plant pathogens when no special pre-treatment technique other than air-drying was applied to the mud samples during isolation (Vikineswary *et al*., 1997). In the current study, the growth of contaminating microorganisms was limited when sample suspensions were heated at 70°C for 30 min. This suggested that the moist-heat pre-treatment methods reviewed by Labeda & Shearer (1990) may be applied to rhizosphere-derived samples to facilitate the isolation of actinomycetes. The applicability of various pre-treatment techniques to rhizosphere soil samples could also facilitate the isolation of interesting actinomycete species with special biosynthetic capabilities (Pisano *et al*., 1986 & 1989).

In another study, pre-selection of chitinolytic strains segregated a group of marine-derived actinomycetes in which 47% were antagonistic towards fungi (Pisano *et al*., 1992). This proportion exceeded the proportion of fungus-antagonistic strains obtained (28%) when chitinolyis was not used as a selective criterion (Pisano *et al*., 1989). Similarly, Okazaki & Okami (1972) also obtained a low percentage of antifungal isolates (23%) when no special pre-treatment methods were applied during the isolation of actinomycetes. Therefore, the use of more selective isolation procedures could enhance the isolation of "rare" actinomycetes and "rare" streptomycetes, particularly species with antimicrobial capabilities.

It has been reported that the rhizosphere of different plant varieties resistant to soil - borne fungi contained members of antagonistic *Streptomyces*. The use of these antagonistic actinomycetes for the biological control of root-attacking fungi was demonstrated with success in many studies (Mohamed, 1982). On the other hand,

Crawford *et al.* (1993) found that there was no difference in the possibilities of isolating antagonistic streptomycetes from rhizosphere soil versus non-rhizosphere soil. However, in the present study, the number of antagonistic actinomycetes isolated from *Colletotrichum*-free rhizosphere soil (5 of 26 antagonistic strains) showed a major difference from the number of actinomycetes isolated from *Colletotrichum*-infested rhizosphere soil (21 of 26 antagonistic strains). This may indicate that the population of cultivable antagonistic actinomycetes increases in the presence of the pathogen. Previous work done by other researchers showed that the population of actinomycetes were isolated from banana plants infested with *Fusarium oxysporum* f.sp. *cubense* was higher in comparison with healthy banana plant (Cao *et al*., 2005). Their results are consistent with the finding of Reiter *et al*. (2002). Although these researchers had isolated actinomycetes from plants instead of soil, there may be a correlation between their findings and the finding in the present study. The isolates isolated from *Colletotrichum*-infested rhizosphere soil showed higher antagonistic activity against test fungi. The results of this study contradicted with the findings of Cao *et al*. (2005) where a higher percentage of antagonistic actinomycetes were found in the roots of healthy plants compared to infested plants. Overall, the occurrence of antagonistic terrestrial actinomycetes in various studies varied from as low as 10% to as high as 47% of the total isolates screened (Table 5.1). This shows that the percentage of occurrence of antagonistic actinomycetes in terrestrial habitats is almost the same as in the coastal or marine habitats. The high proportion of antagonistic *Streptomyces*-like strains obtained in the present study indicated that the rhizospheric areas could also be a potential source for isolating possible biological control agents.

Table 5.1: Comparison of percentage occurrence of fungus-antagonistic actinomycetes isolated from terrestrial environments

Source of actinomycetes	**Actinomycete group**	**Percent (%) antagonistic isolates[a]**	**Reference**
Various soils (Australia)	*Streptomyces* spp	10-47	Broadbent *et al*. 1971
Various soils (India)	ND	40	Reddi & Rao, 1971
Rhizosphere soils of Soybean and Wheat Plants (Egypt)	*Streptomyces* spp	25-45	Mohamed, 1982
Rhizosphere and non-rhizosphere Soils (UK)	*Streptomyces* spp	22	Crawford *et al.*, 1993
Colletotrichum-free rhizosphere soil (Malaysia)	*Streptomyces* spp	5	Current Study
Colletotrichum-infested rhizosphere soil (Malaysia)	*Streptomyces* spp	21	Current Study

[a]: Percentage of the total isolates tested showing antagonism towards one or more fungal plant pathogens, ND: Not determined.

The *in vitro* assay showed that *Colletotrichum* differed greatly in its sensitivity towards the antagonistic effects of different strains of actinomycetes.

The modified 'cross-plug' assay method (Nadaraj, 1996) was used for testing the antagonistic actinomycetes isolates. This allowed a rapid screening of large numbers of potential antagonists. Antagonism of fungi by actinomycetes has frequently been associated with the production of antibiotics (Rangaswami & Ethiraj, 1962; Rangaswami & Vedysekanen, 1963; Knauss, 1976; Omar *et. al*., 2006). The selection of actinomycete biocontrol agent by *in vitro* technique is therefore an appropriate primary screening, as antibiotic production is readily detectable on agar media (Omar *et. al*., 2006). Those shown to be non-antagonistic by plate's assays generally are also inactive in soil (Broadbent *et al*., 1971; Omar *et. al*., 2006).

Streptomyces are known to have a relatively low growth rate on agar plates than most fungi (Yuan & Crawford, 1995). Pre-inoculation (five days) is usually done in the cross-plug assay to allow the actinomycete colonies to establish and sporulate on the agar surface. Based on the large inhibition zones observed on the bioassay plates, the pattern of antagonism indicated that water soluble antifungal metabolites were produced by the active strains.

5.1.3 Inhibition of Linear Growth of Three Different Species of *Colletotrichum* spp. by Isolate P42

In the current study, twelve isolates showed antagonism towards all three *Colletotrichum* species tested (Table 4.4). Out of the twelve, isolate P42 showed very strong inhibition against all three species of the test fungi. In screening for biocontrol agents against the root-rot pathogen of wheat, Stevenson (1956) reported that the most

promising isolates of *Streptomyces* sp. were the ones producing antibiotics against *Helminthosporium sativum*. Yuan & Crawford (1995) also showed that *Streptomyces lydicus* WYEC 108, a potent biocontrol agent for controlling seed and root rot, was selected for its strong *in vitro* activity against *Pythium ultimum*. In this study, isolate P42 was given preference because of its high degree of antagonism towards three various species of *Colletotrichum*.

Virulent strains of three different and dominant species of *Colletotrichum* were obtained from the Institute of Biological Sciences, University of Malaya. Plate assays were carried out to demonstrate the effectiveness of isolate P42 to inhibit different species of the *Colletotrichum*. Control plates supported excellent growth of the fungi and radial growth of the fungal colonies were easily measured over time. Therefore, differences in growth in the test plates could only be attributed to diffusible antifungal metabolites against *Colletotrichum* produced by the streptomycete in agar cultures. For fungal pathogens, tolerance to antibiotics is one of the attributes for successful saprophytic life in the soil. According to Reddi & Rao (1971), reduction in the fungal linear growth rates indicated antibiotic sensitivity. Isolate P42 was grouped as strongly antagonistic to *Colletotrichum* since it showed high reduction in the linear growth (81-94%) of all three species of *Colletotrichum*. *Colletotrichum acutatum* was more sensitive to the antifungal activity of this isolate compared to *C. capsici* and *C. gloeosporioides*. Since these mentioned species are destructive, widespread in distribution, and have a wider host range (Heng & Hamzah, 2011), they were used in the subsequent *in vivo* studies to investigate the disease-suppressing ability of isolate P42 against *Colletotrichum*.

5.1.4 Observation of Morphological Changes of Inhibited Fungal Growth by Scanning Electron Microscope (SEM)

Hyphal tip lysis, folding back, stunted mycelia, disintegrated hyphae, curling and/or bulging of hyphae appearances observed under SEM could be due to antibiosis by the antagonistic actinomycete. Antibiotics have been implicated repeatedly in the antagonism of fungi by actinomycetes (Crawford *et al*., 1993; El-Abyad *et al*., 1993). UV-mutant *S. corchorusii* strain lysed the hyphae of *F. oxysporum* f.sp. *phaseoli* in agar, produced metabolites that significantly inhibited sporulation, spore germination, germ tube elongation and growth of the fungus (El- Abyad *et al*., 1993). Strain P42 also induced malformation such as folding back and bulging hyphae of *Colletotrichum*. Folding back of hyphae was associated with the stunting of hyphal growth (Brian *et al*., 1947). It is known that besides inhibiting spore germination and hyphal growth, some antibiotic substances could induce malformation in fungal germ tubes and hyphae (Rangaswami & Ethiraj, 1962). Antifungal substances with different mechanisms of action could induce similar morphological changes in growing hyphae of fungi. Some antibiotics induced several types of changes, while with others the effects varied with the concentration (Richmond, 1975). For example, the antibiotic griseofulvin produced by *Penicillium janczewskii* induced stunted growth in germ tubes of *Botrytis allii.* In another research, the antibiotic made the hyphae curled or wave-shaped (Brian *et al*., 1947). Morphological changes induced by antifungal substances were also affected by physiological conditions like assay medium composition and growth phase of the indicator strain. Sometimes the presence of an antibiotic substance could suppress the effect of another. For instance, cycloheximide suppressed the swelling of fungal cells induced by tunicamycin (Gunji *et al*., 1983).

Many antifungal compounds produced by *Streptomyces* spp. caused inhibition that was accompanied by bulging of the fungal mycelium (De & Chandra, 1983; Yeo *et al*., 1994). Links *et al*. (1957) described an antifungal antibiotic from a soil isolate of *Streptomyces* sp. that caused bulging at the hyphal tips of several fungi. The site of action of these antibiotics was related to fungal cell wall biosynthesis (Satomi *et al*., 1982). There are many reports related to antibiotic substances that induced such malformation such as stunting, distortion, swelling, and hyphal protuberances of fungal germ tubes (Getha & Vikineswary, 2002). Based on this criterion, Stevenson (1956) found that antibiotics of some soil actinomycetes caused similar effects on hyphae of *Helminthosporium sativum,* both in culture and soil.

5.2 Characterization of Potential Actinomycete Antagonist towards *Colletotrichum* spp.

In the course of screening rhizosphere-derived actinomycetes isolated from chilli farms in two different states of Malaysia, twelve isolates had shown *in vitro* antagonism towards all three types of chilli anthracnose disease pathogens. Eleven out of twelve was categorized in the *Streptomyces*-like group based on their macromorphological characteristics. Nine out of these were from the grey colour group while two belonged to the white colour, and one belonged to the pink colour group. Of the twelve isolates, P42 isolated from *Colletotrichum*-infested chilli farms of Sungai Burung was highly antagonistic towards all three species of test fungi. Therefore, it was important to characterize this promising isolate.

Identification of actinomycetes is a two-stage process. Reliable criteria are needed to assign organisms to families and genera prior to the selection of diagnostic tests for

identification to the species and subspecies levels (Goodfellow, 1992). Identification at the genus level could be achieved using a combination of morphological, cultural and physiological properties (Goodfellow & Haynes, 1984). However, the differentiation of genera solely on the basis of morphological criteria became unsatisfactory when many new genera and families were created, and many species and genera had to be rearranged (Korn-Wendisch & Kutzner, 1992).

5.2.1 Chemotaxonomic and Micromorphological Characteristics of Antagonistic Chilli Rhizosphere-derived Actinomycetes

Chemotaxonomic analyses of the cell wall such as the type of diaminopimelic acid (DAP) isomers had been widely used in the classification of several genera (Goodfellow & Haynes, 1984). The LL-DAP is characteristic of the genus *Streptomyces* (cell wall type I), whereas *meso*-DAP is found in members of other genera such as *Actinomadura, Corynebacterium, Mycobacterium* and *Nocardia*. Morphological features together with the composition of cell wall DAP and whole cell sugars are sufficient for identification of actinomycetes of many genera. However, this approach may fail to correctly identify species of several genera that exhibit similar morphological and chemotaxonomic properties, such as members of *Nonomuria, Microbispora, Microtetraspora* and *Actinomadura* (Wang *et al*., 1999).

In the present study, selected best strains (P8, P39, P42 and P115) contained the LL-DAP isomer in their cell wall. This confirmed that they belonged to the genus *Streptomyces*. *Streptomyces* is the largest genus in terms of number of species among the actinomycetes (Beyazova & Lechevalier, 1993). By using morphological and physiological characteristics, it has been estimated that the genus *Streptomyces* contains

more than 450 validly described species (Al-Tai *et al*., 1999). This genus also contains strains formerly classified as *Chainia*, *Elytrosporangium*, *Kitasatoa*, *Kitasatosporia*, *Actinosporangium* and *Streptoverticillium* based on nucleic acid sequencing and DNA:DNA pairing studies (Embley & Stackebrandt, 1994).

5.2.2 Characterization of Antagonistic Rhizosphere-Derived Actinomycetes by their Spore Chain Morphology

The four selected best strains were from the grey colour group. How close these isolates (P8, P39, P42 and P115) are genetically related, can only be spectulated from the present observations. The four isolates shared some morphological characteristics. Three (P39, P42, P115) out of the four isolates had the spiral spore with smooth surface, while isolate P8 had rectus *flexibilis* spore chain with smooth surface ornamentation. This may, perhaps, indicate some resemblances. In the numerical phonetic survey of Williams *et al*. (1983), a large number of phenotypic traits of streptomycetes were evaluated simultaneously. The four test strains that produced smooth spores, formed in *spirals* (P39, P42 and P115) or straight/flexuous (*rectiflexibiles*) (isolate P8) and grey color spore mass.

