



Efficacy and mode of action of yeast antagonists for control of *Penicillium digitatum* in oranges

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ABSTRACT

Three yeast antagonists (two strains of *Cryptococcus laurentii* and one of *Candida sake*) from orange trees reduced incidence of green mold by 80 to 95% when tested in wounded orange fruits inoculated with *Penicillium digitatum* and incubated at 7°C for 30 days. The yeasts inhibited conidial germination of the pathogen, but did not kill the spores. Effectiveness of the three yeasts as antagonists was associated in part with their ability to rapidly colonize wound sites, despite low nutrient availability. Observations suggested that production of extracellular matrix by the yeasts may have facilitated rapid wound colonization. Germination of *P. digitatum* conidia was significantly inhibited when the pathogen and antagonists were in direct physical contact in a culture suspension. The results supported the view that competition for nutrients is also a mode of action of yeasts against *P. digitatum*.

Key words: *Candida sake*, *Cryptococcus laurentii*, Citrus, Yeast antagonists.

INTRODUCTION

Green mold, caused by *Penicillium digitatum* Sacc., is a foremost postharvest disease of oranges and other citrus fruit. Repeated cycles of infection and sporulation of the pathogen commonly occur in packed fruit. If precautions are not taken, inoculum pressure of *P. digitatum* may increase in packing houses as the picking season advances (Janisiewicz & Korsten, 2002). Synthetic fungicides such as imazalil and thiabendazole are currently used to control postharvest infection (Holmes & Eckert, 1999; Palou et al., 2002) but often result in fungicide residues in the fruit, which may negatively affect human health (Norman, 1988). Frequent use of fungicides has resulted in the development of resistant populations of *P. digitatum* (Wisniewski & Wilson, 1992). Biological agents are receiving attention to control green mold because they are perceived as environmentally safer and more acceptable to the general public (Janisiewicz & Korsten, 2002).

Antagonistic strains of yeasts and bacteria have been evaluated for activity against several pre- and postharvest pathogens (El-Ghaouth et al., 2002; Zheng et al., 2004). Strains of *Bacillus subtilis* (Janisiewicz & Korsten, 2002), *Pseudomonas syringae* (Bull et al., 1998) and *Candida oleophila* (Droby et al., 1989) were shown to effectively control molds and sour rot on citrus. These respective strains have been registered and commercialized in South Africa and the USA as Avogreen (Sebor manufacturing, Johannesburg) (Janisiewicz &

Korsten, 2002), Aspire™ (Ecogen corporation, Langhorne, PA) (Droby et al., 1998) and Biosave 110 (EcoScience Inc., Worcester, MA) (Shachnal et al., 1996). The objectives of the present study were to determine the efficacy and modes of action of three strains of antagonistic yeasts [two *Cryptococcus laurentii* (MeJtw 10-2, TiL 4-2) and one strain of *Candida sake* (TiL 4-3)] against *P. digitatum* in oranges (*Citrus sinensis* L.).

An understanding of the modes of action of antagonists is important for developing protocols for microbial agents in citrus. Commonly recognized modes of action of microbial agents include antibiosis, competition for nutrients and space and induction of host resistance (Lima et al., 1998; Droby et al., 2002; Poppe et al., 2003). Non-antibiotic modes of action are often preferred for biocontrol of postharvest diseases (Janisiewicz et al., 2000).

MATERIAL AND METHODS

The test pathogens

Strain UPPed-1 of the test pathogen *Penicillium digitatum* was obtained from the culture collection of the Department of Microbiology and Plant Pathology, Pretoria University, South Africa. Cultures on potato dextrose agar (PDA; Biolab) medium were incubated under UV light for 7-14 days at 25°C for spore production. Conidial suspensions were prepared by washing the mycelial mat in each Petri dish with 20 mL sterilized distilled water, and the density was adjusted to 1×10^5 conidia mL⁻¹ with the aid of a hemacytometer for use in inoculations.

Antagonist selection

Three yeast strains (MeJtw 10-2 and TiL 4-3 of *C. laurentii* and TiL 4-2 of *C. sake*) were used in experiments. The strains were previously selected from among 242 bacterial and yeast isolates recovered from fruit, twig and leaf tissues of oranges in Ethiopia in 2005/2006 and transferred to South Africa following standard quarantine requirements according to the national legislation and germ-plasm transfer agreements, (import permit number P0017192). Selection was based on rapid growth on three citrus pathogens (*P. digitatum*, *G. candidum* and *Colletotrichum gloeosporioides*) in tests on agar media. The strains were maintained on nutrient agar (NA) medium at 4°C until used.

