Application of Combined Antagonistic Microorganisms for Postharvest Control of Fruit Rot Disease Caused by *Penicillium digitatum* in Mandarin cv. Shogun

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A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การประยุกต์ใช้จุลินทรีย์ปฏิปักษ์ร่วมในการควบคุมโรคผลเน่าหลังการเก็บเกี่ยวที่เกิดจากเชื้อ *Penicillium digitatum* ในส้มโชกุน

ผู้เขียน นางสาวสุมิตรา แสงวนิชย์

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บทคัดย่อ

การประยุกต์ใช้เชื้อจุลินทรีย์ปฏิปักษ์เป็นทางเลือกหนึ่งที่ทั่วโลกนิยมนำมาใช้เพิ่มขึ้นเพื่อแก้ปัญหาโรคหลังการเก็บเกี่ยวของส้ม การศึกษาประสิทธิภาพของเชื้อจุลินทรีย์ปฏิปักษ์และสารละลายโซเดียมไบคาร์บอเนตเพียงอย่างเดียวหรือร่วมกันเพื่อใช้ในการควบคุมโรค ผลเน่าของส้มโชกุน ที่เกิดจากเชื้อรา เขียว *Penicillium digitatum* บนจานอาหารเลี้ยงเชื้อและบนผลส้ม พบว่า เชื้อยีสต์ *Pichia guilliermondii* BCC 5389 เชื้อแบคทีเรีย *Bacillus subtilis* ABS-S14 และสารละลายโซเดียมไบคาร์บอเนตที่ความเข้มข้น 1-2% สามารถยับยั้งการเจริญของเชื้อรา *P. digitatum* และที่ความเข้มข้น 0.5-2.0% ไม่มีผลต่อการเจริญของเชื้อแบคทีเรีย *B. subtilis* ABS-S14 แต่มีผลต่อการเจริญของเชื้อยีสต์ *P. guilliermondii* BCC 5389 ขณะที่เชื้อยีสต์ที่ความเข้มข้น 10^6 ถึง 10^8 เซลล์/มล. และน้ำเลี้ยงเชื้อ ยีสต์สามารถ ยับยั้งการงอกสปอร์และการเจริญของเส้นใยเชื้อรา *P. digitatum* ได้ อย่างไรก็ตามไม่พบการเป็นปฏิปักษ์ ระหว่าง *P. guilliermondii* BCC 5389 กับ *B. subtilis* ABS-S14 เมื่อตรวจสอบบนจานอาหารเลี้ยงเชื้อ ในการทดลองบนผลส้ม สารละลายโซเดียมไบคาร์บอเนตที่ความเข้มข้น 2% สามารถควบคุมโรคผลเน่าสูงสุดและมีเปอร์เซ็นต์การเกิดโรคเท่ากับ 29.8% ในขณะที่ *P. guilliermondii* BCC 5389 ร่วมกับ *B. subtilis* ABS-S14 สามารถควบคุมโรคผลเน่าได้ 100% การศึกษาคุณภาพบนผลส้มเมื่อเก็บส้มที่อุณหภูมิ 25 องศาเซลเซียส นาน 7 วัน พบว่า การ ใช้เชื้อยีสต์และแบคทีเรีย ร่วมกับสารละลายโซเดียมไบคาร์บอเนตไม่มีผลต่อคุณภาพบนผลส้มอย่างมีนัยสำคัญทางสถิติ การศึกษาการเหนี่ยวนำการแสดงออกของยีนที่เกี่ยวข้องกับการป้องกันโรคของผลส้ม ได้แก่ ไคติเนสและกลูคาเนส ทั้งในระดับการสร้างอาร์เอ็นเอและโปรตีนในเนื้อเยื่อผิวส้มที่ ใส่เชื้อจุลินทรีย์ป ฏิปักษ์แบบเดี่ยว หรือร่วมกัน และปลูกเชื้อโรคพืช หรือไม่ปลูกเชื้อโรคพืช ผลการแสดงออกในระดับของยีน โดย วิธี relative quantification `สำหรับ qPCR พบว่าทุกทรีทเมนต์สามารถเหนี่ยวนำการสร้างไคติเนสและกลูคาเนสในเนื้อเยื่อผิวส้มได้

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ABSTRACT

The application of antagonistic microorganisms as an alternative to the use of chemicals control of postharvest diseases in citrus fruit is becoming increasingly popular worldwide. Present study investigated the effectiveness of antagonists and sodium bicarbonate, either individual or in combination to control fruit rot in Mandarin cv. Shogun *in vitro* and *in vivo*. A yeast *Pichia guilliermondii* BCC 5389, and a bacterium *Bacillus subtilis* ABS-S14 and 1-2% (w/v) sodium bicarbonate solutions were antagonists to the growth of *Pencillium digitatum.* Also sodium bicarbonate at 0.5-2.0 % (w/v) had no effect on the growth of *B. subtilis* ABS-S14, but it retarded the growth of *P. guilliermondii* BCC 5389. At 10^6 to 10^8 cells/ml of *P. guilliermondii* BCC 5389 and its culture filtrate significantly affected the germination of spores and hyphal growth of *P. digitatum*. There was no antagonism between *P. guilliermondii* BCC5389 and *B. subtilis* ABS-S14 on agar plates. In an *in vivo* test, the highest level of the chemical control of fruit rot was achieved in the presence of 2% w/v sodium bicarbonate and the disease incidence was shown to be 29.8%. Treatment with a combination of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 showed a complete reduction of disease. After storage at 25^{o}C for 7 days, the combined antagonists with sodium bicarbonate showed no significant effects on the fruit quality parameters. The induction of the defense related gene expression chitinase (*CHI*) and *β*-1,3-glucanase (*GLU*) transcripts), as determined by the relative quantification for qPCR showed that the *CHI* and *GLU* transcripts and enzyme activities were induced after treatment of infected and non infected flavedo tissues of citrus by individual antagonists or in combinations.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	=	ascorbic acid
AGt	=	abnormal germ tube
AH	=	abnormal hyphae
α	=	alpha
AP	=	acetone powder
β	=	beta
bp	=	base pair
°C	=	degree Celsius
DEPC	=	diethylpyrocarbonate
DNS	=	dinitrosalicylic acid
EC	=	enzyme commission
EDTA	=	ethylenediaminetetraacetic acid
EST	=	expressed sequence tags
A_{550}	=	light absorbance at 550 nanometer
CA	=	controlled atmosphere
cDNA	=	complementary deoxyribonucleic acid
CFU	=	colony forming unit
$CHCl_3$	=	chloroform
CHI	=	chitinase gene
cm	=	centimeter
CM-chitosan-RBV	=	carboxymethyl chitosan remazol brilliant violet
CN	=	copy number
C_T	=	threshold cycle
CV	=	coefficient of variation
d	=	day
DMRT	=	Duncan's new multiple range test
DNA	=	deoxyribonucleic acid
DW	=	distilled water
EF-1α	=	elongation factor-1-alpha
ET	=	ethylene

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

g	=	gram
GA_3	=	gibberellic acid
GlcNAc	=	N-acetylglucosamine
GLPs	=	germin-like proteins
GLU	=	glucanase
GRAS	=	generally recognized as safe
Gt	=	germ tube
h	=	hour
H	=	hyphae
HCl	=	hydrochloric acid
HR	=	hypersensitive response
IAA	=	Isoamyl alcohol
ISR	=	induced systemic resistance
JA	=	jasmonic acid
kDa	=	kilodalton
LB	=	Luria-Bertani broth
LSD	=	least significant difference
M	=	molar
µg	=	microgram
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
MCP	=	methylcyclopropene
MeJA	=	methyl jasmonate
meq.wt	=	milliequivalent weight
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	milimolar

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

mm	=	millimeter
mRNA	=	messenger ribonucleic acid
N	=	normal
N	=	newton
NA	=	nutrient agar
NB	=	nutrient broth
NaCl	=	sodium chloride
NYDA	=	nutrient yeast dextrose agar
NYDB	=	nutrient yeast dextrose broth
nmol	=	nanomol
p	=	propability
PAL	=	phenylalanin ammonia-lyase
PCR	=	polymerase chain reaction
PDA	=	potato dextrose agar
PDB	=	potato dextrose broth
%	=	percentage
pH	=	potential of hydrogen ion
*PR*lgf	=	*PR*-like gene families
PRs	=	pathogenesis –related proteins
qPCR	=	quantitative-real time polymerase chain reaction
RNA	=	ribonucleic acid
rpm	=	revolution per minute
RT-PCR	=	reverse transcriptase polymerase chain reaction
Σ	=	summation
SA	=	salicylic acid
SAR	=	systemic acquired resistance
SBC	=	sodium bicarbonate
SD	=	standard deviation
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	=	second

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

Sp	=	spore
T_m	=	melting temperature
TA	=	titratable acidity
Taq	=	*Thermus aquaticus*
TSS	=	total soluble solid
UV	=	ultraviolet
w/v	=	weight/volume

Chapter 1

INTRODUCTION

Postharvest diseases cause considerable losses to harvested fruits and vegetables occur during transportation and storage (Sharma *et al*., 2009). Green mould rot caused by *Penicillium digitatum* is one the most economically important postharvest disease of citrus, wherever it is grown and causes serious losses annually (Eckert and Brown, 1986). *Citrus reticulata* or mandarin orange cv. Sai NumPhung or Shogun, one of the most popular citrus cultivar in Thailand is susceptible to postharvest fungus caused by *P. digitatum* (Inkha and Boonyakiat, 2010). Synthetic fungicides have become the primary means to control postharvest diseases (Eckert, 1990). As a consequence of intensive usage of these synthetic compounds the developments of fungicide-resistant strains of postharvest pathogens in the packing houses have been reported (Kinay *et al*., 2007; Zhang *et al*., 2009a). However, public concerns about environmental contamination and human health risks associated with fungicide residues led to regulatory reviews and potential restrictions of traditional citrus export marketing. Biological control becomes a choice to use for controlling postharvest disease (Janisiewicz and Korsten, 2002). The eco-friendly antagonistic microorganisms such as *B. subtilis* (Obagwu and Korsten, 2003; Leelasuphakul *et al*., 2008) and *P. guilliermondii* (Pacheco *et al*., 2008; Sangwanich *et al*., 2013) have been used to control green mold disease in citrus because of their effectiveness and safe. The advantages of these antagonistic microorganisms are rapid multiplication on fruit wounds that can compete with pathogen (Janisiewicz and Korsten, 2002; Zhao *et al*., 2010). The mechanism of this biological control is based on biocontrol agent-pathogen interactions, such as competition for space and nutrients, mycoparasitism, antibiosis, induction of plant defenses (Saravanakumar *et al*., 2009; Zhu *et al*., 2010), and production of volatile metabolites sometimes associated with enzyme activated reduction of pathogen. Several antagonistic microorganisms, especially antibiotic producing bacteria must be careful to delay the development of antibiotic resistance.

However, the success of biocontrol is the formulation that was developed for commercial application (Jamalizadeh *et al*., 2011). Some of antagonists were commercially produced such as BioSave, Aspire for control postharvest rots of citrus fruit, but there are limited through manipulation of the environment (Janisiewicz and Korsten, 2002). However, several reports used them in combination with food additives such as sodium bicarbonate (SBC, baking soda, $NaHCO_3$) (Obagwu and Korsten, 2003; Smilanick *et al*., 1999), calcium chloride (Gholamnejad and Etebarian, 2009), and salicylic acid (Zhang *et al*., 2008; Yu *et al*., 2007). Moreover, physical method such as heat treatment (Zhao *et al*., 2010; Conway *et al*., 2005; Wszelaki and Mitcham, 2003), controlled atmosphere (Janisiewicz *et al*., 2008; Conway *et al*., 2007), development genetic into biocontrol mechanisms (Papon *et al*., 2013), manipulate formulation, and integrated control with other alternative method (Janisiewicz and Korsten, 2002) have been tried for promoting efficacy to control diseases. Interestingly, sodium bicarbonate treatment still remain use commonly today because of its effectiveness and it is one of the inexpensive food additives allowed with no restrictions for many applications including organic agriculture (Palou *et al*., 2008). Moreover, sodium bicarbonate could eradicate as the primary defense against the pathogen infections, while antagonists could reinforce the pathogen infection on the fruit later on. In general, the antagonist microorganisms beneficially influence the plant by protecting pathogen infection depend upon three main mechanisms: competition for ecological substrate, production of inhibitory allelochemicals, and induction of systemic resistance in host plants. None of these mechanisms are necessarily mutually exclusive, and frequently several modes of action are exhibited by a single biocontrol agent. The simultaneous implementation of several active ingredients in one commercial product is therefore, a way to guarantee the global efficacy under varying conditions. Likewise, a microbial strain can be used together with other strains, with natural extracts or other non-chemically transformed products or with chemical pesticides (Shanmugam and Kanoujia, 2011; Liu *et al*., 2010; Akila *et al*., 2011). These combinations are generally more effective and reliable. For example, combinations of strains can be selected to broaden pathogen spectrum by blending strains with distinct action mechanisms, or to enhance reliability by mixing isolates with different ecological competences (Ramamoorthy *et al*., 2001). Moreover,

the combination of strains can induce synergic effects for biocontrol improving. Nowadays, biopesticides thus play an important role and are legally accepted for use in integrated pest management and organic agriculture such as *B. subtilis* recognized non-pathogenic and well-studied organism which the US Food and Drug Administration (USFDA) has granted the generally regarded as safe (GRAS) status (Cawoy *et al*., 2011).

The aims of this study were to evaluate the use of antagonistic microbes as a stand-alone treatment and as a combination of antagonistic microbes with sodium bicarbonate for the control of green mould. Moreover, the mechanism of induction of plant defense responses via associating hydrolytic enzymes (chitinase, β-1,3-glucanase) was explored through the accumulation of enzyme activities and gene transcripts in flavedo tissues of citrus after treatment with antagonists and pathogen challenge. Their effect on the fruit quality after treatment was also considered.

Review of literatures

1.1 Postharvest practice of citrus

1.1.1 Quality of mandarin orange

Citrus fruits have non-climacteric-respiration that declines slowly throughout the later stages of fruit development and ethylene production of the mature fruit is extremely low. Ripening of citrus fruit is quite different from that of most other fruits. Citrus fruits approaching maturation does not contain starch and must, therefore, achieve internal maturity on the tree, prior to harvest. During maturation, structural and physiological differences occur in the peel and pulp. The pulp is characterized by a gradual change in juice content and in some of its constituents. There is a decline in titratable acidity (TA) brought about by decomposition of citric acid, which is the principal organic acid of citrus juice. On the other hand, there is an increase in sugars, usually expressed as total soluble solids (TSS) with acidity declining. Oranges and mandarins are stored at lower temperatures. A controlled atmosphere is not used with citrus fruits (Spiesel-Roy and Goldschmidt, 1996).

1.1.2 Packinghouse operations

Proper design and operation of the packinghouse line is essential for the maintenance of fruit health. Citrus fruit is comparatively resistant to bruising because of its spongy albedo, which absorbs much of the energy of impact. However, the fruit surface is very prone to scrapes, cuts, and punctures from sharp or abrasive objects. Mechanical damage most commonly occurs when fruit is transferred between pieces of equipment or at corners of the packing line. Properly designed packinghouse lines with limited turns will minimize handling damage to the fruit.

Packing and handling operations in the flow of citrus fruit through a packinghouse are listed in Figure 1.1. Procedures may vary somewhat depending upon the type of fruit and the geographical location. If fungicide treatment and packing are delayed more than 24 h after harvesting (for example, because of

degreening), the fruit should be treated with a fungicide drench or dip to protect against decay. Fruit may be dumped on a roller or belt conveyors or into a dip tank with chlorinated water or fungicide.

After the fruit is dumped, it receives preliminary grading and sizing. A fungicide is often applied during washing. The fruit is rinsed, and excess water is removed before major grading. In addition, fruit unacceptable for the fresh market is then removed for processing into juice and by-products. The remaining fruit destined for packing may receive a color-add treatment before fungicide application. The fungicide maybe applied separately, before waxing, or combined with wax. If the fungicide is applied separately, the fruit may be dried again before the wax treatment. Fungicide and wax-treated fruit is often subjected to a final grading, and some may be removed before packing. Once packed, the fruit is transported to market or held in storage before marketing (Timmer and Ducan, 1999).

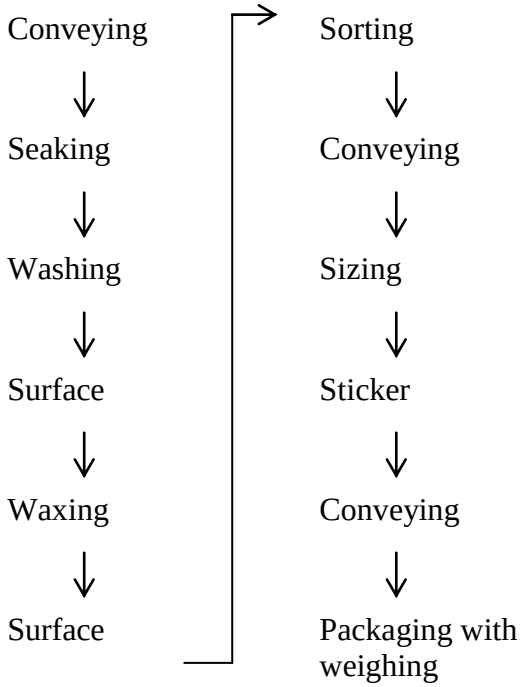

Figure 1.1 Flow chart of operation of Thanathohn packing house

(Jarimopas *et al*., 2005)

1.2 Green mold rot

Green mold disease caused by *P. digitatum* is the major disease of citrus fruit after harvesting (Ballester *et al*., 2006). In many countries, it is the common and serious postharvest disease of citrus. Spores of the causal fungus are produced on the surface of infected fruit and present in the field, packing, storage room, transit containers, and market place (Whiteside *et al*., 1988).

The fungus *P. digitatum* is inform-class Hyphomycetes or imperfect fungi form-order Moniliales, form-family Moniliaceae, and form-genus *Penicillium* (Pitt, 1979). It produces brush-like heads. The conidiophore branches at the tip. At the end of each branchlet is a cluster of spore-producing form phialides. A chain of spores is formed from the tip of each phialide. Size of pores or conidia is about 4-7 X 6-8 μm but may vary in size and shape, even within the same chain. (Whiteside *et al*. 1988). Conidia often appear hyaline or brightly colored in mass, mostly globose or ovoid, in dry basipetal chains (Barnett and Hunter, 1972). Colonies on artificial media are similar in appearance to the mold that develops on infected fruit (Whiteside *et al*., 1988). *P. digitatum* can grow in media with low water potential and temperature below 37 ^{o}C and lower than 5^{o}C (Peterson, 1992) with the optimum temperatures is near 24 ^{o}C and much more slowly above 30 ^{o}C and below 10 ^{o}C. The rotting process is almost completely inhibited at 1^{o}C (Whiteside *et al*., 1988). Interestingly, *P. digitatum* and *P. italicum* do not germinate on a medium lacking a carbon source such as water agar or on the surfaces of intact fruit (Kavanagh and Wood, 1971; Eckert *et al*., 1984), but the spore germination over 80% were found if they were laid over a puncture wound in the peel. Moreover, germination of both pathogens was shown to be stimulated by citrus peel volatile and non-volatile components, as well as growth stimulant was found in ethanol extracts of grapefruit peel (Stange *et al*., 2002). Peel oils from several species of citrus and volatiles emitted from injured oranges were reported to be responsible for stimulating germination of *P. digitatum* conidia (French *et al*., 1978; Eckert *et al*., 1984). Often *Penicillium* species tested, only *P. digitatum* and *P. italicum* spores displayed germination on water agar when exposed to volatiles from injured oranges (Eckert *et al*., 1992).

Initial symptoms of green mold are similar to those of sour rot and blue mold. In the early pinhole stage (Whiteside *et al*., 1988), the fungus colonized rapidly and massively the spongy parenchymatous mesocarp cells before penetrating the more compact cells of the exocarp. Fungus produced cell wall degrading enzymes that diffused at a distance from the areas of penetration, leading eventually to disruption of primary walls and tissue maceration (Barmore and Brown, 1981). The decay appears as a soft, watery, slightly discolored spot, 6-12 mm in diameter. The spot enlarges to 2-4 cm in diameter within 24-36 h at 24^{o}C, and the rot soon involves the juice vesicles. White mycelium appears on the rind surface, and after it reaches a diameter of approximately 2.5 cm, olive green spores are produced. The sporulating area is surrounded by a broad zone of white mycelium and an outer zone of softened rind. The entire fruit is soon encompassed by a mass of olive green spores, which are easily dispersed if the fruit is handled, shaken, or exposed to air currents. If the relative humidity is low, the whole fruit collapses into a soft, decomposing mass. Spores detached from diseased fruit during the opening of packed cartons affect the value of the remaining healthy fruit by settling on them and causing soil age (Whiteside *et al*., 1988).

1.3 Biological control of postharvest diseases of citrus fruits

Postharvest losses of fruits and vegetables including citrus caused by fungal and bacterial infection have been estimated at 25% in the world. For citrus fruits, postharvest decay occurred in the stages like flowering, fruit maturity, during harvesting, subsequent handling and storage (Eckert and Eaks, 1989 reviewed in El Ghaouth *et al.*, 2002). Postharvest citrus infections occur through surface wounds inflicted during harvest until storage. The opportunity pathogens such as *P. digitatum, P. italicum, Geotrichum citri-aurantii,* and *Trichoderma viride* are mainly infected in citrus. However, majority of citrus fruit losses in the world are green mold caused by *P. digitatum* and blue mold caused by *P. italicum.* Synthetic fungicides such as imazalil and thiabendazole are the primary means of controlling postharvest diseases of citrus fruits (El Ghaouth *et al.*, 2002).

Biological control is the most promising alternative non-chemical method using microbial antagonists has applied to postharvest fruit instead of

chemical fungicides (Janisiewicz and Korsten, 2002). Some formulations have been developed and commercialized, such as the registered biological control formulations SHEMER WDG (*Metschnikowia fructicola*; Agro Green, Israel), Bio-Save 10 LP (*Pseudomonas syringae* Strain ESC-10; JET Havest Solutions, Orlando, FL,USA) (Palou *et al*., 2002), Aspire (*C. oleophila* Montrocher I-182), and Yield Plus (*Cryptococcus albidus* (Saito) C.E. Skinner) (Droby *et al*., 2001). Moreover, many reports have investigated some natural antagonists to control citrus fruits. Previous studies revealed that *P. guilliermondii* isolate LCBG-03, biocontrol epiphytic-yeast controlled growth of *P. digitatum* (Larralde-Corona, 2011) or strain Z1 reduced *P. italicum* causes blue mould in citrus (Lahlali *et al*., 2011). Isolate R13 of *P. guilliermondii* has also been found controlling Thai rambutan caused by *Colletotrichum capsici* (Nantawan *et al*., 2010), and anthracnose in chilli fruit (Chanchaichaovivat *et al*., 2008). The whole genome sequencing of the *C. guillermondii* ATCC 6260 reference strain has been obtained, this opened a window to investigate the molecular and biochemical mechanisms dealing with its biotechnological interest, biocontrol potential, pathobiology and antifungal resistance. Recently developed databases will provide precious help in the study of *C. guilliermondii* biosynthetic pathways leading to valuable compounds and for metabolic engineering. Finally, these tools will facilitate the identification of the multiple factors that contribute to its pathogenic potential (Papon *et al*., 2013). *Metschnikowia pulcherrima* was found to suppress *P. digitatum* and *P. italicum* on citrus fruit (Kinay and Yildiz, 2008). *T. viride*, isolated from Spanish citrus packinghouses, showed antagonistic activity against *P. digitatum in vitro* tests. Navelina oranges treated with *T. viride* 48 h or 72 h before inoculation did not produce lesions after 5 days (Borrás and Aguilar, 1990).

