CHAPTER FOUR

DEVELOPING ETHIOPIAN MICROBIAL BIOCONTROL AGENTS FOR CITRUS POSTHARVEST DISEASE CONTROL

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Abstract

An alternate to chemical pesticides, microbial antagonists are used to control pre- and postharvest decay of fruits and vegetables. In this study, three yeast antagonists [two strains of *Cryptococcus laurentii* (MeJtw10-2 and TiL4-3) and one strain of *Candida sake* (TiL4-2)] isolated from twig and leaf surface of an orange tree controlled citrus green mould by 70-95% with higher broad spectrum activity against sour rot and anthracnose caused by *Geotrichum candidum* and *Colletotrichum gloeosporioides*. Possible mode of action of these yeast antagonists [antibiosis, volatiles, phenolics, enzyme production assays, competition for nutrients and space] were studied. All antagonists showed no antibiosis effect against the tested pathogens. Some activity of enzyme production was exhibited by antagonist MeJtw10-2 (*C. laurentii*) unlike antagonist TiL4-2 (*C. sake*) and TiL4-3 (*C. laurentii*). All antagonists showed significant (P <0.05) inhibition of *P. digitatum* spore germination both in cylinder insert and direct well contact experiments. *In vitro* dual culture experiments exhibited 75-100% control of *P. digitatum* spore growth by fast colonization and competition effect on solid medium. All antagonists showed the production of extracellular matrix, which enhanced their rapid colonization on fruit wound site during infection. Germination of *P. digitatum* conidia was significantly (P <0.05) inhibited when pathogens and antagonists were in physical contact. These results may indicate that competition for nutrients is one of the modes of action of these potential antagonists against the pathogen, *P. digitatum*.

Key words: Yeast antagonists; Cylinder insert; *Cryptococcus laurentii*, *Candida sake*

4.1   INTRODUCTION

Biological control with plant extracts and microbial agents has been explored as an alternative to the use of synthetic chemicals for managing postharvest diseases of fruits and vegetables (Wilson and Wisniewski, 1989). Several species of bacteria and yeasts have been already reported to reduced fungi decay of pome fruits (Janisiewicz, 1985; Janisiewicz, 1988; Vinas *et al*., 1998; Mercier and Wilson, 1994; Spadaro *et al*., 2002; Batta, 2004), grape fruit (Droby *et al*., 2002), avocado (Korsten and De-Jager, 1995, Demoz and Korsten, 2006), mango (Korsten *et al*., 1991; Govender and Korsten, 2006), citrus (Obagwu and Korsten, 2003).

The success of some of these microbial antagonists in laboratories and large-scale studies has stimulated the interest of several workers in the development of biological products for postharvest application. Currently, some antagonists such as *Bacillus subtilis* (Avogreen) have been registered in South Africa for the control of pre- and postharvest diseases of avocado (Janisiewicz and Korsten, 2002). Other antagonists such as *Cryptococcus albidus* (YieldPlus) for the control of postharvest diseases of apples and pears, *Pseudomonas syringae* (BioSave 110 and 111) for the control of *Geothricum candidum* on pome and citrus, and *Candida oleophila* (Aspire ^{TM}) for the control of penicillium decay on citrus have been registered by Ecogen Inc. in the USA (Shachnal *et al*., 1996).

Biocontrol systems of antagonistic microorganisms have involved various modes of actions by competition for nutrients and space and/ or induction of host resistance mechanisms. Therefore, the selection and development of microbial antagonists for postharvest application involve the use of in *vitro* and *in vivo* experimental trials including pilot studies.

Because of its fauna and flora diversity and endemism, tropical environments appears to be an ideal source of microbial antagonists (Tewoldebirhan, 1991), which could help the upcoming industry to establish potential biocontrol with no environmental and health problems. The objectives of the present study were to search for effective antagonists from different citrus production regions of Ethiopia, and to investigate *in vitro* and *in vivo* modes of action, recovery and compatibility.

4.2   MATERIAL and METHODS

4.2.1   Sample collection

Samples were collected from healthy looking citrus orchards where disease pressure was supposed to be high. Ten citrus trees per farm and 10 samples from each of the vegetative parts of each tree (leaves, twigs and fruits) were collected from 20 citrus farm units of Ethiopia. Samples collected were kept in brown paper bags and transferred to the Plant Pathology Laboratory, Alemaya University, Ethiopia for preliminary screening. Samples were processed immediately or kept in cooler boxes until use.

4.2.2   Pathogen

Penicillium digitatum Sacc. was used as a test pathogen in the bioassay procedure to select the potential antagonists. The test pathogen was originally isolated from infected citrus fruit obtained from Toni farm, DireDawa, Ethiopia and the pathogen identity was confirmed by Dr. Amare Ayalew (Pathology Division, Plant Science Department, Alemaya University). Once maintained on Potato Dextrose Agar (PDA) (Biolab, Johannesburg, South Africa), the culture was placed under UV light for 7-14 days at 25 °C to sporulate. A conidial suspension of the pathogen was prepared by adding 20 ml of sterilized distilled water onto the surface of the PDA culture plate and spores were harvested by gentle swabbing. Spore concentration was standardized to 1 x 10^5 spores ml^{-1} prior to use using a haemocytometer.

4.2.3   Antagonists

Isolation and screening of the antagonists was done according to Reyes *et al*. (2004) with slight modifications. Samples (fruit, leaves and twigs) were dipped in a sterile jar (1L) with 400 ml Ringer's solution containing a standard concentration of *P. digitatum* (1 x 10^5 spores ml^{-1}) and left on a rotary shaker at 150 *rpm* for ten minutes. Serial dilutions of the wash water were plated out on PDA and Standard 1 Nutrient Agar (STD-1 NA) (Biolab, Johannesburg) to get individual colonies. Culture plates were incubated at 25 ºC for 48-72 h and evaluated for antagonist activity against *P. digitatum*. Fast colonizing microbial isolates without antibiosis effect *in vitro* were randomly selected from culture plates, purified and preserved for further *in vivo* trials.   Potential isolates were taken to Plant Pathology Laboratories, (Pretoria University, South Africa) for further analyses, identification and mode of action study. Samples were brought into the country following standard quarantine requirements according to the national legislation and germ-plasm transfer agreements, (import permit no. P0017192).

4.2.4   *In vivo* antagonist screening assay

Twenty boxes of fresh citrus (*Citrus sinensis* L) fruits with 80 oranges in a box were collected from Crocodile Valley packhouse (Nelspruit, Mpumalanga, South Africa). Fruits were disinfected with 1% sodium hypochlorite for 2 min and air dried prior to wounding. The fruits were wounded on both sides with a picture hook. Wounds were made to a depth of ca 3mm into the fruit rind. An overnight culture of antagonist cell suspension grown in Nutrient Broth (NB) (Biolab, Johannesburg) was standardized to 10^8 cells/ ml^{-1} using the Petroff Hauser counting chamber. Under preventive application, 40µl of the antagonist cell suspension was inoculated onto the wounded area, 12 hours prior to the application of the pathogen, *P. digitatum*. Wound inoculation of *P. digitatum* alone or with NB served as a positive or negative control, respectively. Three fruits per isolates were used and the experiment was repeated twice. Treated fruits were stored at 25 ºC for 7–14 days and moisture was maintained between 80–90% relative humidity (RH). Fruits were evaluated every two days for disease development and data recorded.

4.2.5   *In vitro* antagonist screening assay for broad spectrum activity

In vitro screening of antagonists for broad-spectrum activity was done according to the method described by Spadaro *et al*. (2002) with slight modifications. Three postharvest fungal pathogens: *P. digitatum* (UPPed-1), *G. candidum* (UPGec-1) and *Colletotrichum gloeosporioides* Penz (UPCog-1) obtained from the culture collection of Plant Pathology Laboratories, (University of Pretoria) were used as a test pathogens. Fungal cultures grown on Malt Extract Agar (MEA) (Merck, Johannesburg) for seven days at 25 ºC were placed under UV light for 7-14 days until sporulation. A standard concentration (10^5 spores ml^{-1}) of each pathogen suspension were prepared in Ringer's solution and used immediately in the subsequent trials. Eighteen potential antagonists selected from the *in vivo* experiments on citrus in section 4.2.4 were used. The growth rates of antagonists were tested on four different solid media (PDA, MEA, STD-1NA and citrus peel-agar). For the citrus peel agar medium preparation, 5% v/v of citrus peel were homogenised and filtered through Whatman no. 1 and added to 20g l^{-1} of Agar-Agar (pH 5.5). A standard concentration of antagonist suspension (10^8 cells ml^{-1}) determined using Petroff Hauser counting chamber was used in the challenge test against the three pathogens. Ten micro litres of antagonist cell suspension was streaked on one side of MEA medium in 90 mm Petri dishes, 2mm from the border and three streaks per plate was made at each opposite side at equal distance from the centre. The same volume of pathogen suspension was put at the centre and plates were air dried and incubated at 25 ºC for seven days. Percentage growth inhibition of the pathogen was calculated according to

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Skidmore (1976) using the formula: (C-r) x 100/C, where, C = growth diameter of pathogen alone, r = growth diameter of pathogen grown with an antagonist.

4.2.6   Antagonist identification

Microbial antagonists selected for their postharvest decay control potential were further screened and identified according to the method described by Droby *et al*. (1989). Rose Bengal chloramphenicol agar medium was used as a selective medium to distinguish potential antagonists in their respective categories as bacteria and/ or yeasts. Further identification of potential isolates was done using the API system (I D 32 C Biomerieux, USA). Cultural and microscopic characteristics of isolates were used to confirm identification. Isolates were maintained in 20% glycerol at -70 ℃ and routinely grown on their respective media, STD 1-NA for bacterial and MEA for yeast isolates.

4.2.7   *In vivo* antagonist's activity and disease incidence against *Penicillium digitatum*

Potential antagonists selected for their best performances were further evaluated for disease incidence reduction on citrus fruit. Fruit and inocula preparation was done as described in section (4.2.4). Inoculated fruits were incubated at 7 ℃ for 30 days and fruits were evaluated for disease development by the end of the incubation period, and the data was recorded. The experiment was done twice. Percentage of disease incidence reduction or percentage of intact fruit appearance was calculated using the formula described by Vero *et al*. (2002).

4.2.8   Mechanisms of biocontrol:

4.2.8.1 Antibiosis assay

A streak assay was done as described by Poppe *et al*. (2003). STD 1- NA, MEA and PDA, separately mixed with 10g l^{-1} orange flavedo tissue powder, were used as growth media. An agar disk (4 x 4mm) from a seven days old culture of *P. digitatum* was placed at the centre of a Petri dish containing 20 ml agar medium per Petri dish.. An overnight grown antagonist broth culture was streaked on three sides of the plate at equal distances from the centre. Plates were then incubated at 25 ℃ for seven days and evaluated for formation of an inhibition zone. Five plates were used per treatment and the experiment was repeated twice.