Antagonist activity against green mold

Twenty boxes each containing 80 freshly harvested oranges were collected from the Crocodile Valley packhouse (Nelspruit, Mpumalanga, South Africa). The fruits were disinfested with 1% sodium hypochlorite for 2 min and air dried. Two wounds were made on each fruit, (one each on opposite sides of the fruit) to a depth of 3 mm into the rind by means of a sterilized picture hook. For use in inoculations, test yeasts were grown for 12 h at 24°C in nutrient broth (NB) (Biolab) and the inoculum density was adjusted to 10^8 cells mL⁻¹ using a Petroff Hauser counting chamber. For preventive tests, 40 µL of cell suspension was placed into each wound 12 h prior to inoculation with *P. digitatum* (50 µL). Wounds were inoculated with *P. digitatum* alone or with NB as positive and negative controls, respectively. Treated fruits were stored at 7°C for 30 days and then assessed for incidence of green mold. Reduction in disease incidence and percentage intact fruit were computed. Three fruits were inoculated for each treatment and the experiment was repeated once.

Phenolic production by the antagonists

A 24-h culture of each yeast strain in NB was centrifuged at 5000 x g for 10 min and the culture filtrate was transferred into a 2-mL sterilized Eppendorf tube. Total soluble phenolic compounds were quantified using Folin Ciocalteu's Phenol reagent (Sigma) (Bray & Thorpe, 1954). In the extraction process, twofold volumes of ethyl acetate were added to the culture filtrate, vortexed for 30 s, and allowed to stand for 1 min. The organic phase containing the ethyl acetate and soluble phenolics was transferred to an Eppendorf tube. The extraction process was repeated three times. The supernatants were combined, freeze-dried and re-dissolved in 1 mL distilled water. A comparative study was performed on the culture filtrates by TLC on pre-coated Silica Gel 60 (Merck, Johannesburg) using chloroform/methanol/ethyl acetate/acetone/ water (55:20:20:5:3.5) as a separation solvent system. The TLC plates were loaded with 20 µL of each sample and the experiment was done in triplicate. A sterile broth culture was used as negative control, and standard chemicals such as isoferulic acid and

P-coumaric acid (Sigma), novobiocin, cyclohexamide and chloramphenicol (CAPS Pharmaceuticals, Johannesburg) were used as positive controls.

Growth inhibition assays

A streak assay on agar media in Petri dishes (Poppe et al., 2003) and the TLC plate assay (Castoria et al., 2001) were used to examine the ability of the three yeast strains to suppress the growth of *P. digitatum*. For the assay, orange peel extract (OPE) in a proportion of 10g l⁻¹ was mixed with NA, malt extract agar (MEA) and PDA. An agar disc (3 mm diameter) from a seven-day-old culture of *P. digitatum* was placed on the medium at the center of each Petri dish. Loops of 12-h broth cultures of the yeasts were streaked in a triangular format 15 mm from the center of each Petri dish. The dishes were incubated at 25°C for seven days and examined for zones of growth inhibition of *P. digitatum*. Five dishes were used per treatment and the experiment was repeated twice.

Yeast-pathogen challenge test *in vitro*

Possible effects of the yeasts on *P. digitatum*, such as antibiosis, surface colonization and lytic activity, were assessed on MEA amended with 0.5% citrus juice (v/v) in Petri dishes (Chan & Tian, 2005). Ten µL of pathogen suspension (10^5 conidia mL⁻¹) was placed in the center of the MEA in each dish. After 12 h of incubation at 25°C, 50 µL of each yeast cell suspension (1×10^8 cells mL⁻¹) were spread about 1 cm from the margin of the pathogen inoculum. After incubation at 25°C for 5-7 days the plates were examined for evidence of any antagonistic activity to the pathogen. Any contact of yeast cells and pathogen mycelium was recorded. The experiment was done in triplicate and repeated twice.