B. subtilis isolates F1, L2, and L2-5, isolated from citrus fruit surfaces were evaluated for control of citrus green molds caused by *P. digitatum* artificially inoculated onto ‘Valencia’ and ‘Shamouti’ orange cultivars. Applied alone, all isolates significantly reduced the incidence of green molds as compared with water control, but they were not as effective as the fungicidal (quazatine plus imazalil) treatment. When applied sodium bicarbonate (SB) or *B. subtilis* isolates F1 following

hot water treatment it is as effective as the fungicide treatment, which gave 100% control of both green and blue molds on both cultivars (Obagwu and Korsten, 2003). Moreover, *B. subtilis* SM21 was used to control peach fruit caused by *Rhizopus stolonifer* by reduced lesion diameter and disease incidence by 37.2% and 26.7% compared with the control (Wang *et al*., 2013). Several commercial products based on various *Bacillus* species such as *B. subtilis, B. amyloliquefaciens, B. licheniformis* and *B. pumilus* have been marketed as biofungicides as shown in Table 1.1.

The preharvest application of antagonistic bacterium *Pantoea agglomerans* CPA-2 for controlling postharvest diseases on citrus provided an effective control for orange fruit against natural postharvest pathogen infections and artificial infections of *P. digitatum* with values of decay reduction higher than 50% (Canamas *et al*., 2008). *Aureobasidium pullulans*, a yeast-like fungus, is a potential biocontrol agent for plant and was evaluated for its ability to control postharvest grey mold of apples, and two strains of them (SL250 and SL236), plus a proven antagonist (isolate L47), were able to control *P. digitatum* on grapefruit (Schena, *et al*., 1999). An epiphytic yeast population of grapefruit was identified as *C. guilliermondii, C. oleophila, C. sake,* and *Debaryomyces hansenii,* while *C. guilliermondii* was the most predominant species. They were able to grow under high osmotic conditions and a wide range of temperatures. Epiphytic yeasts exhibited the highest biocontrol activity against *P. digitatum* on grapefruit (Droby *et al*., 1999). Two yeasts, *D. hansenii* and *A. pullulans*, and two bacteria, *P. cepacia* and *P. syringae* were the most effective antagonists from over 100 isolates tested against *P. digitatum* and *P. italicum* rots on citrus fruit. Overall, *P. cepacia* provided the best protection against both of these rots. *P. cepacia* produced antibiotic zones against the two Penicillia rot organisms in culture, whereas *P. syringae, D. hansenii* and *A. pullulans* did not. All four antagonists show promise as biocontrol agents (Wilson and Chalutz, 1989).

Table 1.1 Some commercial formulations of *Bacillus*-based biofungicides (Perez-Garcia *et al*., 2011).

Trade name	*Bacillus* species	Target pathogen/disease	Crop	Manufacturer
Avogreen	*B. subtilis*	*Cercopora* spot	Avocado	Stimuplant, South Africa
Ballad Plus	*B. pumilus*	Rust, powdery mildew, cercospora, brown spot	Soybean	AgraQuest, USA
Biobest	*B. subtilis*	Sheath blight, blast, brown spot	Rice	Appliedchem, Thailand
Companion	*B. subtilis*	*Rhizoctonia, Pythium, Fusarium, Phytophthora, Sclerotinia*	Greenhouse, nursery and ornamental crops	Growth Products, USA
EcoGuard	*B. licheniformis*	Dollar spot, anthracnose	Turf	Novozymes, Denmark
HiStick	*B. subtilis*	*Fusarium, Rhizoctonia, Aspergillus,*	Soybean and peanuts	Becker Underwood, USA
Kodiak	*B. subtilis*	*Rhizoctonia,Fusarium, Pythium, Aspergillus*	Cotton, legumes, soybean and vegetable crops	Bayer CropScience, USA
Laminar	*B. subtilis*	*Alternaria, Botryodiplodia, Colletotrichum, Corticium, Fusarium, Phytophthora*	Vegetables, rice, and field crops	Appliedchem, Thailand

Trade name	***Bacillus* species**	**Target pathogen/disease**	**Crop**	**Manufacturer**
Rhapsody	*B. subtilis*	*Rhizoctonia, Fusarium, Pythium, Phytophthora*	Turf and ornamental, vegetable and fruit greenhouse crops	AgraQuest, USA
Serenade	*B. subtilis*	Rusts, powdery mildews, *Botrytis, Sclerotinia*	Vegetable, wine, nut and fruit crops	AgraQuest, USA
Sonata	*B. pumilus*	Rusts, powdery and downy mildews	Vegetable and fruit crops	AgraQuest, USA
Subtilex	*B. subtilis*	*Rhizoctonia, Fusarium, Aspergillus*	Field, ornamental and vegetable crops	Becker Underwood, USA
Taegro	*B. amyloliquefaciens*	*Rhizoctonia, Fusarium*	Tree seedlings, ornamentals and shrubs	Novozymes, Denmark

1.4 Combination of antagonists with additives

Biological control was only effective when high concentrations of antagonistic yeasts were applied. High concentrations of antagonists would increase production costs and make the control less economical. Moreover, biological control as an alternative to chemical control is not as broad-spectrum and is usually not as effective as chemical fungicides. So the efficacy of antagonistic yeasts in controlling of postharvest disease must be enhanced. Combining organic and inorganic additives with antagonistic yeasts is a very effective method for enhancing the biocontrol efficacy of yeasts (Zhang *et al*., 2008b). The potential of using *Rhodotorula glutinis* in combination with salicylic acid (SA) for the control of postharvest gray mold decay of peach fruit has been reported. The percentage of infection incidence in treated fruit was 16.67%, compared with 46.67% in the water-treated control fruit. SA had no significant effect on quality parameters after 7 days at 20 ^{o}C (Zhang *et al*., 2008a). Moreover, the combined treatment of pear fruit with *C. laurentii* plus SA at 100 μg/ml resulted in a remarkably improved control of *P. expansum* and *B. cinerea* infections. Although, SA at 100 μg/ml neither affected the population growth of *C. laurentii* nor directly inhibited the blue mold, it induced the fruit resistance to the blue and gray mold rots when the time interval between SA treatment and pathogens inoculation was more than 48 h, being associated with a rapid and strong activation of the peroxidase activity in pear fruit (Yu *et al*., 2007).

The combined treatment of *P. membranifaciens* with 2% $CaCl_2$ resulted in a remarkably improved control of the disease in comparison with the treatment of *P. membranifaciens* or $CaCl_2$ alone for control of anthracnose rot caused by *C. acutatum* in postharvest loquat fruit and induced higher activities of two defense-related enzymes chitinase and β-1,3-glucanase than did applying the yeast or $CaCl_2$ alone. *In vitro* experiment showed that the addition of 2% $CaCl_2$ in the suspensions of *P. membranifaciens* significantly inhibited spore germination and germ tube elongation of *C. acutatum* than the yeast or $CaCl_2$ alone. However, adding $CaCl_2$ did

not significantly influence the population of *P. membranifaciens* in NYDB medium or fruit wounds (Cao *et al*., 2008).

Sodium bicarbonate alone or in combination with the antagonists had little effect on apples. The antagonist alone reduced decay caused by *P. expansum* on 'Golden Delicious' apples but tended to be more effective when combined with sodium bicarbonate but completely eliminated decay caused by *C. acutatum* (Conway *et al*., 2005). A mixture of two yeast antagonists, *M. pulcherrima* and *C. laurentii* were used in combination with sodium bicarbonate (SBC) in a pilot test in which treated fruit were stored under a commercially controlled atmosphere (CA) storage conditions, the treatments with the antagonist alone or in combination with SBC were equally effective and reduced blue mold incidence by 84–97% in 2005–2006, and 73–82% in 2006–2007. SBC alone significantly reduced blue mold incidence compared to the non-treated control, but was less effective than the antagonist alone or in combination with SBC. This pilot test showed that the combination of these two antagonists and SBC can be an effective decay control method under commercial CA conditions (Janisiewicz *et al*., 2008). Moreover, application antagonistic bacterium *P. agglomerans* CPA-2 combined with sodium bicarbonate reduced green mold rot caused by *P. digitatum* in navel oranges (Zamani *et al*., 2009).

Gibberellic acid (GA_3) which is naturally associated with fruit ripening and senescence, has no influence on *P. expansum* or *C. laurentii in vivo* and *in vitro*. The combination of *C. laurentii* and GA_3 at 2000 μg /ml was able to control blue mold rot in pear. While *C. laurentii* was applied alone on wound, it was associated with the rapid increase in peroxidase and polyphenol oxidase activities and inhibited lipoxygenase activity and lipid peroxidation. In addition, no phytotoxic injury was observed in fruit tissue treated with GA_3 at 2000 μg /ml (Yu *et al*., 2006).

Application of *C. laurentii*at $1x10^8$ cells/ml combined with Methyl Jasmonate (MeJA) at 100 μmole/l reduced green mold rot caused by *P. digitatum* and this combination was induced mRNA expression level of PR5. Moreover, it can induce natural resistance and stimulate the proliferation of antagonistic yeast on the fruit surface (Guo *et al*., 2014).

The combination of *Candida utilis* TISTR 5001 and *Eugenia caryophyllata* crude extract was the best to reduce green mold of citrus fruit cv. Sai Nam Phung in disease incidence and disease severity. Moreover, this combination had no effect to fruit quality (Sukorini *et al*., 2013).

1.5 Plant defense mechanisms

Plant systemic expression of a broad spectrum and long period disease resistance that inducing by elicitors (Heil and Bostock, 2002). The various structural and chemical components of plant defense mechanisms include the hypersensitive response (HR), synthesis of phytoalexins, lignification of plant cell walls, synthesis of lytic enzymes were induced when applied with biocontrol agent in plant that was challenged by a pathogen (Zhu *et al*., 2010 reviewed in Jamalizadeh *et al*., 2011). A rapid defense response that called the hypersensitive response (HR) includes the programmed cell death of plant cells that are in contact with the pathogen. This HR triggers a system called systemic acquired resistance (SAR) includes the accumulation of the signal molecule salicylic acid (SA) throughout the plant and then expression of a set of defense genes. The expression SAR of the plant to pathogen resistant depends on gene for gene resistance (Glazebrook, 2001). It is a mechanism that confers long-lasting protection against a broad spectrum of microorganisms (Durrant and Dong, 2004). The SAR group includes several pathogenesis-related (PR) proteins such as chitinase (PR3) and glucanase (PR2) (Table 1.2) which are either antifungal or antibacterial that requires SA plays a key role (Ryals *et al*., 1996) that synthesized via the shikimate phenylpropanoid pathway. Meanwhile, the pathway of ISR requires for jasmonic acid (JA) and ethylene (ET) signaling components (Durrant and Dong, 2004). JA induce the expression of genes encoding defense related proteins such as thionins and proteinase inhibitors whereas ET activates several members of PR gene super family (Choudhary and Johri, 2009).

The antagonistic yeast, *P. guilliermondii* stimulated the production of ethylene in loquat fruit able to activate the phenylalanine ammonia-lyase (PAL) (Liu *et al*., 2010 reviewed in Jamalizadeh *et al*., 2011) which is an enzyme involved in the

synthesis of phenols, phytoalexins and lignins (Jamalizadeh *et al*., 2011). Moreover, this yeast which is effective in controlling a wide variety of postharvest diseases in citrus fruits, apples and peaches, has been shown to induce enhanced levels of PAL in citrus fruit peel. In similar, an isolate of the yeast *C. famata* is effective against *P. digitatum* by inducing the formation of the phytoalexin; scoparone and scopoletin in the citrus fruit. Moreover, the antagonistic yeast-like fungus, *A. pullulans* was capable of inducing the activities of β-1,3-glucanase, chitinase and peroxidase in apples inoculated with *B. cinerea* and *P. expansum* (Barkai-Golan, 2001). Thioredoxins were ubiquitous small proteins with a redox-active disulfide bridge. They participate in redox regulation of selected target proteins, such as transcription factors, receptors and various metabolic and regulatory enzymes. Analysis of a grapefruit for a 135 bp polymerase chain reaction (PCR) fragment showed that its expression was enhanced in the fruit peel tissue following the application of *C. oleophila* yeast cells, an elicitation treatment that induces resistance towards the green mold pathogen *P. digitatum* (Hasdai *et al*., 2005). The umbelliferone accumulated in the albedo of immature grapefruit four days after inoculation with *P. digitatum.* It inhibited growth of several other pathogenic fungi *in vitro.* Moreover, umbelliferone plays a role in the defense mechanisms of immature grapefruit against *P. digitatum* (Afek *et al*., 1999). Oranges (*C. sinensis* cv. 'Valencia') treated with inducing agents of elicitors; chitosan (a preparation of ground crab shells), Margosan-O (an oil-based plant derived product from neem seed) Aspire (a water dispersible granule containing an antagonistic yeast) followed by inoculation with *P. digitatum* and *P. digitatum* infection alone increased total soluble proteins in the flavedo (the tissue that forms the outer colored rind) two folds relative to the untreated control. A temporal differential induction of chitinase, β-1,3-glucanase, and peroxidase was corroborated by immuno detection. Lysozyme and a polygalacturonase inhibiting protein were detected at low activity levels. However, the defensive proteins appeared to be constitutive and slightly induced, but did not involve the *de novo* synthesis of novel proteins (Fajardo *et al*., 1998).

Elicitation of ISR by plant-associated bacteria *Pseudomonas* spp. *Bacillus* spp. as elicitors of ISR, such as *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* elicit significant reductions of

various diseases on a diversity of hosts (Kloepper *et al*., 2004). The antagonistic bacteria *Acinetobacter lwoffii* PTA-113 and *P. fluorescens* PTA-CT2 in leaves, and with *A. Iwoffii* PTA-113 and *P. agglomerans* PTA-AF1 in berries, revealed a systemic resistance to *B. cinerea*. This resistance was associated with a stimulation of some plant defense responses such as chitinase and β-1,3-glucanase activities in both leaves and berries (Magnin-Robert *et al*., 2007).

1.6 Pathogenesis-related proteins

The pathogenesis-related proteins (PRs) are defined as proteins that were induced only in pathological or related situations, not only accumulate locally in the infected tissue, but are also induced systemically. This results in the development of SAR against further infection of the plant by fungi, bacteria and viruses. Induction of PRs has been found in many plant species belongs to various families to indicate that there is probably a general role for these proteins in adaptation to biotic stress conditions (Van Loon and Van Strien, 1999). The PRs gene families of citrus have been classified into seventeen families (PR-1 to PR-17) evaluated using the Brazil citrus genome EST CitEST/database (Table 1.2). Changes in expression profiles of clusters for each of the 17 citrus *PR*-like gene families (*PR*lgf) expressed in organs infected by pathogens or drought-stressed citrus species (Campos *et al*., 2007). Germins and germin-like proteins (GLPs) were classified as PR-15 and PR-16. PR-16 was isolated from hot pepper during its resistance response to bacterial and viral infections (Park *et al*., 2004). Moreover, chitinase and β-1,3-glucanase have synergistic effect. Plant chitinases alone usually affect only the hyphal tip and are unable to effectively degrade harder chitin structures of fungi, but whenever these two enzymes are combined, they enhanced levels of protection from pathogen (Ebrahim *et al*., 2011).

Table 1.2 *PR* gene sequences used as query for searching the homologous *PR* genes within the citrus genome EST data bank-CitEST and distribution of the number of citrus *PR*-like ESTs within the 17 *PR* gene families (Campos *et al*., 2007).

PR family	Query sequences	Accession number	Organism	Number of ESTs	Clusters	
					Contigs	Singlets
PR-1	PR-1a	gi|722274	*Brassica napus*	5	2	1
PR-2	β-1,3-glucanase	gi|8980815	*Castanea sativa*	264	33	38
PR-3	Chitinase class I, II, IV, V, VI, VII and endochitinase	gi|23496447	*Citrus jambhiri*	444	24	13
PR-4	Chitinase Hevein-like	gi|19962	*Nicotiana tabacum*	5	2	1
PR-5	Thaumatin-like	gi|4586372	*Nicotiana tabacum*	40	6	6
PR-6	Proteinase inhibitor	gi|170484	*Lycopersicone sculentum*	107	18	6
PR-7	Aspartic proteinase	emb|CAC86003.1	*Theobroma cacao*	178	15	5
PR-8	Chitinase class III	gi|167515	*Cucumis sativus*	12	1	3
PR-9	Lignin-forming peroxidase	gi|170316	*Nicotiana tabacum*	562	22	16
PR-10	Ribonuclease-like	gi|15811629	*Gossypium arboreum*	63	4	4

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PR family	Query sequences	Accession number	Organism	Number of ESTs	Clusters	
					Contigs	Singlets
PR-11	Chitinase, class V	gi|899342	*Nicotiana tabacum*	20	4	7
PR-12	Defensin	gi|11386628	*Raphanus sativus*	11	1	2
PR-13	Thionin	gi|1181531	*Arabidopsis thaliana*	2	1	0
PR-14	Lipid transfer protein	gi|1045201	*Hordeum vulgare*	347	12	5
PR-15	Oxalate oxidase	gi|2266668	*Hordeum vulgare*	503	14	5
PR-16	Oxalate oxidase-like or germin-like	gi|1070358	*Hordeum vulgare*	539	12	6
PR-17	NtPRp27	gi|5360263	*Nicotiana tabacum*	1	-	1

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1.7 Chitinase

Chitinases (EC 3.2.1.14) hydrolyse chitin, a linear homopolymer of β-1, 4 linked N-acetylglucosamine (GlcNAc), which is one of the major cell wall components of most fungi (Wessels and Sietsma, 1981). Most of chitinase have molecular mass in the range of 15 kDa and 43 kDa (Borad and Sriram, 2008). There are well characterized antifungal proteins in the plant defense responses against fungal pathogens (Bol *et al*. 1990). In citrus, several basic and acidic isozymes with chitinase and chitosanase enzyme activity have been purified from Valencia callus (Osswald *et al*. 1994) and they were expressed in the fruit flavedo (the outer pigmented layer of the peel) (McCollum *et al*. 1997). The accumulation of chitinase proteins in grape fruit peel were induced by various treatments such as UV irradiation, hot water, and *C. oleophila* cells to inhibit *P. digitatum* (Porat *et al*. 1999). Plant chitinases have often been grouped into four classes based on primary structure, class I chitinases are enzymes with an N-terminal cysteine-rich and proline/glycine-rich hinge domains; class II chitinases lack the N-terminal domain but have a high sequence identity with the main structure of class I chitinases; class III chitinases have sequence similarity to bacterial and fungal chitinases but not to class I or class II chitinases; class IV chitinases have sequence similarity to class I, but the size of the genes is smaller than of class I because of deletions. Many plant class I chitinases are basic isoforms and class II chitinases are acidic isoforms (Collinge *et al*. 2002). One class II acidic chitinase and seven class III basic chitinases were purified from sweet orange (*Citrus sinensis* L. Osb.cv. Valencia) cell culture and a cDNA that encodes a class II acidic chitinase were identified from sweet orange (Collinge *et al*. 2002; Gomi *et al*. 2002).

cDNA expression of chitinase designated as *chi1,* composes of 875 bp in length, with an open reading frame of 693 bp. The *chi1* gene encodes a predicted polypeptide of 231 amino acids with a predicted molecular mass of 25.1 kDa and a pI of 9. 15. The CHI1 protein shares 60, 58, and 56% identities with the basic chitinase proteins of rice, grape and maize, respectively. Southern blot analysis indicated that

chi1 is present as a low-copy gene. RNA gel blot hybridizations revealed that *chi1* gene expression was markedly induced by various treatments that induce fruit resistance against the green mold (Porat *et al*., 2001).

CHI1 transcription by quantitative RT-PCR was increased when inoculated flavedo tissues of citrus with *P. syringae* pv. *syringae* and *P. digitatum* or co-inoculation. Thus, CHI1 gene is the part of the molecular mechanisms involved in defense responses in citrus fruits (Scuderi *et al*., 2011).

1.8 β-1,3-glucanase

β-1,3-glucanase (β-1,3-glucan 3 –glucanhydrolase, EC 3.2.1.39) is lytic enzymes capable of inhibiting fungi development by degrading the pathogen cell wall, by catalyzing the hydrolysis of β-1,3-d-glucosidic linkages in β-1,3-glucans. That is one of the principal components of the cell wall of fungal pathogen (Alexopoulos, 1996). Molecular mass of β-1,3-glucanase is the range from 33 to 44kDa (Borad and Sriram, 2008). Moreover, it is important for diverse physiological processes, for instance pollen development, stress response, flowering and mobilization of store reserves and is critical in triggering seed germination (Vogeli-Lange *et al*., 1994). The purified enzyme of β-1,3-glucanase was isolated from *Simira glaziovii* secretion and its estimated molecular mass was 35 kDa, analyzed by SDS-PAGE and optimum pH was 5.2 (Vieira *et al*., 2006). Molecular mass of β-1,3-glucanase was 35 kDa, and acidic isoelectric point, pH optimum of 5.2-5.5 was found in pea tissue (Mauch *et al*., 1988). This enzyme with pH optimum of 5 and temperature optimum of 50 ^{o}C was detected in *Phaseolus vulgaris* L. cv. Red Kidney (Abeles and Forrence, 1969).

The combination of *C. laurentii* and UV-C induced the transcription of β-1,3-glucanase based on real-time PCR analysis and their mechanism increased the activity of this enzyme in tomato fruit (Zhang *et al*., 2013). β-1,3-glucanase and chitinase were induced when treated with *P. putida* MGP-1 in papaya fruit to control post-harvest blight caused by *P. nicotianae* (Shi *et al*., 2012). For *Pythium oligandrum*, antagonistic fungus against *B. cinerea* induced RNA transcript levels of β-1,3-glucanase to all treatments in grapevine leaves (Mohamed *et al*., 2007).

Moreover, *B. subtilis* SM21 induced transcription of defense related gene of β-1,3-glucanase and chitinase in peach fruit (Wang *et al*., 2013). *Trichoderma* spp. induced higher systemic expression of β-1,3-glucanase and chitinase in cucumber (Shoresh *et al*., 2005).

1.9 Fruit quality

The quality of the fruits and vegetables when treated with antagonistic microorganisms should be preserved during the process as freshness, firmness, color, flavor, weight loss, texture etc. The main concerns for fruit treated antagonists were nutritional value, consumer acceptance and safety (Warchalewski *et al*., 2011). Bautista-Banos *et al* (2003) showed that chitosan applications did not influence the content of total soluble solids, weight loss of papaya. Zhang *et al* (2009b) investigated the antagonistic yeast *R. glutinis* on apples and reported no significant differences in mass loss, firmness, total soluble solids (TSS), ascorbic acid (AA), and titratable acidity (TA) when the apple fruits were stored at 20 ^{o}C for 5 days or at 4^{o}C for 30 days followed by 20 ^{o}C for 4 days. Meanwhile, on pears, *R. glutinis* combined with hot water showed no effect to mass loss, fruits firmness, TSS, AA and TA when stored at 20 ^{o}C for 15 days or at 4 ^{o}C for 60 days followed by 20 ^{o}C for 15 days (Zhang *et al.,* 2008b). Moreover, Adding of *C. laurentii* in a novel edible bio-film reduced of mold growth and maintenance of strawberry commercial quality throughout storage (Fan *et al.,* 2009). Combined application of *B. amyloliquefaciens* PPCB004 and lemongrass oil protected peach from postharvest diseases and increased the fruit acceptance at market shelf conditions at 20 ^{o}C for 2 days after cold storage at 4 ^{o}C for 14 days (Arrebola *et al*., 2010). Moreover, *B. amyloliquefaciens* PPCB004 with 1-MCP pretreated papaya reduced anthracnose and phomopsis rot and maintenance of fruit quality (Usman *et al.,* 2011). Recently, Sukorini *et al* (2013) showed that there was no effect on fruit quality when bio-fungicides (*C. utilis* TISTR 5001) and plant extract from *Eugenia caryophyllata* were used on citrus fruit cv. Sai NumPhung.