Culture preparation for *in vitro* antibacterial assay was done according to (Castoria *et al*., 2001). A loop full of actively growing antagonists was inoculated into a flask containing 50 ml NB and kept overnight on the shaker at 170 rpm. Separate flasks were prepared for further inoculation assays and Thin Layer Chromatographic study of the culture filtrate active

component. The antagonist suspension was centrifuged at 5000 x g for 10 minutes and the culture filtrate was transferred into another sterilized tube. Total soluble phenolics were extracted as follows: two fold volumes of ethyl acetate was added into the culture filtrate, vortexed for 30 seconds, and left to settle for one minute. The organic phase containing the ethyl acetate and the soluble phenolics was transferred to a clean Eppendorf tube. The extraction was repeated three times. The combined supernatants were left to dry under an air vacuum chamber and re-dissolved with one ml of distilled water.

Total soluble phenolics compounds were quantified using the Folin Ciocaleteu's Phenol reagent (Sigma) (Bray and Thorpe, 1954). A comparative study was performed on the culture filtrates by TLC on pre-coated Silica Gel 60 (Merck, 60F254) using chloroform/methanol/ethyl acetate/acetone and water (55:20:20:5:3.5) as a separation solvent system. The TLC plate was loaded with 20 µl of each sample. Sterile broth culture were used as negative control and standard chemicals such as isoferulic (Sigma), *P*-coummaric acid (Sigma), novobiocin, cyclohexamide and chloramphenicol (CAPS Pharmaceuticals, SA) were used as positive controls.

4.2.8.1.1   Antibacterial assay

The TLC plates prepared as described in section (4.2.7.1) were covered with a nutrient agar and used to test the antibacterial activity of antagonists further. Two millilitres of an overnight grown indicator bacterium (10^8 cell ml^{-1}), in this case, *Erwinia carotovora,* mixed with equal volume of molten STD1- NA (50 °C) and 0.1 ml of 2% (w/v) 2.3.5-triphenyltetrazolium chloride (Sigma) was over laid on the TLC plate and left to solidify. Solidified plates were incubated overnight at 25 ºC and antibacterial activity evaluated. Appearance of red pigmentation on the plate indicates growth of bacteria and the formation of clear zone on the other hand showed pathogen growth inhibition.

4.2.8.1.2   Antifungal assay

The TLC plates prepared as described in (4.2.7.1) were also used for antifungal activity assay. A *P. digitatum* spore suspension (10^5 spore ml^{-1}) was prepared in glucose minimal salt medium and sprayed directly onto the dried TLC plate. Plates were incubated in moist atmosphere (>90RH) for 2-3 days at 25 ºC. The presence of fungitoxic activity of antagonists was noted by the formation of clear zone around the pathogen spore.

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4.2.8.2   Volatile production assay

Fifty microliters of a suspension of each isolate (10^8 spores ml^{-1}) prepared as described in section (4. 2.7.1) was spread plated on 90-mm Petri dishes containing 20 ml aliquots of MEA. Another set of plates containing the same quantity of MEA was inoculated with *P. digitatum* (10^5 spores ml^{-1}) by centrally placing 10µl of spore suspension in the Petri dish. Once the surface dried, the lids were removed and the yeast plates were placed open ended on the fungal plates and sealed with parafilm. Plates were incubated at 25 ºC for 7-14 days and the fungal colony diameter was measured. The control consisted of MEA plates streaked with sterile distilled water instead of yeasts. Each treatment was replicated four times and the experiment was repeated once. Data obtained were statistically analysed.

4.2.8.3   Antagonist enzyme activity assay

Potential antagonists selected for their efficacy were evaluated *in vitro* for production of different enzymes using different synthetic media. Chitinolytic activity was determined according to the method described by Frandberg and Schnurer (1994). Specific media were used to determine the production of amylase, lipase, proteinase, and gelatinase (Norris and Ribbons, 1971). Each specific medium was autoclaved for 20 minutes at 121 ºC and culture plates were prepared for streak inoculation. In all cases, four replicate plates were inoculated for each isolate and the experiment was repeated three times.

4.2.8.4   *In vitro* yeast antagonist-pathogen interaction

The possible interaction of yeasts with the hyphae of *P. digitatum* was assessed using the method described by Chan and Tian (2005) with slight modifications. Petri dishes (90 mm in diameter) containing each 20 ml of MEA amended with 0.5% citrus juice (v/v) was used as an assay medium. Ten micro litres of the pathogen suspension (10^5 spores ml^{-1}) were placed on the centre of the plate. After 12 h of incubation at 25 ºC, 50µl of each yeast cell suspension (1 x 10^8 cells ml^{-1}) were placed at the margin of the fungal inoculum. The dual cultures were incubated at 25 ºC for 5-7 days and plates were evaluated for antagonist–pathogen direct interaction and data was recorded. Experiments were repeated twice.

4.2.8.5   Competition for nutrients and space: cylinder insert trials

The method described by Janisiewicz *et al*. (2000) was used to evaluate the effects of nutrient depletion by antagonists on the germination and growth of *P. digitatum* conidia. Potato Dextrose Broth (PDB) (Oxoid, Johannesburg) (20% or 40%) and orange peel extract (OPE) (0.5 and 5%) diluted in physiological solution was used as source of nutrients (Poppe *et al*.,

2003). Standard concentrations (1×10^8 CFU ml^{-1}) of each antagonist (MeJtw 10-2, TiL4-2, and TiL4-3) were dispensed in the wells of culture plates (0.6 ml per well). The pathogen, *P. digitatum* suspension in a physiological solution (10^5 spores ml^{-1}) were dispensed inside the cylinder inserts (0.4 ml per cylinder) and placed in the wells. Plates were incubated at 25 ºC for 24hr. After incubation, membranes from the cylinder inserts were removed accordingly, blotted with sterilized tissue paper, and cut with a sterilised scalpel. A quarter of a membrane was transferred to a glass slide, stained with lactophenol blue solution (Fluka, Switzerland) and mounted for light microscopy (Zeiss, Germany) to observe spore and/or conidia germination. The percentage of germinating conidia on the membranes was scored using four classes: 1= no germination, 2= germ tube <2x conidia size, 3= germ tube 2 to 4x conidia size; 4= germ tube >4x conidia size. Hundred conidia per treatment were counted (Janisiewicz *et al.*, 2000). Each experiment was carried out twice with four wells per treatment.

After 24 h incubation and removal of the cylinders, two parallel experiments: turbidimetric growth measurements of antagonist populations in the wells were done at 640nm. A quarter of insert membrane was removed, blotted dry on sterilized surface, cut, transferred to MEA plates and incubated at 25 ºC for a period of two weeks. Plates were evaluated for pathogen-antagonist growth and percentage growth diameter of the pathogen and/or the antagonist was recorded and pathogen growth inhibition rate was statistically computed. The trial was also carried out without cylinders, in which the standard concentration of spore suspension was added directly to the well containing the standard concentration of the antagonist to study the direct interaction between the pathogen and antagonist. Evaluation was done by estimating the rate of spore germination in 100µl suspension using the germinating rate scale described above (Meziane *et al.*, 2006).

4.2.9   Effect of antagonists culture filtrate on pathogen conidial germination

To determine the effect of antagonists (MeJtw 10-2, TiL 4-2 and TiL 4-3) on spore germination and germ tube elongation of *P. digitatum,* the method described by Castoria *et al.* (2001) and Spadaro *et al.* (2002) were used. The culture filtrate prepared as described in section 4.2.7.1 was used in this trial. Treatment combinations prepared as heated and not heated culture filtrates were used with or without PDB and/or the pathogen, *P. digitatum.* The application of cyclohexamide (0.1%) (Sigma, Germany) with or without *P. digitatum* spores and PDB with *P. digitatum* spores were regarded as a positive and negative control, respectively.

4.2.10 Minimum *in vitro* inhibitory antagonist concentration against *Penicillium digitatum*

To determine the optimal antagonist concentration at which effective inhibition of pathogens could be achieved, the checkerboard-type titration technique (Korsten, 1993) was used. Fresh Valencia fruits of more or less the same size and maturity were used as described in section 4.2.4. Squares (5 mm x 5mm), spaced 5 mm apart, were drawn with water proof ink in five vertical and five horizontal rows on one side of the fruit. Each square was prick-wounded to a depth of 3 mm using picture hook. As determined in the preliminary experiment, a range of antagonist and pathogen concentrations from 1×10^5 to 10^8 spores ml^{-1}, with the application of 40µl suspension were used accordingly. The higher concentration (1×10^8 cells ml^{-1}) of antagonists applied to the first vertical row from the left and the 10^7 concentration to the second, 10^6 to the third, 10^5 to the fourth row, respectively. The last vertical row served as a control and received 40µl of sterilized Ringer's solution. Fruit were left to air dry at room temperature for 12 h prior to the application of various concentrations of the pathogen, *P. digitatum.* Each square in the top horizontal row was pipette inoculated with 40µl of the 10^8 pathogen spore suspension, successively in the following rows. The last horizontal row (lower bottom) received 40µl of sterilized Ringer's solution only. Four fruits were used for each antagonist-pathogen treatment combination and the experiment was repeated three times. Inoculated fruits were kept in cardboard boxes and incubated at 25 ºC and >85% RH for seven days. Fruits were evaluated for disease development and data was statistically computed.

4.2.11 Colonization and attachment study using *scanning electron microscopy*

Surface colonization and attachment of antagonists at wound sites were determined according to Usall *et al*. (2001). A uniform 3 x 3 mm wound was made at four sites around the equator of fruit using a picture hook. Thirty micro litres of antagonists (C-20, C-28 and C-47) suspension at 1×10^8 cells ml^{-1} were pipetted into each wound site prior to the application of the pathogen. The same volume of *P. digitatum* suspension at 1×10^5 spores ml^{-1} was inoculated, separately into each wound. The separate application of antagonists and/ or the pathogen alone were regarded as a control. The experiments were done in triplicate. Inoculated fruits were either used immediately for scanning electron microscopy (SEM) evaluation or incubated. Fruits were placed at ambient temperature into 400mm x 300mm x 100mm plastic tray wrapped with a high density polyethylene sleeve to maintain high relative humidity (>85% RH). Samples taken by the time of inoculation, 6, 12, 24 and 48 hours were used. The peel tissue from wounds on the surface of citrus fruit was cut (4 x 4 mm) and fixed by immersion in 2.5% glutaraldehyde in 0.075 M phosphate buffer at pH 7.0 for 24 h at room

temperature. Samples were rinsed for 1 h (four or five changes) with 0.075 M sodium phosphate buffer (pH 7. 2) and dehydrated in a series of ethanol concentrations before critical point drying. Dried tissues were mounted on aluminium stubs, coated with gold-palladium, and observed at 6kv with a scanning electron microscope (Joel JSM 840, Tokyo, Japan).