Competition for nutrients *in vitro*

Effects of nutrient depletion by the yeasts on germination and growth of *P. digitatum* conidia were evaluated using a method described by Janisiewicz et al. (2000) in which cylinder inserts in micro-wells were used. Each micro-well was covered with a polytetrafluoroethylene membrane positioned in the well culture. Malt extract broth (MEB) (20 or 40% (w/v) (Oxoid, Johannesburg) and OPE (0.5 and 5%) diluted in Ringer's solution were used as nutrient sources and Ringer's solution alone was used as a negative control (Poppe et al., 2003). A standard nutrient broth suspension of each yeast (1×10^8 cells mL⁻¹ NB) was dispensed (0.6 mL per well) into the wells outside of the insert of the culture plates. A sterilized suspension of *P. digitatum* (10^5 conidia mL⁻¹ water) was dispensed into the well of each cylinder insert (0.4 mL per cylinder) with membrane. After the plates were incubated at 25°C for 24 h, membranes from the cylinder inserts were removed, blotted with sterilized tissue paper, and cut into four equal pieces with a sterilized scalpel. Two of the pieces of each membrane were transferred to a glass slide, stained

with lactophenol blue solution (Fluka, Johannesburg) and mounted for observations of germination of *P. digitatum* for conidia on a light microscope. Germination of conidia on the membranes was scored using four classes: 1 = no germination, 2 = germ tube < 2 x conidia length, 3 = germ tube 2 - 4 x conidia length; 4 = germ tube > 4 x conidia length. One hundred conidia were assessed per treatment. Each experiment was performed twice with four wells per treatment. The other two pieces of insert membranes were transferred to each of separate MEA plates and incubated at 25°C for a period of two weeks. Plates were evaluated for colony growth of the pathogen and yeasts, and mean percentage growth diameter of the pathogen conidia was determined.

In a further experiment, micro-well plates without cylinders were used to study the direct physical contact and interaction between the pathogen and the yeasts. The standard concentration of spore suspension (10^5 conidia mL⁻¹ water) was added directly to the well containing the standard concentration of the antagonist (1×10^8 cells mL⁻¹ NB) and plates were incubated at 25°C for 24 h. The incidence of spore germination was estimated in 100 µL suspension according to Meziane et al. (2006).

Effects of yeast culture filtrates on spore germination of *P. digitatum*

Culture filtrates of the yeasts were prepared as described by Spadaro et al. (2002). Treatment combinations were culture filtrates, boiled (10 min) culture filtrates each with or without potato dextrose broth (PDB; Biolab) and *P. digitatum*; Cyclohexamide (0.1%) (Sigma) with or without *P. digitatum* conidia and PDB with *P. digitatum* conidia, respectively, were used as positive and negative controls. The experiment was done in triplicate and repeated once.

Electron microscopy of inoculated wound sites

Surface colonization and attachment of the yeasts at wound sites were examined according to Usall et al. (2001). A 3 x 3 mm wound was made at each of four sites around the equator of each of 40 orange fruit per treatment using a picture hook. Wounds were treated with the yeasts (30 µL of suspension containing 1×10^8 cells mL⁻¹ water) followed after six hours with 30 µL of *P. digitatum* suspension (1×10^5 conidia mL⁻¹ water). Wounds treated separately with each yeast and the pathogen were used as controls. Fruits were placed in plastic trays (400 x 300 x 100 mm), which were wrapped in high-density polyethylene sleeves to maintain relative humidity of > 85% and incubated at about 25°C. Samples were taken at the time of inoculation and at 6, 12, 24 and 48 h later. Pieces of peel tissue (4 x 4 mm) from wound sites were cut and fixed at room temperature by immersion in 2.5% glutaraldehyde in 0.075 M phosphate buffer at pH 7.0 for 24 h. Samples were rinsed for 1 h (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

aluminum stubs, coated with gold-palladium and observed at 6kV using a Joel JSM 840, SEM Tokyo, Japan. The experiments were done in triplicate. Inoculated fruits were either used immediately for scanning electron microscopy (SEM) evaluation or stored.

Statistical analyses

Disease incidence data from fruit were analyzed with ANOVA (SAS version 8.2, 2001) using Fisher's protected LSD test at $P < 0.05$ and t-grouping. The inhibition rate of pathogen spore germination was analyzed using the non-parametric Kruskal-Wallis test followed by the Mann-Whitney test at $P < 0.05$ (Janisiewicz et al., 2000).