OBJECTIVES

1) To screen antagonistic yeast strains to use as a mixture of antagonistic *B. subtilis* ABS-S14 against *P. digitatum in vitro* and *in vivo*.
2) To monitor the effects of the antagonists and sodium bicarbonate on disease reduction and quality of citrus fruits.
3) To determine the effects of pathogen infection and antagonist on induction of enzyme activities and mRNA transcripts of chitinase and β-1,3-glucanase of citrus flavedo tissue.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments and Equipments

Instruments	Model	Company
Autoclave	ES-315	Tomy
Analytical balance	AB204-S	Metter toledo
Bioharzard carbinet	BSB 3A	Gelaire
Dialysis tubing	D-0655	Sigma
Gel documentation system	Bio chemi system	UVP
Gel electrophoresis apparatus	Miniprotein	Bio-Rad
Haemacytometer		Boeco
Hand refractometer	ATAGO N-1	Atago
Heating block		VWR Scientific
Homogenizer		Kinematica
Hot air oven		Binder
Hot plate	KIKA	Labortechnik
Laminar flow hood	BSB 3A	Gelaire
Micropipet	P20	Gilson
	P200	Gilson
	P1000	Gilson

Instruments	Model	Company
Microscope	compound	Nikon
	stereo	Meiji
Orbital shaker		Gallenkamp
pH meter	713	Metrohm
Real-time PCR	Mx3005P	Agilent Technologies
Refrigerated Centrifuge	J2-21	Beckman
	J-30I	Beckman
	EBA 12R	Hettichzentrifugen
Rotary evaporator	R-210	Buchi
Safety cabinet		Augustin
Spectrophotometer	UV-Vis 8435	Hewlett-Packard
Texture analyser	TA-XT2i	Stable Microsystems
Thermal cycler	Mycycler	Bio-Rad
ThermoE cooling and heating box	CHB-A4-2415	Lio Lab
UV transilluminator	CA91786	BioDoc-It System
	ECX-15M	Vilber Lourmat
Vernier Caliper		
Vortex mixer	Vortex Genie-2	Scientific industries
Water bath		Memmert

2.1.2 Chemicals

Chemicals	Grade	Company
Absolute ethanol	AR	J.T. Baker
Acetic acid	AR	J.T. Baker
Acetone	AR	J.T. Baker
Acrylamide	AR	Fluka
Ammonium persulfate	AR	Merck
Ammonium sulfate	AR	Merck
Bis-acrylamide	AR	Sigma
β-mercaptoethanol	Molecular biology	Sigma
Bovine serum albumin (BSA)	Molecular biology	Sigma
Chitinase	AR	Sigma
Chitosan	food	
Chloroform ($CHCl_3$)	Molecular biology	Sigma
Chloroacetic acid	AR	Sigma
Coomassie brilliant blue G-250	AR	Fluka
Coomassie brilliant blue R-250	AR	Sigma
D-glucose	AR	Univar
Diethyl pyrocarbonate (DEPC)	Molecular biology	Sigma
Dinitrosalicylic acid	AR	Sigma
DNase I	Molecular biology	Biolabs
Ethylenediaminetetraacetic acid	AR	Sigma

Chemicals	Grade	Company
Ethidium bromide	Molecular biology	Bio-Rad
GoTaq qPCR master mix	Molecular biology	Promega
Hydrochloric acid	AR	J.T. Baker
SsoFast Evagreen Supermix	Molecular biology	Bio-Rad
Isoamyl alcohol (IAA)	Molecular biology	Sigma
Larminarin	AR	Sigma
Lithium chloride	AR	Sigma
6x loading dye	Molecular biology	Promega
Methanol	AR	Lab-scan
Phenol pH 4.3	Molecular biology	Amresco
Phenol/chloroform/IAA	Molecular biology	Molecular biology
Phenolphthalein	AR	Sigma
2-propanol	AR	Sigma
Remazol brilliant violet (RBV)	AR	Sigma
Sodium acetate	AR	Sigma
	Molecular biology	Sigma
Sodium bicarbonate	AR	Sigma
Sodium chloride	AR	Lab-Scan
Sodium dodecyl sulfate	AR	Sigma
Sodium hydroxide	AR	Sigma
Sodium phosphate	AR	Sigma
Sodium sulphate	AR	Sigma

Chemicals	Grade	Company
2,3,5-triphenyltetrazolium	AR	Sigma
Tris base	AR	Fisher Scientific

*** AR = Analytical Reagent grade**

2.1.3 Culture medium

Media	Grade	Company
Bacto Agar	Microbiology	Merck
Luria-Bertani broth	Microbiology	Merck
Nutrient agar	Microbiology	Merck
Nutrient broth	Microbiology	Merck
Potato dextrose agar	Microbiology	Difco
Potato dextrose broth	Microbiology	Merck
Yeast extract powder	Microbiology	Himedia

2.1.4 Fruit

Shogun mandarin oranges (*C. reticulata* Blanco cv. Shogun) were used in the efficacy experiments. Green fruits of uniform size and maturity were washed with tap water and sterilized with 2% sodium hypochlorite and air dried prior to wounding.

2.1.5 Fungal isolate

Two strains of *P. digitatum* have been isolated from diseased citrus fruits with typical green mold symptom. KU isolate was obtained from Sangchote's laboratory, Department of Plant Pathology of Kasetsart University and other strain of *P. digitatum* (PSU isolate) was obtained from Department of Biochemistry, Prince of Songkla University. Two strains of *P. digitatum* were used in the efficacy experiments. They were maintained as a spore culture on potato dextrose agar (PDA) with periodic transfers through citrus fruit to maintain pathogenicity (Leelasuphakul *et al.,* 2008). Before starting the experiment, fungus was transferred to sterilized citrus fruit for maintaining an aggressiveness of the pathogen.

The spore suspension was prepared from 7 d-old *P. digitatum* grew on PDA plate at 25 $^{\circ}$C. It was prepared by adding 10 ml of sterile distilled water with 0.01% Tween 80 to the surface of the cultures, and was scrapped from the fungal colony using a sterile spreader. Agar debris and mycelium in suspension were removed by filtration through three layers of Miracloth. The spore concentration was determined using a haemacytometer and the working concentration was adjusted to 10^4 spores/ml by mixing with sterile distilled water.

2.1.6 Microorganisms

Five isolates of yeast cells; *Pichia guilliermondii* BCC 5389, *C. utilis* TSITR 5001, *C. tropicalis* TISTR5010/ATCC 13803, *Pichia membranaefaciens* TISTR 5093, and *Cryptococcus humicola* BCC 7701, obtained from Sangchote's laboratory Department of Plant Pathology of Kasetsart University were screened for the antagonists of *P. digitatum*. They were grown in the 250 ml flasks with 50 ml

nutrient yeast dextrose broth (NYDB) on a rotary shaker at 200 rpm for 24 h at 28°C. Cell suspensions were prepared by centrifugation at 6,000 rpm for 10 min (at 4 ºC) and washed twice with 0.85% NaCl in order to remove the growth medium. Then, cell pellets were suspended in sterilized distilled water and counted by heamacytometer, and adjusted to a concentration of 10^8 cells/ml (Zhao *et al*., 2008).

B. subtilis isolate MK007, NSRS89-24, ABS-S14 and 155, used in this investigation was isolated from soil collecting from groves around the south of Thailand. Their antagonistic properties *in vitro* and *in vivo* were screened (Kuyyogsuy *et al*., 2006; Leelasuphakul *et al*., 2008). Prior to use, these bacterial isolates were stored in glycerol at -70°C. In preparation of aqueous antagonist endospore suspensions, isolates were grown on nutrient agar (NA) at 27 °C for 24 h. A single colony was transferred to a 250 ml flask containing 50 ml of Luria-Bertani broth (LB) and incubated on a rotary shaker (200 rpm) for 72 h at 35°C. The bacterial endospore pellets in culture were harvested by was centrifugation for 20 min at 6,000 rpm and washed twice with 0.85% NaCl. The resulting pellet was resuspended in sterile distilled water and mixed well. Bacterial endospore suspension was adjusted to $1x10^8$ CFU/ml according to comparison with No.0.5 McFarland turbidity standard at 590 nm using a spectophotometric method.

2.2 Method

2.2.1 Screening of antagonistic yeasts against *P. digitatum* by dual culture method

The co-culture of the 5 isolate yeasts and *P. digitatum* hyphae was assessed by dual culture technique (He, *et al.,* 2003). A 0.1 cm agar plug obtained from the margin of actively growing culture *P. digitatum* on a PDA plate, was placed centrally on a fresh PDA plate (for 48 h at 25 ºC). The antagonist test culture was grown in NYDB and streaked on the PDA plate 1 cm away from the fungal plug. Observations of the fungal reactions are recorded after 4 d and the radius of fungal colony were measured in two perpendicular directions by a Vernier Caliper. The radial growth rate of colonies in all treatments was calculated using the formula of Gamliel *et al* (1989). Five replicates were used for each yeast isolate test.

$$\text{Percentage of inhibition} = 100\text{-}[(R^2/r^2) \times 100]$$

R = Radial growth of fungal colony on treatment plate

r = Radial growth of fungal colony on control plate

2.2.2 Growth inhibitory effect of *P. guilliermondii* BCC5389 against *P. digitatum*

2.2.2.1 Effect of *P. guilliermondii* BCC 5389 cell on spore germination of *P. digitatum*

The effect of *P. guilliermondii* BCC 5389 on spore germination of the pathogen was tested in potato dextrose broth (PDB) (Zhang *et al*., 2008). An aliquot of 1 ml of 10^6, 10^7 and 10^8 cells/ml washed cell suspension of *P. guilliermondii* BCC 5389 or sterile distilled water (control) were added into 50 ml test tubes containing 10 ml PDB, respectively. Aliquots (1 ml) of spore suspensions of *P. digitatum* (10^4 spores/ml) were added to each tube. After 24 h incubation at 25 ^{o}C on a

rotary shaker (100 rpm), the percentage of germinated spores was determined by microscopic examination of spores (at least 200 spores per replicate). The number of germinated spore, which was identified by a doubling in length of spore size, were counted and their lengths were measured with a micrometer using a compound microscope.

2.2.2.2 Effect of culture filtrate of *P. guilliermondii* BCC 5389 on mycelial growth of *P. digitatum*

Antifungal activity of culture filtrate was tested by the agar well diffusion assay by the method of Olila *et al.* (2001) with slight modifications. A 0.1 cm agar plug obtained from the margin of actively growing culture *P. digitatum* on a PDA plate, was placed centrally on a fresh PDA plate and maintained for 48 h. Culture filtrate was collected from antagonistic culture in NYDB which pre-incubation for 48 h at 28 ^{o}C, it was pipetted from the top part of the supernatant after centrifugation at 6,000 rpm for 20 min. The 45 µl of culture filtrate was added to the 0.5 cm of two cavities which were made at approximately 1 cm away from the margin of growing mycelium of *P. digitatum,* then incubated at 25 ^{o}C for 72 h. Observations of the fungal reactions and the radial growth rate of colonies were measured and the percentage of fungal colony was calculated as described in 2.2.1.

2.2.2.3 Effect of *P. guilliermondii* BCC 5389 culture filtrate on spore germination of *P. digitatum*

The culture filtrate of *P. guilliermondii* BCC 5389 was prepared in NYDB medium for 48 h at 28 ^{o}C with shaking at 200 rpm (modified from Prapagdee *et al*., 2008). The 2 d-old cell-free culture filtrate was obtained from the top part of the supernatant after centrifugation at 6,000 rpm for 20 min and was further prepared at 2X, 4X, and 8X fold-serial dilutions. The diluted culture filtrates (1 ml) were added into test tubes, and 1 ml aliquots of spore suspension containing 10^4 spores/ml of *P. digitatum* were added to each tube. After 24 h-incubation at 25 ^{o}C on a rotary shaker

(100 rpm), at least 200 spores per replicate were observed microscopically to determine percentage of germination as described in 2.2.2.1.

2.2.3 Growth inhibitory effect of *P. guilliermondii* BCC 5389 and *B. subtilis* strains on *P. digitatum*

The dual culture technique was conducted to test for the antagonistic activity of *P. guilliermondii* BCC 5389 and the four strains of *B. subtilis* on the growth of *P. digitatum*. A 0.1 cm agar plug obtained from the margin of actively growing culture *P. digitatum* on a PDA plate, was placed centrally on a fresh PDA plate (for 48 h at 25 ºC). The antagonistic yeast and bacterial cultures were grown in NYDB and NB, respectively as described in 2.1.6. The antagonistic cells in liquid were harvested by centrifugation at 8,000 rpm for 10 min. The antagonistic pellet was streaked approximately 1 cm away from 2 d-old fungal colony grown on PDA plate. Observations of the fungal reactions are recorded after 4 d at 25 ºC. The radial growth rate of colonies was measured and the percentage of hyphal growth inhibition was calculated as described in 2.2.1.

2.2.4 Antagonism between *B. subtilis* strains and of *P. guilliermondii* BCC 5389

Antagonistic effect was tested between *P. guilliermondii* BCC 5389 and *B. subtilis* isolate ABS-S14, 155, MK007 and NSRS89-24 by strip-paper method of Lorian and Fodor (1974) with some modifications using a sterile strip containing antagonist was placed on spread plate. Each antagonist spread on PDA. Sterile strips with 0.2 ml of yeast inoculum (10^8 cells/ml in NYDB) or bacterial endospore inoculum (10^8 CFU/ml in NB) were placed on the surface of the spread PDA plate 1 cm away from the central at opposite sides of the plate. Clear zone was observed after incubating at 25 ^oC for 4 d.

2.2.5 Effect of sodium bicarbonate on growth of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389

The effect of sodium bicarbonate on growth of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 was tested in NYDB and NB, respectively (some modified from Obagwu and Korsten, 2002). Containing 50 ml of NB or NYDB were obtained 0.5, 1.0, 1.5 and 2% w/v of sodium bicarbonate into 250-ml Erlenmeyer flasks. Aliquots (1 ml) of washed cell suspensions of *P. guilliermondii* BCC 5389 (10^8 cells/ml) or 1 ml-aliquots of *B. subtilis* ABS-S14 (10^8 CFU/ml) endospore suspension were added to each flask. Then, the SBC flask containing yeast cell suspension was incubated at 28 ^oC on rotary shaker at 200 rpm, while the SBC flask containing bacterial endospore suspension was incubated at 35 ^oC on rotary shaker at 200 rpm. Absorbance at 600 nm of growth rates of the antagonists were recorded at 0, 8, 12, 20 and 24 h for *B. subtilis* ABS-S14 and at 0, 8, 12, 16, 20, 24, 30, 36, 42 and 48 h for *P. guiliiermondii* BCC 5389.

2.2.6 Growth inhibitory effect of sodium bicarbonate on *P. digitatum*

2.2.6.1 *In vitro* growth fungal mycelial inhibition assay

The effect of sodium bicarbonate on *P. digitatum* growth was tested on PDA plate supplemented with 0.5, 1.0, 1.5 and 2% (w/v) of sodium bicarbonate using method of Zhang and Swingle (2003) with slight modifications. A 0.1 cm agar plug obtained from the margin of actively growing culture *P. digitatum* on a PDA plate, was placed centrally on a freshly prepared sodium bicarbonate supplemented PDA plate, and incubated at 25 ºC for 3 d. The radial growth rate of colonies was measured and the percentage of hyphal growth inhibition was calculated as described in 2.2.1.

2.2.6.2 *In vitro* fungal spore germination inhibition assay

The effect of sodium bicarbonate on spore germination of the pathogen was tested in potato dextrose broth (PDB) (Smilanick and Margosan, 1999). Four concentrations of each sodium bicarbonate were prepared by dissolving in sterile 50 ml PDB to obtain the proposed concentration of 0.5, 1.0, 1.5 and 2% w/v of sodium bicarbonate. An aliquot (1 ml) of spore suspension of *P. digitatum* (1 x 10^4 spores/ml) was added to each flask. After 24 h-incubation at 25 ^oC on a rotary shaker (50 rpm), the percentage of germinated spores was determined as described in 2.2.2.1.

2.2.6.3 *In vivo* assay of antifungal effect of sodium bicarbonate on *P. digitatum* growth

2.2.6.3.1 Preparation of spore suspension of *P. digitatum*

A suspension of *P. digitatum* spores was prepared as described in 2.1.5.

2.2.6.3.2 Fruit preparation and inoculation

The fruits were prepared as described in 2.1.4. A 0.5 cm circle of five wounds (3 mm depth) were made uniformly at the equator of fruits by puncturing. Aliquots of 20 µl each of 0.5, 1.0, 1.5 and 2% (w/v) sodium bicarbonate and sterile distilled water as control, were dropped into a wound per fruit. Two hours later, the 20 µl suspension of *P. digitatum* (10^4 spores/ml) was inoculated into each wound of fruit in various treatments. The treated citrus fruits were placed in the plastic boxes containing a cup of water to maintain a high relative humidity and stored at 25 ^oC. Disease incidence (lesion diameter) was measured daily by Venier-caliper until 5 d. Each treatment composed of 10 replicates. There were two replicate trials of 20 fruits per treatment with complete randomized block design. A fruit was considered as diseased and decay based on the visible signs of a lesion with a diameter ≥0.5 cm and ≥5.0 cm at the inoculation point, respectively. The percentage

of disease incidence was calculated by summing the number of fruit with visible lesions inoculated fruit (Leelasuphakul *et al*., 2008).

% disease incidence = (Σ no. of fruits with lesion/total no. of fruits) x100

2.2.7 Biocontrol assay of antagonistic yeast and bacteria on citrus fruit against *P. digitatum* compared with sodium bicarbonate

2.2.7.1 Preparation of spore suspension of *P. digitatum*

A suspension of *P. digitatum* spores was prepared as described in 2.1.5.

2.2.7.2 Preparation of *P. guilliermondii* cell suspension and *B. subtilis* endospore suspension

The antagonist suspensions were prepared as described in 2.1.6.

2.2.7.3 Fruit preparation and inoculation

Citrus fruits were prepared and wounding was made as described in 2.1.4. A Treatment set was divided into 7 groups (Table 2.1). Suspension of (1) sterile distilled water (2) 500 ppm imazalil, (3) 2% (w/v) sodium bicarbonate, (4) *P. guilliermondii* BCC 5389 (1 x 10^8 cells/ml), (5) *B. subtilis* ABS-S14 endospores (10^8 CFU/ml), (6) mixtures of *P. guilliermondii* BCC 5389 (1 x 10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml) were dropped into two wound per fruit with aliquots of 20 µl each treatment. Two hours later, a 20 µl suspension of *P. digitatum* (1 x 10^4 spores/ml) was inoculated into each wound of various groups including (7) non-treated wound as a diseased control group. The treated citrus fruits were prepared, the percentages of disease incidence and decay were calculated as described in 2.2.6.3.2.

Table 2.1 Biocontrol assay of antagonistic yeast and bacteria on citrus fruit against *P. digitatum* compared with sodium bicarbonate

Treatment	Treatment agents (20 μl each)
T1	Sterile distilled water
T2	*P. digitatum* spore suspension (10^4 spores/ml)
T3	Imazalil
T4	2% (w/v) sodium bicarbonate
T5	*P. guilliermondii* BCC 5389 cell suspension (10^8cells/ml)
T6	*B. subtilis* ABS-S14 endospore suspension (10^8 CFU/ml)
T7	Mixture of *P. guilliermondii* BCC 5389 cell suspension (10^8cells/ml) and *B. subtilis* ABS-S14 endospore suspension (10^8 CFU/ml)

T3-T7 were followed by *P. digitatum* spore suspension (104 spores/ml) inoculation

2.2.7 Effect of the antagonistic microorganism and sodium bicarbonate on fruit quality

2.2.7.1 Fruit preparation and inoculation

The fruit were prepared as described in 2.1.4.

2.2.7.2 Preparation of *P. guilliermondii* cell suspension and *B. subtilis*

The antagonist suspensions were prepared as described in 2.1.6.

2.2.7.3 Fruit treatments

To evaluate the effect of antagonists on postharvest quality of citrus (Zhang *et al*., 2008), freshly harvested fruits were dipped with mixture of suspensions of washed cells of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml) endospore, and 2% (w/v) sodium bicarbonate and sterile distilled water as a control for 30 sec, and air-dried. The treated citrus fruits were prepared as described in 2.2.6.3.2 and storage at 25 ^{o}C for 7 d. There were two replicate trials of 10 fruits per treatment with complete randomization. The following parameters were measured at the start point and after storage.

2.2.7.4 Fruit quality assay

2.2.7.4.1 Weight loss

Weight loss was measured by an analytical balance (±0.001 g before treatment (A) and after storage (B), respectively, and the weight loss was calculated as (A - B)/A.

2.2.7.4.2 Fruit firmness

Firmness values of each individual citrus fruit were measured at the equatorial region depth 7 mm using commercial brand firmness tester with 8 mm diameter flat probe. The probe was descended toward the sample at 10.0 mm/sec and

the maximum force (N) required was defined as the firmness. The firmness of each citrus fruit was measured twice on opposite sides.

2.2.7.4.3 Total soluble solids

Total soluble solids (TSS) were determined by measuring the refractive index of the juice diluted 1:1 using a hand refractometer and the results were expressed as % ^{o}Brix

2.2.7.4.4 Titratable acidity

Acidity was measured by titratable of 5 ml of juice with 0.1 N NaOH and phenolphthalein as an indicator. Titratable acidity was calculated as a percentage citric acid by the formula

%TA = [(ml NaOH) (N NaOH) (meq.wt.acid)/ml sample] x 100.

2.2.8 Effect on antagonistic yeast and bacteria on defense related enzyme activity

2.2.8.1 *P. digitatum* spore suspension

A suspension of *P. digitatum* spores was prepared as described in 2.1.5.

2.2.8.2 Yeast and bacteria suspensions

The suspensions of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 were prepared as described in 2.1.6.

2.2.8.3 Plant material

The fruit were prepared as described in 2.1.4. A 0.5 cm circle of five wounds (3 mm depth) were made uniformly at the equator of fruits by puncturing. Various treatments were conducted as shown in Table 2.2. Two hours later, a 20 µl suspension of *P. digitatum* (1 x 10^4 spores/ml) was inoculated into two

wounds per fruit. The treated citrus fruits were placed in the plastic boxes containing a cup of water to maintain a high relative humidity and stored at 25 ^{o}C. Flavedo tissues with the areas of 1.0 cm diameter concentric to the inoculation site were taken at 0, 24, 48 and 72 h after incubation. The tissues were frozen in liquid nitrogen. Each treatment was composed of 9 replicates. There were three replicate trials of 9 fruits per treatment with complete randomized block design.

Table 2.2 Effect of *B. subtilis* and *P. guilliermondii* on abundance of defense related gene transcripts and enzyme activities

Treatment	Treatment agents
T1	Sterile distilled water
T2	*P. digitatum*
T3	*B. subtilis* ABS-S14
T4	*P. guilliermondii* BCC 5389
T5	*B. subtilis* ABS-S14+ *P. digitatum*
T6	*P. guilliermondii* BCC 5389+ *P. digitatum*
T7	Mixture of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14
T8	Mixture of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 + *P. digitatum*

Concentrations of *P. digitatum* (10^4 spores/ml)**,** *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. guilliermondii* BCC 5389 (10^8 cells/ml)

2.2.8.4 Acetone powder preparation and protein extraction

The acetone powder (AP) was prepared from flavedo tissues that ground to a fine powder under liquid nitrogen in a mortar and pestle, and then transferred to pre-chill at -20 ^{o}C acetone. The homogenates were filtered though a Whatman No.1 filter paper using vacuum suction pump. The sample residues were washed once with cold acetone and were subsequently transferred to a new tube. The resulting power (AP) was dried at room temperature overnight and kept at -20 ºC.

2.2.8.5 Chitinase activity assay

Activity of chitinase enzyme was determined by the method of Wirth and Wolf (1990) with some modifications using a carboxymethyl chitosan-remazol brilliant violet solution as substrate (CM-chitosan-RBV). 100 mg of AP was mixed with 1 ml of 100 mM sodium acetated buffer pH 5.0. The homogenate was centrifuged at 12,000 rpm at 4^{o}C, for 10 min to remove the insoluble materials. A 50 µl portion of the crude enzyme solution plus 150 µl of distilled water and 100 µl of CM-chitosan-RBV were mixed and incubated for 30 min at 37 ^{o}C. After incubation, 100 µl of 100 mM sodium acetate buffer (pH 5.0) was added. The reaction was terminated by adding 400 µl of 3.0 M HCl. The reaction mixture was cooled on ice for 10 min, then centrifuged at 10,000 rpm for 10 min and the supernatant was kept for measuring light absorbance at 550 nm. CHI activity was calculated as described by Wirth and Wolf (1990) as unit/ml/gAP based on a calibration curve of purified chitinase from *Streptomyces griseus* (Krainsky) (C6137, Sigma). One unit was defined as the amount of enzyme required to catalyze the formation of 1 nmol GlcNAc/min.