4.2.12 *In vitro* integrated treatment of antagonists with plant extracts and commercial chemicals

Various strength of treatment combinations (10^{-1}, 10^{-2} and 10^{-3}) of fresh and six months old plant extract preparations preserved at 4 ºC and commercial chemicals [Prochloraz (AgrEvo, South Africa) (450 g L^{-1}), Guazatine (Rhone-Poulenc, France) (200 g L^{-1}), RSAF-1 (2-furaldehyde) (Illovo, South Africa) (7 %, v:v), Ultracure (Natural Crop Protection, South Africa) (210 g L^{-1}) Quatrokill (N, N Didecyl-N, N- dimethyl ammonium chloride) (Hyper Agrochemicals (Pty) Ltd., Johannesburg) (1.3 g L^{-1}), and Imazalil (Sanachem., Johannesburg) (1.35 g L^{-1})] were used in this trial. Yeast antagonist cultures grown overnight in NB and standardized to 10^6 spores/cells ml^{-1} using a haemacytometer (Janisiewicz *et al*., 2000) and preserved at 4 ºC in an ice box prior to use. One millilitre of antagonists suspension were transferred onto 14 ml molten MEA or STD-1 NA medium in a tube before rotating and poured into a Petri dish and mixing it gently by swirling before solidification. Four plugs (5mm diameter) were punched from actively growing cultures on agar plates to prepare agar wells. Each well was 30 mm from each other and two mm from the edge of the plate. Forty microliters of a plant extract and/ or industrial chemical were transferred into agar wells and plates were incubated at 25 ºC for 48-72 h. Sterilized distilled water alone was regarded as a negative control. The experiment was done in triplicate and repeated twice.

4.2.13 Statistical analyses

Fruit disease incidence data were analysed using analysis of variance (ANOVA) using Fisher's protected LSD test ($P <0.05$) and t- grouping with SAS (version 8.2) 2001. The inhibition rate of pathogen spore germination were analysed using the non-parameteric Kruskall Wallis test followed by Man-Whitney test at P<0.05 with SAS (version 8.2) 2001.

4.3   RESULTS

4.3.1   *In vivo* screening and selection of potential antagonists

Of the 242 microbial epiphytes preliminary isolated from leaf, fruit and twig washes of citrus, 18 potential antagonists were selected for further evaluation (Fig.4.1). Four strains: MeJtw 8-

2, MeJtw 10-2, TiL4-2, and TiL4-3 showed the highest rate of disease incidence reduction (between 50-75%).

4.3.2   *In vitro* antagonists screening assay for broad-spectrum activity

All antagonists showed some degree of antagonistic activity against all three tested pathogens (Fig. 4. 2, 3, 4). About 55.6% of the antagonists exhibited a growth inhibition rate between 30-95%. Six isolates [HF 8-2, MeJtw 10-2, TiL 4-2, TiL 4-3, TiL 1-1 and TiL 8-2] showed high growth inhibition (60-90%) against *P. digitatum* (Fig. 4. 2).
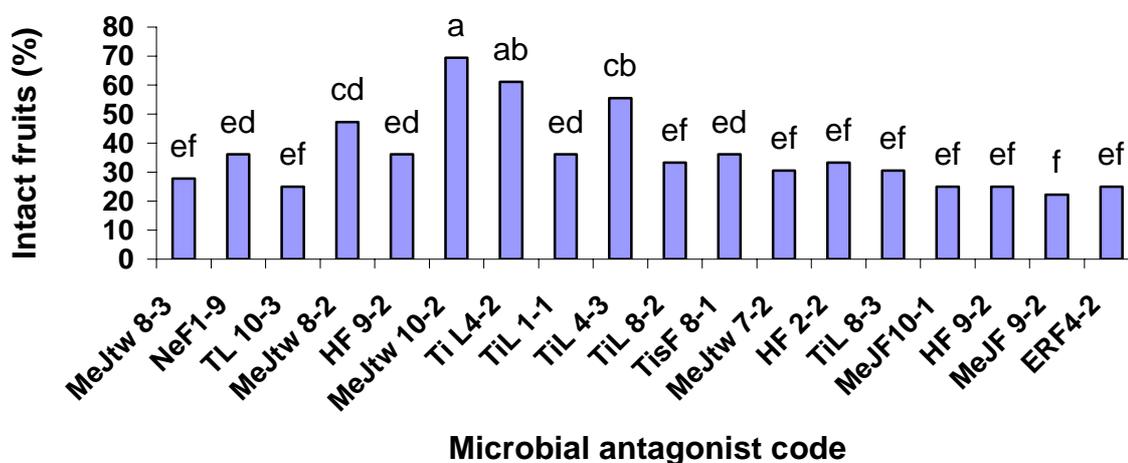
Legend: *= Bars represent percentage intact fruits. Bars with similar letters are not significantly different according to Fisher's protected LSD and t- grouping. Codes given to microbial antagonists referred as follows: MeJtw 8-3= MertiJeju twig sample isolate number 8-3, NeF1-8= NuraEra fruit sample isolate number 1-8, MeJtw 8-2= MertiJeju twig sample isolate number 8-2, HF8-2= Hursso fruit sample isolate number 8-2, MeJtw 10-2= MertiJeju twig sample isolate number 10-2, TiL4-2= Tibila leaf sample isolate number 4-2, TiL1-1= Tibila leaf sample isolate number 1-1, TiL4-3= Tibila leaf sample isolate number 4-3, TiL8-2= Tibila leaf sample isolate number 8-2, TisF8-1= Tisabalima leaf sample isolate number 8-1, MeJtw 7-2= MertiJeju twig sample isolate number 7-2, HF2-2= Hursso fruit sample isolate number 2-2, TiL8-3= Tibila leaf sample isolate number 8-3, MeJF10-1= MertiJeju fruit sample isolate number 10-1, HF8-2= Hursso fruit sample isolate number 8-2, MeJF8-2= MertiJeju fruit sample isolate number 8-2, and ERF4-2= Error Gota fruit sample isolate number 4-2.

Fig. 4.1. *In vivo* microbial antagonist screening assay on citrus fruit for control of *Penicillium digitatum*.

Isolates [MeJtw 10-2, TiL 4-2, TiL 4-3 and HF 2-2] against *G. candidum*, and isolates MeJtw 8-2, Mejtw10-2, TiL 4-2, TiL 4-3, MeJF10-1 and ERF 4-2 against *C. gloeosporioides* showed 30-35% growth inhibition, respectively. Three isolates with code MeJtw 10-2, TiL 4-2 and

TiL 4-3 showed high growth inhibition rate with broad-spectrum activity against the three tested pathogens and were identified as yeasts.
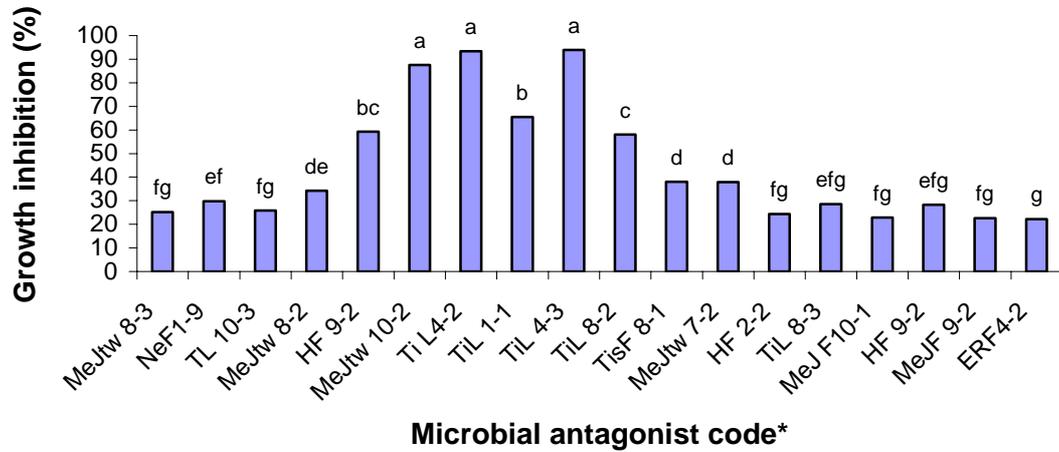
Legend: *= Description refers to figure 1. Bars represent antagonists growth inhibition activity against *Penicillium digitatum*.
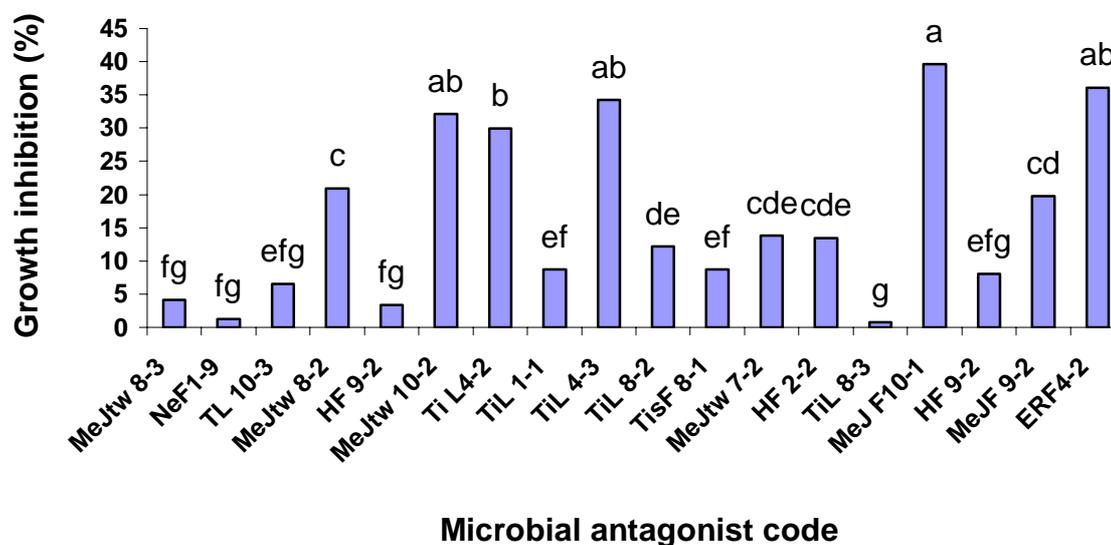
Fig. 4. 2. *In vitro* antagonist activity assay against *Penicillium digitatum*.

Further identification of yeasts with API®C identification system showed that isolate MeJtw 10-2 as *Cryptococcus laurentii*, isolate Til4-2 as *Candida sake* and isolate TiL4-3 as another strain of *Cryptococcus laurentii*.
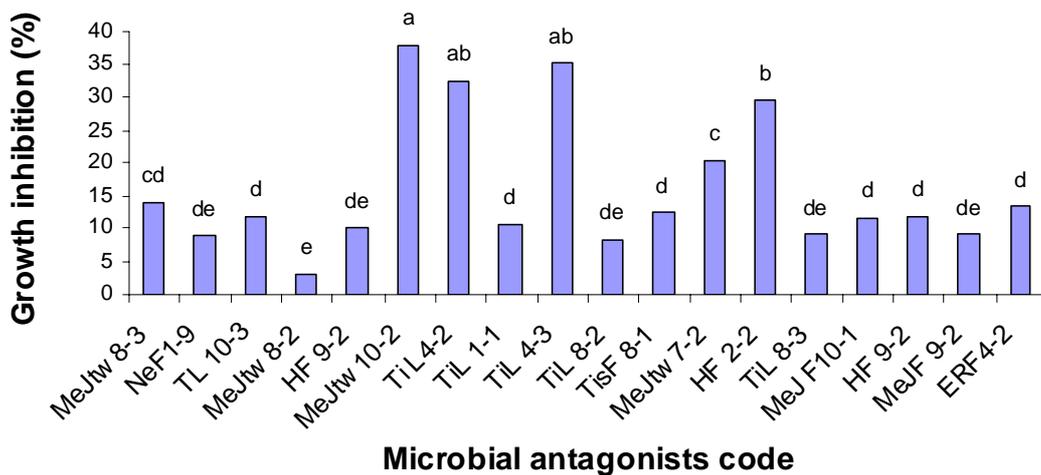
Legend: *= Description refers to figure 1. Bars represent antagonists growth inhibition activity against *Geotrichum candidum* Link ex Pers.