RESULTS

Disease incidence and evaluation of yeast activity against *P. digitatum* in fruits

Treatment of the orange fruit wounds with the yeasts prior to inoculation with *P. digitatum* significantly ($P < 0.05$) decreased estimated incidence of green mold by 65-90% after the fruits were incubated at 7°C for 30 days (Figure 1). The yeast strain TiL 4-2 (*C. sake*) reduced green mold incidence by 95% compared to 65% - 85% of the *C. laurentii* strains: TiL 4-3 and MeJtw 10-2. The addition of NB to MeJtw 10-2 did not significantly ($P < 0.05$) affect the observed reduction in disease incidence. However, green mold incidence increased significantly ($P < 0.05$) when NB was used as a treatment combination with TiL 4-2 or TiL 4-3. All orange fruit treated with yeasts alone and in combination with NB remained symptomless (Figure 1).

Phenolic content assay

Each of the yeasts produced phenolic compounds up to 10 eq mg Gallic acid g⁻¹ dry weight of the culture filtrate. *Candia sake* TiL 4-2 produced significantly more quantities of phenolic compounds compared to the other yeast strains (unpublished data).

Growth inhibition assays

None of the yeast strains measurably reduced colony growth of *P. digitatum*. However, each of the yeasts grew rapidly over *P. digitatum* colonies within 48 h following the challenge inoculation (unpublished data).

Competition for nutrients *in vitro*

Conidia of *P. digitatum* germinated within 24 h at all given concentrations of MEB and OPE (Table 1). Germination was faster at higher concentrations of MEB and OPE. Few conidia germinated in Ringer's solution alone. The yeasts each prevented germination of *P. digitatum* conidia in all treatment combinations with MEB and OPE, and greatly reduced conidial germination at the higher OPE concentration. Yeast strains MeJtw 10-2 and TiL 4-2 reduced conidial germination more effectively than did TiL 4-3. When cylinder insert membranes were transferred to

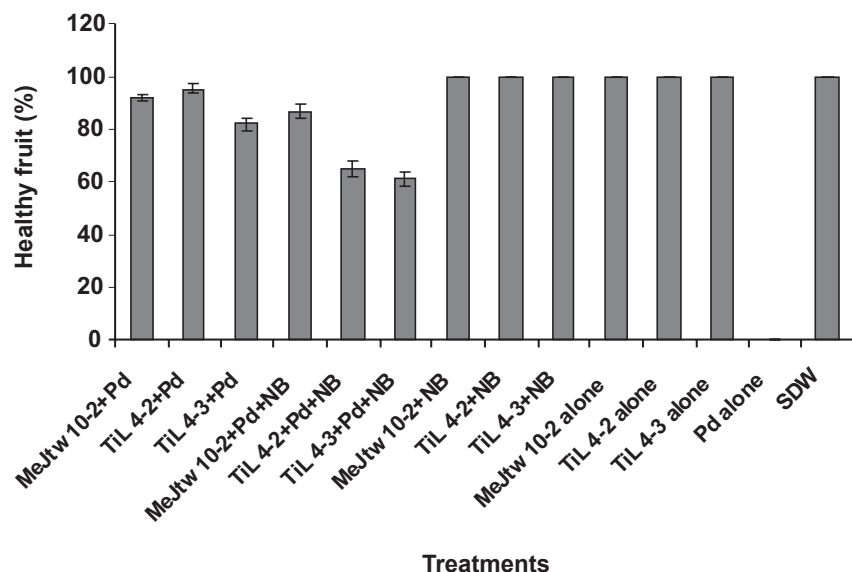


FIGURE 1 - Effect of treating wounds in orange fruit with *Cryptococcus laurentii* strains MeJtw 10-2 and TiL 4-3, and *Candida sake* strain TiL 4-2, with or without nutrient broth (NB), or with NB only or sterilized distilled water (SDW), 12 h before the wounds were inoculated with *Penicillium digitatum* (Pd) on incidence of healthy fruit estimated following storage at 7°C for 30 days. 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 = *C. sake*, TiL 4-3 = *C. laurentii*, Pd = *Penicillium digitatum* only and SDW = Sterilized distilled water (control).