2.2.8.6 β-1,3-glucanase activity assay

Measurement of β-1,3-glucanase activity from Burner (1964). Ten µl of sample was mixed with 90 µl of 4 mg/ml laminarin. The mixture tube was immersed in water bath at 35 ^{o}C and shaking for 30 min prior to boiling for 2 min to denature the enzyme. Then, 0.2 ml of dinitrosalicylic acid (DNS) solution and 0.2 ml of 0.1 M acetate buffer pH 5 were added and boiled for 5 min, followed by chilling on ice for 5 min. Then, 0.9 ml of distilled water was added. Light absorbance was measured at 540 nm. The enzyme activity was calculated from the amount of reducing sugar released from substrate digestion by enzyme and this was obtained from glucose standard curve. One unit of enzyme activity was equivalent to one µmol of reducing sugar derived from laminarin degradation in one min.

2.2.9 Effect on antagonistic yeast and bacteria on defense related gene transcripts

2.2.9.1 *P. digitatum* spore suspention

A suspension of *P. digitatum* spores was prepared as described in 2.1.5.

2.2.9.2 Yeast and bacteria suspension

Antagonist suspensions of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 were prepared as described in 2.1.6.

2.2.9.3 Plant material

The citrus fruit and experimental treatment were described in 2.2.8.3 and Table 2.2. The peels of citrus fruit were collected at 0, 24, 48 and 72 h after inoculation of *P. digitatum.*

2.2.9.4 Total RNA extraction

Total RNA was extracted from 0.1 g of flavedo tissue. Tissues were ground to a fine powder under liquid nitrogen in a mortar and pestle.

The homogenate was transferred and mixed to 0.5 ml of RNA extraction buffer (100 mM Tris-HCl pH 8, 10 mM EDTA, 100 mM LiCl and 2% SDS) and 0.25 ml of water saturated phenol (pH 4.0), 0.25 ml of Chloroform: Isoamyl alcohol ($CHCl_3$:IAA) (24:1, v/v), and centrifuged at 4,200 rpm for 20 min. After centrifugation, the aqueous phase was transferred to a new tube, and equal volume of phenol: $CHCl_3$:IAA (25:24:1, v/v) was added, mixed, and centrifuged at 4,200 rpm for 20 min. The aqueous phase was transferred to a new tube, equal volume of $CHCl_3$:IAA (24:1, v/v) was added and mixed, centrifuged at 4,200 rpm for 20 min and transferred the aqueous phase to a new tube. Equal volume of isopropanol and 1/10 volume of 3M sodium acetate pH 5.2 were added, and incubated at -20 ^{o}C for 16 h. The nucleic acids were precipitated by centrifugation at 10,000 rpm at 4 ^{o}C for 10 min and re-suspended in 500 µl RNase free-water (DEPC treated water). Total RNA was precipitated with 500 µl of 8 M LiCl with slowly shaking and incubated at 4 ^{o}C for 16 h. The RNA pellets were collected by centrifugation at 13,000 rpm at 4 ^{o}C for 10 min and washed twice with cold 70% ethanol. Total RNA was dried at room temperature and suspended in 100-200 µl of RNase free-water and the RNA solution was kept at -80 ^{o}C.

2.2.9.5 DNase I Treatment

RNA pellets were suspended in 40 µl of 1x DNase I buffer containing 1 unit of DNase I and incubated at 37 ^{o}C for 1 h. DNase I was removed using phenol-chloroform extraction. Afterward, the homogenate's volume was adjusted to 160 µl with RNase free-water it was extracted twice with 200 µl of phenol: $CHCl_3$:IAA (25:24:1) and once with 200 µl of $CHCl_3$:IAA (24:1). The aqueous phase was transferred to a new tube, and RNA was precipitated by adding 20 µl of 3 M sodium acetate (pH 5.2) and 550 µl of pre chilled absolute ethanol and

stored at -80 ^{o}C for 15 min. RNAs were collected by centrifugation at 13,000 rpm at 4 ^{o}C for 20 min. The RNA pellets were washed with 1 ml of 75% ethanol, air-dried at room temperature, and suspended in 20 µl of RNase free-water. The concentration of total RNA was determined by measuring the absorbance at 260 and 280 nm (A260/A280).

2.2.9.6. cDNA synthesis

cDNA was synthesized from 1 µg of total RNAs using the ImProm-II Reverse Transcription System (Promega Corp. Madison, USA.). The reaction mix was prepared as recommended by the manufacturer. The RNAs were annealed with primer oligodT at 70 ^{o}C for 5 min, chilled on ice for 4 min, and then mixed with 15 µl of PCR reaction mix. Reverse transcription PCR cycling program was performed using MyCycler thermal cycler (Bio-Rad) at 42 ^{o}C for 60 min, the reaction was stopped by incubating at 70 ^{o}C for 15 min, and chilled on ice at least 1 minute, respectively. Finally, the cDNA products were kept at -20 ^{o}C.

2.2.9.7 Standard curve of target gene

Standard curves for each gene were developed by 10X-fold serial dilutions of cDNA (10^4-10^0) with RNase–free-water. Primers of chitinase (*CHI*), glucanase (*GLU*) and elongation factor 1 alpha (*EF-1*α) were synthesized from Integrated DNA Technologies and their sequences as presented in Table 2.3. The 8-tube strips were placed in the PCR capillaries in Mx3005P qPCR system (Agilent Technologies, USA) for amplification. PCR cycling program was started at 95 ^{o}C for 10 min for activating Taq DNA polymerase, and three step amplification including denaturation step at 95 ^{o}C for 15 sec, annealing step at 55 ^{o}C for 45 sec and extension step at 72 ^{o}C for 45 sec with a total of 40 cycles. The standard curves of each cDNA (10^4-10^0 dilution) were expressed as C_T value (axial Y) and plotted against the logarithm of cDNA dilution (axial X), and linear regression equation was analyzed using Microsoft Excel program.

2.2.9.8 Determination of gene expression by quantitative RT-PCR

Citrus gene expression in citrus peel treated with various treatments (Table 2.2) and determined by real-time quantitative PCR using Mx3005P qPCR system (Agilent Technologies, USA) and the SsoFast EvaGreen Supermix (Bio-Rad). The primers of *EF1α*, *GLU* and *CHI* were used in this experiment as shown in Table 2.3. The transcript level was calculated by standard curve and normalized against *EF1α* as an internal control. Negative controls without cDNA were routinely included (Distefano *et al*., 2008).

Table 2.3 Gene sequences downloaded from GenBank (NCBI) with accession numbers. These sequences have been used for the designing of the primers adopted in quantitative real time PCR

Gene name	Primer	GenBank accession number
Chitinase (*CHI*)	Forward 5'-AATGATGAACGATGCCCTGCCA-3'	Z70032.1
	Reverse 5'-CCACTTGATGCTGTCTCCAA-3'	
β-1,3-glucanase (*GLU*)	Forward 5'-ACCTCCGAAGAATCGCTTCCAA-3'	AJ000081.1
	Reverse 5'-TGTTTCTCATGGCGGGAACA-3'	
Elongation factor 1-alpha (*EF1α*)	Forward 5'-ACATGATTACCGGTGCCTCACA -3'	AY498567.1
	Reverse 5'-ACACCAAGGGTGAAAGCAAGCA-3'	

2.2.10 Statistical analysis

All data obtained from the experiments were subjected to statistical analysis. The average difference was compared by the Least Significant Difference Test (LSD) and Duncan's new multiple range test (DMRT).

Chapter 3

RESULTS

3.1 Screening of antagonistic yeasts against *P. digitatum* by dual culture method

The *in vitro* inhibitory effectiveness of 5 yeast isolates against citrus fruit rot pathogen, *P. digitatum* was evaluated. The antagonistic yeasts were grown at 25°C for 4 d. The radial growth rate of fungal colony was inhibited by yeast antagonists when compared with the control plate. *P. guilliermondii* BCC 5389 showed the highest percentage of radial growth inhibition (31%) among tested yeasts (Table 3.1 and Figure 3.1). Based on the highest potential of antifungal activity of *P. guilliermondii* BCC 5389 detected, therefore, *P. guilliermondii* BCC 5389 was chosen to be antagonistic yeast used in the following experiments.

Table 3.1 Percent inhibition of radial mycelial growth of *P. digitatum* by antagonistic yeasts on PDA plate after 4 d-incubation at 25 ^{o}C

Treatment	Radial growth inhibition(%)[1]
P. digitatum	00.0±0.0a
Candida utilis TSITR 5001	57.8±1.5b
Candida tropicalis TISTR5010/ATCC 13803	57.8±1.2b
Pichia guilliermondii BCC 5389	85.0±0.8cd
Cryptococcus humicola BCC 7701	75.0±1.2c
Pichia membranaefaciens TISTR 5093	75.0±1.6c

[1] Mean values of 5 replications within columns followed by the same letter are not significantly different according to the Least Significant Difference test (LSD) at $P \leq 0.05$.

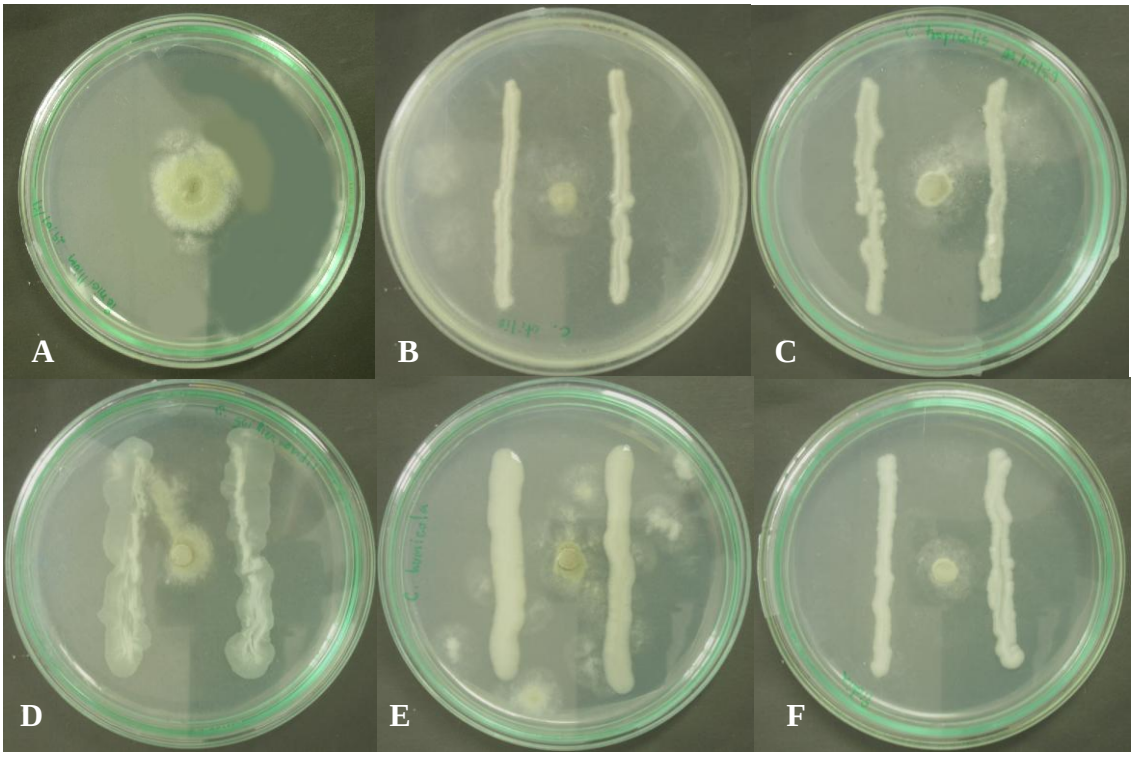

Figure 3.1 Effect of antagonistic yeasts on mycelial growth of *P. digitatum* on PDA plate after 4 d-incubation at 25 ^{o}C. A: *P.digitatum* (control); B: *C. utilis* TSITR 5001; C: *C. tropicalis* TISTR5010/ATCC 13803; D: *P. guilliermondii* BCC 5389; E: *C. humicola* BCC 7701 and F: *P. membranaefaciens* TISTR 5093.

3.2 Growth inhibitory effect of *P. guilliermondii* BCC5389 against *P. digitatum*

3.2.1 Effect of *P. guilliermondii* BCC 5389 cell on spore germination of *P. digitatum*

The *in vitro* antagonistic effect of *P.guilliermondii* BCC5389 at concentrations of 10^6 10^7 and 10^8 cells/ml to spore germination of *P. digitatum* was conducted in PDB for 24 h (Table 3.2 and Figure 3.2). Complete inhibition of spore germination of *P. digitatum* was demonstrated when the cell suspension of *P. guilliermondii* BCC 5389at 10^7 and 10^8 cells/ml were used, whereas 10^6 cells/ml of *P. guilliermondii* BCC 5389 showed 42% inhibition. Observation was made at 24 h under compound light microscope revealed abnormal germ tubes of *P. digitatum* (Figure 3.2B) while normal germination of germ tube and mycelium of *P. digitatum* were shown in the control tube (Figure 3.2A).

Table 3.2 *In vitro* inhibition of spore germination of *P. digitatum* by *P. guilliermondii* BCC 5389 cell suspension after 24 h-incubation at 25 °C

***P. guilliermondii* BCC 5389 (cells/ml)**	**Inhibition of *P. digitatum* spore germination (%)[1]**
0	0±0.0c
10^6	42±0.8b
10^7	100±0.0a
10^8	100±0.0a

[1]Values within columns followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$.

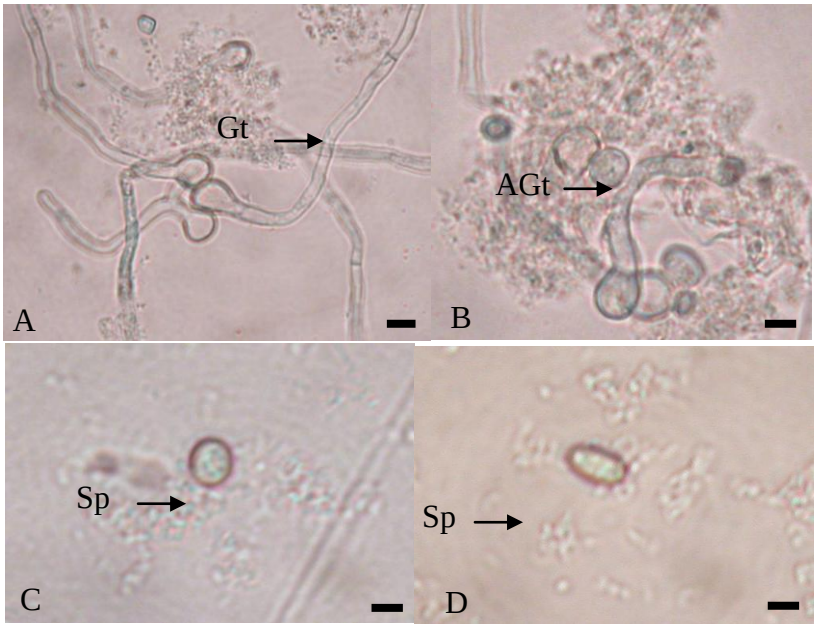

Figure 3.2 Appearances of *P. digitatum* spores and germ tubes in PDB after incubation with various concentrations of *P. guilliermondii* BCC 5389 under microscopic examination. A: *P. digitatum* germ tubes at 24 h (control) B: washed cell suspension of *P. guilliermondii* BCC 5389 (10^6 cells/ml) and *P. digitatum*. C and D: washed cell suspension of *P. guilliermondii* BCC 5389 (10^7 and 10^8 cells/ml, respectively). Sp=spore; Gt=germ tube; and AGt=abnormal germ tube. Bars = 5 μm.

3.2.2 Effect of culture filtrate of *P. guilliermondii* BCC 5389 on mycelial growth of *P. digitatum*

The percentage of the radial growth inhibition of *P. digitatum* by culture filtrate of *P. guilliermondii* BCC 5389 after 72 h incubation in PDA was 46.7% compared with untreated fungal colony (Figure 3.3). A significant change of *P. digitatum* morphology was observed under light microscopy with lactophenol cotton blue staining. Observations revealed morphological abnormalities in fungal structure, swelling of hyphal tips of *P. digitatum* (Figure 3.3B and 3.3D) when compared with normal mycelium (Figure 3.3A and 3.3C).

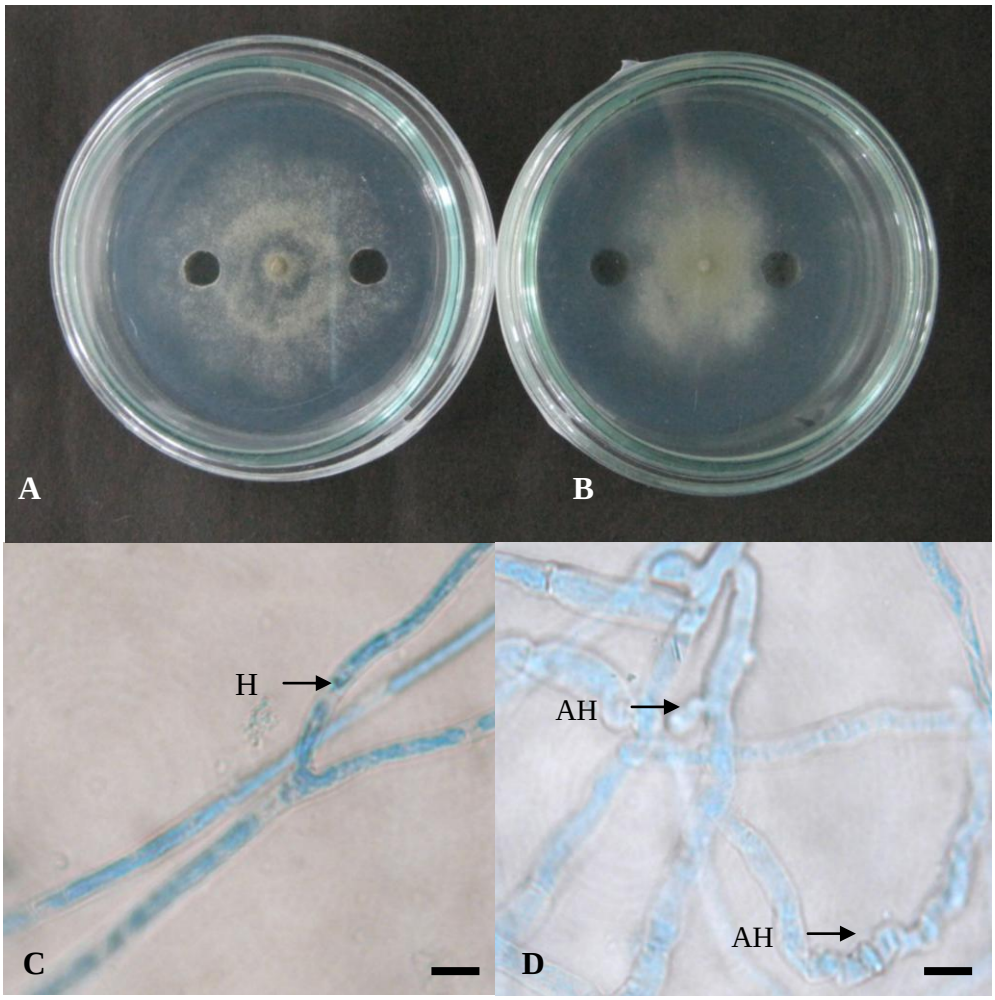

Figure 3.3 Inhibitory test of *P. guilliermondii* BCC 5389 culture filtrate on *P.digitatum* mycelium growth on PDA medium. A: *P. digitatum* colony on PDB (control plate); B: treated with culture filtrate of *P. guilliermondii* BCC 5389; C and D: fungal mycelia were observed under light microscope from A and B, respectively. H=hyphae; AH=abnormal hyphae. Bars=10μm.

3.2.3 Effects of *P. guilliermondii* BCC 5389 culture filtrate on spore germination of *P. digitatum*

In vitro antagonistic effect of culture filtrate obtained from 10^8 cells/ml culture of *P. guilliermondii* BCC 5389 were tested against *P. digitatum* spore germination. Fungal spores were observed after incubating in the PDB medium containing various concentrations of undiluted, 2x, 4x, and 8x fold-serial dilutions of yeast culture filtrate for 24 h. At undiluted and 2x fold-serial dilution of *P. guilliermondii* BCC 5389 culture filtrate completely inhibited spore germination of *P. digitatum*, whereas 4x and 8x diluting culture filtrate showed no effect on hyphal growth (Table 3.3, Figure 3.4).

Table 3.3 Percent inhibition on spore germination of *P. digitatum* by culture filtrate of *P. guilliermondii* BCC 5389 in PDB after 24 h-incubation at 25 °C

***P. guilliermondii* BCC 5389 culture filtrate dilution**	**Inhibition of *P. digitatum* spore germination (%) [1]**
undiluted	100a
2x	100a
4x	0b
8x	0b

[1]Values within columns followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$.

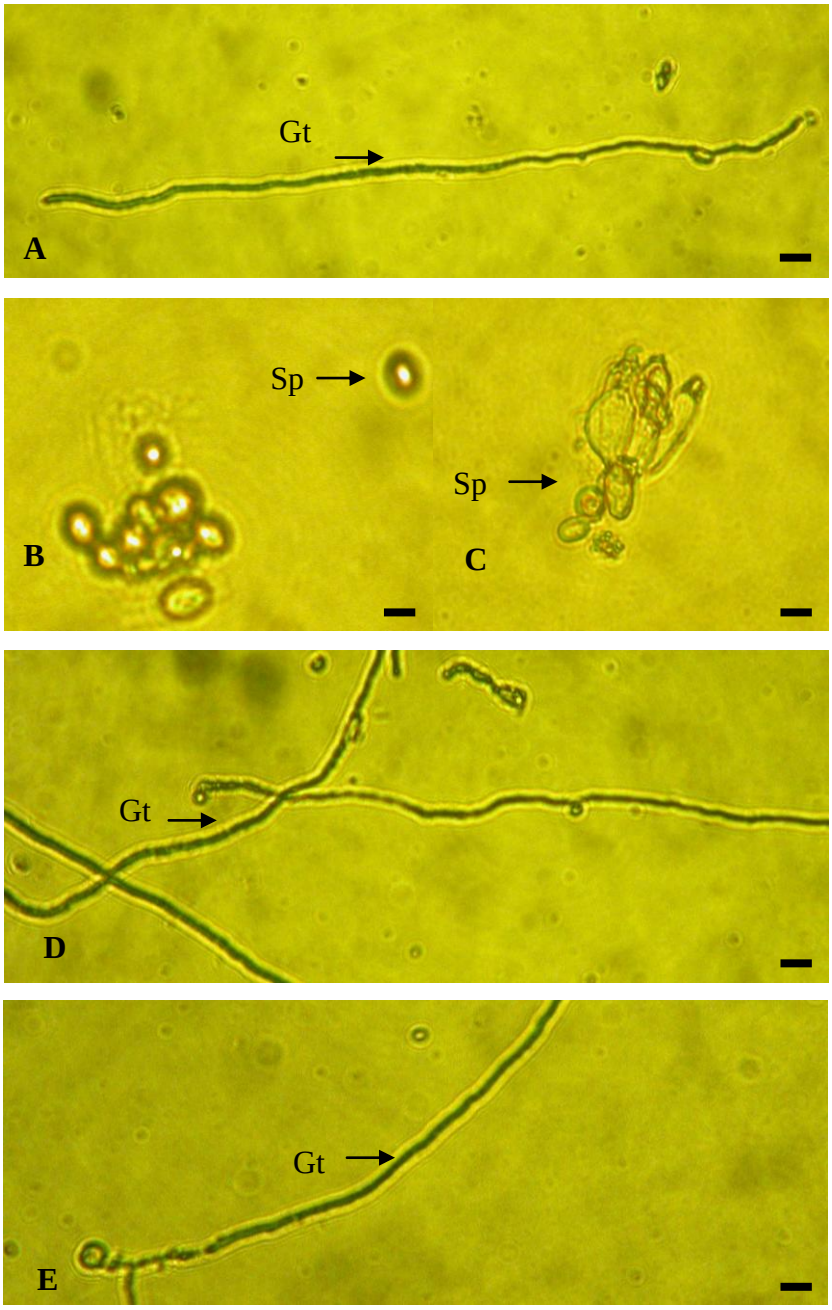

Figure 3.4 Microscopic morphology of germinated hypha of *P. digitatum* spores after exposure to culture filtrate of *P. guilliermondii* BCC 5389 in PDB at 24 h-incubation. Hyphal morphology A: untreated *P. digitatum* (control); B-E: undiluted, 2x, 4x and 8x fold-serial dilution of *P. guilliermondii* BCC 5389 culture filtrate, respectively. Sp=spore; Gt=germ tube. Bars=5μm.

