



## ISOLATION, SCREENING AND IDENTIFICATION OF PROMISING YEAST ISOLATES USED FOR BIOLOGICAL CONTROL OF ORANGE GREEN MOULD

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### ABSTRACT

Ninety nine yeast isolates were isolated from surface of apple, grape, orange and tomato fruits. The isolates were tested *in vivo* in preliminary study for biocontrol potential against green mould of navel orange fruit. According to primary screening, twenty two isolates were selected to continue the secondary screening (phase one) using different concentrations of the washed yeast cells in water suspension to evaluate their biocontrol efficacy at  $21\pm 1^\circ\text{C}$ . Among twelve yeast isolates were passed to the secondary screening (phase two) at  $7\pm 1^\circ\text{C}$ , washed cells of yeast isolates CT 503, CT 507, CT 508, CT 512 and CT 550 at  $1\times 10^9$ ,  $2\times 10^8$  and  $1\times 10^8$  CFU/ml produced complete protection for 21 days to wounded navel orange fruits inoculated with spore suspension of *Penicillium digitatum* ( $1\times 10^4$  conidia/ml). Meantime, no lesions developed on the navel orange fruits treated with the yeast isolates CT 503, CT 507 (*Debaryomyces hansenii* var. *hansenii* strain C) and CT 512 (*Endomycopsella vivi*) at  $6.6\times 10^7$  CFU/ml, while the percentage of rot reduction of the isolate CT 550 (*Candida edax*) was 99.81%. Culture filtrate of twenty two different yeast isolates used in secondary screening (phase one) did not prevent decay of wounded navel orange fruits but had an inhi-

bitory effect on rot development. The relative abilities of the promising yeast isolates (CT 503, CT 507, CT 512 and CT 550) to induce disease resistance against *P. digitatum* on navel orange fruits were studied. Inoculation of promising yeast isolates significantly triggered induction of resistance in navel orange fruits. The lesion diameters of green mould 66 hours later after inoculation by spore suspension of *P. digitatum* in a neighbouring wound that was made approximately 6 mm away from the initial wound which inoculated with the isolates CT 512, CT 550, CT 507 and CT 503 were reduced by 25.5%, 20.5%, 16.7% and 14.1%, respectively. In this respect, there were no significant differences among the three different isolates CT 503, CT 507 and CT 550.

### INTRODUCTION

Citrus is the World's premiere fruit crop, grown commercially in more than 135 countries on six continents (Naqvi, 2004). Citrus fruit is enjoyed around the world for its taste, nutritional value and relatively cheap price. Egypt is among the first ten countries in terms of navel orange (*Citrus sinensis* L.) production with 4.1% of total orange production in the world (FAOSTAT, 2012). Meanwhile, navel orange is the main fresh fruit exported by the Egyptian horticultural industry. In 2011 record export volumes of 1,042,291 tonnes were achieved with gross value of 538.156 million US\$ (FAOSTAT, 2012). Export involves a need for extended storage during transport from Egypt to these importing countries. Therefore, effective control of