TABLE 1 - Effects of yeast strains TiL 4-2 (*Candida sake*) and TiL 4-3 and MeJtw 10-2 (*Cryptococcus laurentii*) on incidence of *Penicillium digitatum* conidia in Ringer's solution alone or amended with two concentrations of malt extract broth (MEB), or orange peel extract (OPE), after 24 h and 48 h of incubation on polytetrafluoro-ethylene membranes in micro-wells at 24°C

Treatments		Incidence of germination per rating category (%)*							
Yeast Strain	Ringer's solution amendment (+/-)**	24 h incubation				48 h incubation			
		1	2	3	4	1	2	3	4
None	+	98 ^b	2 ^k	0 ^k	0 ^g	96 ^a	2 ^k	2 ^m	0 ^p
	MEB (20%)	19 ^k	21 ^c	23 ^b	37 ^d	0 ^m	4 ^j	14 ^h	82 ^d
	MEB (40%)	10 ^l	5 ^h	27 ^a	58 ^c	0 ^m	0 ^l	11 ^j	88 ^c
	OPE (0.5%)	9 ^m	11 ^f	17 ^c	63 ^b	0 ^m	0 ^l	7 ^l	93 ^b
	OPE (5%)	0 ⁿ	3 ^j	9 ^d	88 ^a	0 ^m	0 ^l	0 ⁿ	100 ^a
MeJtw 10-2	+	100 ^a	0 ^m	0 ^k	0 ^g	64 ^e	18 ^c	12 ^j	6 ^m
	MEB (20%)	95 ^c	5 ^h	0 ^k	0 ^g	18 ^g	23 ^a	30 ^d	29 ^k
	MEB (40%)	96 ^d	4 ⁱ	0 ^k	0 ^g	12 ^j	21 ^b	31 ^c	36 ^g
	OPE (0.5%)	91 ^f	7 ^g	2 ⁱ	0 ^g	19 ^f	19 ^d	24 ^f	38 ^f
	OPE (5%)	98 ^b	1 ^l	1 ^j	0 ^g	0 ^m	0 ^l	0 ⁿ	100 ^a
TiL 4-2	+	100 ^a	0 ^m	0 ^k	0 ^g	76 ^b	12 ^h	8 ^k	4 ⁿ
	MEB (20%)	97 ^c	3 ^j	0 ^k	0 ^g	11 ^k	19 ^d	37 ^a	33 ⁱ
	MEB (40%)	98 ^b	2 ^k	0 ^k	0 ^g	13 ⁱ	23 ^a	26 ^c	38 ^f
	OPE (0.5%)	98 ^b	2 ^k	0 ^k	0 ^g	9 ^l	11 ⁱ	34 ^b	46 ^c
	OPE (5%)	97 ^c	3 ^j	0 ^k	0 ^g	0 ^m	0 ^l	0 ⁿ	100 ^a
TiL 4-3	+	100 ^a	0 ^m	0 ^k	0 ^g	54 ^d	20 ^c	23 ^g	3 ^o
	MEB (20%)	68 ⁱ	27 ^b	4 ^g	1 ^f	31 ^e	17 ^f	26 ^c	26 ^l
	MEB (40%)	78 ^g	16 ^c	3 ^h	3 ^e	14 ^h	21 ^b	24 ^f	31 ^j
	OPE (0.5%)	65 ^j	29 ^a	6 ^c	0 ^g	18 ^g	16 ^g	31 ^c	35 ^h
	OPE (5%)	76 ^h	18 ^d	5 ^f	1 ^f	0 ^m	0 ^l	0 ⁿ	100 ^a

*Germinating rating scale: 1 = no germination, 2 = germ tube < 2x conidia length, 3 = germ tube 2 to 4 x conidia size, 4 = germ tube > 4x conidia length (100 conidia per treatment were counted). Codes given to antagonists: MeJtw 10-2 (*Cryptococcus laurentii*), TiL 4-2 (*Candida sake*) and TiL 4-3 (*Cryptococcus laurentii*). Means with the same letter in each column are not significantly different ($P < 0.05$) according to Duncan's Multiple Range test and t-grouping. ** = amended (+), not amended (-).

new wells containing the corresponding growth medium without the yeasts, all conidia germinated in 0.5% and 5% OPE and in 20% and 40% MEB in the second 24 h incubation period (Table 1). In the studies using the well plates, each of the yeasts greatly inhibited germination of *P. digitatum* conidia during the initial 24 h of incubation, but most conidia germinated during the subsequent 24 h. The strongest inhibition was exhibited by isolate TiL 4-2 as determined by SAS means comparison analysis.