3.3 Growth inhibitory effect of *B. subtilis* strains and *P. guilliermondii* BCC 5389 on *P. digitatum*

In vitro inhibitory effectiveness of *P. guilliermondii* BCC5389 and *B. subtilis* strains against citrus fruit rot pathogen; *P. digitatum* was evaluated by dual-culture plate assay. Complete fungal inhibition was obtained when fungal pathogen exposed to *B. subtilis* 155 and ABS-S14 as shown in Table 3.4 and Figure 3.5.

Table 3.4 Percent inhibition of *B. subtilis* and *P. guilliermondii* toward *P. digitatum* mycelial growth after 3 d-incubation at 25 ^{o}C

Strains of antagonists	% inhibition[1]
	Dual-culture test
B. subtilis NSRS89-24	97.1±1.2a
B. subtilis MK007	94.2±1.1b
B. subtilis 155	100.0±1.8a
B. subtilis ABS-S14	100.0±0.9a
P. guilliermondii BCC5389	80.0±1.5bc

[1]Values within columns followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$.

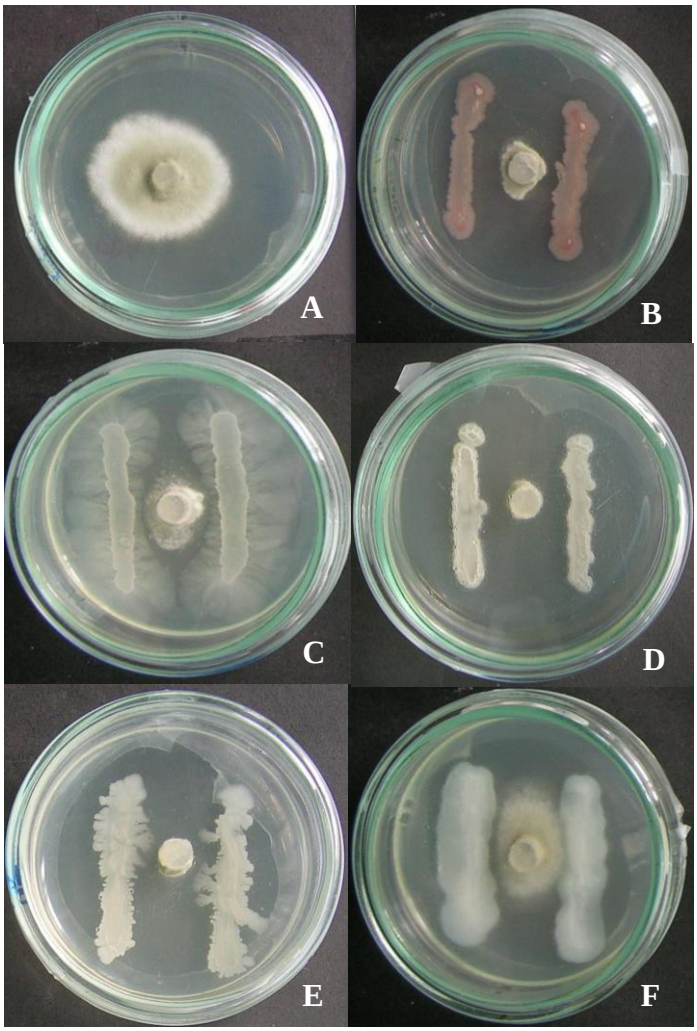

Figure 3.5 Effect of *B. subtilis* strains and *P. guilliermondii* on growth of *P. digitatum* after 3 d-incubation at 25 ^{o}C. A: *P. digitatum* (control); B-E: *B. subtilis* NSRS89-24 MK007, 155 and ABS-S14, respectively; F: *P. guilliermondii* BCC 5389.

3.4 Antagonism assay between *B. subtilis* strains and *P. guilliermondii* BCC 5389

The *in vitro* antagonistic effect among *P. guilliermondii* BCC5389 and *B. subtilis*; ABS-S14, 155, MK007 and NSRS89-24 were investigated by strip-paper plate assay. After 4 d-incubation at 25°C, growths of both bacterial strains and *P. guilliermondii* BCC 5389 colonies showed no clear zone between two microorganisms both on agar plates, and the culture dipping strips. Therefore, yeast and *B. subtilis* had no antagonistic effect toward each other (Figure 3.6).

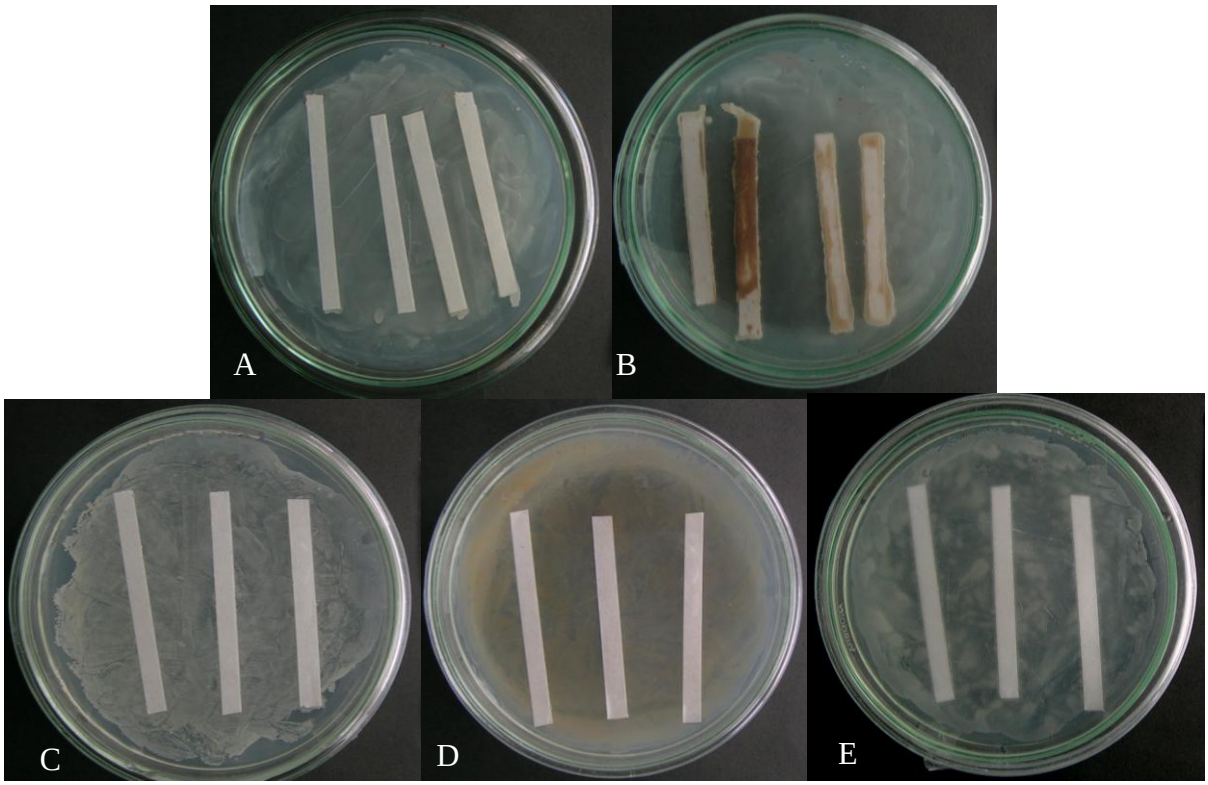

Fig 3.6 The antagonism test of various *B. subtilis* strains and *P. gulliermondii* BCC 5389. A and B plates; culture of *P. guilliermondii* BCC 5389 was overlaid on the agar as background, and A: various strains of *B. subtilis* were cultured on the filter paper strips ABS-14 (left), 155 (right); B: MK007 (left), NSRS89-24 (right); below C, D, E : cultures of *B. subtilis* strain ABS-S14, MK007 and 155 were overlaid on the agar as background, respectively, and *P. guilliermondii* BCC 5389 was cultured on the paper strips.

3.5 Effect of sodium bicarbonate on growth of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389

Non-significant differences of *B. subtilis* cultures in NB medium supplementing with sodium bicarbonate at 0.0, 0.5, 1.0, 1.5 and 2.0% (w/v) over 0-24 h, 35 ^{o}C was detected in bacterial growth curves as shown Figure 3.7A. Whereas, distinctive growth curves of *P. guilliermondii* BCC 5389 in NYDB containing 0.0, 0.5, 1.0, 1.5 and 2.0% (w/v) sodium bicarbonate over 0-48 h at 28 ^{o}C were observed. At log phase (0-12 h) yeast cells were grown in 1% (w/v) of sodium bicarbonate supplemented growth medium over the normal growth and reached the stationary phase as quick as 12 h, instead of 16-24 h as a normal growth period was. Sodium bicarbonate at 1.5% had no effect on *P. guilliermondii* growth. In opposite, the growths of *P. guilliermondii* BCC5389 in the presence of 0.5 and 2.0% sodium bicarbonate were slower than normal growth in log phase and reached stationary phase as late as 24 h (Figure 3.7B).

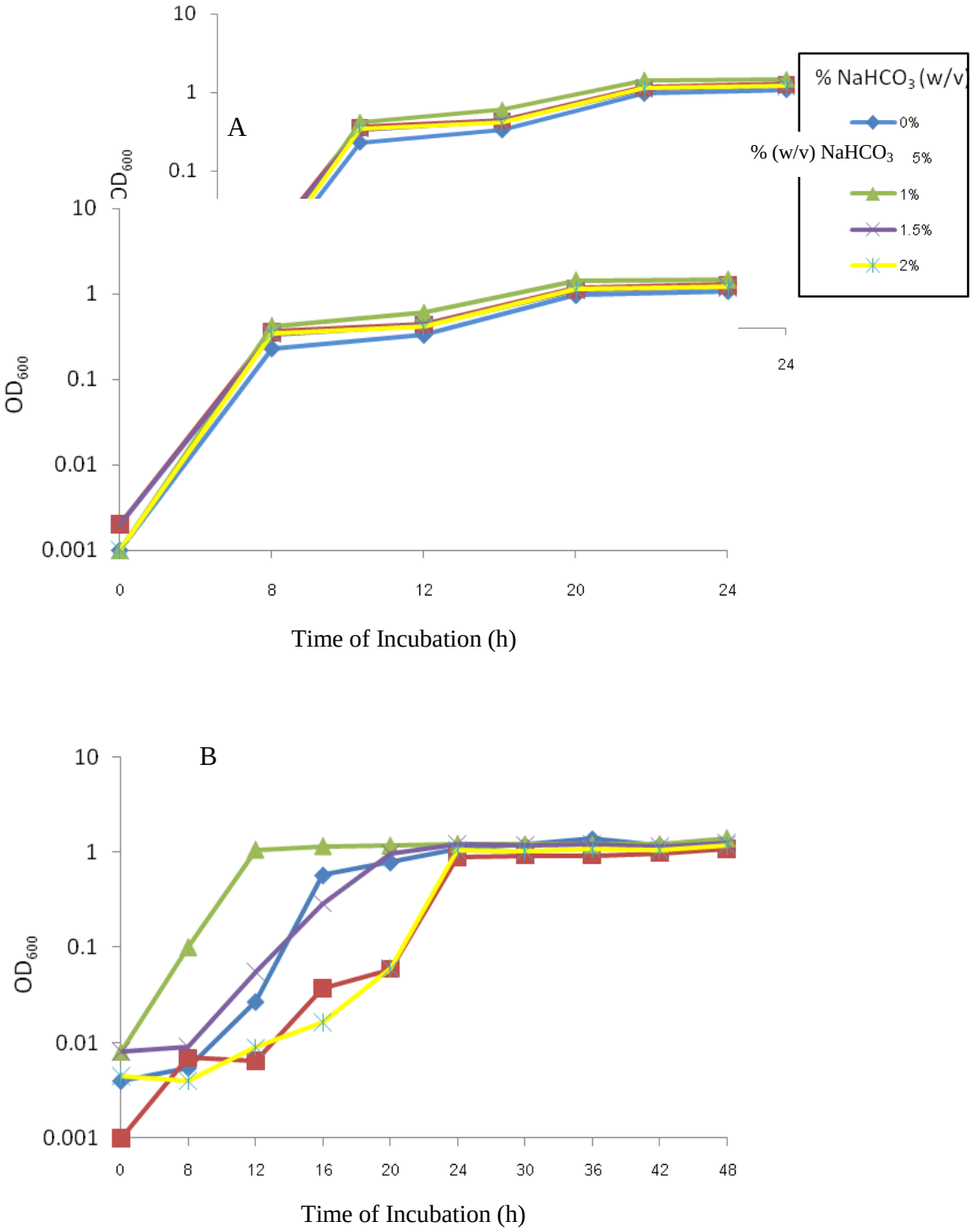

Figure 3.7 Growth curves of antagonistic microorganisms cultures in medium supplemented with sodium bicarbonate. Absorbance at 600 nm of A: *B. subtilis* ABS-S14 at 0-24 h, 37 ^{o}C in NB, B: *P. guilliermondii* BCC5389 at 0- 48 h, 28^{o}C in NYDB. Both A and B contained 0-2% w/v of sodium bicarbonate.

3.6 Growth inhibitory effect of sodium bicarbonate on *P. digitatum*

3.6.1 *In vitro* growth fungal mycelial inhibition

Screening of inhibition effect of various concentrations of sodium bicarbonate on *P. digitatum* mycelial growth on PDA plate was conducted (Figure 3.8). At 3 d-incubation complete fungal growth inhibitions were shown on the plates supplemented with sodium bicarbonate. Non-significant difference in concentration of sodium bicarbonate on *P. digitatum* mycelial growth was displayed (Table 3.5). The mycelial growth of the pathogen was completely effected by the 1.0-2.0 % (w/v) of sodium bicarbonate supplementing in the PDA plates. *P. digitatum* mycelia were rarely produced at concentration of 0.5% (w/v) sodium bicarbonate.

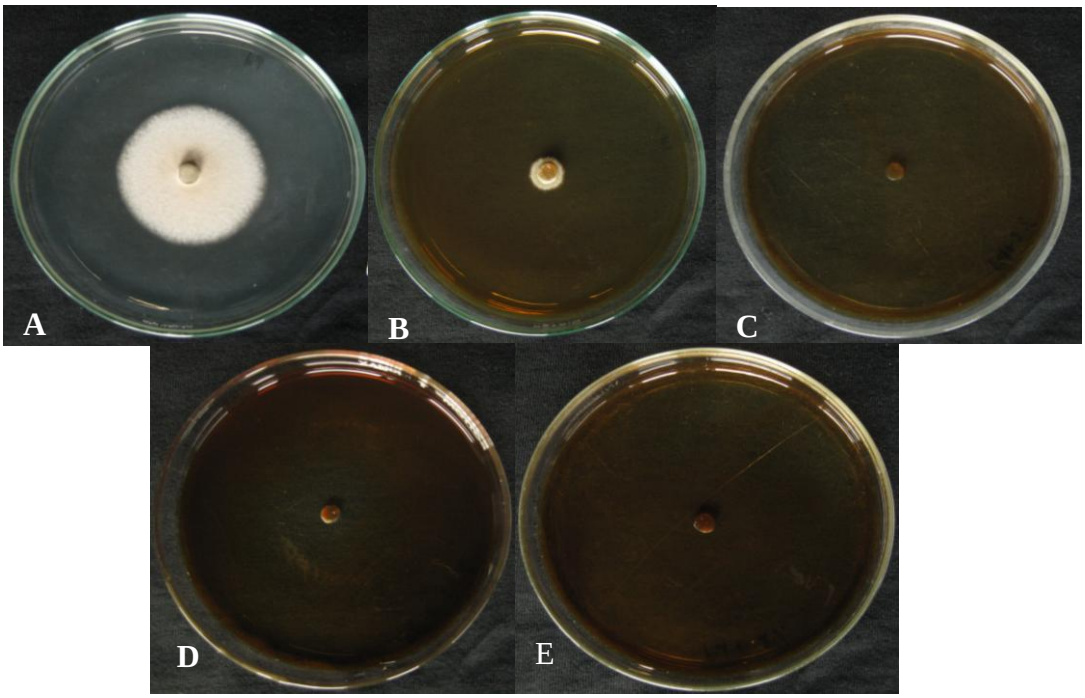

Figure 3.8 Effect of sodium bicarbonate on mycelial growth of *P. digitatum* incubation at 25 ^{o}C for 3 d. A: *P. digitatum* on PDA control plate, B, C, D, and E: plates supplementing with 0.5, 1, 1.5 and 2% (w/v) sodium bicarbonate, respectively.

Table 3.5 Effect of various concentrations of sodium bicarbonate on mycelial growth of *P. digitatum* after incubation at 25 ^{o}C for 3 d

Concentration of $NaHCO_3$ (%w/v)	% hyphal growth inhibition of *P. digitatum* [1]
0.5	99±0.1a
1.0	100±0.0a
1.5	100±0.0a
2.0	100±0.0a

[1]Values within columns followed by the same letter are not significantly different according to the LSD test at $P \leq 0.05$.

3.6.2 *In vitro* spore germination inhibition

Germination of *P. digitatum* spores incubated in PDB with 0.5, 1.0, 1.5 and 2 %(w/v) sodium bicarbonate were observed under a compound light microscope after 24 h-exposure (Figure 3.9). At 24 h 100 % spore germination of *P. digitatum* normal growth in the control tube was revealed (Figure 3.9A). However, complete spore germination inhibition of *P. digitatum* was detected in the medium supplemented with all concentrations of sodium bicarbonate (Figure 3.9B).

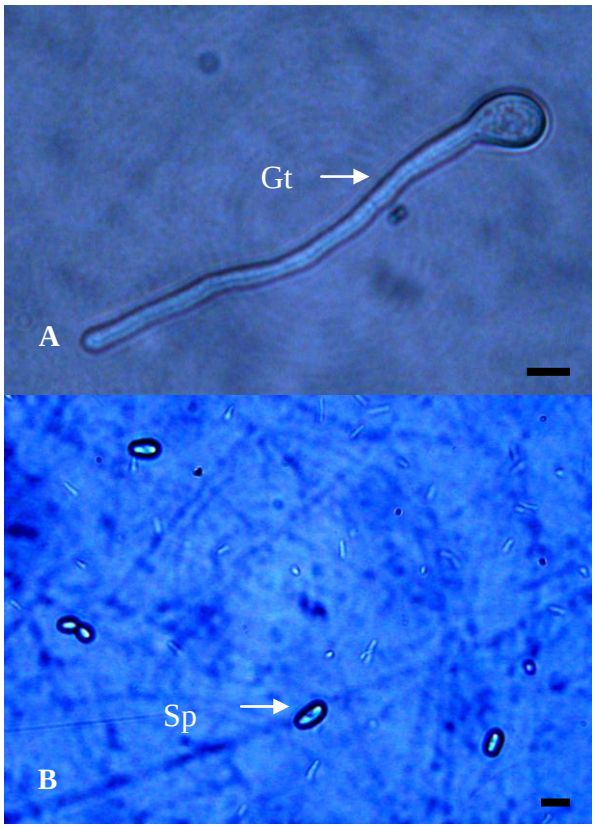

Figure 3.9 Microscopic examination of *P. digitatum* spores and germ tubes incubated in PDB containing 0, 0.5, 1, 1.5 and 2 % (w/v) sodium bicarbonate and lactophenol cotton blue staining. A: *P. digitatum* germ tubes after 24 h-exposure in non-treated medium; B: treated with 0.5-2% (w/v) sodium bicarbonate. Sp=spore; Gt=germ tube. Bars=5µm.

3.6.3 *In vivo* assay of antifungal effect of sodium bicarbonate on *P. digitatum* growth

In vivo fungal inhibition of sodium bicarbonate against *P. digitatum* were examined by co applying mandarin cv. Shogun simultaneously with sodium bicarbonate at concentrations of 0.5, 1, 1.5, 2% (w/v) and following with *P. digitatum* spore suspension (10^4 spores/ml) inoculation. Five days following application the significant disease reduction of treated fruits with sodium bicarbonate at all concentrations (Table 3.6). The highest level of control fruit rot was achieved at 2% (w/v) sodium bicarbonate treatment and disease incidence was significantly low at 29.81%, while the *P. digitatum* infected fruit showed 100% disease incidence. Treatments with 1 and 1.5% (w/v) sodium bicarbonate also affected *P. digitatum* growth markedly with 35.7 and 37.7% disease incidences respectively. Furthermore, on the day 5 after storage (Figure 3.10) delay green mold rot symptoms were exhibited in most of citrus fruits treated with sodium bicarbonate at all concentrations whereas disease was shown on control fruits within 3 d.

Table 3.6 Disease incidence (%) on citrus fruits after treated with sodium bicarbonate at different concentrations at 25 °C for 5 d

Treatment	Disease incidence (%)
P. digitatum (10^4 spores/ml)	100.0±0.5d
0.5%$NaHCO_3$+*P.digitatum*	57.6±1.2c
1%$NaHCO_3$+*P.digitatum*	35.7±0.3ab
1.5%$NaHCO_3$+*P.digitatum*	37.7±0.2ab
2%$NaHCO_3$+*P.digitatum*	29.8±0.5a

[1]Mean values within columns followed by same letter are not significantly different according to the LSD test at $P \leq 0.05$.

Figure 3.10 Effect of various concentrations of sodium bicarbonate on green mold rot in mandarin cv. Shogun, incubated at 25 ^{o}C under high relative humidity for 5 d. A: Fruit was inoculated with *P. digitatum* (control), B, C, and D, Fruits were treated with 0.5, 1, 1.5 and 2.0% (w/v) sodium bicarbonate, respectively and following with *P. digitatum* inoculation.

3.7 Biocontrol assay of antagonistic yeast and bacteria on citrus fruit against *P. digitatum* compared with sodium bicarbonate

In vivo antagonistic microorganisms against *P. digitatum* were examined by treating citrus mandarin cv. Shogun simultaneously with cell suspension of *P. gulliermondii* BCC 5389 (10^8 cell/ml), *B. subtilis* ABS-S14 endospores (10^8 CFU/ml), mixing antagonists of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 (10^8 cell/ml), and *P. digitatum* spore suspension (10^4 spores/ml). Imazalil (500 ppm) and 2% (w/v) sodium bicarbonate were used as fungicide and food additive control agents. Complete control of fruit rot disease was accomplished in five days following co application of antagonists of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 treatment. Meanwhile, fruits treated with 2% (w/v) sodium bicarbonate, *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 were 49.5, 56.2 and 70.3% disease incidence, respectively. Meanwhile, 92.9% disease incidence was detected in *P. digitatum* inoculated citrus fruit (Table 3.7). Interestingly, mixture of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 had greater effect on controlling fruit rot than using *P. guilliermondii* BCC 5389 or *B. subtilis* ABS-S14 individually. Sodium bicarbonate showed nearly the same effect as yeast cell did on fruit rot control. Furthermore, (Figure 3.11) on the day 5 after storage, decay symptom was not exhibited in citrus fruits treated with the mixture of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14. Same phenomenon was observed in non-treated fruits and fruits treating with imazalil.

Table 3.7 Disease incidence (%) on wounded citrus fruits with different treatments and incubation at 25 ^{o}C for 5 d

Treatment	Disease incidence (%)[1]
Sterile distilled water	0.0 ±0.0a
Imazalil (500 ppm)+ *P. digitatum**	0.0 ±0.0a
*P. digitatum**	92.9 ±0.5d
2%(w/v) $NaHCO_3$+ *P. digitatum**	49.5 ±1.9bc
P.guilliermondii +*P. digitatum**	56.2 ±2.1c
B. subtilis + *P. digitatum**	70.3 ±1.7cd
Mixture of *P guilliermondii* and *B. subtilis* +*P. digitatum**	0.0 ±0.0a

**P. digitatum* (10^4 spores/ml); *B. subtilis* ABS-S14 (10^8 CFU/ml); *P. guilliermondii* BCC 5389 (10^8 cells/ml); and mixture of *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml) and *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml).

[1]Mean values within columns followed by same letter are not significantly different according to the LSD test at $P \leq 0.05$.