**Fig. 4. 3.** *In vitro* antagonistic activity against *Geotrichum candidum*.

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Legend: *= Description refers to figure 1. Bars represent antagonists growth inhibition activity against *Colletotrichum gloeosporioides* Penz.

**Fig. 4. 4.** *In vitro* antagonists assay against *Colletotrichum gloeosporioides*.

4.3.3   *In vivo* antagonists activity and disease incidence against *Penicillium digitatum*

From the *in vitro* experiments conducted in section 4.3.2, three antagonists [MeJtw 10-2, TiL 4-2 and TiL 4-3] were selected for their broad spectrum and overall effective antifungal activity. Fast and competitive colonisation of antagonists correlated directly with the high percentage of intact fruit. Wound application of antagonists against *P. digitatum* showed a significant ($P < 0.05$) rate of disease incidence reduction by (60-90%) on fruits incubated at 7 ºC for 30 days (Fig. 4. 6). A higher rate of disease incidence reduction was observed by antagonist MeJtw 10-2 (*C. laurentii*) (>85%). Antagonist TiL4-3 (strain of *C. laurentii*) and TiL4-2 (*C. sake*) showed 65-85% disease incidence reduction unlike antagonist MeJtw10-2 (Fig. 4. 6). No significant ($P <0.05$) changes were observed in the reduction of disease incidence by the addition of NYDB to antagonist MeJtw10-2 (*C. laurentii*). The rate of fruit infection increased significantly ($P <0.05$) with the addition of NYDB to a treatment combination with TiL4-2 (*C. sake*) and/ or TiL4-3 (*C. laurentii*) (Fig. 4. 5 and 6). Fruits remained 100% intact with antagonist treatments alone and with or without a NYDB combination (Fig. 4. 5 and 6).
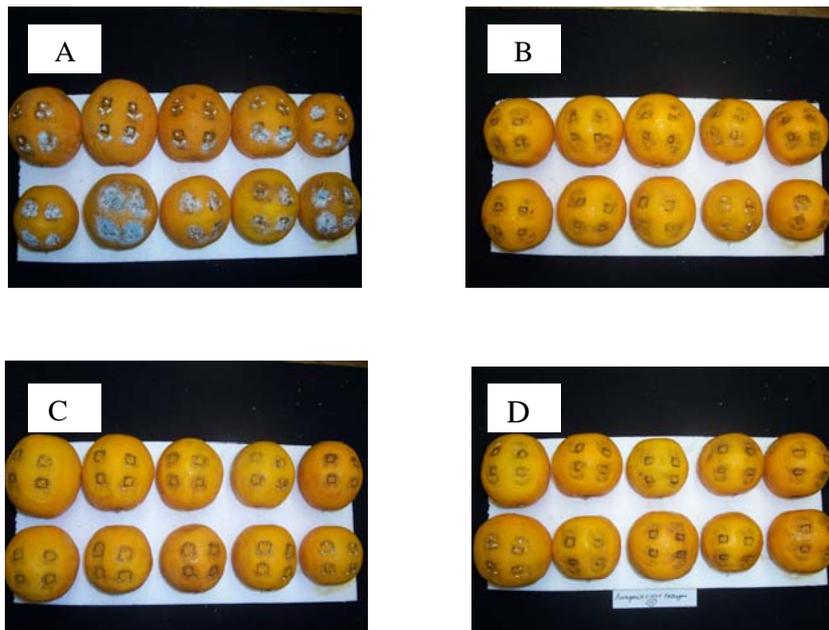
Legend: Treatment codes given are described as follows: A= *Penicillium digitatum* infection (control), B= MeJtw 10-2 + *Penicillium digitatum*, C= TiL 4-2 + *Penicillium digitatum,* D= TiL 4-3 + *Penicillium digitatum*.

Fig. 4. 5. Isolates: MeJtw 10-2 (*Cryptococcus laurentii*), TiL 4-2 (*Candida sake*) and TiL4-3 (*Cryptococcus laurentii*) activity *in vivo* against *Penicillium digitatum*.
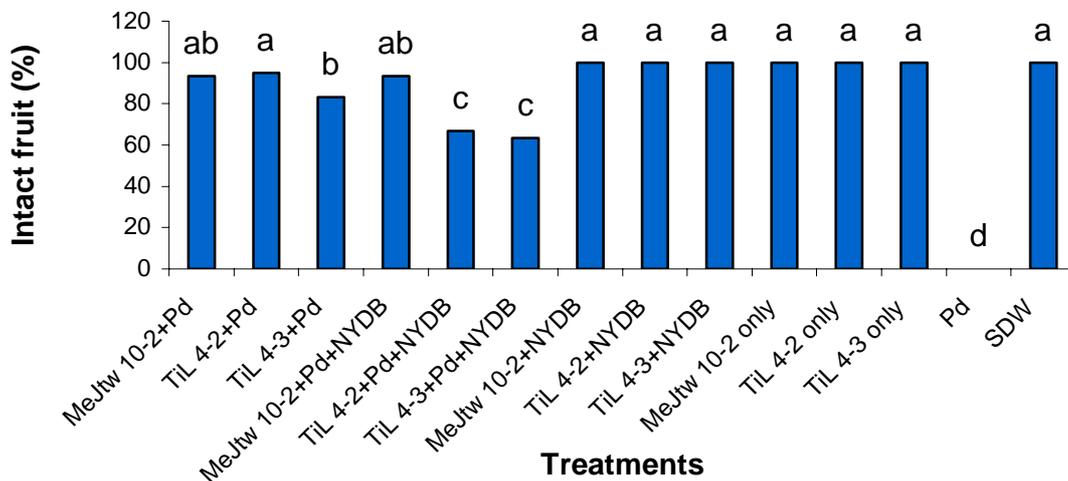

Legend: Bars with the same letter are not significantly different (*P* <0.05) according to Fisher's LSD test and t- grouping. Designated codes are referred as follows: MeJtw 10-2 = *Cryptococcus laurentii*, TiL 4-2 = *Candida sake*, TiL 4-3 = *C. laurentii*, Pd = *Penicillium digitatum* and SDW = Sterilised distilled water.

Fig. 4.6. *In vivo* yeast antagonists activity against *Penicillium digitatum*.

4.3.4   Antibacterial and antifungal assay

In the dual culture assay, all potential antagonists had no antibiosis activity against *P. digitatum*. Instead, rapid surface colonization activity on medium was noticed. (Fig. 4. 7).
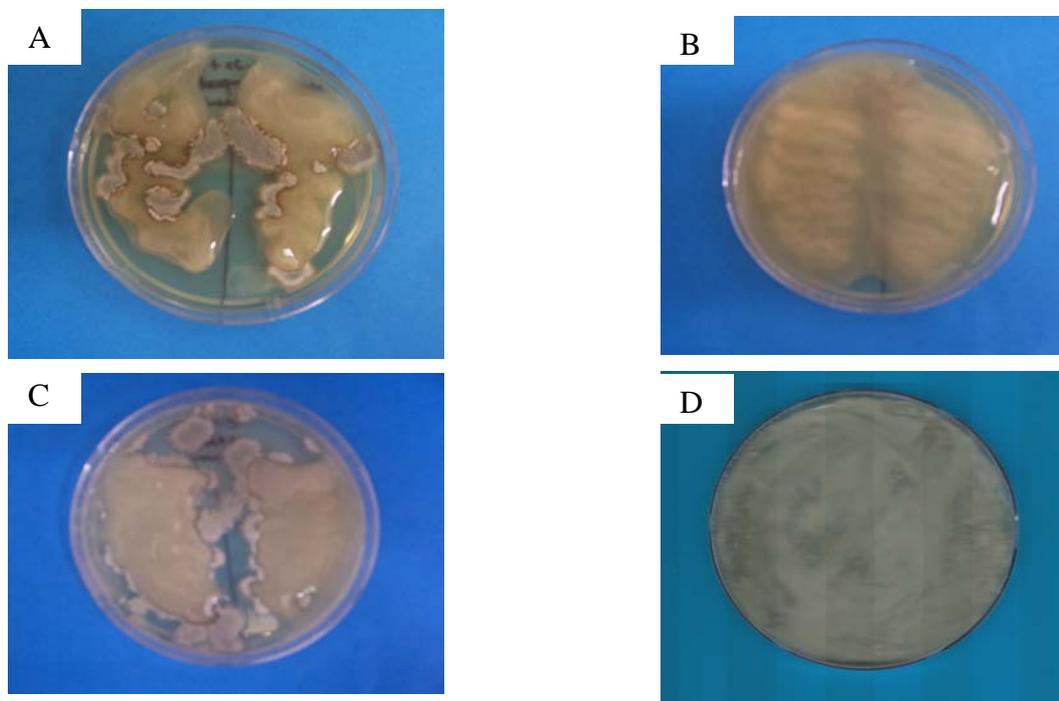
Legend: Pictures from A-D depict antagonists colonization effect over the growth of *Penicillium digitatum*. A= activity of antagonist MeJtw (*Cryptococcus laurentii*) (80% efficacy) B= activity of antagonist TiL4-2 (*Candida sake*) (100% efficacy), C = activity of antagonist TiL4-3 (*Cryptococcus laurentii*) (85% efficacy) against the growth of *Penicillium digitatum* and D= growth of *Penicillium digitatum* alone (control).

Fig. 4. 7. *In vitro* yeast antagonists activity on MEA plate against *Penicillium digitatum*.

4.3.5   Antagonist volatile production assay

None of the three potential antagonists produced volatiles ($P < 0.05$) as compared to the control.