Effects of culture filtrates on spore germination of *P. digitatum*

Culture filtrates of each of the yeast antagonists amended with PDB significantly ($P < 0.05$) inhibited spore germination of *P. digitatum* (Figure 2). A treatment combination with the culture filtrate of TiL 4-2 showed 46.6% inhibition followed by MeJtw 10-2 (38.3%) and TiL 4-3 (35.8%). No inhibition was observed with boiled yeast cell culture suspensions.

SEM observations of wound sites

The SEM observations indicated that germination incidence of *P. digitatum* conidia in orange fruit wounds was much lower in wounds treated with *Candida sake* strain (TiL 4-2) and *Cryptococcus laurentii* strains (MeJtw 10-2 and TiL 4-3) than in untreated control wounds. The scanning electron micrographs (Figure 3) showed the activity of *Candida sake* strain TiL 4-2 at wound sites in orange fruits inoculated with *Penicillium digitatum* (A-E) or inoculated with *P. digitatum* only (F-J). The sequence of micrographs in each column was taken after 0, 6, 12, 24, and 48 h of incubation at 24°C and at 1 µm magnifications. Each of the micrographs under each column showed the germination, attachment, overgrowth, and wrapping of the yeast strain around the germ tube of *P. digitatum*, and hyphal wall decomposition, respectively (A-E). In the absence of the yeast strain, germination incidence of *P. digitatum* conidia was high with abundant germ tube elongation and hyphal

growth (F and J). Observations also indicated that wounds of the *Candida sake* (TiL 4-2) treatment were healing.

DISCUSSION

The two strains of *C. laurentii* (MeJtw 10-2 and TiL 4-2) and the strain of *C. sake* (TiL 4-3) tested in this study each substantially suppressed incidence of green mold. Each strain was also found to inhibit germination of *P. digitatum* conidia in cylinder insert tests and in orange fruit wounds. While inhibited, the conidia were not killed but were able to germinate in the absence of the yeasts. The living yeasts exhibited greater efficacy than killed cells in reducing germination of *P. digitatum* conidia. In recent tests the yeasts were found to suppress spore germination of two other citrus pathogens, *G. candidum* and *C. gloeosporioides* (Unpublished data).

Several previous reports demonstrated the potential value of yeast antagonists for controlling postharvest diseases of citrus and other fruits and vegetables (Wisniewski and Wilson, 1992; Janisiewicz and Bors, 1995). Yeast strains were found to suppress diseases caused by several pathogens including *Penicillium* spp. (Teixido et al., 1998; Vero et al., 2002; Abadias et al., 2003; Zhang et al., 2003; Zhang et al., 2005), *G. candidum* (Chalutz & Wilson, 1990) and *C. gloeosporioides* (Koomen & Jeffries, 1993). Strains of *C. laurentii* were reported to suppress diseases in *Arbutus* berries (Zheng et al., 2004), pears (Nunes et al., 2001), strawberries, kiwi fruit and table grapes (Lima et al., 1998), and those of *C. sake* in apples (Usall et al., 2001). Taken together, the findings indicate that yeasts can be effective in diverse crops and have wide temperature tolerances for use in biological control.

The three yeast strains in the present study exhibited greater efficacy than was reported for strains of *C. sake* on apple (Usall et al., 2001; Vero et al., 2002) and were of similar efficacy (80% control) reported for a strain of *C. laurentii* against blue mold in orange fruit (Zhang et