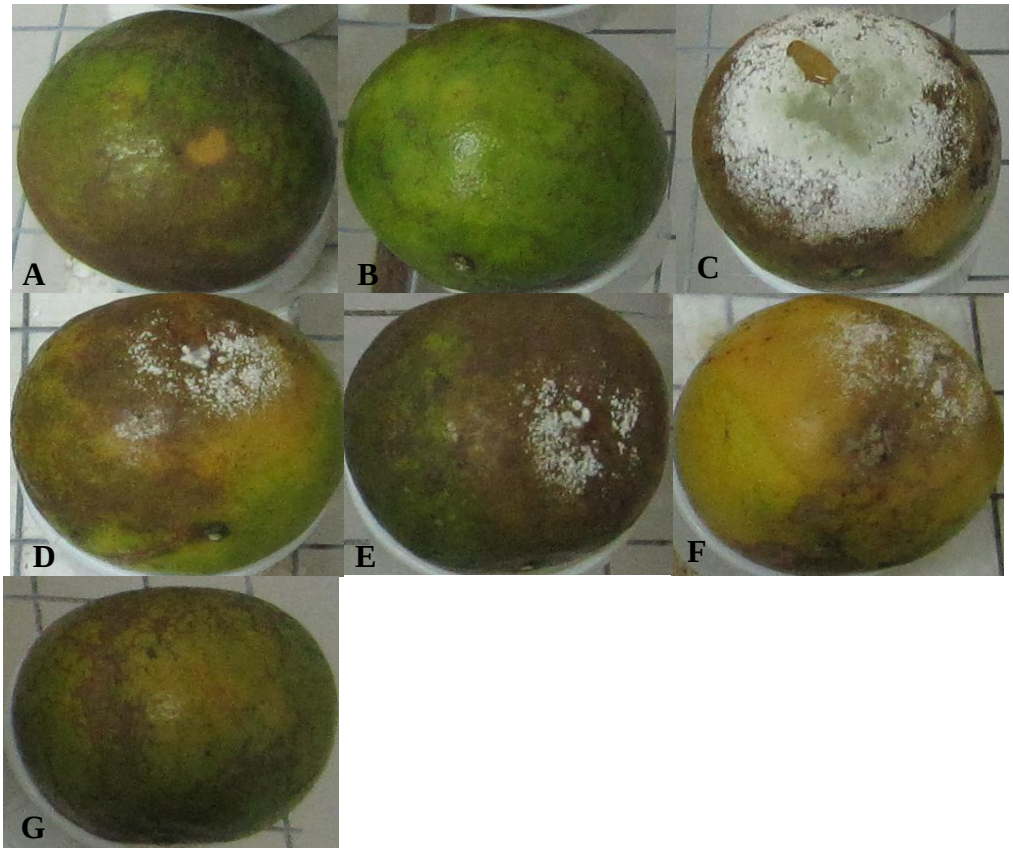

Figure 3.11 Effect of the antagonists on green mold rot control in mandarin cv. Shogun, incubation at 25 ^{o}C under high relative humidity for 5 d. A: non-treated fruit; B: Imazalil (500 ppm); C: *P. digitatum* (10^4 spores/ml); D: sodium bicarbonate (2% w/v); E: *P. guilliermondii* BCC 5389 (10^8 cells/ml); F: *B. subtilis* ABS-S14 (10^8 CFU/ml) and G: Mixture of *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml) and *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml).

3.8 Effect of the antagonistic microorganisms and sodium bicarbonate on fruit quality

Effect of antagonists on postharvest quality of mandarin cv. Shogun fruits was evaluated by dipping citrus fruits in the mixture solution of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 (10^8 cells/ml) and combined antagonists and sodium bicarbonate (2% w/v). Sterile distilled water was used as control, and stored for one week at 25 ^{o}C under high relative humidity.

3.8.1 Effect on weight loss of citrus fruit.

Following applications of mixture of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 or mixture of antagonists combined with sodium bicarbonate they showed non-significant differences in quality on weight loss of citrus fruits. Weight loss by 0.030 - 0.038% was obtained among these treated fruits (Table 3.8).

Table 3.8 Effect of antagonistic microorganisms in combination with sodium bicarbonate on weight loss of citrus fruit after incubating at 25 ^{o}C for 7 d

Treatment	**Weight loss (%)[1]**
Sterile distilled water	0.04±0.16a
P. guilliermondii + *B. subtilis**	0.04±0.51a
P .guilliermondii + *B. subtilis** + 2%(w/v) $NaHCO_3$	0.03±0.22a

*Mixture of *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml) and *B.subtilis* ABS-S14 (equal volume of 10^8 CFU/ml)

[1]Values within columns followed by the same letter are not significantly different according to LSD test at $P \leq 0.05$.

3.8.2 Effect on fruit firmness of citrus fruit.

Following application of mixture of *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 or mixture of antagonists combined with sodium bicarbonate they had no effect on fruit firmness in fruits before and after treatments (Table 3.9).

Table 3.9 Effect of antagonistic microorganisms in combination with sodium bicarbonate on fruit firmness of citrus fruit after incubating at 25 ^{o}C for 7 d

Treatment	Fruit firmness (g)[1]	
	Before treatment	After treatment
Sterile distilled water	665±49.17a	820±69.14a
P. guilliermondii + *B. subtilis**	635±49.17a	705±69.14a
P .guilliermondii + *B. subtilis** + 2%(w/v) $NaHCO_3$	580±49.17a	705±69.14a

*Mixture of *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml) and *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml)

[1]Values within columns followed by the same letter are not significantly different according to LSD test at $P \leq 0.05$.

3.8.3 Effect on total soluble solids content on citrus fruit.

Non-significant effect on total soluble solid content change of the various treated fruits was detected after storage. However, mixture *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 before treatment showed significant variation in TSS content at 4.96% (Table 3.10).

Table 3.10 Effect on total soluble solid content of citrus fruit after incubating at 25 ^{o}C for 7 d

Treatment	Total soluble solid contents[1]	
	Before treatment	After storage
Sterile distilled water	5.34±0.18a	5.20±0.36a
P. guilliermondii + *B. subtilis**	4.96±0.18b	5.62±0.36a
P .guilliermondii + *B. subtilis** + 2%(w/v) $NaHCO_3$	5.16±0.18a	5.42±0.36a

*Mixture of *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml) and *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml)

[1]Values within columns followed by the same letter are not significantly different according to LSD test at $P \leq 0.05$.

3.8.4 Effect on titratable acidity of citrus fruit.

Non-significant differences of titratable acidity of treated fruit and control fruits were detected. Slightly decrease in titratable acidity was founded in the treated fruits (Table 3.11).

Table 3.11 Effect on titratable acidity of citrus fruit after incubating at 25 ^{o}C for 7 d

Treatment	Titratable acidity (%citric acid)[1]	
	Before treatment	After treatment
Sterile distilled water	0.51±0.03a	0.55±0.96a
P. guilliermondii + *B. subtilis**	0.49±0.03a	0.47±0.96a
P .guilliermondii + *B. subtilis** + 2%(w/v) $NaHCO_3$	0.40±0.03a	0.42±0.96a

*Mixture of *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml) and *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml)

[1]Values within columns followed by the same letter are not significantly different according to LSD test at $P \leq 0.05$.

3.9 Effect of antagonistic yeast and *B. subtilis* on defense related enzyme activities of citrus fruit followed by *P. digitatum* inoculation

The citrus flavedo tissues were collected from mandarin cv. Shogun treated with the individual antagonist, or in combination with and without following pathogen inoculation at different time points to investigate the level of chitinase and *β*-1,3-glucanase activities. The induction of chitinase enzymes in treated flavedo tissues by antagonistic yeast and bacteria was evaluated. The chitinase patterns presented in Figure 3.12, Table 3.12 and 3.13 revealed that at 24 h incubation the chitinase activity was significantly induced by individual antagonist with and without pathogen inoculation. Subsequently, the enzyme level gradually declined at 48 h and 72 h incubation. The highest chitinase activity in *B. subtilis* ABS-S14 treated tissues with non-pathogen inoculation was shown at 48 h and then it was declined at 72 h. (Figure 3.12A). None of other treatment showed significant difference in chitinase induction in either with or without pathogen infection.

The induction of β-1,3-glucanase enzymes by antagonistic yeast and bacteria was evaluated as presented in Figure 3.13, Table 3.14 and 3.15. It was revealed that the β-1,3-glucanase activity was significantly increased during 0- 24 h in tissue treated with individual antagonist with and without following pathogen inoculation. The highest activity was observed at 48 h and then declined at 72 h. Surprisingly its level was significantly elicited by *P. digitatum* inoculation and *B. subtilis* ABS-S14 without pathogen inoculation (Figure 3.13A) at 24 h while other treatments showed enzyme induction at 48 h particularly in *P. digitatum* treated tissues. Then, the enzyme activities in all treatments were lowered at 72 h, especially *B. subtilis* ABS-S14 and distilled water treatment (Figure 3.13A). The induction of β-1,3-glucanase by co-inoculation of antagonist and *P. digitatum* was illustrated that in all treatments the glucanase activity levelof the flavedo tissues reached the highest peak at 48 h following *P. digitatum* inoculation treatment (Figure 3.13B). At 48 h-treatment *P. guillermondii* BCC 5389 following *P. digitatum* inoculation showed the highest enzyme activity elicitation. Obviously, the levels of glucanase activity in citrus flavedo were declined in all treatments.

Table 3.12 Chitinase activity in citrus flavedo in response to antagonist inoculation

Treatment	Chitinase activity (Unit/ml/gAP) in citrus flavedo[1]			
	0 h	24 h	48 h	72 h
Sterile distilled water	0.06±0.00ab	0.07±0.01fgh	0.06±0.02cde	0.07±0.01gh
P. digitatum	0.06±0.01ab	0.07±0.00fgh	0.07±0.01e-h	0.07±0.01e-h
B. subtilis	0.05±0.01a	0.07±0.01h	0.07±0.04h	0.07±0.02fgh
P. guilliermondii	0.06±0.01ab	0.07±0.01fgh	0.07±0.02d-g	0.07±0.01e-h
B. subtilis + P.guilliermondii	0.06±0.03abc	0.07±0.02e-h	0.07±0.01def	0.06±0.01bcd

[1]Chitinase activity in wounded citrus flavedo treated with sterile distilled water; *P. digitatum* (10^4 spores/ml); *B. subtilis* ABS-S14 (10^8 CFU/ml); *P. guilliermondii* BCC 5389 (10^8 cells/ml); mixture of *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml) and *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml). Mean±S.E. values bearing a different alphabet in the same column and row show significant differences according to DMRT at $P \leq 0.05$.

Table 3.13 Chitinase activity in citrus flavedo in response to antagonist and *P. digitatum* inoculation

Treatment	Chitinase activity (Unit/ml/gAP) in citrus flavedo[1]			
	0 h	24 h	48 h	72 h
Sterile distilled water	0.06±0.00a	0.07±0.01g	0.06±0.02bc	0.07±0.01g
P. digitatum	0.06±0.01a	0.07±0.00g	0.07±0.01c-f	0.07±0.01c-f
B. subtilis + *P. digitatum*	0.06±0.01a	0.07±0.00fg	0.07±0.02b-g	0.06±0.01b
P.guilliermondii + *P. digitatum*	0.06±0.02a	0.07±0.01g	0.07±0.01efg	0.06±0.01b-e
B. subtilis + *P.guilliermondii* + *P. digitatum*	0.06±0.02a	0.07±0.01d-g	0.07±0.01b-f	0.06±0.01bcd

[1]Chitinase activity in wounded citrus flavedo treated with sterile distilled water; *P. digitatum* (10^4 spores/ml); *B. subtilis* ABS-S14 (10^8 CFU/ml); *P. guilliermondii* BCC 5389 (10^8 cells/ml); and *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml) and *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml). Mean±S.E. values bearing a different alphabet in the same column and row show significant differences according to DMRT at $P \leq 0.05$.

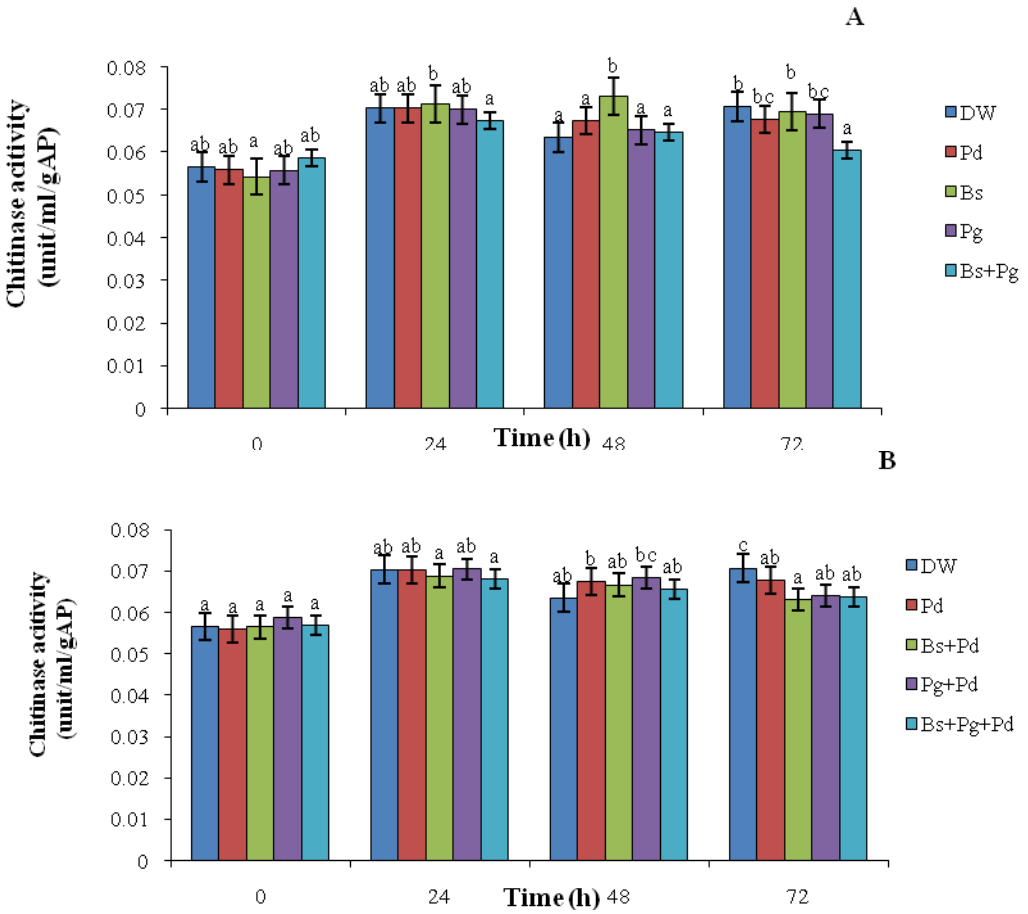

Figure 3.12 Changes in chitinase activity in flavedo tissues of citrus fruit of various treatments. A: Antagonist treatments; B: Antagonist treatments with following pathogen inoculation, wounded-peels treated with DW=distilled water, Pd=*P. digitatum*, Bs=*B. subtilis* ABS-S14, Pg= *P. guilliermondii* BCC 5389, Bs+Pg= mixture of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389. Bars represent the standard deviations of the mean. Values on bars with different letters are statistically different according to DMRT at $P \leq 0.05$.

Table 3.14 β-1,3-glucanase activity in citrus flavedo in response to antagonist inoculation

Treatment	β-1,3-glucanase activity (Unit/ml/gAP) in citrus flavedo[1]			
	0 h	24 h	48 h	72 h
Sterile distilled water	1.08±0.02a	6.25±0.01b	9.13±0.02fg	6.49±0.03bc
P. digitatum	1.20±0.00a	7.94±0.01de	9.52±0.01g	7.19±0.00cd
B. subtilis	1.08±0.01a	8.27±0.00ef	8.33±0.01ef	5.92±0.01b
P. guilliermondii	1.18±0.00a	7.13±0.01cd	8.62±0.03ef	7.19±0.00cd
B. subtilis + *P.guilliermondii*	1.10±0.00a	7.12±0.01cd	8.62±0.02ef	7.18±0.01cd

[1] β-1,3-glucanase activity in wounded citrus flavedo treated with sterile distilled water; *P. digitatum* (10^4 spores/ml); *B. subtilis* ABS-S14 (10^8 CFU/ml); *P. guilliermondii* BCC 5389 (10^8 cells/ml); mixture of *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml) and *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml). Mean± S.E. values bearing a different alphabet in the same column and row show significant differences according to DMRT at $P \leq 0.05$.

Table 3.15 β-1,3-glucanase activity in citrus flavedo in response to antagonist and *P. digitatum* inoculation

Treatment	β-1,3-glucanase activity (Unit/ml/gAP) in citrus flavedo[1]			
	0 h	24 h	48 h	72 h
Sterile distilled water	1.08±0.02a	6.25±0.01bc	9.13±0.02ef	6.49±0.03bc
P. digitatum	1.20±0.00a	7.94±0.01cde	9.52±0.01ef	7.19±0.00bcd
B. subtilis + *P. digitatum*	1.11±0.01a	7.04±0.00bc	10.24±0.01f	6.92±0.01bc
P.guilliermondii + *P. digitatum*	1.22±0.01a	8.96±0.02def	10.42±0.01f	6.87±0.01bc
B. subtilis + *P.guilliermondii* + *P. digitatum*	1.18±0.00a	7.71±0.00b	7.89±0.01cde	6.72±0.01bc

[1] β-1,3-glucanase activity in wounded citrus flavedo treated with sterile distilled water; *P. digitatum* (10^4 spores/ml); *B. subtilis* ABS-S14 (10^8 CFU/ml); *P. guilliermondii* BCC 5389 (10^8 cells/ml); *B. subtilis* ABS-S14 (equal volume of 10^8 CFU/ml) and *P. guilliermondii* BCC 5389 (equal volume of 10^8 cells/ml). Mean±S.E. values bearing a different alphabet in the same column and row show significant differences according to DMRT at $P \leq 0.05$.

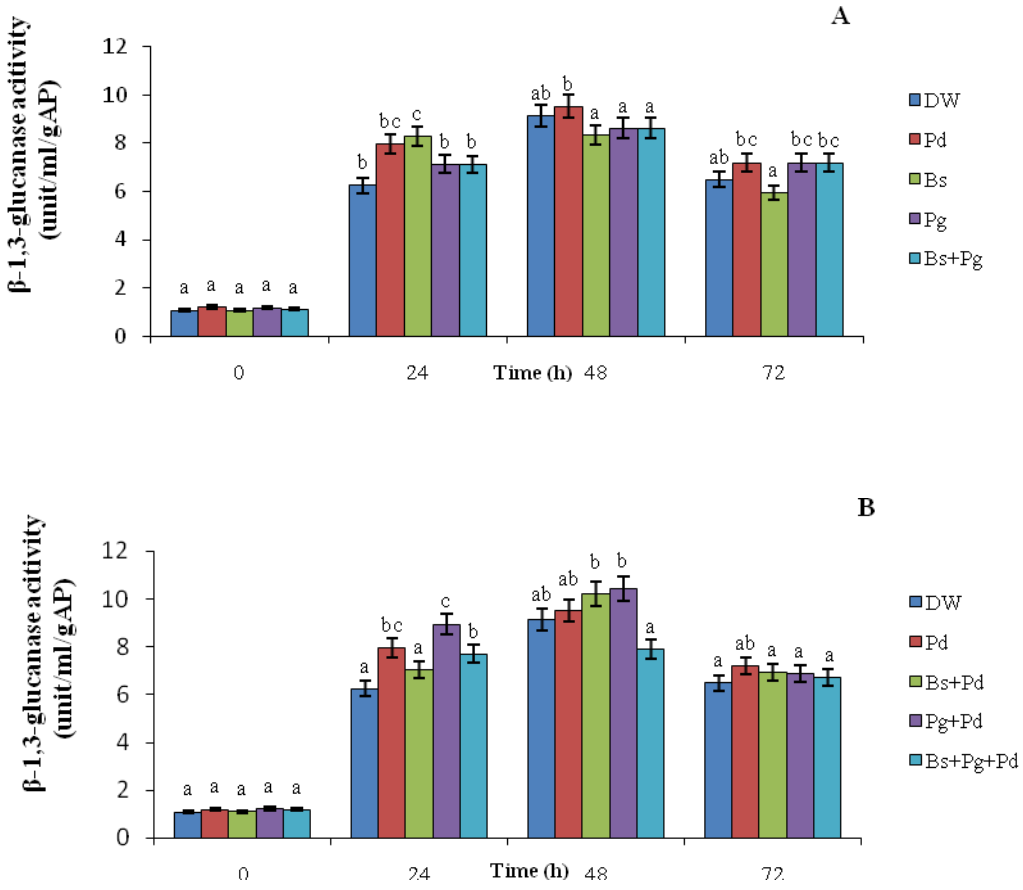

Figure 3.13 Changes in *β*-1,3-glucanase activity in flavedo tissues of citrus fruit of various treatments. A: Antagonist treatments; B: Antagonist treatments with following pathogen inoculation, wounded-peels treated with DW= distilled water, Pd =*P. digitatum* only, Bs=*B. subtilis* ABS-S14, Pg= *P. guilliermondii* BCC 5389, Bs+Pg= mixture of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389. Bars represent the standard deviations of the mean. Values on bars with different letters are statistically different according to DMRT at $P \leq 0.05$.

3.10 Effect of antagonistic yeast and *B. subtilis* on defense related gene transcription followed by *P. digitatum* inoculation

The citrus flavedo tissues were collected from mandarin cv. Shogun treated with the individual antagonist, or in combination with and without following pathogen inoculation at different time points to investigate the level of *CHI and GLU* gene expression.

The transcription level of *CHI* gene in treated flavedo tissues by antagonistic yeast and bacteria was shown in Figure 3.14. An individual antagonist following with and without pathogen inoculation could elicit the *CHI* gene, but at different times. The highest *CHI* gene level was significantly elicited at 24 h in treated fruit treating with *B. subtilis* ABS-S14 (Figure 3.14A) and *P. guilliermondii* BCC5389 following *P. digitatum* inoculation (Figure 3.14B), then continuously declined at 48 h and gradually increased again at 72 h, whereas the flavedo tissues obtained from other treatments expressed high level at 48 h and declined at 72 h (Figure 3.14A and B).

The transcription level of *GLU* gene was showed in Figure 3.15. No significantly different in time period of *GLU* expression. Unexpectedly, a few *GLU* gene was induced at 24, 48 and 72 h except tissues treated with pathogen and co-inoculation of yeast and bacteria which highly expressed with significant different at 24 h, then declined at 48 h. Only co-inoculation of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389 induced gene transcription twice at 72 h in citrus flavedo tissues (Figure 3.15A). The transcription level of *GLU* gene gradually increased 48 h in treatment of co-inoculation of *B. subtilis* ABS-S14 and *P. digitatum* and subsequently, the transcription level declined at 72 h, other treatments showed slight *GLU* gene expression (Figure 3.15B).