Grey isolates in this study had some differences in the occurrence of spirals in their spore chain such as the number of turns and interval of turns (compact or open *spirals*). Some also differed from one another in the shades of their grey aerial spore mass colour (Table 5.2). These observations may be reasonable to some extent, although colour may not be a basic criterion in the systematics of *Streptomyces* (Lechevalier *et al*., 1971).

The hyphae formed are normally non-fragmenting and are divided by septa into long cells with length 20 µm or longer. Held at the end of the filaments are conidiophores, a sort of thin-walled asexual spore. They are known as sporangiospores if they are kept in a sporangium (Figure 5.1). Arrangements of sporangia and conidia depend on the genera. Some genera form single conidium while some may have conidia arranged in short chains. The arrangement of sporangia and conidia are also known as spore-bearing arrangements. This is an important characteristic in strain identification.

Normally, on the agar media plate, the mature, spore-bearing hyphae in the centre of the colony could not be seen due to the density of hyphae. Only the young, immature aerial hyphae at the margins of the colony with adequate light levels could be observed with a transmitted light microscope (Plates 4.10A, 4.10C; 4.11 A, 4.11C.).

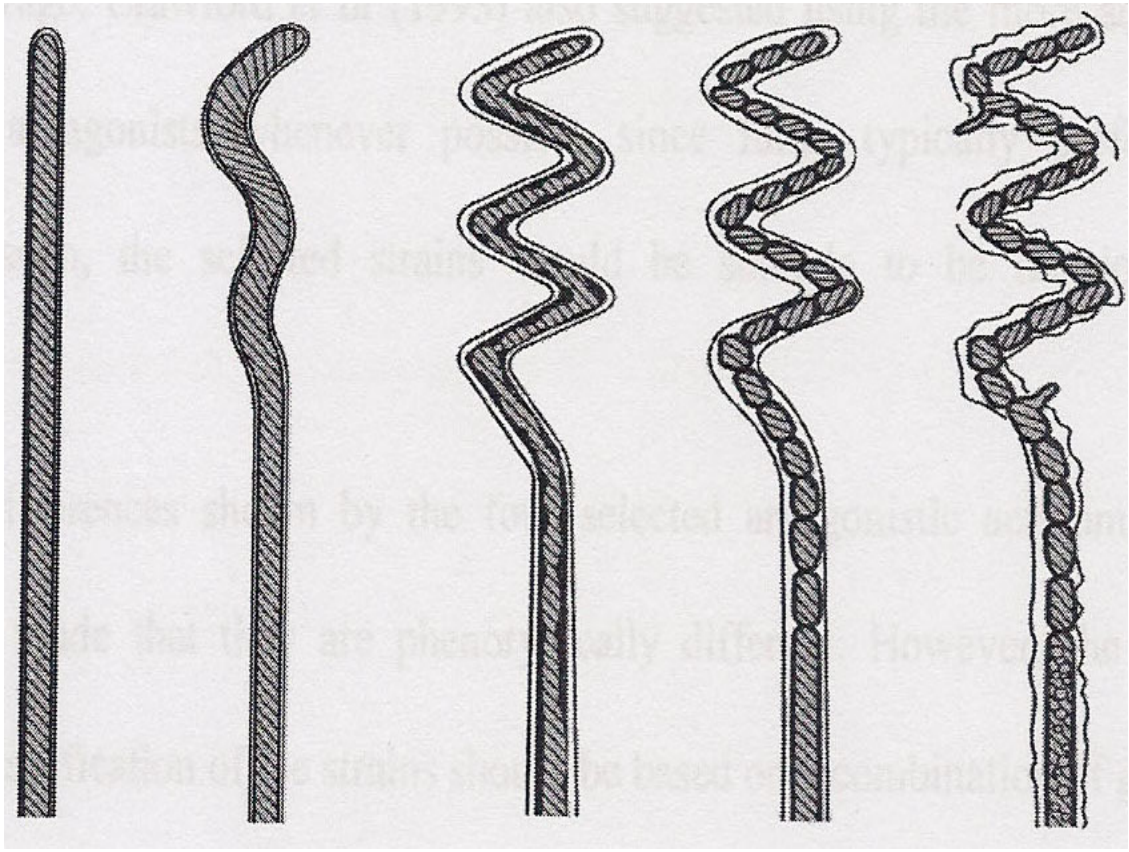

Figure 5.1: The formation of asexual spore of actinomycetes. (http://organicsoiltechnology.com/most-important-microorganisms.html)

Table 5.2: Spore chain morphology and pigmentation observed after 14 days of incubation at 28 ± 2ºC on oatmeal agar (ISP3) of selected rhizosphere-derived actinomycetes that showed antagonism towards *Colletotrichum* spp.

Strain	**Spore Chain Morphology**	**Spore Surface Ornamentation**	**Aerial Spore Mass**	**Substrate Mycelium**	**Soluble Pigment**
P8	*Rectiflexibiles*	Smooth	Dark Grey	Dark Brown	Brown
P39	*Spirals*	Smooth	Grey	Reddish Brown	None
P42	*Spirals*	Smooth	Grey	Grey	None
P115	*Spirals*	Smooth	Grey White	Pale Brown	None

5.2.3 Morphological Characteristics of Antagonistic Actinomycetes

5.2.3.1 Cultural and Physiological Characteristics of Antagonistic Actinomycetes

Actinomycetes are Gram-positive bacteria that form branching filamentous hyphae and asexual spores. Most of them are aerobic, but some genera, such as *Actinomyces*, are strictly anaerobic (Waksman, 1967). When growing in solid substratum, such as agar, the branching network of hyphae developed extensively on the surface of the substratum, as well as into it to form a root-like mycelium. The former forms the aerial mycelium, whereas the latter is commonly known as substrate mycelium.

The overall morphology of actinomycetes depends upon the nature of the strains, the composition of the media, conditions of growth (especially aeration) and the presence of growth-stimulating or growth-inhibiting agents. All the four selected antagonistic actinomycetes exhibited different growth morphology on different growth media used in this study (Table 4.14). Upon plating on agar media, colonies of actinomycetes are easily distinguished from bacteria or fungi colonies. They form compact colonies, often with powdery or leathery dry surface, giving a conical appearance (Plate 4.13, 4.14). The morphology of actinomycetes observed on the plate usually provides a rapid clue to its identification. Certain features of the strain can only be observed when the growth is thin or in differentiation medium. The development and differentiation of characteristics such as spores and pigments for certain actinomycetes require specific media. Brown soluble pigments can only be seen when strain P8 was cultured on ISP2, ISP3 and ISP6 agar plates.

Results of degradation activity of strains revealed that strain P8 was not able to degrade L-tyrosine, xylan, and xanthine. However strains P39, P42 and P115 were able to degrade L-tyrosine and xanthine (Table 4.15). Casein could only be degraded by strain P115, while all four tested strains were able to degrade starch. Since these compounds are the fungal cell wall compounds, strains with the ability of degrading these compounds will be suitable for biocontrol purposes.

Three out of four selected strains (P8, P42 and P115) were able to grow in a wide range of pH from 4.0 to 10.0 (Table 4.16). These results concurred with the findings of Crawford *et al.* (1993) where strong antagonists could grow at a wider pH range. Crawford *et al.* (1993) also suggested using the more acidophilic actinomycetes as antagonists wherever possible since fungi typically prefer acidic environments, as such, the selected strains would be suitable to be developed into biocontrol agents.

One of the important characteristics of a suitable antagonist is tolerating a wide range of temperature. Strains P39, P42 and P115 showed the ability to grow at different temperatures from 10 ºC to 40ºC (Table 4.16). These strains have the ability to grow at high temperatures as they all have a tropical origin. The temperature test could help to select the strains able to tolerate, grow and inhibit the pathogen at a wide range of temperatures from low to high.

Another important characteristic of a good antagonist is the ability to tolerate and continue growing in salty soils. Strain P8 could not grow in more than 2% salinity. The other three strains (P39, P42 and P115) were able to grow in up to 7% salinity level (Table 4.17). Strain P39 could not grow in more than 7% salinity level, but strans P42

and P115 showed ability to grow in 8% and 10% NaCl level respectively. However, they did not form sporulating aerial hyphae, and growth was poor and slow. NaCl tolerance of these actinomycete isolates was related to their origin. Previously reported studies have revealed that terrestrial regions have been one of the sources to collect and explore different types of actinomycetes including NaCl tolerant types. Okazaki & Okami (1976) found that most actinomycete spores were removed easily from terrestrial soil and might be transported to the sea. Fluctuating NaCl concentrations could also induce halo-tolerance and adaptation to increased NaCl concentration in rhizosphere-derived (terrestrially-derived) actinomycetes (Okazaki & Okami, 1976). This induction of halo-tolerance might be the reason that some of the test isolates grew at various NaCl concentrations in this study.

A distinct carbon utilization pattern was observed for each of the actinomycete isolates tested. Each isolate exhibited an almost uniform utilization pattern, differing by utilization/non-utilization of one or more carbon sources (Table 4.19). All isolates did not grow on ISP9 without carbon sources as the medium only contained mineral salts (Appendix A1.15). On the other hand, all isolates had good growth with sporulation on ISP9 with D-glucose, D-xylose and galactose as sole carbon sources. This showed that the isolates could metabolize and utilize a wide range of simple sugars, especially simple carbohydrates such as glucose, xylose, galactose, mannitol, inositol and arabinose.

In general, the production of H_2S was done only by isolate P8; strains P39, P42 and P115 were able to reduce nitrate to nitrite, strains P8, P42 and P115 showed gelatinise activity, and all four strains demonstrated different levels of susceptibility to antibiotics.

The present study investigated the potential of actinomycetes isolated from different healthy and *Colletotrichum*–infected soil to control anthracnose disease caused by *Colletotrichum* spp. As chitin is a major component of the pathogens' cell wall (El-Tarabily *et al*., 2000), the chitinase-producing actinomycetes could be implemented as an antagonist in biological control studies. Therefore, the production of this enzyme would be useful for the selection of actinomycetes as potential antagonists against *Colletotrichum* spp. (El-Tarabily *et al*., 2000). The isolates with high levels of chitinase production are more antagonistic to the fungus compared to the lower producers of chitinase. This concurs with earlier findings by Gupta *et al*. (1995) who reported similar activity against several phyto-pathogenic fungi, and Saadoun *et al*. (2000) against several food-associated fungi and moulds. These findings confirm the importance of the chitinase producing *Streptomyces* isolates as biocontrol agents, and emphasizes the importance of rhizosphere-derived *Streptomyces* as biocontrol agents against this fungus and probably several other fungal pathogens. High levels of chitinase activity of the isolates P8, P39 and P42 *in vitro* corresponded with high levels of antagonistic activity against mycelium growth as well as fungicidal effect against *Colletotrichum*. This confirmed the effectiveness of chitinase as a fungicidal mechanism against mycelial germination and growth of *Colletotrichum*. The non-chitinase producers on the other hand showed a fungistatic inhibition of fungus mycelia only via other inhibitory mechanisms that may be produced by the non-chitinolytic isolates. Hence, the non-chitinase producers and weak chitinase producers should not be over-looked as some may be good biological agents against some other plant pathogenic fungi.

Based on the results of colour grouping, micro-morphological observation and whole-cell diaminopimelic acid analysis, it can be concluded that selected actionomycete isolates belonged to *Streptomyces* group. Being halo-tolerance, the isolates indicated that they have a terrestrial origin. The isolates showed a versatile carbon metabolism, being able to utilize a wide range of simple sugars as well as complex carbohydrates such as starch. Representative among the best strains (Strain P42) was selected to use in next stages of the study (phytotoxicity and *in vivo* trials).

5.2.4 Phylogenetic Analysis of Antagonistic Actinomycetes

Phylogenetic characterization of sequences of gene encoding 16S ribosomal RNA is useful in determining species identity of actinomycetes (Embley & Stackebrandt, 1994; Tamura *et al*., 1998). However, individual approaches in bacterial classification have various strengths and weaknesses. The 16S rRNA sequencing is commonly used to infer evolutionary relationships but is needs to be evaluated and refined using other taxonomic criteria, notably from chemotaxonomic and DNA:DNA hybridization studies. Phenotypic tests also provide valuable information for separating closely related species and for identifying strains to validly describe taxa. This integrated use of genotypic and phenotypic characteristics to study the taxonomy of groups of organisms is known as polyphasic taxonomy (Goodfellow & O'Donnell, 1993).

By comparing the morphological, cultural and some physiological properties of strains P8, P39, P42 and P115 with other species in the literature, the strains are considered to belong to the family Streptomycetaceae that characteristically produce smooth ornamented spores in *spirals* (strains P39, P42 and P115) and *rectiflexibiles* (strain P8) spore chain and grey aerial mycelia (Shirling & Gottlieb, 1968). It is also clear from the

phylogenetic analysis carried out in this study that strains P8, P39, P42 and P115 are assigned to different clades in the 16S rRNA tree. It is, therefore, encouraging that in the present study good agreement was found between the phenotypic properties (notably morphological and pigmentation features) and phylogenetic data to assign strains to their operational taxonomic unit (OTU).