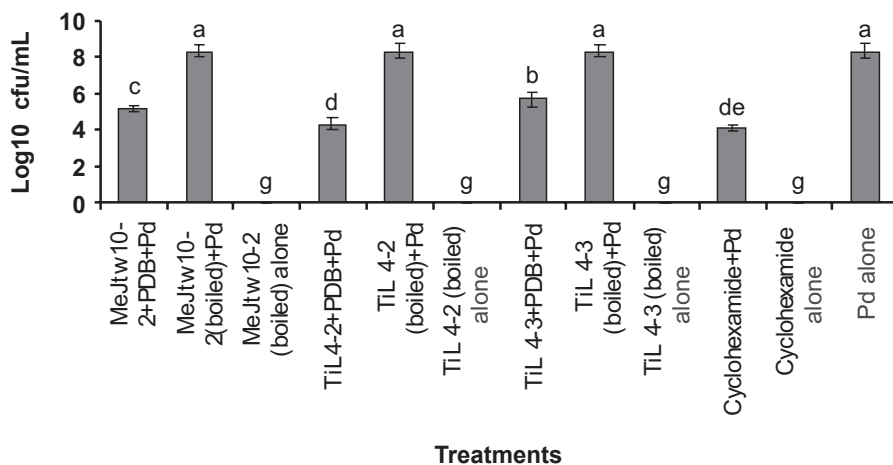


FIGURE 2 - Effects of culture filtrates of yeast strains: *Cryptococcus laurentii* (MeJtw 10-2 and TiL 4-3) and *Candida sake* strain TiL 4-2 grown in potato dextrose broth (PDB), boiled filtrates (Bf) of the same cultures, on the density of colony forming units (CFU) of *Penicillium digitatum* spore germination. 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.