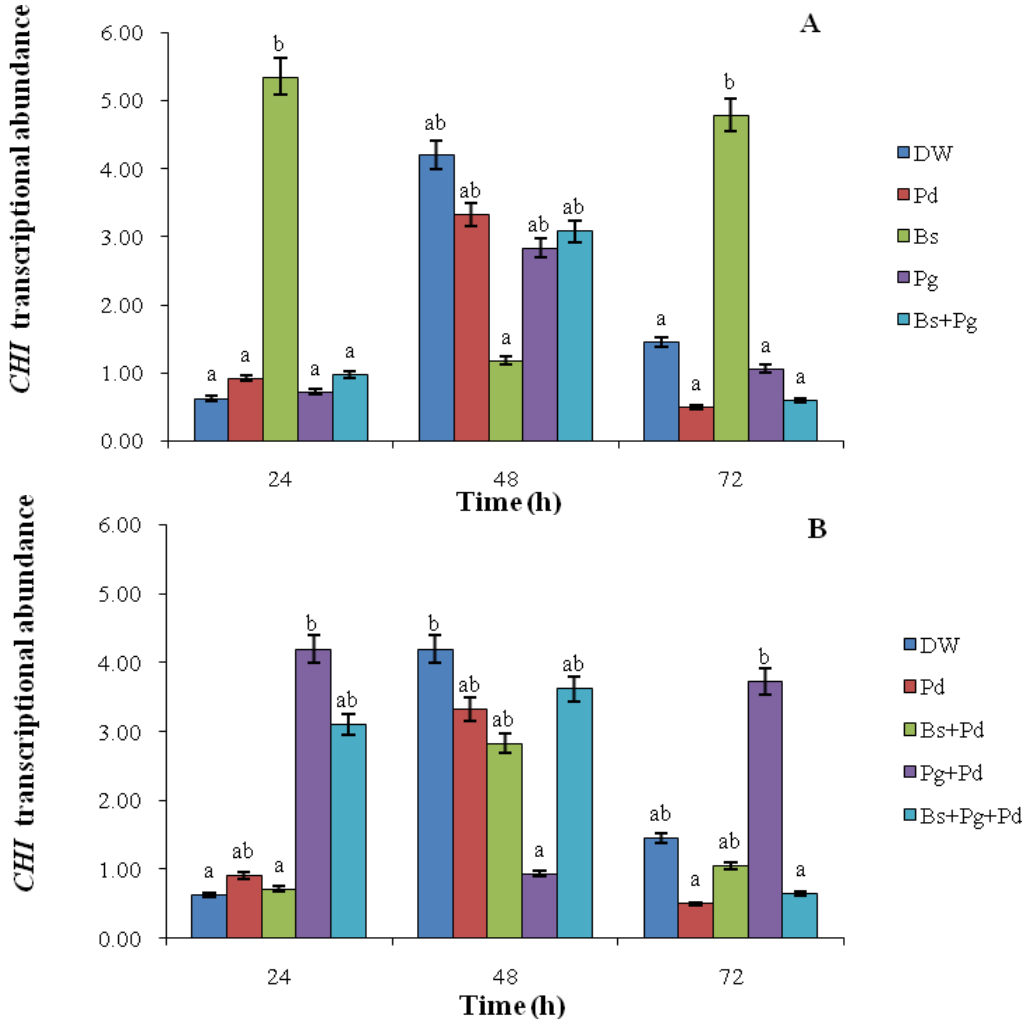

Figure 3.14 Expression level of chitinase gene in flavedo tissues of citrus fruit of various treatments. A: Antagonist treatments; B: Antagonist treatments with following pathogen inoculation, wounded-peels treated with DW= distilled water, Pd =*P. digitatum* only, Bs=*B. subtilis* ABS-S14, Pg= *P. guilliermondii* BCC 5389, Bs+Pg= mixture of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389. Bars represent the standard deviations of the mean. Values on bars with different letters are statistically different according to DMRT at $P \leq 0.05$.

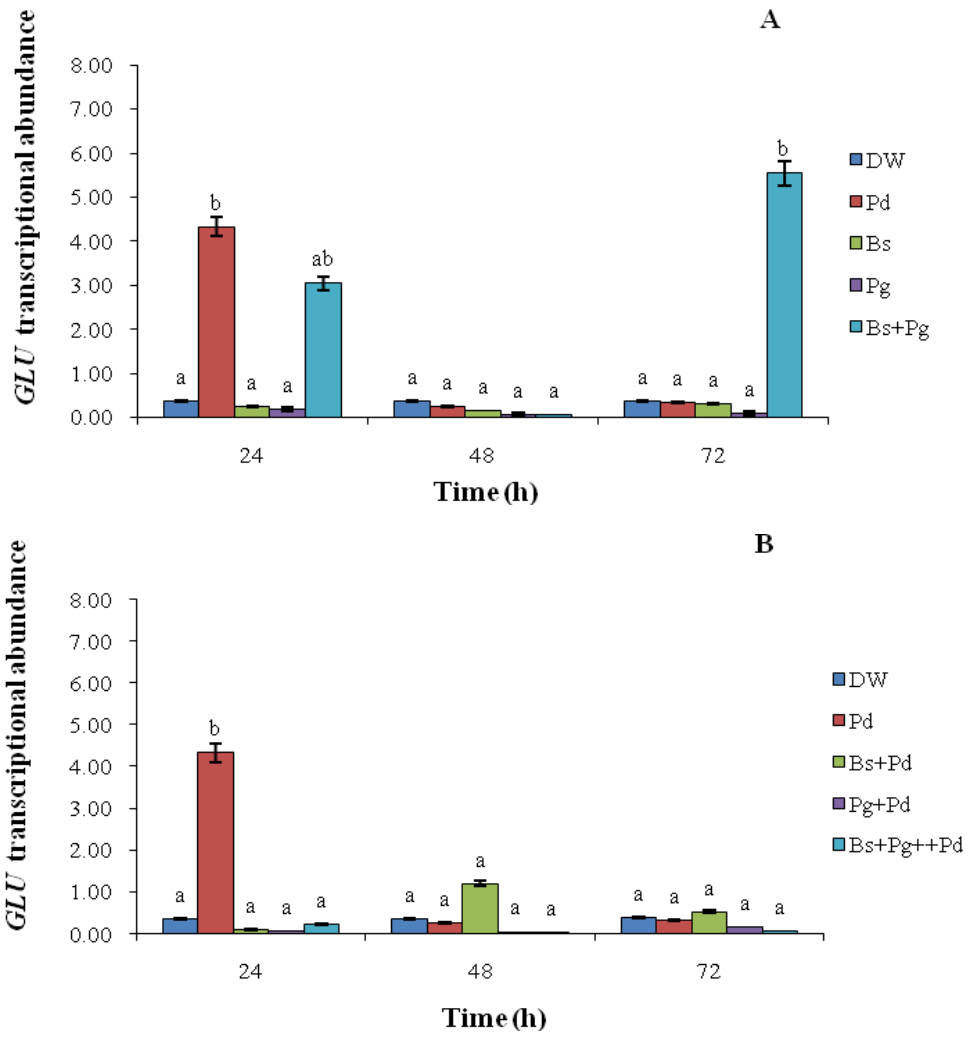

Figure 3.15 Expression level of glucanase gene in flavedo tissues of citrus fruit of various treatments. A: Antagonist treatments; B: Antagonist treatments with following pathogen inoculation, wounded-peels treated with DW= distilled water, Pd =*P. digitatum* only, Bs=*B. subtilis* ABS-S14, Pg= *P. guilliermondii* BCC 5389, Bs+Pg= mixture of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389. Bars represent the standard deviations of the mean. Values on bars with different letters are statistically different according to DMRT at $P \leq 0.05$.

Chapter 4

DISCUSSION

4.1 *In vitro* screening of antagonistic yeast isolates and *B. subtilis* to citrus fruit rot pathogen, *P. digitatum.*

The potential to elicit biocontrol efficiency of fungal diseases has been studied for more than 40 years (Papon *et al*., 2013) because of its biotechnological and clinical significance. Many reports have found that the yeast *P. guilliermondii* was a good biological agent to control postharvest fruit rot, such as citrus rot caused by *P. digitatum* (Larralde-Corona *et al*., 2011), *P. italicum* (Lahlali *et al*., 2011), blue mold caused by *P. expansum* (Gholamnejad and Etebarian, 2009), chilli anthracnose caused by *C. capsici* (Chanchaichaovivat *et al*., 2008, Nantawan *et al*., 2010) and gray mold caused by *Botrytis cinerea* (Wszelaki and Mitcham, 2003; Zhao *et al*., 2010). As mentioned in the literature review, *B. subtilis* is also a good biocontrol agent for many different types of fruits such as citrus (Obagwu and Korsten, 2003) and avocado (Demoz and Korsten, 2006). In this study it was found that *P. guilliermondii* BCC 5389 and all strains of *B. subtilis* were tested effective in inhibiting *P. digitatum in vitro* situation. *B.subtilis* and *P. guilliermondii* grew together with no signs of antagonism. Furthermore, an efficacy was enhanced when applied together.

The effect of culture filtrates of *P. guilliermondii* BCC 5389 on the radial growth of *P. digitatum* was investigated. Undiluted culture filtrates of *P. guilliermondii* BCC 5389 were effective in retarding the growth of *P. digitatum*. The abnormalities in fungal structure such as swelling of the hyphal tips of *P. digitatum* were shown after exposure to undiluted culture filtrates. These results were consistent with the other studies that had indicated that *P. guilliermondii* became strongly attached to the hyphae of *R. stolonifer* in cherry tomato fruit (Zhao *et al*., 2010).

The germination of *P. digitatum* spores incubated in PDB with *P. guilliermondii* was significantly inhibited by cell suspension of *P. guilliermondii*. A

cell suspension of *P. guilliermondii* at 10^7 and 10^8 cells/ml completely inhibited germination, whereas 10^6 cells/ml of *P. guilliermondii* showed a 42.0% inhibition. Observations made at 24 h revealed abnormal germ tube structures of *P. digitatum* compared with a normal germinating control. Chanchaichaovivat *et al*. (2008) showed that *P. guilliermondii* strain R13 suppressed *C. capsici* spore germination and germ tube length. Poppe *et al*. (2003) found that *P. agglomerans* CPA-2 completely inhibited spore germination of *P. digitatum*. Lahlali *et al*. (2011) found that *P. guillermondii* strain Z1 at a concentration $1x10^8$ cells/ml significantly inhibited the growth of *P. italicum*. This indicated that the mode of action was by competition for nutrient. In our study spore germination of *P. digitatum* was shown to be completely inhibited by the culture filtrate of *P. guilliermondii* BCC 5389 at an undiluted and a 2X fold serial dilution, whereas a 4X and 8X fold serial dilution were not effective. Observations made at 24 h revealed abnormal germ tube structures of *P. digitatum* when compared to the control with normal germination in contrast to the report of Zhao *et al*. (2008) who observed that a culture filtrate of *P. guilliermondii* had no effect on the *R. nigricans* pathogen even though its living cells did control *R. nigricans* on tomato fruit. Luo *et al*. (2012) indicated that a culture filtrate of *P. membranefaciens* had no effect, but living yeast cell and washed cell suspension did inhibit *P. digitatum* and *P. italicum* after treated with a cell suspension of *P. membranefaciens* at $1x10^8$ cells/ml.

4.2 Effect of sodium bicarbonate on the growth of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389

Postharvest chemicals used to treat pathogens of tropical fruits have resulted in a serious problem of developing resistant strains. Various additives have been shown to increase the effectiveness of some antagonists in controlling postharvest decay such as sodium bicarbonate (Zhang and Swingle, 2003; Obagwu and Korsten, 2003; Conway *et al*., 2005; Conway *et al*., 2007; Janisiewicz *et al*., 2008), salicylic acid (Yu *et al*., 2007; Zhang *et al*., 2008), calcium chloride (Cao *et al*., 2008; Gholamnejad and Etebarian, 2009). The most interesting finding was that

1% sodium bicarbonate was effective in controlling the mycelium growth of the *P. digitatum*. It consisted with other research that demonstrated that 1% sodium bicarbonate inhibited colonial growth by inducing the development of abnormal mycelia (Ferreira-Pinto *et al*., 2008). Present study demonstrated that the growth of *P. guilliermondii* BCC 5389 in the presence of 0.5 and 2.0% sodium bicarbonate was lower than the control treatment whereas there was no effect on the growth of *P. guilliermondii* BCC 5389 at 1.0 and 1.5% sodium bicarbonate. Puawongphat *et al*. (2008) reported that *C. utilis* grew in the presence of 2% sodium bicarbonate. Also the growth pattern of *B. subtilis* during its log phase to stationary phase showed no significant inhibition with any treatment, but some grew better than the control. Obagwu and Korsten (2003) reported that sodium bicarbonate at 1.0-3.0% showed no effect on *B. subtilis* F1 growth while the growth of *B. subtilis* F1 was inhibited by 5.0% sodium bicarbonate. The germination of *P. digitatum* spores incubated in PDB containing 0.5, 1.0, 1.5 and 2% sodium bicarbonate were observed after 24 h of incubation. All concentrations of sodium bicarbonate showed complete inhibition of the germination of spores of *P. digitatum*. A similar result was found by Smilanick and Margosan (1999) who observed that sodium bicarbonate concentrations were effective in inhibiting the germination of *P. digitatum*. The mechanism of carbonic acid salt reduced the fungal turgor pressure resulting in the collapse and shrinkage of hyphae causing subsequent inhibition of sporulation (Fallik *et al*., 1997). Furthermore, pH 8.5 of sodium bicarbonate has been reported to injure the fungus *Sclerotium rolfsii* (Punja and Grogan, 1982).

4.3 Assay for the biocontrol by antagonistic yeast and bacteria on *P. digitatum* infected citrus fruit compared to sodium bicarbonate.

The highest level of control of fruit rot of mandarin cv. Shogun was achieved with 2% sodium bicarbonate. The disease incidence of citrus fruits treated with 2% sodium bicarbonate was a significant reduced 29.8% after 5 days at 25^{o}C. Zhu *et al.* (2013) showed that 5% sodium bicarbonate with 10^{7} cells/ml of *Rhodosporidium paludigenum* was as effective as 500 ppm fungaflor to control the

green mould rot of citrus fruit (*Citrus reticulata* Blanco cv. Subcompressa) and 2% sodium bicarbonate with 10^8 cells/ml of *R. paludigenum* was effective in controlling green mold rot of citrus fruit (*C. reticulata* Blanco cv. Ponkan). Moreover, Obagwu and Korsten (2003) indicated that the treatment comprising *B. subtilis* F1 combined with sodium bicarbonate applied following hot water treatment was as effective as the fungicide treatment that controlled green and blue mould by 100 %.

The highest level of control of fruit rot disease of Shogun mandarin was achieved with mixtures of *P.guilliermondii* BCC 5389 and *B. subtilis* ABS-S14. The disease incidence of citrus fruits treated with 1 x 10^8 spores/ml of combined *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 was 0% after 5 days at 25^{o}C, which was significantly lower than that of untreated (92.94%) and all other treatments. Similar result from Zong *et al.* (2010) indicated that the combination of antagonists, *C. guilliermondii*, and *P. membranaefaiens* and hot water treatment controlled *Botrytis cinerea* in tomato fruit better than the untreated. Liu *et al.* (2010) also reported that *P. guilliermondii* combined with heat treatment decreased anthracnose rot in loquat fruit by significantly inhibited spore germination and mycelial growth. Lu *et al*. (2013) also indicated that application of *R. paludigenum* at concentrations of 1x10^8 and 1x10^9 cells/ml reduced an infection from *P. digitatum* in citrus fruits.

In order to evaluate the efficacy of the combined antagonists and with sodium bicarbonate, they showed no significant effect on the quality of fruit parameters; weight loss, fruit firmness, total soluble solids, titratable acidity after storage at 25^{o}C for 7 days. In addition, it was shown that the combination did not impair the fruit quality parameters under commercial conditions and indicated that there was a significant potential for commercialization. Similarly, Zhao *et al*. (2011) showed that preharvest spraying with *P. guilliermondii* did not affect the quality parameters of fruit. In conclusion, the result in this research has highlighted the effectiveness of a combination of yeast and bacteria for use as a non-chemical alternative for treatment against postharvest diseases of Shogun mandarins. Future research will be aimed at investigating the mode of action of the combination on controlling postharvest diseases of fruits.

4.4 Effect of antagonistic yeast and bacteria on the induction of plant defense-related enzyme activities.

The induction of defense-related proteins had been reported in several plants to protect against pathogens or insect attack. The 17 families of PRs have been classified and most of them are induced through salicylic acid, jasmonic acid, or ethylene signals. Many defense-related proteins are induced in the senescence period, by wounding or cold stress (Van Loon *et al*., 2006; Jiang *et al*., 2009). The induction of defense responses occurs by treatment with several elicitors such as salicylic acid (SA), oxalic acid, calcium chloride, and antagonistic yeast that could enhance β-1,3-glucanase, phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase activities (Tian *et al*., 2006). Chitinase has been suggested to be involved in plant defense responses against fungal infections (Sharma *et al*., 2011). Droby *et al*. (2002) revealed that the induction of pathogen resistance from *P. digitatum* in citrus fruit appeared 24 h after being elicited by *C. oleophila.*

The induction of chitinase enzymes by antagonistic yeast and bacteria was evaluated. The chitinase patterns obtained in this work revealed that the chitinase activity gradually increased 24 h after incubation with individual antagonists or by pathogen attack and after that the enzyme continuously declined at 48 and 72 h. With an exception for treatment with *B. subtilis* ABS-S14 increased up to 48 h and decreased at 72 h. Moreover, it was shown in this study that the induction of β-1,3-glucanase activity was the highest at 48 h particularly after treatment with *P. guilliermondii* BCC 5389, or with a combined *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 , also after a co-inoculation of *B. subtilis* ABS-S14 and *P. digitatum* and co-inoculation of *P. guillermondii* BCC 5389 and *P. digitatum* except that for *B. subtilis* ABS-S14 that induced the enzyme at 24 and 48 h and then continuously declined at 72 h. There have been several similar reports showed that *P. guilliermondii* induced chitinase and β-1,3-glucanase activity in several fruits (Zhao *et al*., 2008; Liu *et al*., 2010; Zhao *et al*., 2011; Xu and Du, 2012). Also, activity of β-1,3- glucanase was induced in jujube fruit wounding between 24 and 48 h after treated with *C. laurentii* (Tian *et al*., 2007). Furthermore, Xu *et al*. (2008) showed that *C. guilliermondii* stimulated chitinase and β-1,3-glucanase activities in peach fruit. Luo

et al. (2012) also showed that *P. membranefacienns* induced chitinase and β-1,3-glucanase activities in citrus fruit peel. In addition, Ballester *et al.* (2010) found that chitinase and β-1,3-glucanase activities including PR proteins were increased in the albedo and flavedo of citrus fruits after inoculating oranges with *P. digitatum* and 1 d later fruits were exposed to a hot air treatment at 37 ^{o}C for 3 d. Ippolito *et al.* (2000) found that chitinase and β-1,3-glucanase were induced at 24 h after treated with *A. pullulans* on apple fruit and then the high level increased at 48 and 96 h. However, chitinase activity was lower than that of β-1,3- glucanase activity. Moreover, these enzyme activities could induce by wounding although the level of enzyme was markedly lower than in treated fruit whereas in non-wounded treatment showed small increase in these enzyme activites. Also, Droby *et al*. (2002) reported that the accumulation of chitinase had smaller effect than β-1,3-glucanase when exposure of grapefruit to UV light. All of these reports are in agreement with the phenomena occurring in this work that wounding and pathogen (*P. digitatum*) inoculation on the citrus peel could elicit the enzyme induction and β-1,3-glucanase in flavedo tissues of citrus fruit. This enzyme seems to be rather sensitive to this physical treatment, pathogen attack and antagonist application than chitinase. Weak induction of enzyme activity could be associated with the developmental stage of the plant that chitinase activity was highest at the immature stage of fruit development whereas glucanase activity was at its lowest or the ability to synthesize defense responses would decline when fruit ripening (McCollum *et al*., 1997) El Ghaouth *et al.* (2002) showed the increase of chitinase and β-1,3-glucanase activities in fresh apples were higher than in stored fruit when treated with *C. saitoana* in wounding apple fruit. Meanwhile, Droby *et al*. (2002) reported that viable yeast cells of *C. oliophila* at concentrations of $1x10^{8}$ and $1x10^{9}$ cells/ml could induce resistance mechanism in citrus fruit which chitinase protein accumulation had a much smaller effect on the accumulation of β-1,3-glucanase. Both chitinase and β-1,3-glucanase activities were moderately increased at 48 h of water-treated control whereas 24 h of fruit dipped in *C. oleophila* an increase continued and intensified over a period of up to 7 days.

It is well known that the gene-for-gene theory is a pattern of plant disease resistance. A specific resistance (R) gene from plants recognizes a pathogen that carries the corresponding avirulence gene. These situation activated defense

responses including the hypersensitive response (HR) that continuously enhanced a systemic acquired resistance (SAR) (Glazebrook, 2001). All treatments conducted in present study could elicit the *CHI* gene but at different times. Upon challenge by a pathogen *CHI* transcription in citrus fruit treated with *P. guilliermondii* BCC5389 or *B. subtilis* ABS-S14 showed a similar pattern. Their transcription level was gradually increased until 24 h then declined at 48 h after incubation. After that the transcription level increased again at 72 h whereas with other treatments, the *CHI* transcription level in citrus fruit increased to its highest level at 48 h then continuously declined at 72 h after incubation. Meanwhile, the highest level of *GLU* expression occurred at 24 h in fruit treated with pathogen or in combination with the antagonistic yeast and bacteria then decreased at 48 h. However, *GLU* expression induced by the combination increased again at 72 h. Similarly, Xu *et al*. (2008) reported that the expressions of *CHI* and *GLU* genes were produced during storage when treated with four antagonistic yeasts, *C. guilliermondii*, *C. laurentii*, *P. membranaefaciens* and *R. glutinis.* The expression of *CHI* gene was significantly increased 48 h after inoculation and treated with *P. membranaefaciens* and *C. laurentii* whereas in the control fruit with pathogen inoculation, expression of *CHI* increased at the first day then gradually decreased. Meanwhile, the expression of *GLU* gene was induced at 48 h in the disease control fruit but there was no obvious difference among different yeast treatments. Zhang *et al*. (2013) founded that UV-C enhanced efficacy of *C. laurentii* in tomato fruit and induced the β-1,3-glucanase gene increasing at 72 h after incubation. However, Lui *et al.* (2013) found that *PR*-8 or *CHI* gene induction was induced by treatment of the wounds with *C. oleophila* and *B. cinerea* on apple fruit at 8-48 h. Moreover, Tian *et al*. (2007) supported that *Glu-1* and *Glu-2* genes were expressed and responded to wounding by increased gene expression in jujube fruit when treated with *C. luarentii* and expression pattern after yeast inoculation found that *Glu-1* expression level was induced at 48 h whereas *Glu-2* expression was unaffected by yeast inoculation.

In conclusion, the results present in this work showed that antagonistic yeast had direct and indirect inhibitory effects on *P. digitatum* growth. A combination of yeast and bacteria significantly control fruit rot and did not impair quality parameters of citrus fruits. All these results suggested that the combination of yeast

and bacteria may have potential for commercialization for integrated management of citrus disease. However, the important limitations of biocontrol are a lack of eradicative activity and a narrow spectrum of activity compare with synthetic fungicides (Conway *et al*., 2004). These results suggested that sodium bicarbonate application pre-protected the fruit in processing of packing house for pathogen eradication that contaminated from the orchard whereas treatment of antagonistic combination could preserve harvested fruit to prolong shelf life through ending up on the market. Considering that the expression of gene (*CHI* and *GLU* gene) and enzyme activity (chitinase and β-1,3-glucanase) in citrus fruit in response to *P. digitatum* or without pathogen infection were higher induced in response to *B. subtilis* ABS-S14 or *P. guillermondii* BCC 5389 than to the pathogen. More detailed studies will be required to understand a mechanism of the antagonistic yeast and bacteria could induce gene expression and developing the formulation is consequently needed to produce the effective biocontrol agent under large-scale operations.

Chapter 5

CONCLUSIONS

1. *P. guilliermondii* BCC 5389, *B. subtilis* ABS-S14 and 1-2% (w/v) sodium bicarbonate solution are antagonists to *P. digitatum* by suppression the fungal growth on agar plate and on infected citrus fruit. The highest level of control fruit rot was achieved by 2% (w/v) sodium bicarbonate treatment and disease incidence was shown to be 29.8%. Combination treatment of citrus with *P. guilliermondii* BCC 5389 and *B. subtilis* ABS-S14 showed complete disease control reduction.

2. At 10^6 to 10^8 cells/ml of *P. guilliermondii* BCC 5389 and its culture filtrate significantly affected spore germination of *P. digitatum*. An abnormality and severe damage of mycelial wall and absence of germ tube germination were observed when they exposed to *P. guilliermondii* at 10^7 and 10^8 cells/ml.

3. No antagonism between *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389 was detected on agar plate. Also sodium bicarbonate at 0.5-2.0 % (w/v) showed no effect on growth of *B. subtilis* ABS-S14, but it retarded the growth of *P. guilliermondii* BCC 5389.

4. The combined antagonists and sodium bicarbonate showed non-significant effect on the quality of fruit parameters: weight loss, fruit firmness, total soluble solids, titratable acidity.

5. The induction of chitinase enzymes in citrus flavedo tissues was enhanced in response to all treatments at 24 h and declined at 48 h except in a treatment with *B. subtilis* ABS-S14 a decrease activity was shown at 72 h. For β-1,3-glucanase activity, all treatments triggered enzyme elicitation starting from 24 h and reached the highest level at 48 h after application of antagonists, their combination or inoculation of the pathogen then all were declined at 72 h.