In this study strain P8 belonged to the family Streptomycetaceae that form straight/flexuous (*rectiflexibiles*) spores that were initially assigned to four validly described species, namely, *S. purpeofuscus*, *K. azatica*, *S. misakiensis* and *K. putterlickiae*, based on the pairwise sequence similarity, and phylogenetic tree on the basis of 16S rRNA sequence data (Figure 4.4).

Comparing the morphological and biochemical characteristics of *S. purpeofuscus*, *K. azatica*, *S. misakiensis* and *K. putterlickiae* with strain P8 revealed that this strain closely resembled to *S. purpeofuscus* (Table 4.20). This species is also known to produce antifungal antibiotics (Anupama *et al*., 2007; Jain & Jain, 2005), antibacterial antibiotics (Anupama *et al*., 2007), antiviral antibiotics (Kawamura *et al*., 1976), antitrichomonal antibiotics (Yamaguchi & Saburi, 1955), antioxidative substances (Laursen & Nielsen, 2004) and neuronal cell protecting substances (Kunigami *et al*., 1998) *in vitro*.

This study revealed that strain P39 belonged to the genus *Streptomyces* that form closed/compact spiral spores that were initially assigned to three validly described species, namely, *S. violaceorubidus*, *S. tendae* and *S. tritolerans* based on the pairwise sequence similarity and phylogenetic tree on the basis of 16S rRNA sequence data (Figure 4.4).

Comparing morphological and biochemical characteristics with strain P39 revealed that this strain closely resembled *Streptomyces violaceorubidus* (Table 4.21). Strain P39 showed inhibitory activity against the growth of *Colletotrichum* spp. The same species revealed to be antagonistic against yeast and bacteria (Bensultana *et al*., 2010) *in vitro*.

This study suggested that strain P42 belonged to the genus *Streptomyces* that form open spiral spores that were initially assigned to four validly described species, namely, *S. rochei*, *S. enissocaesilis*, *S. plicatus* and *S. vinaceusdrappus* based on the pairwise sequence similarity and phylogenetic tree on the basis of 16S rRNA sequence data (Figure 4.4).

Comparing morphological and biochemical characteristics with strain P42 revealed that this strain closely resembled *S. rochei* (Table 4.22).

Strain P42 (*S. rochei*) revealed the best inhibitory activity against all three *Colletotrichum* spp. among the other strains tested (Table 4.6), therefore used for the *in vivo* studies. This species has been shown to be active against fungi and bacteria (Reddy *et al*., 2011; Vijayalakshmi & Vijayalakshmi, 2007). Previous studies on *S. rochei* exhibited biodegradation activity of this species *in vitro* (Yokota *et al*., 1995; Zaborina *et al.*, 1997). *Streptomyces rochei* in another study in Russia revealed considerable progress in the degradation of polychlorinated phenols which are the most persistent pollutants of the environment (Golovleva *et al*., 1991). *Streptomyces rochei* also showed to be able to produce an extracellular inulin-degrading enzyme that can degrade inulin into inulotriose as the main end product (Yokota *et al*., 1995).

Streptomyces rochei was reported to be able to produce antibiotics such as lankacidin that exhibit significant antibacterial activities against a wide variety of bacteria and commercially used in livestock industries for the treatment of porcine infected with *Serpulina* (*Treponema*) *hyodysenteriae*, an anaerobic spirochete (Arakawa *et al*., 2005). *Streptomyces rochei* has also been shown to have the ability to produce active metabolites against dermatophytes (Augustine *et al*., 2005).

In Spain, *S. rochei* was used as a biocontrol agent in conjunction with *Trichoderma harzianum* against *Phytophthora in vivo* and it revealed successful control against root rot of pepper (*Capsicum annuum* L.) (Ezziyyani *et al*., 2007).

The phylogenetic analysis revealed that strain P115 did not cluster well with the closest pairwise sequences of the related species as suggested by EzTaxon-e server (Figure 4.4). This may due to the nature of the 16S ribosomal RNA gene (Labeda *et al*., 2011). There is insufficient variability to determine the exact phylogenetic relationships within the genus *Streptomyces* (Kim *et al*., 2004). Therefore it is suggested that additional molecular tools are utilised for further clarification of these closely related species.

Hence, for the identification of this strain, high pairwise similarity method was used according to the strains that showed most similarity with strain P115 in EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; (Kim *et al*., 2012). Three most similar strains to strain P115 were *S. rubrogriseus*, *S. tricolor* and *S. lienomycini*. Strain P115 was able to form open spiral spores, but the differential morphological and biochemical characteristics of *S. rubrogriseus*, *S. tricolor* and *S. lienomycini* did not correspond with the characteristics of strain P115 (Table 4.23).

5.3 Evaluation of the *in vivo* Biocontrol Ability of Strain P42 against Three Different Species of *Colletotrichum*

5.3.1 Phytotoxicity Test of Selected *Streptomyces rochei* (Strain P42) by Seed Germination Assay

Phytotoxicity is the capacity of a compound (s) to cause temporary or long-lasting crop damage to the plants. The assessment of the phytotoxicity of a plant protection product to a crop plant or plant product is an essential element in this efficacy evaluation. The basic principles for assessing phytotoxicity are the same whether the compound tested is a herbicide, fungicide, insecticide or other type of plant protection product. Phytotoxicity effects may be observed on the crop at emergence or during its growth or may be expressed at harvest. They may be temporary or lasting. The symptoms may affect the whole plant or part of plant roots, shoots, leaves, flowers or fruits.

Several techniques have been devised to evaluate the damaging effects and toxicity of compost (Wong, 1985). Germination and root growth are two sensitive and important parameters that can be affected by phytotoxicity effects (Tam & Tiquia, 1994). According to Zucconi and his co-workers (1981), having the ability above 80% to germinate and grow indicated the disappearance of phytotoxicity in compost, and no injury on olive plant growth was found when growth and germination were above 50%. An assumption has been made that the same physiological response was exhibited by the plant when toxic substances were present in the media, regardless of the source of the substances. Therefore, the substances which are toxic to the plant, if any, produced by the antagonistic actinomycetes will diffuse into the agar and contact with the seed, subsequently retarding the seed germination as well as the root elongation. ISP2 agar,

which is an actinomycete growing medium with only 2% (w/v) of agar content was used as the germination medium in this study. The agar medium is relatively soft and rich in water. The use of ISP2 agar as the germination media in this study was due to the need to grow actinomycetes on a suitable medium. This allows the targeted microorganism to grow under normal physiological processes with the nutrients supplemented in the agar. The ISP2 agar content did not show any negative effects on the maize seed germination. This is shown in the control replicate with only ISP2 agar without microbial inoculums (Table 4.24).

In this study, higher plant height (>20 cm), longer main roots (>38 cm), more leaves (>2) and secondary roots (>6) in low dosage P42-treated maize seeds was observed (Table 4.24). The difference among plant height, main root length and number of leaves was significant compared to control seeds. However, the number of secondary roots treated with low dosage of P42 did not show any significant difference compared to the control. These differences might be attributed to the nature of the maize to germinate in an environment with suitable moisture content regardless of other factors such as light sources and media ingredient. The higher plant height, longer main roots, more number of leaves and secondary roots in low dosage treated seeds might also be due to the plant growth promoting effect of strain P42. *Streptomyces rochei* is able to produce plant growth regulators such as auxins, gibberellins and cytokinins (Mansour, 1994). In another study done by Aldesuquy *et al*., (1998), shoot length, shoot fresh mass, dry mass and diameter of wheat showed significant increase when *S. olivaceoviridis* and *S. rochei* were applied for treatment. Hormone extraction, purification, and bioassay showed that both species produced substantial amounts of growth-regulating substances, including auxins, gibberellins, and cytokinins. This demonstrated that

selected *Streptomyces* spp. produces at least one class of compounds that directly influence plant growth (Aldesuquy *et al*., 1998). Zhao *et al*. (2012) reported that the antagonists *S. pactum* and *S. globisporus* subsp. *globisporus* have the ability to promote plant growth and they are able to increase mean root length and aerial parts of melon up to 44.2 and 40.3% respectively.

Shorter main roots (<19 cm) and plant height (<13 cm), lesser leaves (<2) and secondary roots (<5) had been observed in the high dosage P42-treated seeds as compared to control seeds (significant difference $p<0.05$). This might have been due to the high amount of metabolites that were produced by the antagonistic actinomycetes which were inhibitory to the root elongation of maize seeds. There is also a possibility where the inhibition was caused by competition for nutrients between the germinating actinomycetes and germinating seed. By reducing the concentration of strain P42 to a tolerable level, which needs to be further determined, it may perform better as a biocontrol agent with no adverse effect at all. However, a more detailed study has to be conducted on the appropriate concentration of inoculums.

5.3.2 *In vivo* Biocontrol Assay

Greenhouse experimental trials (*in vivo*) were implemented to confirm the *in vitro* results. In this study, 8 different treatments were used and arranged based on completely randomized design in greenhouse. Each treatment included 10 plants (1 plant in each plastic bag) and the experiment repeated 3 times. The results of 240 plants were analysed.

Anthracnose symptoms in plants inoculated with the *Colletotrichum* was significantly suppressed when the chilli seeds and plants were treated with strain P42. The disease symptom was expressed severely in non-treated plants after 9 days of inoculation of fruits with the pathogen. The *in vivo* biocontrol assays showed that there was a reduction of 52 - 54% in the DSI values of the treated plants compared to the untreated plants. This provided preliminary evidence that strain P42 has the ability to interfere with the anthracnose disease cycle and may be a potential biocontrol agent.

Several studies have reported the antagonistic effects of *Streptomyces* spp. on plant pathogens, *S. rochei* on *Phytophthora capsici* on pepper by producing 1-propanone (Ezziyyani *et al*. 2007); *S. griseoviridis* on *Fusarium* root rot and wilt of tomato (Minuto *et al*. 2006); *S. platensis* on *R. solani* leaf blight/seedling blight of rice (Wan *et al*. 2008); *S. hygroscopicus* on *Colletotrichum gloeosporioides* anthacnose of several crops (Prapagdee *et al*. 2008); *Streptomyces* sp. on *Fusarium oxysporum* by producing isochainin (Bouizgarne *et al*., 2006). According to Bell & Mace (1981), antifungal metabolites produced by antagonists could reduce the invasion and subsequent development of pathogens and thus play a role in plant resistance. However, the antifungal metabolites would have to be produced at the right time and at high enough levels to protect the infection sites from the pathogen (Marois & Ploetz, 1990). Besides antibiosis, competition and mycoparasitism have been described as mechanisms that may be involved in the biological control of plant pathogens by biocontrol agents (Cook & Baker, 1983).

El-Tarabily *et al*., (2000), Bressan & Figueiredo (2005) showed that bioactive secondary metabolites derived from *S. rochei* play significant roles in the biocontrol ability of this potent biocontrol agent. To date, several *Streptomyces* antibiotics, which

are known to be active against fungal pathogens, have been isolated (Ezziyyani *et al*., 2007). In the present study, it was speculated that the antifungal activity of *Streptomyces* sp. strain P42 could have been one of the major factors that may have induced the reduction in incidence of chilli anthracnose disease by *Colletotrichum* sp. The reduction in anthracnose disease severity was significant with the three different *Colletotrichum* inoculums used in this study. This observation suggested that the antagonistic effect of strain P42 in the rhizosphere was enough to prevent spread of the pathogen, resulting in the generally healthy appearance of the strain P42-treated plants after 9 days.

In the present study chilli seeds were treated with antagonist Streptomycete (Strain P42) prior to planting. It was believed that seed treatment with the antagonist can be an effective way to promote the plant growth and also decrease the incidence of disease. This was supported by Merriman *et al*., (1974) who reported the successful use of antagonist Streptomycete as a seed treatment of barley, carrot, oat and wheat in order to increase their growth and biological control of *Rhizoctonia solani*. El-Abyad *et al*. (1993) described the use of three *Streptomyces* spp. in the control of bacterial, *Fusarium* and *Verticillium* wilts, early blight, and bacterial canker of tomato. The isolates used were *S. pulcher*, *S. canescens*, and *S. citreofluorescens*. As seed-coating, all three strains were effective at variable levels in controlling the test pathogens. In addition, tomato growth was observed to be significantly improved with the antagonistic *Streptomyces* spp. as a seed-coating. An increased availability of growth regulators produced by the inoculum was the reason proposed for the improvement in tomato growth.

In this study, 1.16×10^5 cfu/ml concentration of strain P42 was bioprepared and put in the soil (rhizosphere area) when the chilli plants were 45 days old. The biopreparation is made of already germinated micro-organisms with abundant mycelial mass, which enables them to adapt readily to a growth medium containing plantation soil. This facilitates its adherence and adaptation to the soil and makes it more effective than ungerminated spores, which would have to overcome the competition from other micro-organisms of the basal flora of the plant rhizosphere (Ezziyyani *et al*., 2007).