4.3.6   Antagonist total phenolic content determination

The antagonists exhibited production of some phenolic compounds between 6 – 10 equivalent mg Gallic acid/g dry weight (Fig. 4. 8). A strain of *C. laurentii* (TiL4-3) contained relatively higher amounts of phenolic compounds (Fig. 4. 9).
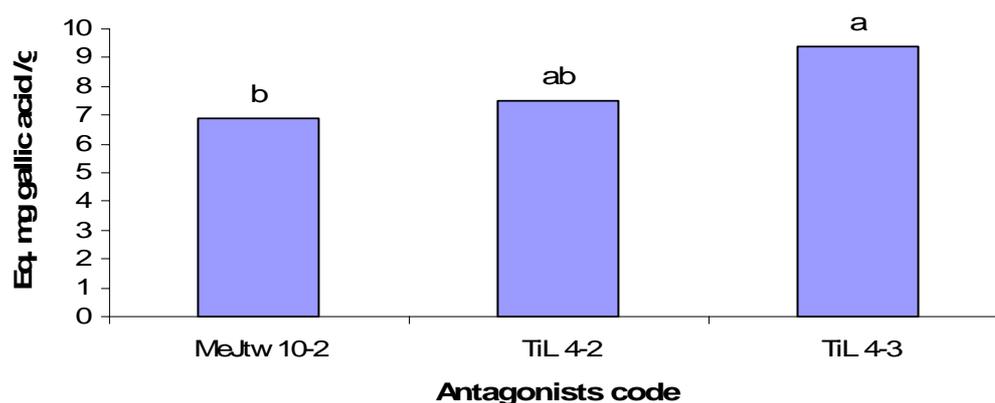
Legend: Bars with the same letter are not significantly different ($P < 0.05$) according to Fisher's protected LSD test and t- grouping.

Fig. **4. 10.** Antagonists total phenolics content determination.

4.3.7   Antagonists enzyme activity assay

Among the potential antagonists tested, one isolate *C. laurentii* (MeJtw10-2) showed some degree of extra cellular amylase, lipase and proteinase activity unlike *C. sake* (Til4-2) and *C. laurentii* (TiL4-3)] isolates (Table 4.1).

Table 4.1   Enzymatic activities of three yeasts, *in vitro*

Yeast antagonist code	Chitinolytic activity	Extracellular amylase activity	Lipase activity	Proteinase activity	Gelatinase activity
MeJtw 10-2	-	+	+	+	-
TiL 4-2	-	-	-	-	-
TiL 4-3	-	-	-	-	-

Legend: + = activity present
-    = no activity

4.3.8   *In vitro* competition for nutrients and space

Conidia of *P. digitatum* germinated at various concentrations of MEB and OPE within the first 24 h (Table 4.2a). At the higher concentration of the OPE, more conidia germinated. On the other hand, almost no conidia germinated in Ringer's solution (Table 4.2a). The antagonists prevented germination of conidia in all treatment combinations with 20 and 40% of MEB and 0.5 and 5% of OPE. All antagonists greatly reduced conidia germination in the higher OPE concentrations (Table 4.2a). Antagonists MeJtw10-2 (*C. laurentii*) and Til4-2 (*C.*

sake) showed a higher reduction rate of conidia germination compared to isolate Til4-3 (*C. laurentii*).

Cylinder insert membranes that moved from the original treatment to new wells containing the corresponding growth medium but without antagonists resulted in germination of all conidia in 5% OPE and the majority of the conidia in 0.5% OPE and 20 and 40% of MEB in the second 24 h incubation period (Table 4.2b).

Table 4. 2a   Percentage germination of *Penicillium digitatum* conidia on Polytetra-
fluoroethylene membranes

Treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer 's solution	98^b	2^k	0^k	0^g
20% MEB	19^k	21^c	23^b	37^d
40% MEB	10^l	5^h	27^a	58^c
0.5% orange peel extract (OPE)	9^m	11^f	17^c	63^b
5% OPE	0^n	3^j	9^d	88^a
With antagonists:				
Ringer's solution + antagonist MeJtw 10-2	100^a	0^m	0^k	0^g
20% MEB + antagonist MeJtw 10-2	95^e	5^h	0^k	0^g
40% MEB + antagonist MeJtw 10-2	96^d	4^i	0^k	0^g
0.5% OPE + antagonist MeJtw 10-2	91^f	7^g	2^i	0^g
5% OPE + antagonist MeJtw 10-2	98^b	1^l	1^j	0^g
Ringer's solution + antagonist TiL 4-2	100^a	0^m	0^k	0^g
20% MEB + antagonist TiL 4-2	97^c	3^j	0^k	0^g
40% MEB + antagonist TiL 4-2	98^b	2^k	0^k	0^g
0.5% OPE + antagonist TiL 4-2	98^b	2^k	0^k	0^g
5% OPE + antagonist TiL 4-2	97^c	3^j	0^k	0^g
Ringer's solution + antagonist TiL 4-3	100^a	0^m	0^k	0^g
20% MEB + antagonist TiL 4-3	68^i	27^b	4^g	1^f
40% MEB + antagonist TiL 4-3	78^g	16^e	3^h	3^e
0.5% OPE+ antagonist TiL 4-3	65^j	29^a	6^e	0^g
5% OPE + antagonist TiL 4-3	76^h	18^d	5^f	1^f

Legend: *Germinating rating scale: 1= no germination; 2= germ tube <2x conidia size; 3= germ tube 2 to 4x conidia size; 4= germ tube >4x conidia size: 100 conidia per treatment were counted. Code given to antagonists referred as follows: MeJtw 10-2 (*Cryptococcus laurentii*) = Merti-Jeju farm twig sample 10-2, TiL 4-2 (*Candida sake*) = Tibila farm leaf sample 4-2, TiL 4-3 (*Cryptococcus laurentii*) = Tibila farm leaf sample 4-3. Means with the same letter in the column are note significantly different (*P* <0.05) according to Duncan's Multiple Range test and grouping.

Table 4. 2b Percentage germination of *Penicillium digitatum* conidia on Polytetrafluoroethylene membranes in cylinders

Original treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer 's solution	96^a	2^k	2^m	0^p
20% MEB	0^m	4^j	14^h	82^d
40% MEB	0^m	0^l	11^j	88^c
0.5% OPE	0^m	0^l	7^l	93^b
5% OPE	0^m	0^l	0^n	100^a
With antagonists:				
Ringer's solution + antagonist MeJtw 10-2	64^c	18^e	12^i	6^m
20% MEB + antagonist MeJtw 10-2	18^g	23^a	30^d	29^k
40% MEB + antagonist MeJtw 10-2	12^j	21^b	31^c	36^g
0.5% OPE + antagonist MeJtw 10-2	19^f	19^d	24^f	38^f
5% OPE+ antagonist MeJtw 10-2	0^m	0^l	0^n	100^a
Ringer's solution + antagonist TiL 4-2	76^b	12^h	8^k	4^n
20% MEB + antagonist TiL 4-2	11^k	19^d	37^a	33^i
40% MEB + antagonist TiL 4-2	13^i	23^a	26^e	38^f
0.5% OPE + antagonist TiL 4-2	9^l	11^i	34^b	46^e
5% OPE+ antagonist TiL 4-2	0^m	0^l	0^n	100^a
Ringer's solution + antagonist TiL 4-3	54^d	20^c	23^g	3^o
20% MEB + antagonist TiL 4-3	31^e	17^f	26^e	26^l
40% MEB + antagonist TiL 4-3	14^h	21^b	24^f	31^j
0.5% OPE + antagonist TiL 4-3	18^g	16^g	31^c	35^h
5% OPE + antagonist TiL 4-3	0^m	0^l	0^n	100^a

Legend: For germinating rating scale refer to table 4.2a above. Further analysis of antagonist impact on *Penicillium digitatum* conidia germination was assessed by inserting the cylinder membranes to the new wells containing the same growth medium (Ringer's solution, 20% of MEB, 40% of MEB, 0.5% OPE and 5% of OPE) as used for original treatment to each antagonist. But, this time, the medium used was without antagonists. Plates were incubated for additional 24 h at 25 ºC. Means with the same letter in the column are note significantly different ($P < 0.05$) according to Duncan's Multiple Range test and grouping.

4.3.9   Turbidimetric measurements of antagonists growth in different growth mediums

All three antagonists showed a higher rate of population growth in 5% OPE in the first 24 and second 48 h of incubation (Fig. 4. 10, 11, 12). Antagonist Til4-2 (*C. sake*) exhibited a higher rate of population growth than the two *C. laurentii* strains (MeJtw10-2 and TiL4-3) (Fig. 4.10, 11, 12).
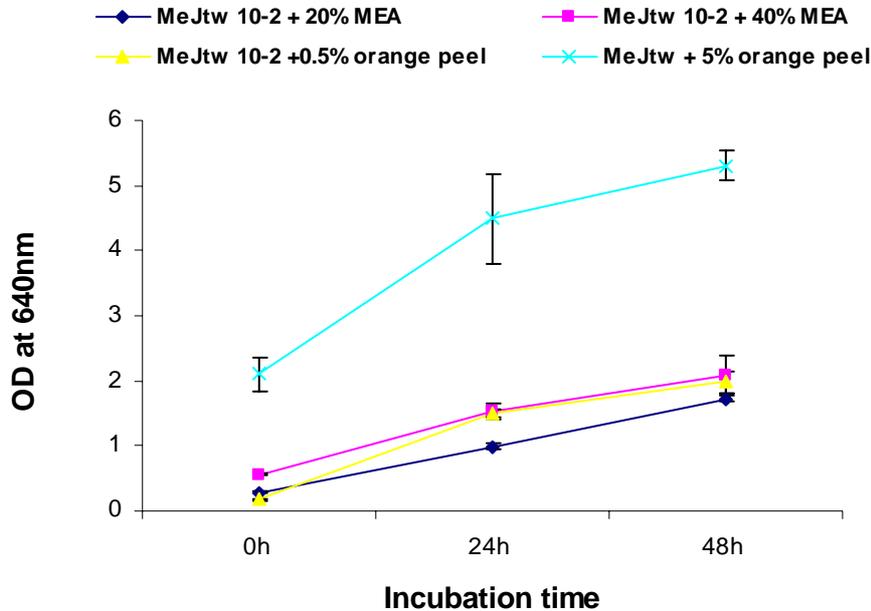

Fig. 4. 10. Turbidimetric measurement of antagonist MeJtw 10-2 (*Cryptococcus laurentii*) growth in different culture growth media.
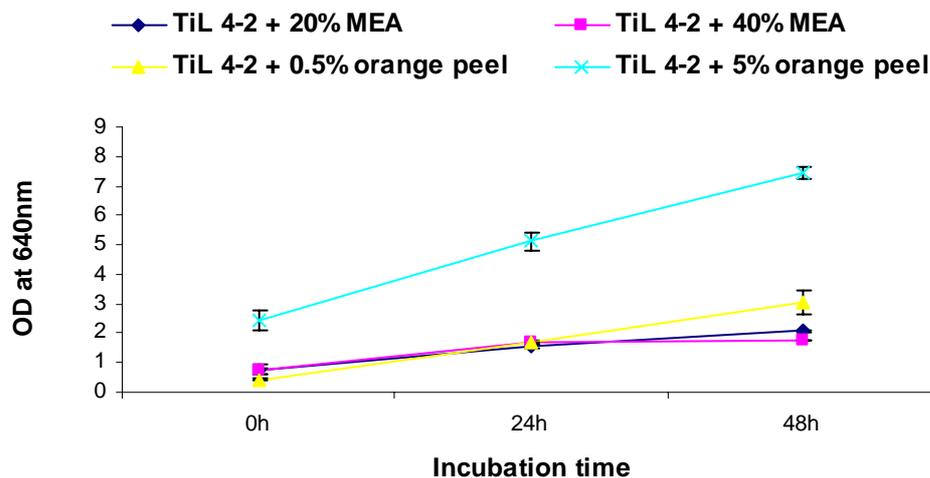