6. The expression level of *CHI* gene at 24 h and 72 h was strongly induced by *B. subtilis* ABS-S14 whereas, *P. guilliermondii* BCC 5389 and co-inoculation of *B.*

subtilis ABS-S14 and *P. guilliermondii* BCC 5389 elicited *CHI* gene at 24 and 72 h when it was followed with pathogen inoculation.

7. The abundances of *GLU* transcripts in flavedo were strongly enhanced in co-application of *B. subtilis* ABS-S14 and *P. guilliermondii* BCC 5389 at 24 and 72 h. *P. digitatum* also triggered the expression of *GLU* gene level at 24 h. Only *B. subtilis* ABS-S14 triggered *GLU* gene level at 48 h after pathogen inoculation.

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APPENDIX

1. Culture media preparation

1.1 Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB)

The formulation of 1 liter of PDA composed of 39.0 g of PDA. Suspend the ingredients altogether in distilled water and autoclaved at 121 ^{o}C for 15 minutes. For PDB preparation, the medium is composed of 24.0 g of PDB in 1 liter of distilled water and autoclave at 121 ^{o}C for 15 minutes.

1.2 Nutrient Yeast Dextrose Agar (NYDA) and Nutrient Yeast Dextrose Broth (NYDB)

The formulation of 1 liter of NYDA is composed of 8 g of Nutrient broth, 5 g of yeast extract and 10 g of glucose and 20 g of agar. Suspend the ingredients altogether in distilled water and autoclave at 121 ^{o}C for 15 minutes. For NYDB preparation, the medium is identical to NYDA except agar is excluded.

1.3 Nutrient Agar (NA) and Nutrient Broth (NB)

The formulation of 1 liter of NA is composed of 20.0 g of NA. Suspend the ingredients altogether in distilled water and autoclaved at 121 ^{o}C for 15 minutes. For NB preparation, the medium is composed of 8.0 g of NB in 1 liter of distilled water and autoclave at 121 ^{o}C for 15 minutes.

2. Preparation of carboxy methyl chitosan remazol brilliant violet (CM-chitosan-RBV)

Chitosan (10 g) was added into a flask containing 2-propanol (125 ml) and chloroacetic acid (15 g) at 20 ^{o}C for 60 minutes under stirring and then, filtered with whatman no. 1 filter paper, kept the CM-chitosan and washed by 2-propanol (125 ml). The pH was adjusted 7.0 with HCl solution and then, stirring at 20 ^{o}C for 24 h. Weight dye of RBV 5 (g) was added into CM-chitosan with 100 g fine sodium sulfate at 50^{o}C for 45 minutes. Sodium phosphate (7.84 g) was added to CM-chitosan-RBV at 50 ^{o}C for 75 minutes under stirring. Salt was separated by dialysis tubing in

distilled water for 48 h. Then, the solution was centrifuged at 4200g for 5 minutes and kept at 4 ^{o}C.

3. RNase Free water

RNase-free water is pure, quality-tested water suitable for use in all experiments, including PCR, RT-PCR, and real-time PCR. 0.1% Diethylpyrocarbonate (DEPC) was added to 1 ml of to 1000 ml distilled water, and mixed well and let set at room temperature for overnight. Thereafter, it was autoclaved at 121 °C for 35 min and cooled down to room temperature prior to use. RNase free water was kept at 4 °C for long period storage.

4. 10X reaction DNase I reaction buffer

RNase-free water contains 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, and 5 mM $CaCl_2$.

5. DNase I storage buffer

RNase-free water was supplied in a 50% glycerol solution containing 10 mM Tris-HCl (pH7.5), 50 mM $CaCl_2$, and 10 mM $MgCl_2$.

6. Chitinase activity in citrus flavedo in response to antagonist inoculation

Time (h)	Treatment	Chitinase (Unit/ml/gAP)				SD	SE
		Rep 1	Rep 2	Rep 3	Average		
0	T1	0.43	0.43	0.42	0.42	0.00	0.00
	T2	0.43	0.41	0.42	0.42	0.01	0.01
	T3	0.39	0.40	0.42	0.41	0.01	0.01
	T4	0.43	0.40	0.43	0.42	0.01	0.01
	T5	0.48	0.39	0.45	0.44	0.04	0.03
24	T1	0.53	0.51	0.54	0.53	0.02	0.01
	T2	0.52	0.53	0.53	0.53	0.01	0.00
	T3	0.53	0.55	0.54	0.54	0.01	0.01
	T4	0.52	0.54	0.51	0.53	0.01	0.01
	T5	0.53	0.51	0.48	0.51	0.03	0.02
48	T1	0.49	0.50	0.44	0.48	0.03	0.02
	T2	0.50	0.51	0.52	0.51	0.01	0.01
	T3	0.62	0.51	0.52	0.55	0.06	0.04
	T4	0.52	0.46	0.49	0.49	0.03	0.02
	T5	0.50	0.47	0.48	0.49	0.01	0.01
72	T1	0.54	0.526	0.52	0.53	0.01	0.01
	T2	0.52	0.50	0.51	0.51	0.01	0.01
	T3	0.53	0.54	0.50	0.52	0.02	0.02
	T4	0.51	0.52	0.53	0.52	0.01	0.01
	T5	0.45	0.45	0.47	0.45	0.01	0.01

T1 = Wounded-peels treated with distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml)

7. Chitinase activity in citrus flavedo in response to antagonist and *P. digitatum* inoculation

Time (h)	Treatment	Chitinase activity (Unit/ml/gAP)				SD	SE
		Rep 1	Rep 2	Rep 3	Average		
0	T1	0.43	0.43	0.42	0.42	0.00	0.00
	T2	0.43	0.41	0.42	0.42	0.01	0.01
	T3	0.41	0.42	0.44	0.42	0.02	0.01
	T4	0.44	0.42	0.46	0.44	0.02	0.01
	T5	0.42	0.50	0.40	0.43	0.03	0.02
24	T1	0.53	0.51	0.54	0.53	0.02	0.01
	T2	0.52	0.53	0.53	0.53	0.01	0.00
	T3	0.513	0.518	0.519	0.52	0.00	0.00
	T4	0.54	0.523	0.523	0.53	0.01	0.01
	T5	0.53	0.51	0.48	0.51	0.01	0.01
48	T1	0.49	0.50	0.44	0.48	0.03	0.02
	T2	0.50	0.51	0.52	0.51	0.01	0.01
	T3	0.47	0.498	0.533	0.50	0.03	0.02
	T4	0.512	0.525	0.501	0.51	0.01	0.01
	T5	0.498	0.477	0.502	0.49	0.01	0.01
72	T1	0.54	0.526	0.52	0.53	0.01	0.01
	T2	0.52	0.50	0.51	0.51	0.01	0.01
	T3	0.494	0.457	0.47	0.47	0.02	0.01
	T4	0.49	0.486	0.468	0.48	0.01	0.01
	T5	0.488	0.493	0.456	0.48	0.02	0.01

T1 = Sterile distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *P. digitatum* (10^4 spores/ml) T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml).

8. β-1,3-glucanase activity in citrus flavedo in response to antagonist inoculation

Time (h)	Treatment	β-1,3-glucanase activity (Unit/ml/gAP)				SD	SE
		Rep 1	Rep 2	Rep 3	Average		
0	T1	0.07	0.02	0.04	0.04	0.03	0.02
	T2	0.05	0.05	0.05	0.05	0.01	0.00
	T3	0.04	0.06	0.02	0.04	0.02	0.01
	T4	0.04	0.04	0.05	0.05	0.01	0.00
	T5	0.04	0.05	0.05	0.04	0.00	0.00
24	T1	0.24	0.26	0.24	0.25	0.01	0.01
	T2	0.30	0.33	0.32	0.32	0.02	0.01
	T3	0.33	0.33	0.34	0.33	0.00	0.00
	T4	0.29	0.30	0.27	0.29	0.01	0.01
	T5	0.29	0.27	0.30	0.29	0.01	0.01
48	T1	0.36	0.40	0.34	0.37	0.03	0.02
	T2	0.36	0.40	0.39	0.38	0.02	0.01
	T3	0.35	0.33	0.33	0.33	0.01	0.01
	T4	0.37	0.38	0.29	0.34	0.05	0.03
	T5	0.38	0.33	0.33	0.35	0.03	0.02
72	T1	0.30	0.24	0.24	0.26	0.02	0.01
	T2	0.28	0.29	0.29	0.29	0.04	0.03
	T3	0.25	0.22	0.24	0.24	0.01	0.00
	T4	0.29	0.29	0.29	0.29	0.01	0.01
	T5	0.30	0.29	0.28	0.29	0.01	0.01

T1 = Wounded-peels treated with distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and T5 = mixture of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml).

9. β-1,3-glucanase activity in citrus flavedo in response to antagonist and *P. digitatum* inoculation

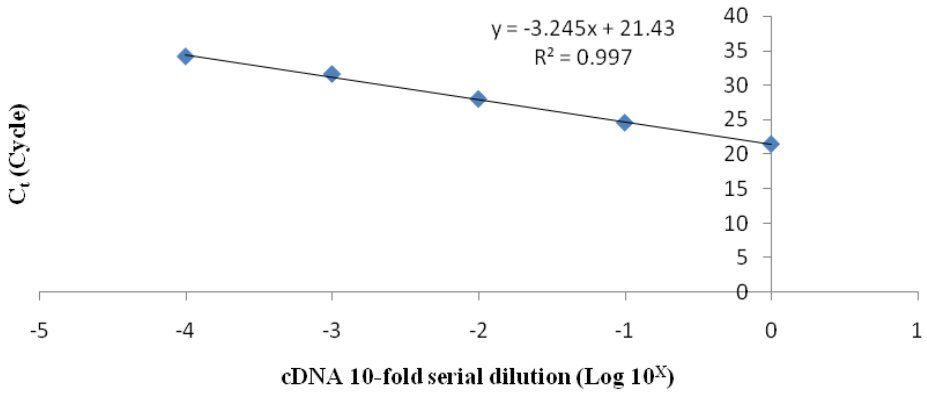
Time (h)	Treatment	β-1,3-glucanase activity (Unit/ml/gAP)				SD	SE
		Rep 1	Rep 2	Rep 3	Average		
0	T1	0.07	0.02	0.04	0.04	0.03	0.02
	T2	0.05	0.05	0.05	0.05	0.01	0.00
	T3	0.05	0.03	0.05	0.04	0.01	0.01
	T4	0.06	0.05	0.04	0.05	0.01	0.01
	T5	0.05	0.05	0.05	0.05	0.00	0.00
24	T1	0.24	0.26	0.24	0.25	0.01	0.01
	T2	0.30	0.33	0.32	0.32	0.02	0.01
	T3	0.28	0.28	0.28	0.28	0.00	0.00
	T4	0.36	0.33	0.38	0.36	0.03	0.02
	T5	0.31	0.31	0.31	0.31	0.00	0.00
48	T1	0.36	0.40	0.34	0.37	0.03	0.02
	T2	0.36	0.40	0.39	0.38	0.02	0.01
	T3	0.42	0.40	0.41	0.41	0.01	0.01
	T4	0.41	0.40	0.44	0.42	0.02	0.01
	T5	0.31	0.30	0.33	0.32	0.02	0.01
72	T1	0.30	0.24	0.24	0.26	0.04	0.03
	T2	0.28	0.29	0.29	0.29	0.01	0.00
	T3	0.28	0.29	0.27	0.28	0.01	0.01
	T4	0.29	0.29	0.25	0.28	0.02	0.01
	T5	0.28	0.25	0.27	0.27	0.02	0.01

T1 = Sterile distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *P. digitatum* (10^4 spores/ml) T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml).

10.Chitinase standard curve

Dilution	C_{T1}	C_{T2}	MEAN	SD	%CV
10^{0}	21.53	21.32	21.43	0.15	0.69
10^{-1}	24.14	24.92	24.53	0.55	2.25
10^{-2}	27.95	27.97	27.96	0.01	0.05
10^{-3}	31.26	31.89	31.58	0.45	1.41
10^{-4}	34.36	33.9	34.13	0.33	0.95

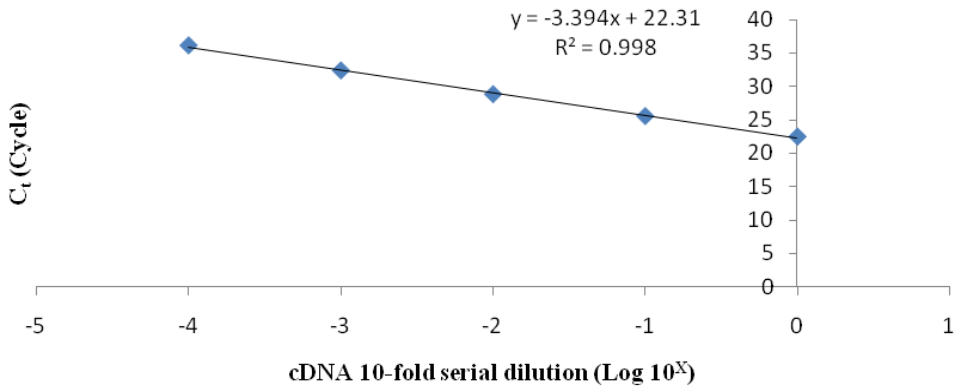
Serial Dilution Standard of *CHI*



11. Glucanase standard curve

Dilution	C_{T1}	C_{T2}	MEAN	SD	%CV
10^{0}	22.53	22.56	22.55	0.02	0.09
10^{-1}	25.59	25.6	25.60	0.01	0.03
10^{-2}	28.82	28.9	28.86	0.06	0.20
10^{-3}	32.47	32.33	32.4	0.10	0.31
10^{-4}	36.01	36.23	36.12	0.16	0.43

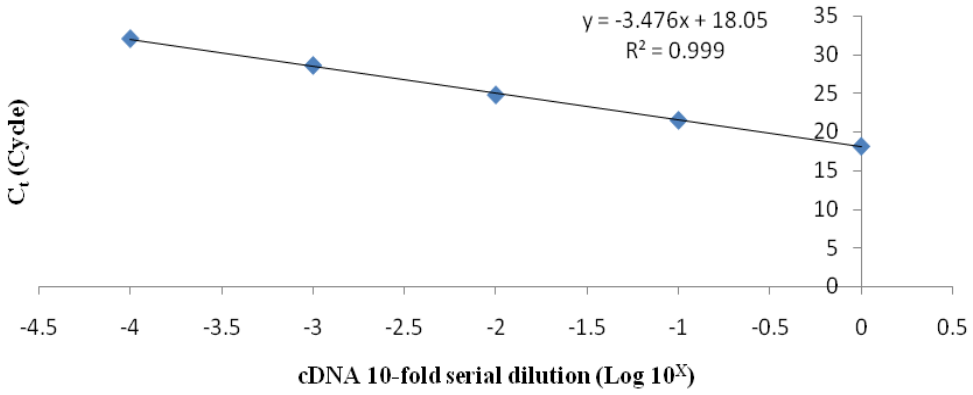
Serial Dilution Standard of *GLU*



12. Elongation factor 1 alpha standard curve

Dilution	C_{T1}	C_{T2}	MEAN	SD	%CV
10^{0}	18.25	18.07	18.16	0.13	0.70
10^{-1}	21.56	21.46	21.51	0.07	0.33
10^{-2}	24.92	24.63	24.78	0.21	0.83
10^{-3}	28.48	28.66	28.57	0.13	0.45
10^{-4}	31.78	32.24	32.01	0.33	1.02

Serial Dilution Standard of *EF-1α*



13. Abundance of transcripts for chitinase genes induced by antagonist inoculation in flavedo tissues

Time (h)	Treatments	C_{T1}	C_{T2}	Mean	SD	% CV
24	T1	23.25	22.73	22.99	0.37	1.60
	T2	25.41	26.05	25.73	0.45	1.76
	T3	22.58	22.36	22.47	0.16	0.69
	T4	25.13	25.48	25.305	0.25	0.98
	T5	24.57	24.25	24.41	0.23	0.93
48	T1	24.43	24.34	24.385	0.06	0.26
	T2	21.61	21.83	21.72	0.16	0.72
	T3	23.03	23.12	23.075	0.06	0.28
	T4	21.33	21.5	21.415	0.12	0.56
	T5	21.76	21.18	21.47	0.41	1.91
72	T1	20.62	20.7	20.66	0.06	0.27
	T2	22.41	22.35	22.38	0.04	0.19
	T3	24.82	24.89	24.855	0.05	0.20
	T4	22.36	22.53	22.445	0.12	0.54
	T5	22.33	22.53	22.43	0.14	0.63

T1 = Wounded-peels treated with distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml).

14. Abundance of transcripts for chitinase genes induced by antagonist and *P. digitatum* in flavedo tissues

Time (h)	Treatments	C_{T1}	C_{T2}	Mean	SD	% CV
24	T1	23.25	22.73	22.99	0.37	1.60
	T2	25.41	26.05	25.73	0.45	1.76
	T3	24.87	24.81	24.84	0.04	0.17
	T4	21.84	21.66	21.75	0.13	0.59
	T5	22.59	22.3	22.445	0.21	0.91
48	T1	24.43	24.34	24.385	0.06	0.26
	T2	21.61	21.83	21.72	0.16	0.72
	T3	20.69	20.36	20.525	0.23	1.14
	T4	22.53	22.33	22.43	0.14	0.63
	T5	22.88	21.35	22.115	1.08	4.89
72	T1	20.62	20.7	20.66	0.06	0.27
	T2	22.41	22.35	22.38	0.04	0.19
	T3	21.21	21.2	21.205	0.01	0.03
	T4	20.93	20.81	20.87	0.08	0.41
	T5	19.83	19.65	19.74	0.13	0.64

T1 = Sterile distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *P. digitatum* (10^4 spores/ml) T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml).

15. Abundance of transcripts for glucanase genes induced by antagonist inoculation in flavedo tissues

Time (h)	Treatments	C_{T1}	C_{T2}	Mean	SD	% CV
24	T1	23.67	24.23	23.95	0.40	1.65
	T2	26.77	26.85	26.81	0.06	0.21
	T3	23.62	23.57	23.595	0.04	0.15
	T4	23.88	23.87	23.875	0.01	0.03
	T5	24.93	24.92	24.925	0.01	0.03
48	T1	27.48	27.33	27.405	0.11	0.39
	T2	23.76	23.79	23.775	0.02	0.09
	T3	26.52	26.09	26.305	0.30	1.16
	T4	25.74	25.81	25.775	0.05	0.19
	T5	25.56	25.56	25.56	0.00	0.00
72	T1	24.83	25.05	24.94	0.16	0.62
	T2	24.49	24.54	24.515	0.04	0.14
	T3	26.72	27.1	26.91	0.27	1.00
	T4	25.42	25.32	25.37	0.07	0.28
	T5	26.15	25.98	26.065	0.12	0.46

T1 = Wounded-peels treated with distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml).

16. Abundance of transcripts for glucanase genes induced by antagonist and *P. digitatum* in flavedo tissues

Time (h)	Treatments	C_{T1}	C_{T2}	Mean	SD	% CV
24	T1	23.67	24.23	23.95	0.40	1.65
	T2	26.77	26.85	26.81	0.06	0.21
	T3	24.19	24.87	24.53	0.48	1.96
	T4	23.67	24.65	24.16	0.69	2.87
	T5	24.02	24.04	24.03	0.01	0.06
48	T1	27.48	27.33	27.405	0.11	0.39
	T2	23.76	23.79	23.775	0.02	0.09
	T3	26.37	26.45	26.41	0.06	0.21
	T4	25.91	25.94	25.925	0.02	0.08
	T5	26.51	26.15	26.33	0.25	0.97
72	T1	24.83	25.05	24.94	0.16	0.62
	T2	24.49	24.54	24.515	0.04	0.14
	T3	25.21	25.25	25.23	0.03	0.11
	T4	24.48	24.37	24.425	0.08	0.32
	T5	24.62	24.36	24.49	0.18	0.75

T1 = Sterile distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *P. digitatum* (10^4 spores/ml) T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml).

17. Abundance of transcripts for *EF-1α* genes induced by antagonist inoculation in flavedo tissues

Time (h)	Treatments	C_{T1}	C_{T2}	Mean	SD	% CV
24	T1	22.35	22.74	22.55	0.28	1.22
	T2	26.18	26.14	26.16	0.03	0.11
	T3	21.98	21.9	21.94	0.06	0.26
	T4	23.97	23.75	23.86	0.16	0.65
	T5	23.08	22.88	22.98	0.14	0.62
48	T1	25.52	25.48	25.5	0.03	0.11
	T2	20.46	20.39	20.43	0.05	0.24
	T3	22.71	22.97	22.84	0.18	0.8
	T4	22.27	22.33	22.3	0.04	0.19
	T5	21.29	21.32	21.31	0.02	0.1
72	T1	21.08	20.6	20.84	0.34	1.63
	T2	21.69	21.65	21.67	0.03	0.13
	T3	25.83	25.84	25.84	0.01	0.03
	T4	21.08	21.15	21.12	0.05	0.23
	T5	22.04	21.92	21.98	0.08	0.39

T1 = Wounded-peels treated with distilled water, T2= P. digitatum (10^4 spores/ml), T3 = B. subtilis ABS-S14 (10^8 CFU/ml), T4 = P. guilliermondii BCC 5389 (10^8 cells/ml) and T5 = co-inoculum of P. guilliermondii BCC 5389 (10^8 cells/ml) and B. subtilis ABS-S14 (10^8 CFU/ml).

18. Abundance of transcripts for *EF-1α* genes induced by antagonist and *P. digitatum* in flavedo tissues

Time (h)	Treatments	C_{T1}	C_{T2}	Mean	SD	% CV
24	T1	22.35	22.74	22.55	0.28	1.22
	T2	26.18	26.14	26.16	0.03	0.11
	T3	22.38	22.19	22.29	0.13	0.6
	T4	21.79	21.63	21.71	0.11	0.52
	T5	21.46	20.97	21.22	0.35	1.63
48	T1	25.52	25.48	25.5	0.03	0.11
	T2	20.46	20.39	20.43	0.05	0.24
	T3	21.85	21.82	21.84	0.02	0.1
	T4	21.99	22.1	22.05	0.08	0.35
	T5	21.8	21.59	21.7	0.15	0.68
72	T1	21.08	20.6	20.84	0.34	1.63
	T2	21.69	21.65	21.67	0.03	0.13
	T3	22.33	22.3	22.32	0.02	0.1
	T4	21.56	21.62	21.59	0.04	0.2
	T5	20.6	20.53	20.57	0.05	0.24

T1 = Sterile distilled water, T2= *P. digitatum* (10^4 spores/ml), T3 = *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml), T4 = *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *P. digitatum* (10^4 spores/ml) T5 = co-inoculum of *P. guilliermondii* BCC 5389 (10^8 cells/ml) and *B. subtilis* ABS-S14 (10^8 CFU/ml) and *P. digitatum* (10^4 spores/ml).

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A scholarship from the Postharvest Technology Innovation Center, Prince of Songkla University

List of Publications and Proceedings

Publications

S. Sumitra., Sangchote, S. and Leelasuphakul, W. 2012. Biocontrol of citrus green mould and postharvest quality parameters. International Food Research Journal 20 (6): 3381-3386.

S. Sumitra., Sangchote, S. and Leelasuphakul, W. 2010. An application of antagonistic microorganisms and sodium bicarbonate to control green mold rot disease (*Penicillium digitatum*) of citrus. Agricultural Science Journal 41 (2) (Suppl.): 57-60.

Proceedings

S. Sumitra., Sangchote, S. and Leelasuphakul, W. 2010. An application of antagonistic microorganisms and sodium bicarbonate to control green mold rot disease (*Penicillium digitatum*) of citrus. The 9th National Horticultural Congress, KrungSri Rever Hotel, KrungSri Ayutthaya. May 11-14. pp. 57-60.

S. Sumitra., Sangchote, S. and Leelasuphakul, W. 2012. Effect of *Pichia guilliermondii* on *Penicillium digitatum* and green mold rot in Mandarin 'Shogun' from Thailand. International conference on Postharvest Pest & Disease Management in Exporting for Horticultural Crops, Golden Tulip Sovereign Hotel, Bangkok. February 2-6. Pp. 77-80.