This study revealed that an anthracnose disease symptom on P42-treated chilli plants was significantly lower compare to those which did not receive any seed or plant treatment. Other studies have reported antagonistic effects of *Streptomyces* spp. on different plant diseases including *S. rochei* on *Phytopathora* root rot of pepper (Ezziyyani *et al*., 2007); *S. griseoviridis* on *Fusarium* root rot and tomato wilt (Minuto *et al*., 2006); *S. pactum* and *S. globisporus* subsp. *globisporus* on gummy stem blight of melon (Zhao *et al*., 2012). In Thailand, it was revealed up to 50% success in the suppression of chilli anthracnose disease caused by *C. gloeosporioides*, by the use of antagonists *Bacillus amyloliquefaciens*, *B. pumilus* and *B. sphaericus* (Jetiyanon & Kloepper, 2002). Heng & Hamzah (2011) showed the successful *in vitro* inhibition of *C. gloeosporioides* by two *Streptomyces* strains known as PM2 and PM4. Intanoo & Chamswarng (2007) showed that two antagonist bacterial strains, DGg13 and BB133, were found to be effective against *C. capsici*, the major anthracnose pathogen in Thailand. *Bacillus subtilis* and *Candida oleophila* are two other biological agents that have been successfully effective against *C. acutatum* (Wharton & Dieguez-Uribeondo, 2004).

In the present study mean fresh weight of treated chilli plants showed significant difference compared to the untreated plants. However the strain P42-treated pathogen-free control plants (P) showed the highest fresh weight compared to untreated pathogen -free control (C) and other treatments. The effects of actinomycetes in improving crops fresh weight and yield have been previously reported (Doumbou *et al*., 2001). By using *S. hygroscopicus* in greenhouse for the treatment of radish, the wet weight increased up to 13% greater than untreated controls. Radishes treated with *S. violaceusniger* YCED9 and grown under greenhouse conditions showed statistically significant increases in total plant wet weight compared to untreated controls (19%) (Doumbou *et al*., 2001). Growth promotion was not limited to *S. violaceusniger* YCED9. The use of *S. lydicus* WYEC108 radishes showed 10% greater fresh weight compared to untreated controls (Crawford *et al*., 1993). The mean fresh weight of melons treated with *S. pactum* and *S. globisporus* subsp. *globisporus* showed up to 40.4% improvement compared to control (Zhao *et al*., 2012).

Even though it is always difficult to anticipate the biocontrol activity of a given strain from the laboratory to natural environment (Hermosa *et al*., 2000), the results of the current study noticeably revealed the potential of *S. rochei* for controlling chilli anthracnose disease caused by *Colletotrichum* sp. If the antagonists provide sustained biocontrol effect in field trial on a semi-commercial scale, the *S. rochei* can be extensively used as a biocontrol agent for anthracnose disease of chilli.

CHAPTER 6
OVERALL DISCUSSION, CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Biocontrol of Anthracnose Disease of Cilli

Species of *Colletotrichum* is one of the most essentially important plant pathogens that cause anthracnose disease and is effective on decreasing the value of crop yield production between 10% to 80% in tropical developing countries like Pakistan, India, Thailand, Mexico and Malaysia (Freeman *et al*., 1998; Than *et al*., 2008). Although synthetic chemicals have been used to inhibit these pathogens, the excessive use of harmful chemicals has polluted the environment. Furthermore, with the development of resistant pathogens, the effectiveness of these chemicals is continually decreasing. Therefore, research to generate alternative and environmental friendly approaches for disease control, including biological control, is being conducted globally (Saravanan *et al*., 2003).

Biological control has been actively practiced for more than 100 years and the history of biocontrol, its failures and successes, has been extensively reviewed by many researchers (Stiling & Cornelissen, 2005). History has shown that the introduction of natural enemies to control the native and exotic species is effective in a number of instances and many were completely successful, i.e., it has provided permanent control of the target species (Stiling & Cornelissen, 2005). The development of biological control agents as a key component of integrated disease management has tremendous potential for application for the reduction of losses from plant disease. Several biological control agents can suppress diseases as effective as fungicides, which are

often prohibitively expensive to resource-poor farmers (Bandyopadhyay & Cardwell, 2003).

6.2 Isolation And *in vitro* Antagonism of Rhizosphere-derived Actinomycetes

Streptomyces strains have been reported to control plant diseases caused by nematodes, fungi and bacteria, indicating their broad spectrum of activity against other microorganisms. They have been isolated from various types of soil (Kim *et al*., 1998), rice paddy farmland (Xu *et al*., 1996), lake mud and water (Jiang & Xu, 1996), deciduous forest, tropical forest, wasteland (Xu *et al*., 1996), cave soils and mangrove mud (Vikineswary *et al*., 1997; Getha & Vikineswary, 2002). Production of antibiotics has been suggested as the principal mechanism of action of many actinomycetes in biological control (Jones & Samac, 1996). Since, as far as is known, rhizosphere-derived actinomycetes have not been much studied for their possible use as biocontrol agents against plant pathogenic fungi, indigenous strains of *Streptomyces* spp. isolated from healthy and *Colletotrichum*-infested rhizosphere-derived soils were screened for this purpose. In the current study the mechanism of inhibition could be due to the production of anticbiotics by the Streptomyces strain.

The actinomycete strains were isolated from various samples collected from chilli farms in Peninsular Malaysia. Simple moist-heat and dry-heat pre-treatment methods were carried out on the air-dried samples and selective isolation was done using starch-casein (SC), raffinose-histidine (RH) and humic acid-vitamin (HV) agar medium incorporated with antibiotics cycloheximide, nystatin and nalidixic acid (Chapter 3). The use of three different media was useful to select more and various types of actinomycete colonies. Air-drying of soil samples at room temperature eliminates most of the unwanted Gram-negative bacteria that produce mucoid, spreading colonies on soil-dilution plates. Moist-

heat treatment of soil is an effective method for inhibiting non-filamentous bacterial growth (Labeda & Shearer, 1990) and also allowed the recovery of large numbers of actinomycetes in the present study. Starch, degraded by most streptomycetes, as the carbon source, and nitrate or casein as the nitrogen source, are effective for the isolation of actinomycetes from soil (Kustar & Williams, 1964). The media are clear and allowed good development of aerial mycelium and pigmentation (Williams & Davies, 1965; Maldonado *et al*., 2005).

Approximately 81.5% of the strains isolated in the present study fell into the *Streptomyces*-like group (Chapter 4). It is generally known that isolation procedures and human biases in selecting colonies are among the major factors affecting the relative frequency of different groups of actinomycetes isolated from soil samples (Wang *et al*., 1999). Vickers *et al*. (1984) considered SC agar as a 'non-selective' medium that mainly supported the growth of streptomycetes, notably those belonging to the *Streptomyces* albidoflavus group. Therefore, the use of SC agar in the isolation procedures could have also caused the underestimation of actinomycete groups present. Although these factors may have influenced the results of this study to some extent, it was reported that nearly all of the actinomycetes observed in various rhizosphere habitats (Jensen *et al*., 1991; Genilloud *et al*., 1994) could be assigned to the major genera observed, namely *Streptomyces*. Heng & Hamzah (2011) also showed that *Streptomyces* was the major genera in isolates from rhizosphere soil of Malaysian crops.

Streptomyces-like isolates from the grey colour group dominated (84.9%) the present study (Chapter 4). Okazaki & Okami (1976) had shown that approximately 60% of the streptomycetes isolated from shallow sea mud samples were from the grey spore colour

group. A high percentage of streptomycetes with grey aerial mycelium was previously isolated from Malaysian coastal mangrove mud (Vikineswary *et al*., 1997). Hatano (1997) reported that 77% of isolates from mangrove rhizosphere soil belonged to the grey colour group. In other studies, grey spore-coloured streptomycetes had been isolated frequently from Jordanian soil (Saadoun & Gharaibeh, 2002) and river soil suspensions (Batra *et al*., 1972). Okazaki & Okami (1976) suggested that this similarity between the terrestrial and marine-derived streptomycetes could be due to the fact that most of the strains originated from land soil.

In the current study, actinomycetes were isolated from rhizosphere-derived soil. This source has the potential to harbour different types of actinomycetes used for antagonistic purposes (Boukaew *et al*., 2011). The rhizosphere habitats are known to be areas highly influenced by roots and their metabolites (Heng & Hamzah, 2011). These metabolites may be a possible factor that attracts actinomycetes to be gathered more in the rhizosphere area. However, factors contributing to their occurrence and ecology in rhizosphere habitats need to be thoroughly investigated before further conclusions can be made.

Many attempts were made to correlate the efficiency of potential biocontrol agents with some specific trait(s) which are easy to measure *in vitro* (Turhan & Grossmann, 1986; Crawford *et al*., 1993). Despite these efforts, it was argued that there was no general relationship between the *in vitro* antagonism of biocontrol candidates and disease suppression *in vivo* (Alabouvette & Couteaudier, 1992). In contrast, potential biocontrol *Streptomyces* spp. seem to be exceptions to this view (Turhan, 1981b; Tahvonen, 1982). Streptomycetes which were shown to be antagonists of fungi in agar plate assays may or

may not be active antagonists in soil (Broadbent *et al*., 1971). Those shown to be non-antagonists by plate assays were also inactive in soil.

Many antibiotics produced by *Streptomyces* spp. had been used directly to control fungal infection in plants. Hwang *et al*. (2001) reported that a strain of *S. violaceoniger* produced the nucleoside antibiotic tubercidin. Soil drenched with the crude antibiotics and mixed with zoospore suspensions of *Phytophthora capsici* effectively suppressed the development of *Phytophthora* blight in pepper plants. Faeriefungin, a novel antibiotic from *S. griseus* var. *autotrophicus*, effectively controlled root rot and stem wilt in asparagus plants caused by *Fusarium oxysporum* f.sp. *asparagi* (Smith *et al*., 1990). Antibiotics produced by *S. violaceusniger* YCED9 had been shown to be responsible for the suppression of damping-off disease in lettuce caused by *Pythium ultimum* (Trejo-Estrada *et al*., 1998). Others had reported that the frequency of isolating fungus-antagonistic *Streptomyces* spp. from plant rhizosphere soil was much greater than other inhibitory bacteria (Axelrood *et al*., 1996). Based on these reports, and the fact that streptomycetes are fast growers compared to the other actinomycetes genera, the *Streptomyces*-like isolates obtained in the present study were screened for *in vitro* antagonistic activity against plant pathogenic fungi (Chapter 3). The strategy adopted was, to first screen the strains for the production of strong inhibitory substances against a variety of fungal pathogens *in vitro* and identify the potential strain that might function in soil. The second step was to determine its success *in vivo*.

Out of a total of 106 *Streptomyces*-like strains isolated in this study, 26 strains selected in accordance with differences in the colony morphology were tested for potential antagonistic activity against plant pathogenic fungi. Out of these isolates, 18 (69%)

showed very strong to moderate inhibition against one or more test fungi. A total of twelve isolates (46%) showed varying degrees of inhibition against all fungal pathogens tested (Chapter 4). The high proportion of antagonistic isolates obtained confirmed the previous findings that recognized Malaysian rhizosphere habitats as a rich source of actinomycetes with antifungal properties (Heng & Hamzah, 2011). These antagonistic strains could be a potential source of biological control agents for plant diseases. Isolate P42, isolated from *Colletotrichum*-free rhizosphere soil, was selected as the best potential candidate for biocontrol studies because of its wide antifungal spectrum and strong antifungal activity against three species of *Colletotrichum* tested in this study. The antifungal compounds produced by isolate P42 were responsible for the high degree of reduction in the linear growth of *Colletotrichum* spp. (Chapter 4).

6.3 Characterization of Antagonistic Streptomyces Strains

Many of the antagonistic isolates (84.9%) fell into the grey-color group of aerial mycelium (Chapter 4). This was not surprising as many other researchers reported the same incidence (Getha *et al*., 2005; Saadoun *et al*., 1998). Getha *et al*. (2005) reported 60% of the antagonistic isolates in her study belonged to the grey-color group of aerial mycelium. All four selected antagonistic strains in this study were from the grey-color group. Whole- organism hydrolysates of the four isolates contained major amounts of LL-DAP, confirming their identity as members of the genus *Streptomyces* (Anderson & Wellington, 2001). Isolate P8 possessed *rectiflexibiles* spore chain while the other three (isolates P39, P42 and P115) possessed *spirals* with smooth spore surface ornamentation (Chapter 4). According to Hatano (1997), 80% of the grey colored *Streptomyces* isolates belonged to the spiral chain spore group.