Fig. 4. 11. Turbidimetric measurement of antagonist TiL 4-2 (*Candida sake*) growth in different culture growth media.
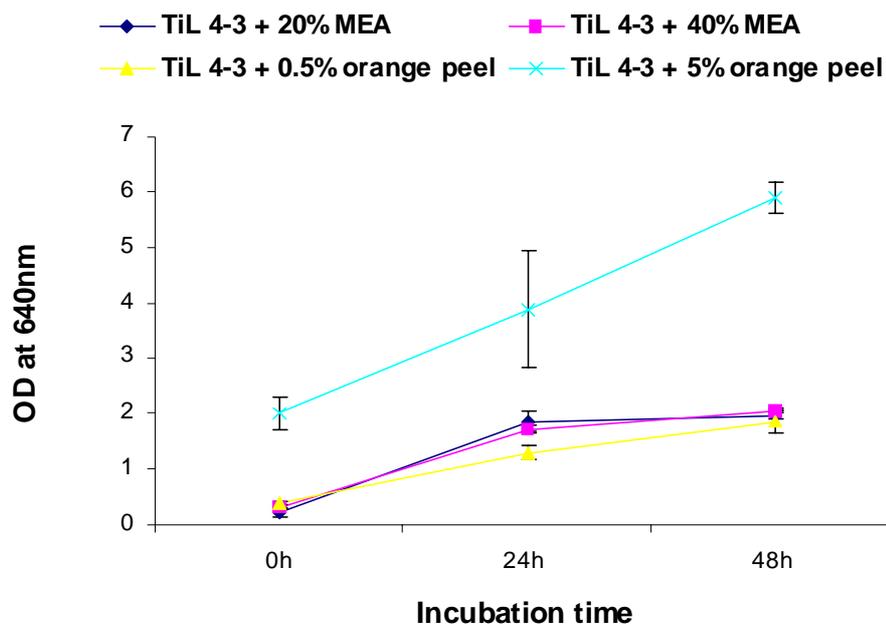
Fig. 4. 12. Turbidimetric measurement of antagonist TiL 4-3 (*Cryptococcus laurentii*) growth in different culture growth media.

4.3.10. *In vitro* study of antagonist-pathogen interaction using micro well plates

Conidia of *P. digitatum* germinated in all culture broths except in Ringer's solution when incubated for 24 and 48 h at 25 ºC. All three antagonists [MeJtw10-2 (*C. laurentii*), TiL4-2 (*C. sake*) and TiL 4-3 (*C. laurentii*) greatly decreased *P. digitatum* conidia germination for the first 24 h incubation, where the highest inhibition was exhibited by antagonists TiL4-2 (Table 4.3a). Almost all conidia of *P. digitatum* germinated in the following 24 h cycle at 25 ºC. The antagonist prevented conidia germination between 48-82% with the highest rate recorded for the antagonist TiL 4-2 (*C. sake*) (82%) and the least by antagonist TiL4-3 (*C. laurentii*) (Table 4. 3b) (48%).

Table 4. 3a   Percent germination of *Penicillium digitatum* conidia in micro wells direct interaction with antagonists exposed for 24 h incubation at 25 ºC

Treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer 's solution	96^e	4^i	2^e	0^e
20% MEB	9^l	16^a	28^a	47^d
40% MEB	9^l	11^e	26^b	54^c
0.5% orange peel extract (OPE)	6^m	12^d	17^c	65^b
5% OPE	0^n	8^f	11^d	81^a
With antagonists:				
Ringer's solution + antagonist MeJtw 10-2	100^a	0^m	0^f	0^e
20% MEB + antagonist MeJtw 10-2	92^h	8^f	0^f	0^e
40% MEB + antagonist MeJtw 10-2	94^f	6^h	0^f	0^e
0.5% OPE + antagonist MeJtw 10-2	96^e	4^i	0^f	0^e
5% OPE + antagonist MeJtw 10-2	97^d	3^j	0^f	0^e
Ringer's solution + antagonist TiL 4-2	100^a	0^m	0^f	0^e
20% MEB + antagonist TiL 4-2	97^d	3^j	0^f	0^e
40% MEB + antagonist TiL 4-2	98^c	2^k	0^f	0^e
0.5% OPE + antagonist TiL 4-2	98^c	2^k	0^f	0^e
5% OPE + antagonist TiL 4-2	99^b	1^l	0^f	0^e
Ringer's solution + antagonist TiL 4-3	100^a	0^m	0^f	0^e
20% MEB + antagonist TiL 4-3	86^k	14^b	0^f	0^e
40% MEB + antagonist TiL 4-3	89^i	11^e	0^f	0^e
0.5% OPE+ antagonist TiL 4-3	87^j	13^c	0^f	0^e
5% OPE + antagonist TiL 4-3	93^g	7^g	0^f	0^e

Legend: *Germinating rating scale: 1= no germination; 2= germ tube <2x conidia size; 3= germ tube 2 to 4x conidia size; 4= germ tube >4x conidia size: 100 conidia per treatment were counted. For code given to antagonists refer to table 4.2a legend. Means with the same letter in the column are note significantly different (*P* <0.05) according to Duncan's Multiple Range test and grouping.

Table 4. 3b Percent germination of *Penicillium digitatum* conidia in micro wells direct interaction with antagonists exposed for additional 24 h incubation at 25 ºC

Original treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer 's solution	94^b	2^k	4^m	0^m
20% MEB	1^m	3^j	14^f	82^d
40% MEB	0^n	3^j	4^m	93^c
0.5% orange peel extract (OPE)	0^n	2^k	3^n	95^b
5% OPE	0^n	0^l	0^o	100^a
With antagonists:				
Ringer's solution + antagonist MeJtw 10-2	100^a	0^l	0^o	0^m
20% MEB + antagonist MeJtw 10-2	48^l	22^a	18^c	12^g
40% MEB + antagonist MeJtw 10-2	51^j	18^d	21^a	10^i
0.5% OPE + antagonist MeJtw 10-2	55^i	19^c	15^e	11^h
5% OPE + antagonist MeJtw 10-2	68^f	18^d	8^j	6^k
Ringer's solution + antagonist TiL 4-2	100^a	0^l	0^o	0^m
20% MEB + antagonist TiL 4-2	68^f	15^f	11^h	6^k
40% MEB + antagonist TiL 4-2	72^e	13^g	9^i	6^k
0.5% OPE + antagonist TiL 4-2	79^d	9^h	7^k	5^l
5% OPE + antagonist TiL 4-2	82^c	7^i	6^l	5^l
Ringer's solution + antagonist TiL 4-3	100^a	0^l	0^o	0^m
20% MEB + antagonist TiL 4-3	49^k	18^d	19^b	14^f
40% MEB + antagonist TiL 4-3	51^j	21^b	17^d	11^h
0.5% OPE+ antagonist TiL 4-3	56^h	16^e	12^g	16^e
5% OPE + antagonist TiL 4-3	61^g	19^c	11^h	9^j

Legend:*Germinating rating scale and other descriptions, see table 4. 2a legend. Means with the same letter in the column are not significantly different ($P < 0.05$) according to Duncan's Multiple Range test and grouping.

4.3.11   Effects of the culture filtrate against spore germination of *Penicillium digitatum*

All yeast antagonists: MeJtw10-2 (*C. laurentii*), TiL4-2 (*C. sake*) and TiL4-3 (*C. laurentii*) culture suspensions amended with PDB showed significant ($P < 0.05$) inhibition against *P. digitatum* spore germination (Fig. 4.13). A treatment combination with antagonist TiL4-2

showed 46.6% inhibition followed by MeJtw10-2 (38.3%) and TiL4-3 (35.8%) (Fig. 4.13). No inhibition was observed with autoclaved spore culture suspensions.
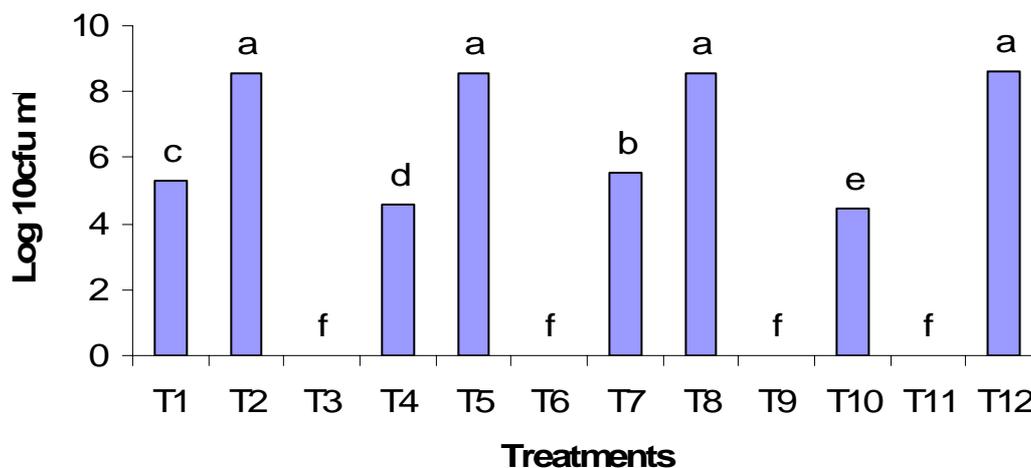

Legend: Mean values are expressed in Bars. Bars designated with the same letter are not significantly different according to Fisher's LSD test (P <0.05) and t-grouping. Treatments are described as follows: T1= Antagonist MeJtw 10-2 + PDB (Potato Dextrose Broth) (Oxoid, Johannesburg) + *P. digitatum* spore, T2= Antagonist MeJtw 10-2 boiled culture + *P. digitatum* spore, T3= Antagonist MeJtw 10-2 boiled culture alone, T4 = Antagonist TiL 4-2 + PDB (Oxoid) + *P. digitatum* spore, T5= Antagonist TiL 4-2 boiled culture + *P. digitatum* spore, T6= Antagonist TiL 4-2 boiled culture alone, T7= Antagonist TiL 4-3 + PDB (Oxoid) + *P. digitatum* spore, T8= Antagonist TiL 4-3 boiled culture + *P. digitatum* spore, T9= Antagonist TiL 4-3 boiled culture alone, T10= Cyclohexamide (Sigma, Germany) + *P. digitatum* spore, T11= Cyclohexamide alone and T12= *P. digitatum* alone.

Fig. 4. 13. *In vitro* activity of antagonists against *Penicillium digitatum* spore germination.

4.3.12   Minimum inhibitory concentration of antagonists against *Penicillium digitatum*
All spore concentrations of antagonists [10^5, 10^6, 10^7 and 10^8] significantly (P <0.05) reduced disease incidence as compared to the control (Fig. 4. 14, 15, 16). A high rate of disease incidence reduction was observed and an increased application of antagonist spore suspension was found with lower concentrations (10^5 and 10^6) of the pathogen, *P. digitatum*. Antagonist MeJtw10-2 (*C. laurentii*) showed complete control of Penicillium infection when applied at 10^7 and 10^8 spore concentrations challenged to 10^5 and 10^6 spore concentration of the pathogen. All spore concentrations of antagonist TiL4-2 (*C. sake*) [10^5, 10^6, 10^7 and 10^8] showed complete control of Penicillium spore when challenged at lower (10^5) concentration.