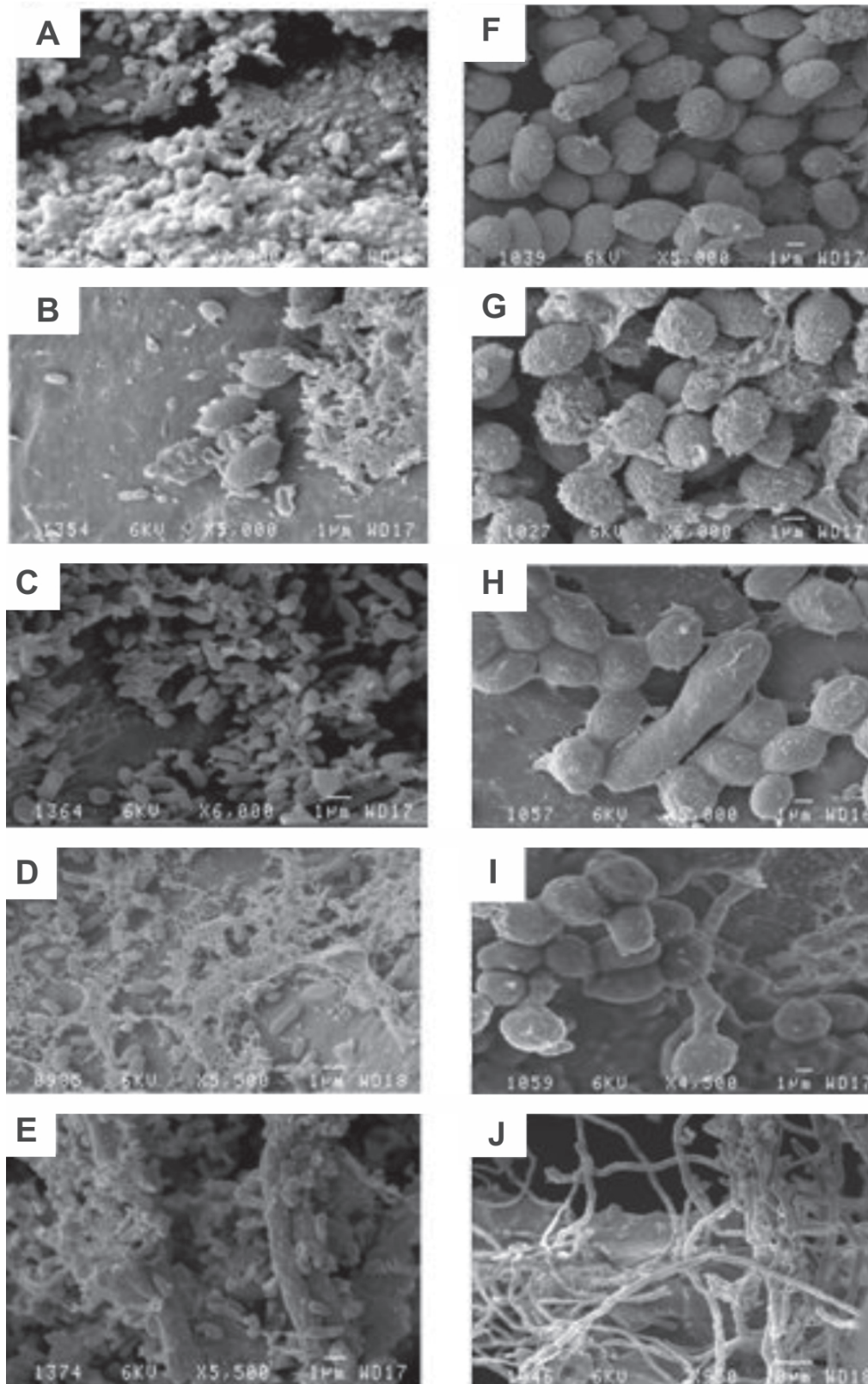


FIGURE 3 - Scanning electron micrographs of wound sites in orange fruits treated with *Candida sake* strain TiL 4-2 and inoculated with *Penicillium digitatum* (A-E) or inoculated with *P. digitatum* only (F-J). The sequence of micrographs in each column was taken after 0, 6, 12, 24, and 48 h of incubation at 24°C and at 1 µm magnifications.

al., 2005). The strain TiL 4-2 of *C. sake* suppressed green mold more effectively (by 95%) than did strains TiL 4-3 and MeJtw 10-2 of *C. laurentii* (by 80-90%).

In the *in vitro* studies, the three yeast strains rapidly colonized surfaces of *P. digitatum* colonies. Fast colonization and competitive ability of yeast antagonists were previously demonstrated by the non-destructive *in vitro* cylinder insert method (Janisiewicz et al., 2000). The inhibition of spore germination by the pathogen during the first 24 h of the cylinder insert experiments, and subsequent germination of the spores when transferred to fresh nutrient solutions, suggested that competition for nutrients by the yeast strains could have been one of the modes of action, which agrees with similar studies by Janisiewicz et al. (2000) and Grebenisan, et al. (2008). The application of different yeast antagonists such as *Debaryomyces hansenii* (Droby et al., 1989), *Pichia guilliermondii* (Arras et al., 1998) and *Aureobasidium pullulans* (Janisiewicz et al., 2000; Castoria et al., 2001) against *Penicillium* spp. gave similar results.

Our observations that the application of NB to fruit wound sites treated with *C. sake* strain TiL 4-2 or *C. laurentii* strain TiL 4-3 suppressed effectiveness of the strains against green mold were consistent with the view that nutrient competition is an important mode of action of the yeasts against the pathogen. However, NB did not significantly affect the effectiveness of *C. laurentii* MeJtw 10-2 against fruit decay, which may indicate that this strain could have competed for nutrition against the pathogen. While the nutrient environment of NB-amended wounds was not representative of natural (unamended) fruit wounds, the findings indicated that levels of microbial nutrients in wounds can influence biocontrol effectiveness of the yeasts. Unlike the report of Nunes et al. (2001), our study demonstrated that amendment of the nutritional environment at the wound site could favor growth of a pathogen rather than the yeast antagonists. Our observations are in agreement with the report of Vero et al. (2002), which indicated that addition of a nitrogen source to apple wounds limited the growth of the antagonists. Our results suggest that the effectiveness of the three yeasts as antagonists is due in part to their ability to rapidly colonize wound sites despite low nutrient availability. The SEM observations indicated that the production of extracellular matrix may also facilitate rapid colonization of wound sites by the yeasts, a principle discussed by Janisiewicz (1988). On the other hand, in some instances extracellular matrices of antagonists lyse pathogen hyphae and thereby increase the availability of simple carbon sources which may stimulate antagonist growth rates (Chan & Tian, 2005; Zhang et al., 2010).

Our observations support the view that rapid growth, competition for nutrients, and production of extracellular matrix by the three yeast strains at wound sites are important for the suppression of *P. digitatum* and green mold in orange fruit. The rapid growth of the yeasts in unamended wounds

suggested that additional nutrients may not be needed for optimal effectiveness against the pathogen, which would simplify any commercial use of these strains. All three strains used in the studies merit further testing for control of green mold and other fruit diseases affecting oranges and other citrus crops.

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