A polyphasic taxonomic approach was used in this study where the phenotypic, biochemical and molecular traits of the isolates were examined in an integrative manner to identify the potential antagonistic isolate (Anderson & Wellington, 2001). Strains grew relatively well in most descriptive media (Shirling & Gottlieb, 1966). Strain P8 showed diffusible pigment in ISP2, ISP3 and ISP6 while others did not reveal any pigmentation activity. Degradation results showed that strain P8 was not able to degrade L-tyrosine, xylan, and xanthine, while the other three strains were able to degrade L-tyrosine and xanthine. Degradation of casein was done by strain P115, while all four strains were able to degrade starch. Strains P8, P42 and P115 could grow in wider pH range (4.0-10.0) and strains P39, P42 and P115 were able to grow in different range of temperature from 10 to 40ºC (Chapter 4). Salinity tolerance of strain P8 was not more than 2%, while the other three strains could grow in up to 7% salinity level. Exploring carbon utilization pattern of isolates showed that they were not able to grow on ISP9 without carbon source, while they demonstrated good growth with glucose, xylose and galactose. H_2S was only produced in strain P8; reduction of nitrate to nitrite was observed in strains P39, P42 and P115; P8, P42 and P115 showed gelatinise activity, and they revealed different levels of susceptibility to antibiotics. Strains P8, P39 and P42 demonstrated high levels of chitinase activity. As this compound is one the compounds in fungal cell wall, identifying strains with chitinase activity could be beneficial for biocontrol purposes.

Characterization of the well-conserved region of 16S rRNA is effective for quick classification and identification of large numbers of different taxonomic groups of actinomycetes (Embley & Stackebrandt, 1994). An integrated use of phenotypic and genotypic characteristics clearly assigned strain P8 to the *S. purpeofuscus*; strain P39 to

S. violaceorubidus and strain P42 to *S. rochei*. Strain P115 could not cluster well with the closest pairwise sequences in the phylogenetic analysis, so for identification of this strain, most similar strains in pairwise similarity method was used (Chapter 5). Although all four strains belonged to the grey-color group, the molecular studies showed they belong to different clades. Biocontrol and some other properties of these strains have been reported by different researchers. The best selected strain in this study strain P42 (*S. rochei*) has revealed many and important biocontrol properties (Doumbou *et al*., 2001).

6.4 Evaluation on Phytotoxic Property of Antagonistic Actinomycetes

In the selection of suitable biocontrol agent, the antagonistic microorganism should not pose any threat to the plant health. Antagonistic actinomycetes are believed to produce secondary metabolites, with visible inhibition zone on bioassay plates (Crawford *et al*., 1993), which is toxic to other organisms, particularly the pathogen. It is necessary to evaluate the possible phytotoxic effect on the plant health. An experiment has been designed to evaluate the phytotoxicity of the selected antagonistic strain by evaluating the effect of high and low dosage of strain P42 inoculum on plant height, main root length, number of leaves and number of secondary roots on maize seeds. Implementing a high dosage of strain P42 showed a significant ($p<0.05$) decrease in plant growth factors, while low dosage revealed no toxic effect and also promote maize seed growth significantly. These differences allowed the selection of an antagonist with no phytotoxic effect on plants.

6.5 Biological Control of Anthracnose Disease of Chilli by Strain P42

In the present study, low dosage of strain P42 revealed the best results. In addition, it also showed plant growth promoter ability, hence used for biocontrol purposes *in vivo*.

Seeds of chilli were treated with low dosage of strain P42 before plantation. Seed treatment prior to planting has revealed successful results in control of diseases like *Fusarium* and *Verticillium* wilts, earli blight and bacterial canker of tomato (El-Abyad *et al*., 1993). Three *Streptomyces* spp. were used to treat seeds against these pathogens. In addition to the successful control of pathogens, the mean fresh weight of the plants was significantly higher than the control.

In the present study, there was a significant difference between fruit fresh weights of P42-treated plants compared to untreated plants. This confirmed the plant growth promoting effect of strain P42 (*S. rochei*) as well as its antagonistic effect against three dominant *Colletotrichum* species tested. The plant growth promoting effect of *S. rochei* has been observed on shoot length and shoot fresh mass of wheat which were successfully increased (Aldesuquy *et al*., 1998).

Strain P42 significantly decreased Fruit Symptom Index in the current study. The use of this *Streptomyces* strain could be recommended for biocontrol purposes of anthracnose disease in chilli plants.

6.6 Conclusions

The aim of this study was to find antagonistic actinomycetes of potential value as biocontrol agents against plant pathogenic fungi from rhizospheric habitats. Isolates were first screened for the production of metabolites with strong inhibition against the *in vitro* growth of three dominant *Colletotrichum* pathogens in tropical countries, to identify the potential isolate that might function and be beneficial to inhibit the pathogen. In principle, such isolate should have a higher antifungal capacity than those found with low activity or none at all. The second step was to determine its success *in vivo*.

All four objectives of this project have been achieved.

a) 130 isolates were isolated from healthy and *Colletotrichum*-infested soils from chilli farms of Peninsular Malaysia.

b) All isolates were screened for biocontrol activity against *Colletotrichum* spp. 26 isolates showed biocontrol activity, at varying degrees, against at least one *Colletotrichum* species tested. Out of these 26 isolates, 4 isolates were active against all three *Colletotrichum* species tested.

c) The four most active isolates were identified as P8, P39, P42 and P115 using molecular approaches.

d) The most bioactive isolate, strain P42, was successful in *in vivo* control tests.

It was evident from the literature that the present study is the first report that (1) investigated the potential of an antagonistic *Streptomyces* strain isolated from rhizosphere-derived sources of healthy and *Colletotrichum*-infested chilli plants to

control anthracnose disease of chilli, (2) evaluated the possible phytotoxic effects of the antagonistic actinomycetes by the use of two different concentrations of antagonist, and (3) identified and used the best strain that had the ability to produce antifungal metabolites and enzymes. Based on the results obtained, it was possible to conclude that findings of the present study would contribute greatly to similar studies looking at the possibilities of using actinomycetes to control other plant pathogenic fungi with economic importance. Further, this study would also contribute to the knowledge on possible uses of fungus-antagonistic actinomycetes which could be isolated from unexploited ecological niches such as the Malaysian terrestrial habitats for biotechnological applications.

6.7 Future Recommendations

The early beliefs that biological control agents offer more variable and less effective protection than fungicides have been refuted (Harman & Taylor, 1990). However, good knowledge of the host-pathogen-environment interaction in specific agro ecosystem in which the biological control agent has to act is essential for a successful biological control (Harman & Taylor, 1990). In the present study, the potential biocontrol agent *Streptomyces* sp. strain P42 was selected following the *in vitro* study for confirmation of its antagonistic effect *in vivo*. As an alternative screening approach, Xiao *et al*. (2002) had noted the significant positive correlation between alfalfa seedling weights when inoculated with antagonistic *Streptomyces* spp. in sterile vermiculite in the absence of the root rot pathogen *Phytophthora* sp., and those inoculated with antagonists in pathogen-infested field soil. Direct enhancement of alfalfa growth by *Streptomyces* may be one of the mechanisms by which *Streptomyces* antagonists enhance plant health in field soil. Thus, antagonist-screening approaches that consider plant growth in the

absence of any pathogens may provide a complement to more traditional antibiotic screening assays. This approach could be used in future studies to determine if *Streptomyces rochei* could enhance the growth characteristic of other crops.

In the present study, strain P42 was used in sterile soil and revealed its successful action in sterile environment. Different mechanisms in natural conditions can effect on biocontrol agents (Goodfellow & Williams, 1983). Further *in vivo* biocontrol studies using naturally infested field soil under controlled conditions are needed to determine whether strain P42 is competitive enough with other soil microbes that may be present in natural soils.

The induction of host defense responses leading to enhanced systemic resistance may also play a role in successful biocontrol (Jones & Samac, 1996). Many researchers have shown that actinomycetes found in the rhizosphere population (Crawford *et al*., 1993; Gesheva, 2002), and endophytic streptomycetes found inside root tissues (Sardi *et al*., 1992), have antagonistic potential and play an important role in the plant health. Some rhizosphere-colonizing and endophytic microorganisms have shown the ability to reduce the susceptibility of plants to the pathogen by inducing systemic resistance in the host (Marois & Ploetz, 1990). The possible impact of induced host resistance in the mechanism of disease suppression by strain P42, in addition to antibiosis, should be investigated by using "killed" cells of strain P42 as a control treatment in future biocontrol studies.

Additional research is also needed on soil physical and chemical factors that may influence both root colonization and the expression of traits important to antagonism of

strain P42 in the rhizosphere. By identifying these factors, it may be possible to manipulate these in the field so as to enhance root colonization and attain maximum biocontrol potential of the antagonistic strain. Many factors are likely to affect biological control of root diseases, including environmental conditions such as soil temperature and morphology, water status, nutrient availability and pathogen population densities. Indigenous soil microbes can also interact with inoculated antagonists, thereby influencing their activities in soil.

Consequently, it seems unlikely that a single antagonist will be capable of providing consistent and long-lasting control in different field locations under varying environmental conditions (Xiao *et al*., 2002). Although the results presented here are promising, this study looked at the biocontrol properties of a single antagonist on only one chilli cultivar. It is likely that support and action of biocontrol agents is determined to some extent by the genotype of the plant (Jones & Samac, 1996). Further work to evaluate the biocontrol ability of strain P42 should consider multiple chilli cultivars varying in their anthracnose resistance and planted in multiple field soils varying in pathogen population densities and environmental conditions.

The interaction considered in this study concerns a single pathogen and a single biocontrol agent. However, it is known that multiple interactions are the normal situation in the rhizosphere. One way of improving biocontrol in the rhizosphere may be to add mixtures or combinations of biocontrol agents, particularly if they exhibit different or complementary modes of action or abilities to colonize root microsites. Combinations of *Paenibacillus* sp. and a *Streptomyces* sp. suppressed *Fusarium* wilt of cucumber better than when either was used (Whipps, 2001). Mixtures of different

species of microorganisms may result in better plant colonization, better adaptation to environmental changes, present a large number of pathogen suppressive mechanism, and/or may protect against a broad range of pathogens (Hervas *et al*., 1998).

Future studies are needed to examine the possibility of integrating chemical and biological agents to give additional control of the plant pathogenic fungi. Several combinations of biocontrol agents and pesticides have been shown to stimulate the antagonists that suppress soil pathogens. E1-Shanshoury *et al*. (1996) reported that a combination of antagonistic *Streptomyces* spp. with pendimethalin was more effective than the single treatment with antagonists or the herbicide against tomato wilt pathogens, *Pseudomonas solanacearum* and *F. oxysporum*. The results suggested a possible induction of biochemical defense within the host plant in the presence of herbicides and antagonistic microorganisms. In another study, field experiments were performed to evaluate the effect of an antagonistic *Streptomyces* strain alone and in combination with the fungicide metalaxyl on alfalfa seedling disease control. The combined treatment increased the frequency of healthy plants with no or slight symptoms of *Phytophthora* root rot (Jones & Samac, 1996). Studies should be carried out to examine the parallel effects of strain P42 and chemical treatments on the chilli growth and anthracnose disease control.

Protoplast fusion is a technique that could provide stable changes in the genome of *Streptomyces* spp. to modify the phenotypic traits. This technique has been used to induce expression of silent genes of antibiotic biosynthesis in a strain (Hopwood *et al*., 1977). Agbessi *et al*. (2003) carried out intraspecific protoplast fusion on *S. melanosporofaciens* EF-76, an antagonist of raspberry root rot caused by *Phytophthora*

fragariae var. *rubi*. The recombinant strain FP-54 produced exhibited higher antagonistic activities against *Bacillus cereus*, *Streptomyces scabies* and *P. fragariae* than did the wild-type strain. Although both FP-54 and EF-76 produced geldanamycin, two other antimicrobial compounds produced by FP-54 were absent in the culture supernatants of the wild-type strain. The ability of the recombinant strain to biosynthesize new compounds, obtained by intraspecific protoplast fusion, contributed to the higher antagonistic property of the strain (Agbessi *et al*., 2003). Therefore, this technique can be used to modify the efficiency of an antagonist such as strain P42. Continued bioprospecting is required to diversify the potential applications of a biocontrol agent, as well as to replace more widely used biocontrol products should resistance develop. Extended screening should be undertaken in the future to look for more efficient strains of streptomycetes as biocontrol tools.

Finally, future research should also not overlook the possible negative impacts linked to the use of biocontrol agents. Since biocontrol is often associated with antibiosis, pathogens might develop resistance to antibiotics produced by antagonistic actinomycetes. Antibiotics produced by actinomycetes often have a large spectrum of action. The effect of introduced actinomycetes on non-target organisms should be evaluated. Evidence for horizontal transfer of virulence genes between common potato scab-inducing strains of *S. scabies* and saprophytic *Streptomyces* species has been presented (Bukhalid *et al*., 2002). The horizontal transfer of genes between introduced actinomycetes and indigenous microorganisms is another risk associated with biocontrol that needs to be assessed.