Antagonists at 10^8 concentration showed complete control of a pathogen challenged at 10^6 concentration (Fig. 4. 15). The antagonist TiL4-3 (*C. laurentii*) on the other hand showed complete control of *P. digitatum* fruit decay when applied at higher concentration (10^7 and 10^8) challenged against lower concentrations of the pathogen spore suspension (10^5 and 10^6), respectively.
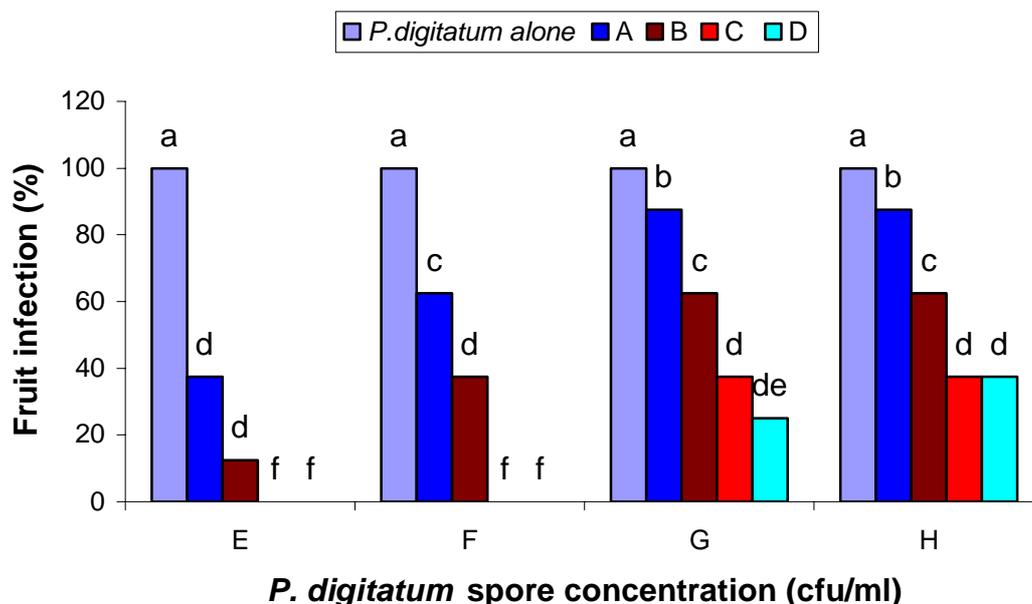
Legend: Bars represent mean of experiments. Means with the same letter are not significantly different at *P* <0.05 using Fisher's LSD t- grouping.

Bars with different colour referred percentage intact fruit and antagonist concentrations used in the test, accordingly:
⬛A= 1 x 10^5, ⬛B= 1 x 10^6, ⬛C= 1 x 10^7, and ⬜D= 1 x 10^8 spores ml $^{-1}$.

*Capital alphabets on the horizontal line of the figure referred to *P. digitatum* spore concentrations:
E= 1 x 10^5, F= 1 x 10^6, G= 1 x 10^7, and H= 1 x 10^8 spores ml^{-1}. In the preliminary experiment, all antagonists at 10^4 concentration were found ineffective to the range of pathogen concentrations tested and were therefore excluded for simplicity.

Fig. 4.14. *In vivo* efficacy of MeJtw 10-2 (*Cryptococcus laurentii*) minimum inhibitory concentrations against *Penicillium digitatum* spores growth .
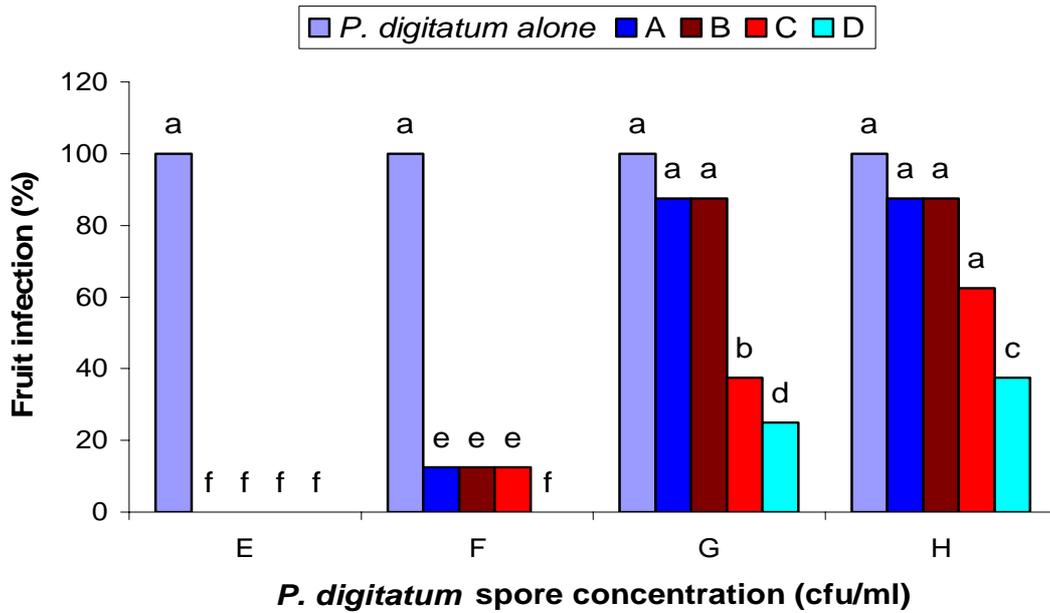
Legend: Bars with the same letter are not significantly different according to Fisher's LSD test ($P <0.05$) and t- grouping. For other letter descriptions refer the figure 4. 14.

Fig. 4. 15. *In vivo* antagonist (TiL 4-2) minimum inhibitory concentrations determination against spore germination of *Penicillium digitatum* on citrus fruit.
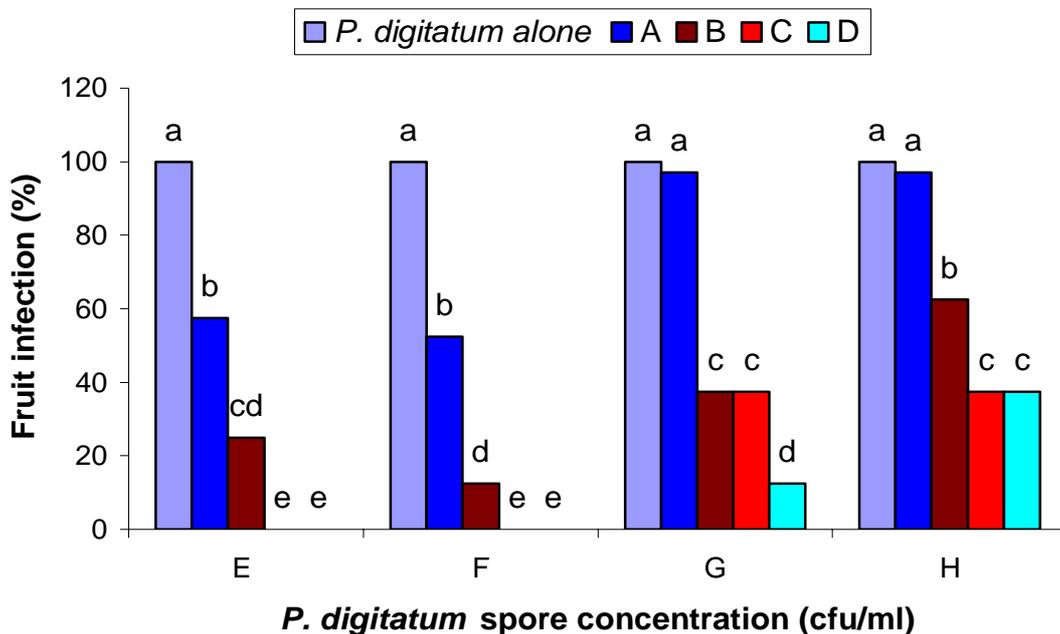
Legend: Means with the same small alphabet letters are not significantly different at $P <0.05$ using Fisher's LSD t- grouping. For the rest of letters descriptions refer the above figure 4.14.

Fig. 4. 16. *In vivo* antagonist TiL 4-3 (*C. laurentii*) minimum inhibitory concentrations determination against *Penicillium digitatum* spore growth on citrus fruit.