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APPENDIX A: MEDIA AND REAGENTS

A1: MEDIA

A1.1 Yeast extract - SDS solution (Hayakawa and Nonomura, 1989)

Yeast Extract	6.0 g
Sodium dodecyl sulphate (SDS)	0.05 g
Distilled water	1000 ml

(pH adjusted to 7.3 prior to autoclaving at 121ºC for 15 min)

A1.2 Starch-casein agar (SC; Kustar and Williams, 1964)

Soluble starch	10.0 g
K_2HPO_4	2.0 g
KNO_3	2.0 g
Casein	0.3 g
NaCl	3.0 g
$MgSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
$CaCO_3$	0.02 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

Antibiotics used: Cycloheximide (50 µg/mL), Nystatin (50 µg/mL) and Nalidixic acid (20 µg/mL)

A1.3 Humic acid-vitamin agar (HV; Hayakawa and Nonomura, 1987)

Humic acid	0.5 g
Na_2HPO_4	0.5 g
KCl	1.7 g
$MgSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	10 µg
$CaCO_3$	10 µg
Agar	15.0 g
Distilled water	1000 ml

(0.5 g of Humic acid was dissolved in 10ml of 0.2 N NaOH prior to use)
Autoclaved at 121ºC for 15 min

0.1 ml of stock solution of vitamins was added into 1000 ml of sterile HV medium. The stock solution consists of:

Thiamine-HCl	5.0 mg/ml
Riboflavin	5.0 mg/ml
Niacin	5.0 mg/ml
Pyridoxin HC	5.0 mg/ml
Inositol	5.0 mg/ml
Ca. Pantothenate	5.0 mg/ml
2 amino benzoid acid	5.0 mg/ml
Biotin	2.5 mg/ml

A1.4 Raffinose-histidine agar (RH; Getha, 2005)

Raffinose	10.0 g
Histidine	1.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.5 Yeast extract-malt extract agar (ISP2; Shirling and Gottlieb, 1966)

Yeast extract	4.0 g
Malt extract	10.0 g
Glucose	4.0 g
Agar	18.5 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

Antibiotics supplement when needed:

Antibiotics used: Cycloheximide (50 µg/mL), Nystatin (50 µg/mL) and Nalidixic acid (20 µg/mL).

A1.6 Inorganic salts-starch agar (ISP4; Shirling and Gottlieb, 1966)

Soluble starch	10.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	1.0 g
NaCl	1.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	2.0 g
$FeSO_4.7H_2O$	0.001 g
$MnCl_2.4H_2O$	0.001 g
$ZnSO_4.7H_2O$	0.001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.7 Sporulation agar

Yeast extract	4.0 g
$CaCO_3$	1.0 g
Soluble starch	20.0 g
Glucose	15.0
K_2HPO_4	0.1 g
$MgSO_4.7H_2O$	0.1 g
Casein	5.0 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.8 Potato Dextrose agar (PDA)

PDA	39.0
Distilled water	1000 ml

(Autoclaved for 121ºC for 15 min)

A1.9 Non-sporulating agar (Sanglier *et al*., 1993)

Casamino acids	20.0 g
Soluble starch	20.0 g
Yeast extract	4.0 g
Agar	18.0 g
Deionized distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.10 Oatmeal agar (ISP3; Shirling and Gottlieb, 1966)

Oatmeal	20.0 g
$FeSO_4.7H_2O$	0.001 g
$MnCl_2.4H_2O$	0.001 g
$ZnSO_4.7H_2O$	0.001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.11 Glycerol-asparagine agar (ISP5; Shirling and Gottlieb, 1966)

Glycerol	10.0 g
L-Asparagine	1.0 g
K_2HPO_4	1.0 g
$FeSO_4.7H_2O$	0.001 g
$MnCl_2.7H_2O$	0.001 g
$ZnSO_4.7H_2O$	0.001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.12 Peptone-yeast extract-iron agar (ISP6; Shirling and Gottlieb, 1966)

Peptone	15.0 g
Proteose peptone	5.0 g
Yeast extract	1.0 g
K_2HPO_4	1.0 g
Ammonium Iron (II) Citrate	0.5 g
Sodium Thiosulfate	0.08 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.13 Tyrosine agar (ISP7; Shirling and Gottlieb, 1966)

Glycerol	15.0 g
L-Tyrosine	0.5 g
L-Asparagine	1.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4.7H_2O$	0.011 g
$MnCl_2.4H_2O$	0.001 g
$ZnSO_4.7H_2O$	0.001 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.14 Modified Benett's agar (MBA; Jones, 1949)

Beef extract	1.0 g
Yeast extract	1.0 g
Tryptone	2.0 g
Glycerol	10.0 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.15 Pridham-Gottlieb agar medium (ISP9; Shirling and Gottlieb, 1966)

$(NH_4)_2SO_4$	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4.7H_2O$	1.00 g
$FeSO_4.7H_2O$	0.0011 g
$MnCl_2.4H_2O$	0.0079 g
$ZnSO_4.7H_2O$	0.0015 g
$CuSO_4.5H_2O$	0.0064 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A1.16 Starch-casein-nitrate agar (SCN; Kustar and Williams, 1964)

Soluble starch	10.0 g
K_2HPO_4	2.0 g
KNO_3	2.0 g
Casein (vitamin free)	0.3 g
NaCl	3.0 g
$MgSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
$CaCO_3$	0.02 g
Agar	18.0 g
Distilled water	1000 ml

(pH adjusted to 7.0 - 7.2 with 1M NaOH prior to autoclaving at 121ºC for 15 min)
Antibiotics: Cycloheximide (50 µg/mL), Nystatin (50 µg/mL)

A1.17 Colloidal chitin agar (CCA; Hus and Lockwood, 1975)

i. Preparation of bleached chitin

60 g coarse, unbleached chitin was stirred with 300 m1 commercial bleach (5.25% sodium hypochlorite) for 10 min. The mixture was added to 5 L tap water and filtered with a coarse filter paper (Whatman) under reduced pressure to remove the bleach. This bleach removal step was repeated five times. The chitin was air-dried overnight and subsequently ground in a Waring blender.

ii. Preparation of powdered colloidal chitin

40 g bleached chitin powder was dissolved in 400 ml concentrated HCl by stirring for 30-50 min. The mixture was added slowly to 2 L cold water (2-5°C) to precipitate the chitin as a colloidal suspension. The suspension was collected by filtration under reduced pressure onto a coarse filter paper and washed by re-suspending it in 5 L tap water and re-filtered The washing step was repeated five times until the pH of the suspension was about 3.5. The suspension was freeze coated in round bottom flasks (100 ml/flask) with cold denatured ethanol and dry ice, left overnight at -20°C and freeze-dried (Christ, model Beta 1-8) for 1-2 days into dry powder form.

iii. Preparation of chitin agar

Powdered colloidal chitin	4.0 g
K_2HPO_4	0.7 g
KH_2PO_4	0.3 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
$ZnSO_4.7H_2O$	0.001 g
$MnCl_2.4H_2O$	0.001 g
Agar	20.0 g
Distilled water	1000 ml

(pH adjusted to 7.5 - 8.5 with 1M NaOH prior to autoclaving at 121ºC for 15 min)

A2: REAGENTS

A2.1 Ninhydrin reagent

Ninhydrin	0.2 g
Acetone	100 ml

A2.2 Lugol's iodine solution

Iodine	0.1 g
Potassium iodide	0.2 g
Distilled water	100 ml

A2.3 Nitrate broth

Peptone	5 g
Meat extract	3 g
Potassium	1 g

Final pH 7.0 ± 0.2 at 37ºC

APPENDIX B: EXPERIMENTAL METHODS

B1: Determination of the 16S rRNA sequences of *Streptomyces* sp. strains

B1.1 DNA extraction

Total genomic DNA from four strains tested was extracted using a using Machery Nagel NucleoSpin DNA extraction kit according to the manufacturer's specification. A small cell pellet of biomass was scrapped from seven day old cultures on non-sporulating agar (Appendix A1.9).

1. Pre-lyse sample

- Suspend 5 loopful of actinobacteria cells in **180 µl buffer T1**
- Add **25 µl Proteinase K** and **20 mg/ml lysozyme**
- Vortex vigorously
- Incubate at 56ºC until complete lysis is obtained. Vortex occasionally during incubation period (at lease 1-3 hours or incubate overnight)

2. Lyse sample

- Vortex the samples
- Add **200 µl Buffer B3** and vortex vigorously
- Incubate at 70ºC for 10 min and vortex briefly
- Centrifuge for 5 min at 12000 rpm and transfer the supernatant to a new microcentrifuge tube

3. Adjust DNA binding conditions

- Add **210 µl ethanol (96-100%)** and vortex vigorously

4. Bind DNA

- Place a **NucleoSpin Tissue Column** into a **Collection Tube**
- Load all samples into the column
- Centrifuge for 1min at 12000 rpm
- Discard the flow-through and place the column back into the Collection Tube

5. Washing steps

- Add **500 µl Buffer BW** and centrifuge for 1 min at 12000rpm
- Discard the flow-through and place the column back into the Collection Tube
- Add **600 µl Buffer B5** and centrifuge for 1 min at 12000 rpm
- Discard the flow-through and place the column back into the Collection Tube

6. Dry silica membrane

- Centrifuge the column for 1 min at 12000 rpm

7. Elute DNA

- Place the NucleoSpin Tissue Column into a 1.5 ml microcentrifuge tube
- Add **100 µl pre-warmed Buffer BE (70ºC)**
- Incubate at room temperature for 1 min and centrifuge for 1 min at 12000 rpm

B1.2 Gel electrophoresis (to check DNA quality and quantity)

- 1% agarose gel (0.4 g) in 0.5x TBE[a] (40 ml)
- Heat in microwave (low power for 3.5 min)
- Cool and pour gel into electrophoresis apparatus
- Insert comb and let gel to harden
- Remove comb and pour 0.5x TBE (~500ml)

- Loading buffer[b] : sterile distilled water: DNA sample (or DNA marker[c]) (2 µl : 2 µl : 2 µl)
- Load into well and run at 100V (30-35 min)
- Stain with EtBr[d] (5 µl in 200ml distilled water) by shaking gently for 30 min
- Check under UV light (retain with another 5 µl EtBr/30 min if bands not clear

[a] : Prepare 500 ml (50 ml 5x TBE[e] + 450 ml sterile distilled water)

[b] : Loading buffer = 0.2x TBE + 2 M sucrose (6.8 g in 10 ml) + bromophenol blue (0.26 g in 10 ml)

[c] : DNA marker (Gene Ruler^{TM} , Fermentas), 100bp DNA Ladder Plus 0.5 mg DNA/ml

[d] : Ethidium bromide stock = 10 mg/10 ml

[e] : 5x TBE = 54 g Tris + 27.5 g Boric acid + 20ml 0.5M EDTA (pH 8.0) + 1 L distilled water

APPENDIX C: EXPERIMENTAL AND STATISTICAL DATA

C1: Inhibition of mycelial growth of three species of *Colletotrichum* spp. by isolate P42

Type of *Colletotrichum*	Incubation period (days)	γ_o (mm)	γ (mm)	% inhibition of mycelial growth[a]
C. acutatum	2	11 10 10.5 11	1 1 1 1.5	89.44 ± 2.08
	4	22 22 22 22.5	1.5 1.5 1.5 2.0	92.66 ± 1.04
	6	30 30.5 30.5 30	2.0 2.0 2.0 2.0	93.39 ± 0.06
	8	37.5 37.5 37 37.5	2.0 2.0 2.0 2.5	94.32 ± 0.66
C. capsici	2	13 13 13.5 13	2 1.5 2 2.5	84.76 ± 3.15
	4	24 24 24.5 24.5	2.5 2.0 2.5 3.5	89.19 ± 2.50
	6	33 34.5 34.5 33	3 2.5 3 3.5	91.09 ± 1.38
	8	40.5 40 40 41	4.0 3.5 4.0 4.5	90.10 ± 0.91
C. gloeosporiodes	2	12.5 12.5 13 13	2.5 2.5 2 2.5	81.35 ± 2.21
	4	24 24 24 24.5	3.5 3.5 3.0 3.5	86.01 ± 1.00
	6	35 35 35.5 35	4.0 4.0 3.5 4.0	88.96 ± 0.78
	8	41.5 41 41 41.5	4.5 4.5 5.0 4.5	88.79 ± 0.66

γ_o : Fungal colony radius control plate.

γ : Distance of fungal colony growth in the direction of strain P42 culture on paired culture plate

[a] : Mean % inhibition of mycelial growth from four readings ± standard deviation {% inhibition of mycelial growth = $[1 - (\gamma / \gamma_o)] \times 100$}

C2: Analysis of variance on the effect of 3 different species of *Colletotrichum* on the inhibition of mycelial growth of Anthracnose disease by isolate P42 at different incubation periods

C2.1 ANOVA on day 2

Source	Sum of square	d.f.	Mean square	F value
Colletotrichum spp.	131.9630167	2	65.9815083	10.32*
Error	57.5313500	9	6.3923722	
Total	189.4943667	11		

* **Significant, p<0.05**

Multiple range analysis using LSD at 95% confidence intervals

***Colletotrichum* spp.**	**% inhibition (average)**	**Homogeneous groups***
C. acutatum	89.438	A
C. capsici	84.760	B
C. gloeosporioides	81.348	B

* Means by a common latter are not significantly different at 5% level

C2.2 ANOVA on day 4

Source	Sum of square	d.f.	Mean square	F value
Colletotrichum spp.	88.5030167	2	44.2515083	15.93*
Error	25.0049500	9	2.7783278	
Total	113.5079667	11		

* **Significant, p<0.05**

Multiple range analysis using LSD at 95% confidence intervals

***Colletotrichum* spp.**	**% inhibition (average)**	**Homogeneous groups***
C. acutatum	92.663	A
C. capsici	89.190	B
C. gloeosporioides	86.013	C

* Means by a common latter are not significantly different at 5% level

C2.3 ANOVA on day 6

Source	Sum of square	d.f.	Mean square	F value
Colletotrichum spp.	39.13685000	2	19.56842500	23.23*
Error	7.58285000	9	0.84253889	
Total	46.71970000	11		

* **Significant, p<0.05**

Multiple range analysis using LSD at 95% confidence intervals

***Colletotrichum* spp.**	**% inhibition (average)**	**Homogeneous groups***
C. acutatum	93.3850	A
C. capsici	91.0875	B
C. gloeosporioides	88.9625	C

* Means by a common latter are not significantly different at 5% level

C2.4 ANOVA on day 8

Source	Sum of square	d.f.	Mean square	F value
Colletotrichum spp.	66.78781667	2	33.39390833	58.89*
Error	5.10387500	9	0.56709722	
Total	71.89169167	11		

* **Significant, p<0.05**

Multiple range analysis using LSD at 95% confidence intervals

***Colletotrichum* spp.**	**% inhibition (average)**	**Homogeneous groups***
C. acutatum	94.3150	A
C. capsici	90.0975	B
C. gloeosporioides	88.7850	C

* Means by a common latter are not significantly different at 5% level

C3: 16S rRNA sequence of four tested strains

C3.1: 16S rRNA sequence of *Streptomyces* sp. strain P8

GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAA
ACGGGGTCTAATACCGGATATGACCTGGGACCGCATGGTTCTGGGTGTAAA
GCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTA
ATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATG
ACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACG
GTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGC
GGCCTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATT
CGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCT
CTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGAC
ATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAG
TACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
AGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCT
TGACATATGCCGGAAACGTCTAGAGATAGGCGCCCCCTTGTGGTCGGTATA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGA
TGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
ACGTCAAATCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGG
TCGGTACAAAGGGCTGCGATGCCGTGAGGCGGAGCGAATCCCAAAAAGCC
GGCCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGGAGTTGC
TAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAA
CC

C3.2: 16S rRNA sequence of *Streptomyces* sp. strain P39

GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAA
ACGGGGTCTAATACCGGATACTGATCCTCGCAGGCATCTGTGAGGTTCGAA
AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGT
AATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGAC
GGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
CGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGT
CGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCT
CTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAA

CATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGA
GTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGC
TTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGT
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGC
TGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGG
CCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCG
GTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCT
AGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAA
CC

C3.3: 16S rRNA sequence of *Streptomyces* sp. strain P42

GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAA
ACGGGGTCTAATACCGGATACTGATCCTCGCAGGCATCTGCGAGGTTCGAA
AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGAC
GGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
CGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGT
CGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCT
CTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAA
CATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGA
GTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGC
TTGACATACACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGT
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGC
TGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
ACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGG
CCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCG
GTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCT
AGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAA
CC

C3.4: 16S rRNA sequence of *Streptomyces* sp. strain P115

GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAA
ACGGGGTCTAATACCGGATACTGACTCTCGCAGGCATCTGTGAGGGTCGAA
AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGT
AATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGAC
GGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
CGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGT
CGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCT
CTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAA
CATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGA
GTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGC
TTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGT
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAACTCTTCGGAGGTT
GGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGA
CGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGC
CGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGG
TCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTA
GTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAAC
C

C4: Results of *in vivo* biocontrol assay in 1st time

C4.1: Analysis of variance (ANOVA) on the effect of different treatment on Fruit Symptom Index (FSI)

Source	Sum of squares	d.f.	Mean square	F value
Treatment	232.7875000	7	33.2553571	69.00*
Error	34.7000000	72	0.4819444	
Total	267.4875000	79		

* Significant, p<0.05.

Multiple range analysis using LSD at 95% confidence intervals

Treatment	FSI (average)	Homogeneous groups*
C	0.0	C
P	0.0	C
Ca	4.1	A
PCa	1.4	B
Cc	4.3	A
PCc	2.0	B
Cg	4.4	A
PCg	1.9	B

* Means followed by a common letter are not significantly different at 5% level

C4.2: Analysis of variance (ANOVA) on the effect of different treatment on mean fruit fresh weigh

Source	Sum of squares	d.f.	Mean square	F value
Treatment	1221.630875	7	174.518696	162.42*
Error	77.363000	72	1.074486	
Total	1298.993875	79		

* Significant, $p<0.05$.

Multiple range analysis using LSD at 95% confidence intervals

Treatment	fruit fresh weight (g) (average)	Homogeneous groups*
C	11.93	B
P	13.02	A
PCa	11.82	B
Ca	3.86	D
PCc	8.97	C
Cc	3.14	D
PCg	9.38	C
Cg	3.15	D

* Means followed by a common letter are not significantly different at 5% level

C4.3: Summary of Fruit Symptom Index (FSI) and Disease Severity Index (DSI) and fresh weight of chilli plants with and without strain P42 treatment (recorded after 9 days)

Colletotrichum Species	Strain P42 treatment[a]	Fruit Fresh Weight (g)[b]	FSI[c]	DSI(%)[d]	A	B
-	Untreated (C)	11.93 ± 0.36B[e]	0.0C	0	0	0
-	Treated (P)	13.02 ± 0.29A	0.0C	0	0	0
C. acutatum	Untreated (Ca)	3.86 ± 1.07D	4.1A	82	10	3
C. acutatum	Treated (PCa)	11.82 ± 0.48B	1.4B	28	10	0
C. capsici	Untreated (Cc)	3.14 ± 0.60D	4.3A	86	10	4
C. capsici	Treated (PCc)	8.97 ± 1.70C	2.0B	40	10	0
C. gloeosporioides	Untreated (Cg)	3.15 ± 0.87D	4.4A	88	10	6
C. gloeosporioides	Treated (PCg)	9.38 ± 1.73C	1.9B	38	10	0

[a]: C, seeds and plants were not treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension and fruits immersed in sterile distilled water; P, chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in sterile distilled water; Ca,Cc and Cg seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension; PCa, PCc and PCg chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension.

[b]: Average fruit fresh weight of ten plants for each treatment (mean ± standard deviation).

[c]: Fruit Symptom Index, FSI (average of ten plants for each treatments).

[d]: Disease Severity Index, DSI (average of ten plants for each treatments).

[e]: Means with the same letter within a column are not significantly different ($p<0.05$).

A: Number of plants inoculated with the *Colletotrichum* species; B: Number of dead chilli fruits.

C5: Results of *in vivo* biocontrol assay in 2nd time

C5.1: The effect of strain P42 on pathogen-free chilli plants (recorded after 70 days)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
Untreated chilli plants (C)	1	12.2	0
	2	12.3	0
	3	11.0	0
	4	12.3	0
	5	12.0	0
	6	11.7	0
	7	11.5	0
	8	11.5	0
	9	12.4	0
	10	12.0	0
Strain P42-treated chilli plants (P)	1	13.4	0
	2	13.2	0
	3	12.9	0
	4	13.1	0
	5	12.7	0
	6	13.0	0
	7	12.8	0
	8	13.0	0
	9	12.4	0
	10	13.5	0

C5.2: The effect of strain P42 on chilli plants inoculated with *C. acutatum* (recorded after 9 days of inoculation with fungus suspension)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
P42-free chilli plants (Ca)	1	4.9	3
	2	3.0	5
	3	4.1	4
	4	4.0	4
	5	2.9	5
	6	3.5	4
	7	4.2	4
	8	2.7	5
	9	2.5	5
	10	4.0	4
Strain P42-treated chilli plants (PCa)	1	11.7	2
	2	12.2	1
	3	12.0	1
	4	11.5	2
	5	12.0	1
	6	12.1	1
	7	11.7	2
	8	9.9	2
	9	11.0	2
	10	11.9	1

C5.3: The effect of strain P42 on chilli plants inoculated with *C. capsici* (recorded after 9 days of inoculation with fungus suspension)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
P42-free chilli plants (Cc)	1	2.9	5
	2	2.5	5
	3	4.0	4
	4	2.7	5
	5	4.5	3
	6	2.6	5
	7	3.9	4
	8	4.1	4
	9	3.8	4
	10	2.2	5
Strain P42-treated chilli plants (PCc)	1	11.0	1
	2	11.3	1
	3	9.5	2
	4	8.7	2
	5	11.5	1
	6	9.0	2
	7	11.2	1
	8	10.9	1
	9	9.9	2
	10	7.5	3

C5.4: The effect of strain P42 on chilli plants inoculated with *C. gloeosporioides* (recorded after 9 days of inoculation with fungus suspension)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
P42-free chilli plants (Cg)	1	2.6	5
	2	3.5	4
	3	2.5	5
	4	2.8	5
	5	2.6	5
	6	3.6	4
	7	3.4	4
	8	2.5	5
	9	2.7	5
	10	3.8	4
Strain P42-treated chilli plants (PCg)	1	8.6	3
	2	9.9	2
	3	10.9	1
	4	8.5	3
	5	11.3	1
	6	11.0	1
	7	9.6	2
	8	11.5	1
	9	11.8	1
	10	10.0	2

C5.5: Analysis of variance (ANOVA) on the effect of different treatment on Fruit Symptom Index (FSI)

Source	Sum of squares	d.f.	Mean square	F value
Treatment	257.5875000	7	36.7982143	110.86*
Error	23.9000000	72	0.3319444	
Total	281.4875000	79		

* Significant, $p<0.05$.

Multiple range analysis using LSD at 95% confidence intervals

Treatment	FSI (average)	Homogeneous groups*
C	0.0	C
P	0.0	C
Ca	4.3	A
PCa	1.5	B
Cc	4.4	A
PCc	1.6	B
Cg	4.6	A
PCg	1.7	B

* Means followed by a common letter are not significantly different at 5% level

C5.6: Analysis of variance (ANOVA) on the effect of different treatment on mean fruit fresh weigh of Chilli fruits

Source	Sum of squares	d.f.	Mean square	F value
Treatment	1281.241875	7	183.034554	264.76*
Error	49.775000	72	0.691319	
Total	1331.016875	79		

* Significant, $p<0.05$.

Multiple range analysis using LSD at 95% confidence intervals

Treatment	fruit fresh weight (g) (average)	Homogeneous groups*
C	11.89	B
P	13.0	A
PCa	11.60	B
Ca	3.58	D
PCc	10.05	C
Cc	3.32	D
PCg	10.31	C
Cg	3.0	D

* Means followed by a common letter are not significantly different at 5% level

C5.7: Summary of Fruit Symptom Index (FSI), Disease Severity Index (DSI) and fruit fresh weight of chilli plants with and without strain P42 treatment

Colletotrichum Species	Strain P42 Treatment[a]	Fruit Fresh Weight (g)[b]	FSI[c]	DSI(%)[d]	A	B
-	Untreated (C)	11.89 ± 0.45B[e]	0.0C	0	0	0
-	Treated (P)	13.00 ± 0.33A	0.0C	0	0	0
C. acutatum	Untreated (Ca)	3.58 ± 0.78D	4.3A	86	10	4
C. acutatum	Treated (PCa)	11.6 ± 0.69B	1.5B	30	10	0
C. capsici	Untreated (Cc)	3.32 ± 0.82D	4.4A	88	10	5
C. capsici	Treated (PCc)	10.05 ± 1.35C	1.6B	32	10	0
C. gloeosporioides	Untreated (Cg)	3.0 ± 0.51D	4.6A	92	10	6
C. gloeosporioides	Treated (PCg)	10.31 ± 1.17C	1.7B	34	10	0

[a]: C, seeds and plants were not treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension and fruits immersed in sterile distilled water; P, chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in sterile distilled water; Ca,Cc and Cg seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension; PCa, PCc and PCg chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension.

[b]: Average fruit fresh weight of ten plants for each treatment (mean ± standard deviation).

[c]: Fruit Symptom Index, FSI (average of ten plants for each treatments).

[d]: Disease Severity Index, DSI (average of ten plants for each treatments).

[e]: Means with the same letter within a column are not significantly different ($p<0.05$).

A: Number of plants inoculated with the *Colletotrichum* species; B: Number of dead chilli fruits.