4.3.13   Wound site colonization and attachment of antagonists

Under SEM observations of orange fruit wounds inoculated with antagonists and the pathogen *P. digitatum*, a significant reduction of conidia germination and different mechanisms of wound healing could be seen (Fig. 4-17). Mode of actions that involved secretion of extracellular fluid and sticking of the pathogen (Fig. 4. 17A-F) were the major activities identified. All antagonists produce extracellular fluid when applied alone. The application of *P. digitatum* alone showed higher degree of conidia germination Fig. 4.17 E-G), which later changed into germ tube elongation and hyphae growth (Fig. 4.17H and I).
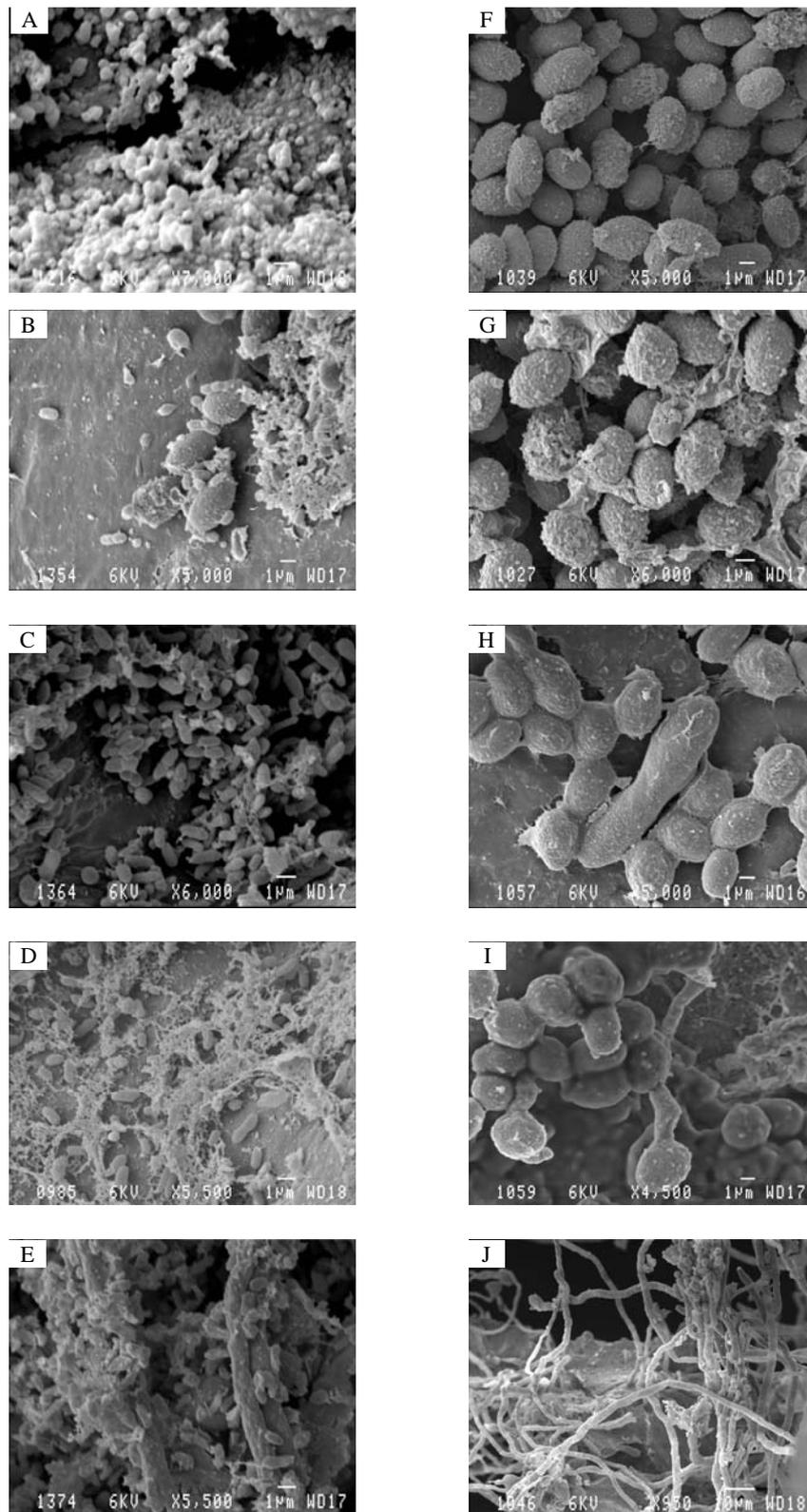
Fig. 4. 19. Antagonists mode of action against *Penicillium digitatum* on fruit wound viewed through scanning electron microscope. Images from A-F describe antagonists (MeJtw 10-2, TiL 4-2 and TiL4-3) activity at 0, 6, 12, 24 and 48h attachment against *Penicillium digitatum* at the wound site, respectively. Images from G-J refer to the development of infection by *P. digitatum* at the wound site at 0, 6, 12, 24 and 48 h of attachment.

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4.3.14 *In vitro* **integrated treatment between yeast antagonists, plant extracts and commercial fungicide**

All antagonists showed higher rate of growth with fresh and old preparations of plant extracts at various treatment concentrations (Table 4.4). Although they exhibited high rate of recovery (data not shown here), maximum rate of antagonist growth inhibition (67%) was observed with the application of Procloraz (10^{-1} dilution) to antagonist TiL 4-2 and Guazatine (10^{-1} dilution) to antagonist MeJtw 10-2.

Table 4. 4 Integrated treatment *in vitro* between yeast antagonists, plant extracts and commercial fungicide

Treatments	Dilution	Yeast antagonists*		
		MeJtw10-2	TiL4-2	TiL4-3
Extract A fresh preparation	Control	0	0	0
	10^{-1}	++	++	++
	10^{-2}	0	+	0
	10^{-3}	0	0	0
Extract A old preparation	Control	0	0	0
	10^{-1}	++	++	++
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Extract B fresh	Control	0	0	0
	10^{-1}	+	++	++
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Extract B old	Control	0	0	0
	10^{-1}	+	+	0
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Prochloraz	Control	0	0	0
	10^{-1}	+++	+++++	+++
	10^{-2}	++	++++	+++
	10^{-3}	++	+++	++
Guazatine	Control	0	0	0
	10^{-1}	+++++	++++	++++
	10^{-2}	++++	+++	++
	10^{-3}	+++	+	0
RSAF-1	Control	0	0	0
	10^{-1}	+	+	++++
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Ultracure	Control	0	0	0
	10^{-1}	+++	+++	++
	10^{-2}	+++	++	+
	10^{-3}	+++	+	+
Quatrokill	Control	0	0	0
	10^{-1}	+++	++++	+++
	10^{-2}	++	+++	++
	10^{-3}	++	++	++
Imazilil	Control	0	0	0
	10^{-1}	++++	+++	+++
	10^{-2}	+++	++	++
	10^{-3}	+++	+	+

Legend: The formation of inhibition zone were tabulated in to the following categories: 0 = No Inhibition, + = 1-2 mm diameter, ++ = 3-6 mm diameter =, +++ = 7-10 mm diameter, ++++ = 11-14 mm diameter and +++++ = 15-18 mm diameter. * = Antagonist MeJtw10-2 (*Cryptococcus. laurentii*), TiL4-2 (*Candida sake*) and TiL 4-3 (*C. laurentii*). Extract A = *Acacia seyal* Del. Var. Seyal, B = *Withania somnifera* L. Dunal.

4.4   DISCUSSION

In this study, three potential yeast antagonists [two strains of *C. laurentii* (Megtw10-2 and TiL4-2) and one strain of *C. sake* (TiL4-3) exhibited high inhibition of *P. digitatum* growth rate. In addition, these isolates have a broad spectrum activity against *G. candidum* and *C. gloeosporioides*. Several previous reports demonstrated the potential use and application of yeast antagonists to control postharvest decay of fruits and vegetables (Wisniewski and Wilson, 1992; Janisiewicz and Bors, 1995). The successful application of *C. laurentii* on arbutus berries (Zheng *et al*., 2004), pear (Zhang *et al*., 2005a), oranges (Zhang *et al*., 2005b), apples (Roberts, 1990), strawberries, kiwi fruits and table grapes (Lima *et al*., 1998) and *C. sake* on apple (Usall *et al*., 2001), pears (Nunes *et al*., 2001) has been studied. These isolates proofed effective against a range of pathogens including *Penicillium* spp. (Teixido *et al*., 1998; Abadias *et al*., 2002; Vero *et al*., 2002; Zhang *et al*., 2003; Zhang, *et al*., 2005a), *G. candidum* (Chalutz and Wilson, 1990) and *C. gloeosporioides* (Koomen and Jeffries, 1993).

It is evident from the *in vitro* study that the selected potential antagonists did not show antibiosis or volatile production against the pathogens tested. On the other hand, i*n vivo* wound treatment application of these antagonists showed significant (P <0.05) reduction of disease incidence between 65-95% on fruits incubated at 7 ºC for 30 days. Fast colonization and competitive ability of antagonists were previously demonstrated by the non-destructive *in vitro* cylinder insert experiment (Janisiewicz *et al*., 2000). Inhibition of pathogen spore germination during the first 24 h of cylinder insert experiments, and its germination when transferred to fresh nutrient solution without antagonists confirmed that competition for nutrients and space by the antagonists were the main mode of actions which is in agreement with Janisiewicz *et al*. (2000). Reports with the application of different yeast antagonists such as *Debaryomyces hansenii* (Droby *et al*., 1989), *Pichia guilliermondi* (Arras *et al*., 1998) and *Aureobasidium pullulans* (Janisiewicz *et al*., 2000; Castoria *et al*., 2001) against *Penicillium* spp indicated similar results.

A higher rate of disease incidence reduction was observed through activity of antagonists TiL4-2 (*C. sake*) (95%) followed by *C. laurenti,* isolate MeJtw10-2 and TiL4-3 (70-90%). This result showed higher efficacy as compared to reports made by Usall, *et al*. (2001) and Vero *et al*. (2002) with *C. sake* (CPA-1) on apple (70 and 80%, respectively), Zhang *et al*. (2005) with *C. laurentii* on orange fruits with (80%) efficacy against blue mould.

The addition of NYDB as growth additive to yeast antagonists on fruit wound sites suppressed the activity of the antagonists against *P. digitatum*. Significant ($P < 0.05$) infection rate of fruits was observed on fruits treated with antagonists TiL4-2 (*C. sake*) and/ or Til4-3 (*C. laurentii*) using NYDB as a growth substrate medium. The application of NYDB to the antagonist MeJtw10-2 (*C. laurentii*), however did not exhibit significant ($P < 0.05$) change in fruit decay. Unlike the report made by Nunes *et al*. (2001), this study demonstrated that the nutritional environment amended at the wound site could favour growth of a pathogen rather than the antagonists. On the other hand, this result is in agreement with the report made by Vero *et al*. (2002) indicating the growth limitation of antagonists with the addition of a nitrogen source medium as a growth substrate on apple wounds. This proofed the great potential of the yeast antagonists for their rapid colonization for space and nutrients when minimum nutrients are available at the wound site and without additional expenses.

Boiled culture filtrates of all antagonists failed to control spore germination of *P. digitatum*. It is evident from this experiment that only live cells were effective in controlling *P. digitatum* spore germination involving competition for nutrients and space rather than antibiosis. Similar reports by Droby *et al*. (1989) indicated effective competition of a yeast antagonist on grapefruits for nutrients against *P. digitatum*. This explanation for the rapid colonization of antagonists on the wound site with minimum nutrients available is also supported by the scanning electron microscope observation and *in vitro* dual culture study of antagonists on solid MEA media suggesting the production of an extracellular matrix on which they grow faster. According to Janisiewicz (1988), the rapid growth of antagonists is facilitated by the production of these extracellular polysaccharides over the surface. Chan and Tian (2005) on the other hand explained that the extracellular matrix produced by the antagonists may have a lytic effect towards the pathogen and provides higher amounts of simple carbon sources for the antagonists (Lima *et al*., 1998). Therefore, the rapid growth of the yeast antagonists without any additives at the wound site indicates their ability and considerable potential to be used as a biocontrol agent (Vero *et al*., 2002).

Results from MIC determination of antagonists demonstrated that the efficacy of the yeast antagonists depends on the inocula concentration of both the pathogen and antagonists. All antagonists effectively decreased disease incidence (100%) at concentration of 10^8 cells ml^{-1} against *P. digitatum* (10^6 spores ml^{-1}). The efficacy of antagonists however decreased as the concentration of the pathogen inoculum was increased. Antagonist TiL4-2 (*C. sake*) suppressed *P. digitatum* growth at a minimum concentration of 10^5 spores ml^{-1} of both

antagonist and pathogen, which is a better result compared to the report made by Droby *et al*. (1989).

In conclusion, although the mechanisms by which yeast biocontrol agents provide decay control are not fully understood, the mode of action of several yeast antagonists doesn't involve antibiosis as was found in this study. Instead, competition for nutrients (Benbow and Sugar, 1999; Janisiewicz *et al*., 2000) and space (Janisiewicz *et al*., 2000) at the wound site is more likely the mode of action.   In this study, the rapid colonization effect of yeast antagonists through production of the extracellular matrix that sticks to the pathogens and/ or having a lytic effect against the pathogen was also found as was confirmed with the *in vitro* dual culture experiments supported by the electron microscopy study.   Such peculiar characteristics of these yeast antagonists signify their great potential for industrial use in the postharvest disease control arena.

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