C6: Results of *in vivo* biocontrol assay in 3rd time

C6.1: The effect of strain P42 on pathogen-free chilli plants (recorded after 70 days)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
Untreated chilli plants (C)	1	12.1	0
	2	11.4	0
	3	12.2	0
	4	11.8	0
	5	12.3	0
	6	11.9	0
	7	12.5	0
	8	11.6	0
	9	11.5	0
	10	12.0	0
Strain P42-treated chilli plants (P)	1	13.0	0
	2	13.5	0
	3	12.9	0
	4	13.2	0
	5	13.4	0
	6	13.1	0
	7	12.9	0
	8	13.4	0
	9	13.6	0
	10	13.5	0

C6.2: The effect of strain P42 on chilli plants inoculated with *C. acutatum* (recorded after 9 days of inoculation with fungus suspension)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
P42-free chilli plants (Ca)	1	3.9	4
	2	2.5	5
	3	3.1	5
	4	4.1	4
	5	4.4	4
	6	5.2	3
	7	2.7	5
	8	4.0	4
	9	5.3	3
	10	3.9	4
Strain P42-treated chilli plants (PCa)	1	11.5	1
	2	12.0	1
	3	11.9	1
	4	11.3	2
	5	10.0	2
	6	12.1	1
	7	12.0	1
	8	10.9	2
	9	11	2
	10	11.2	2

C6.3: The effect of strain P42 on chilli plants inoculated with *C. capsici* (recorded after 9 days of inoculation with fungus suspension)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
P42-free chilli plants (Cc)	1	3.2	4
	2	2.5	5
	3	3.6	4
	4	3.3	4
	5	2.4	5
	6	2.6	5
	7	3.1	4
	8	3.9	4
	9	2.7	5
	10	3.0	4
Strain P42-treated chilli plants (PCc)	1	9.5	2
	2	9.0	2
	3	9.8	2
	4	11.0	1
	5	11.4	1
	6	8.9	2
	7	10.5	1
	8	11.1	1
	9	7.2	3
	10	10.8	1

C6.4: The effect of strain P42 on chilli plants inoculated with *C. gloeosporioides* (recorded after 9 days of inoculation with fungus suspension)

Treatment group	Plant #	Fruit Fresh weight (g)	Damage scale
P42-free chilli plants (Cg)	1	3.7	4
	2	2.8	5
	3	2.0	5
	4	2.8	5
	5	3.0	4
	6	3.3	4
	7	3.8	4
	8	2.8	5
	9	3.2	4
	10	2.7	5
Strain P42-treated chilli plants (PCg)	1	8.1	3
	2	11.5	1
	3	11.2	1
	4	9.0	2
	5	11.0	1
	6	10.9	1
	7	8.4	3
	8	11.4	1
	9	9.2	2
	10	8.5	3

C6.5: Analysis of variance (ANOVA) on the effect of different treatment on Fruit Symptom Index (FSI) of Chilli plants

Source	Sum of squares	d.f.	Mean square	F value
Treatment	244.1875000	7	34.8839286	103.36*
Error	24.3000000	72	0.3375000	
Total	268.4875000	79		

* Significant, $p<0.05$.

Multiple range analysis using LSD at 95% confidence intervals

Treatment	FSI (average)	Homogeneous groups*
C	0.0	C
P	0.0	C
Ca	4.1	A
PCa	1.5	B
Cc	4.4	A
PCc	1.6	B
Cg	4.5	A
PCg	1.8	B

* Means followed by a common letter are not significantly different at 5% level

C6.6: Analysis of variance (ANOVA) on the effect of different treatment on mean fruit fresh weigh of Chilli fruits

Source	Sum of squares	d.f.	Mean square	F value
Treatment	1309.175500	7	187.025071	261.78*
Error	51.440000	72	0.714444	
Total	1360.615500	79		

* Significant, $p<0.05$.

Multiple range analysis using LSD at 95% confidence intervals

Treatment	fruit fresh weight (g) (average)	Homogeneous groups*
C	11.93	B
P	13.25	A
PCa	11.39	B
Ca	3.91	D
PCc	9.92	C
Cc	3.03	D
PCg	9.92	C
Cg	3.01	D

* Means followed by a common letter are not significantly different at 5% level

C6.7: Summary of Fruit Symptom Index (FSI), Disease Severity Index (DSI) and fruit fresh weight of chilli plants with and without strain P42 treatment in the *in vivo* biocontrol assay (recorded after 9 days)

***Colletotrichum* Species**	**Strain P42 Treatment[a]**	**Fruit Fresh Weight (g)[b]**	**FSI[c]**	**DSI(%)[d]**	**A**	**B**
-	Untreated (C)	11.93 ± 0.36B[e]	0.0C	0	0	0
-	Treated (P)	13.25 ± 0.26A	0.0C	0	0	0
C. acutatum	Untreated (Ca)	3.91 ± 0.94D	4.1A	82	10	3
C. acutatum	Treated (PCa)	11.39 ± 0.66B	1.5B	30	10	0
C. capsici	Untreated (Cc)	3.03 ± 0.49D	4.4A	88	10	4
C. capsici	Treated (PCc)	9.92 ± 1.31C	1.6B	32	10	0
C. gloeosporioides	Untreated (Cg)	3.01 ± 0.52D	4.5A	90	10	5
C. gloeosporioides	Treated (PCg)	9.92 ± 1.39C	1.8B	36	10	0

[a]: C, seeds and plants were not treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension and fruits immersed in sterile distilled water; P, chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in sterile distilled water; Ca,Cc and Cg seeds and plants were not treated with *Streptomyces* sp. strain P42 suspension and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension; PCa, PCc and PCg chilli seeds treated with low dosage (1.16×10^5 cfu/ml) of *Streptomyces* sp. strain P42 suspension prior to planting, plants were treated with same dosage of strain P42 45 days after planting and fruits immersed in 10^5 *C. acutatum, C. capsici* and *C. gloeosporioides* suspension.

[b]: Average fruit fresh weight of ten plants for each treatment (mean ± standard deviation).

[c]: Fruit Symptom Index, FSI (average of ten plants for each treatments).

[d]: Disease Severity Index, DSI (average of ten plants for each treatments).

[e]: Means with the same letter within a column are not significantly different ($p<0.05$).

A: Number of plants inoculated with the *Colletotrichum* species; B: Number of dead chilli fruits.

C7: Two-sample analysis for the effect of strain P42 treatment on Fruit Symptom Index (FSI) of chilli plants in the *in vivo* biocontrol assay in 1st Time

	Untreated (Ca)	**Treated (PCa)**	**Pooled**
Number of observations	10	10	20
Average	4.1	1.4	2.75
Variance	0.5444373796	0.26666896	0.4055531698
Standard deviation	0.73786	0.51640	0.62713

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 2.640 and significant level = 0.05. Reject H_0

	Untreated (Cc)	**Treated (PCc)**	**Pooled**
Number of observations	10	10	20
Average	4.3	2.0	3.15
Variance	0.4555575025	0.8888906961	0.6722240993
Standard deviation	0.67495	0.94281	0.80888

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 2.560 and significant level = 0.05. Reject H_0

	Untreated (Cg)	**Treated (PCg)**	**Pooled**
Number of observations	10	10	20
Average	4.4	1.9	3.15
Variance	0.7111042929	0.9888910249	0.8499976589
Standard deviation	0.84327	0.99443	0.91885

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 2.472 and significant level = 0.05. Reject H_0

C8: Two-sample analysis for the effect of strain P42 treatment on mean fruit fresh weight of chilli plants in the *in vivo* biocontrol assay in 1st Time

	Untreated (Ca)	**Treated (PCa)**	**Pooled**
Number of observations	10	10	20
Average	3.86	11.82	7.84
Variance	1.1404531264	0.2262238969	0.68333851165
Standard deviation	1.06792	0.47563	0.771775

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 2.033 and significant level = 0.05. Reject H_0

	Untreated (Cc)	**Treated (PCc)**	**Pooled**
Number of observations	10	10	20
Average	3.14	8.97	6.055
Variance	0.3648884836	2.9045703184	1.634729401
Standard deviation	0.60406	1.70428	1.15417

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 5.876 and significant level = 0.05. Reject H_0

	Untreated (Cg)	**Treated (PCg)**	**Pooled**
Number of observations	10	10	20
Average	3.15	9.3800	6.265
Variance	0.7538927929	2.9951148096	1.87450380125
Standard deviation	0.86827	1.73064	1.299455

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 4.504 and significant level =0.05. Reject H_0

C9: Two-sample analysis for the effect of strain P42 treatment on Fruit Symptom Index (FSI) of chilli plants in the *in vivo* biocontrol assay in 2nd Time

	Untreated (Ca)	**Treated (PCa)**	**Pooled**
Number of observations	10	10	20
Average	4.3	1.5	2.9
Variance	0.4555575025	0.2777817025	0.3666696025
Standard deviation	0.67495	0.52705	0.601

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 4.870 and significant level = 0.05. Reject H_0

	Untreated (Cc)	**Treated (PCc)**	**Pooled**
Number of observations	10	10	20
Average	4.4	1.6	3
Variance	0.4888946241	0.4888946241	0.4888946241
Standard deviation	0.69921	0.69921	0.69921

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 1.357 and significant level = 0.05. Reject H_0

	Untreated (Cg)	**Treated (PCg)**	**Pooled**
Number of observations	10	10	20
Average	4.6	1.7	3.15
Variance	0.26666896	0.6777734929	0.47222122645
Standard deviation	0.51640	0.82327	0.669835

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 6.992 and significant level = 0.05. Reject H_0

C10: Two-sample analysis for the effect of strain P42 treatment on mean fruit fresh weight of chilli plants in the *in vivo* biocontrol assay in 2nd Time

	Untreated (Ca)	**Treated (PCa)**	**Pooled**
Number of observations	10	10	20
Average	3.58	11.6	7.59
Variance	0.6106641025	0.4777712641	0.5442176833
Standard deviation	0.78145	0.69121	0.73633

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 1.634 and significant level = 0.05. Reject H_0

	Untreated (Cc)	**Treated (PCc)**	**Pooled**
Number of observations	10	10	20
Average	3.32	10.05	6.685
Variance	0.6706627236	1.8183443716	1.2445035476
Standard deviation	0.81894	1.34846	1.0837

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 1.355 and significant level = 0.05. Reject H_0

	Untreated (Cg)	**Treated (PCg)**	**Pooled**
Number of observations	10	10	20
Average	3.0	10.31	6.655
Variance	0.2622259264	1.3787691241	0.82049752525
Standard deviation	0.51208	1.17421	0.843145

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 3.937 and significant level = 0.05. Reject H_0

C11: Two-sample analysis for the effect of strain P42 treatment on Fruit Symptom Index (FSI) of chilli plants in the *in vivo* biocontrol assay in 3rd Time

	Untreated (Ca)	**Treated (PCa)**	**Pooled**
Number of observations	10	10	20
Average	4.1	1.5	2.8
Variance	0.5444373796	0.2777817025	0.41110954105
Standard deviation	0.73786	0.52705	0.632455

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 3.164 and significant level = 0.05 . Reject H_0

	Untreated (Cc)	**Treated (PCc)**	**Pooled**
Number of observations	10	10	20
Average	4.4	1.6	3
Variance	0.26666896	0.4888946241	0.37778179205
Standard deviation	0.51640	0.69921	0.607805

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 2.051 and significant level = 0.05. Reject H_0

	Untreated (Cg)	**Treated (PCg)**	**Pooled**
Number of observations	10	10	20
Average	4.5	1.8	3.15
Variance	0.2777817025	0.8444507236	0.56111621305
Standard deviation	0.52705	0.91894	0.722995

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 4.265 and significant level = 0.05. Reject H_0

C12: Two-sample analysis for the effect of strain P42 treatment on mean fruit fresh weight of chilli plants in the *in vivo* biocontrol assay in 3rd Time

	Untreated (Ca)	**Treated (PCa)**	**Pooled**
Number of observations	10	10	20
Average	4.00	11.39	7.695
Variance	0.7866625636	0.4321090225	0.60938579305
Standard deviation	0.88694	0.65735	0.772145

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 2.388 and significant level = 0.05. Reject H_0

	Untreated (Cc)	**Treated (PCc)**	**Pooled**
Number of observations	10	10	20
Average	2.91	9.92	6.415
Variance	0.4054505625	1.7039908369	1.0547206997
Standard deviation	0.63675	1.30537	0.97106

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 1.205 and significant level = 0.05. Reject H_0

	Untreated (Cg)	**Treated (PCg)**	**Pooled**
Number of observations	10	10	20
Average	2.74	9.92	6.33
Variance	0.2493304489	1.9395575824	1.09444401565
Standard deviation	0.49933	1.39268	0.946005

Confidence interval for difference in means at p=0.05
Hypothesis test for H_0:$\mu_1 = \mu_2$; vs H_{Alt}: $\mu_1 \neq \mu_2$
Computed t-statistic = 2.912 and significant level = 0.05. Reject H_0

C13: 16S rRNA sequence of *Streptomyces* sp. strain P42 isolated from experimental chilli plants *in vivo*

GGGGACTGGCGGTATTGCTTAACACATGCAAGTCGAACGATGAACCACTTC
GGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGC
ACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGATCCTCG
CAGGCATCTGCGAGGTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGC
CTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCC
GGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGC
AGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGG
GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGG
GAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC
ACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAA
GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGG
TGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGC
ATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAAC
GCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACCCTGGAGACAGG
GTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
CCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAAC
TCGgAGGAAGGTGGGgACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGC
TGCACaCgTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGA
GCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACC
CCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAAC
ACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGCAGCTGTCCGAAGGTGG
GACTGGCGACTGGGACGCAAGTCGTAACAAGGTTAGCCCTCCCCGGG