postharvest diseases is especially important for Egypt to compete in the citrus world export markets. Among postharvest losses, those of pathological origin are typically of considerable economical importance. Green mould, caused by *Penicillium digitatum* (Pers.: Fr.) Sacc. is the most economically important postharvest diseases of citrus in all production areas that are characterized by a Mediterranean-type climate with low summer rainfall (Eckert and Eaks, 1989). Actual losses due to green mould are variable and depend upon climate and orchard factors, citrus cultivar, the extent of physical injury to the fruit during harvest and subsequent handling, the effectiveness of antifungal treatments, and the postharvest environment (Smilanick et al 2006). Green mould can be responsible for up to 90% of production losses during postharvest handling (Macarasin et al 2007). Pelser (1977) showed that *Penicillium* moulds accounted for about 75% of total decay present in South African 'Valencia' oranges shipped to London. Typically, the disease has been controlled worldwide for many years solely by the application of fungicides. Synthetic fungicides such as imazalil, thiabendazole and sodium o-phenylphenol (SOPP) are traditionally used (more than 30 years) to control green mould, and have played an important role for the management of *P. digitatum* of citrus fruit (Ismail and Zhang, 2004; Smilanick et al 2006; and Ladaniya, 2008). Continuous and sometimes incorrect use of imazalil in citrus packing houses has led to the proliferation worldwide of resistant strains of *P. digitatum* (Holmes and Eckert, 1999; Kinay et al 2007; and Pérez et al 2011), resistance is also a major problem related to thiabendazole use for many years (Schmidt et al 2006; Sánchez-Torres and Tuset, 2011), the lack of continued approve of some effective fungicides (Jamalizadeh et al 2008); also a growing concern is increased for human safety and public perception that pesticides are harmful to human health and the environment (Gullino & Kuijpers, 1994; Ragsdale & Sisler, 1994; Mari et al 2007; and Abano & Sam-Amoah, 2012), all these factors together have prompted researchers to search for alternative disease control methods. Biological control is one of the alternatives, either alone or as a part of an integrated control strategy (Wisniewski and Wilson, 1992). The evaluation of epiphytic yeasts that naturally occurring on fruit surfaces as potential antagonists against postharvest fungal pathogens of many commodities have been reported (Chalutz and Wilson, 1990; Chand-Goyal and Spotts, 1997; Lima et al 1998; Ippolito et al

2000; Shehata et al 2006; Abraham et al 2010; Janisiewicz et al 2010; Oro et al 2014 and Shehata, 2014). Antagonistic yeasts grow rapidly, colonize fruit surfaces and limit nutrient availability to pathogens that would cause damage to fruits and vegetables (Richard and Prusky, 2002). These advantages should aid registration of antagonistic yeasts. *Debaryomyces hansenii* has been reported to control green and blue mold of citrus fruits (Singh, 2002; and Chalutz & Wilson, 1990). The yeasts *Pichia anomala* and *Pichia guilliermondii* (Wilson & Chalutz, 1989; Lahlali et al 2004; and 2011); *Kluyveromyces marxianus* (Geng et al 2011), *Metschnikowia andauensis* (Manso and Nunes, 2011) *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* (Platania et al 2012); *Pichia membranefaciens* (Luo et al 2013); *Rhodospodium paludigenum* (Lu et al 2013) are commonly tested for controlling postharvest green mold of citrus fruits. Some antagonistic yeasts such as *Candida oleophila*, *Cryptococcus albidus*, *Metschnikowia fructicola* and *Candida sake* are available on the market (Janisiewicz and Korsten, 2002; Fravel, 2005 and Lahlali et al 2011).

The present study aimed to isolate natural epiphytic yeasts from surface of different fruits, investigate their biocontrol activity against green mould of navel orange fruit as well as identification of the most promising yeast isolates and test their induction of resistance against *P. digitatum* in citrus fruit.

## MATERIALS AND METHODS

### Plant material

Navel orange fruits (*Citrus sinensis* L.) were harvested at typical commercial maturity from orchards in Qalyubia Governorate. Fruits were uniform in shape, size and free from obvious mechanical damage or pathological symptoms. Fruits were used in the same day of harvest, or held at 21°C, and about 90% relative humidity for no longer than 2 days before use.

### Pathogen

The fungus *Penicillium digitatum* (Pers.: Fr.) Sacc. was isolated from infected orange fruits during its marketing, confirmed its pathogenicity and identified on the basis of cultural and microscopic morphological characters according to Pitt (2000). The fungus was maintained on potato dextrose agar (PDA) slants and stored under a phosphate buffer (pH 6.5) at 4± 0.5 °C until needed (Boeswinkel, 1976).

### Preparation of inoculum (spore suspension)

The fungal isolate of *P. digitatum* was grown on PDA plates, at  $21\pm 1^\circ\text{C}$  for 10 days. Conidia were harvested by flooding a sporulating culture with 10 ml of sterile distilled water containing 0.03% Tween-80 and dislodging conidia with a glass rod. Conidial suspension was filtered through a double layer of sterile cheesecloth and vortexed for 30 second to break spore chains into individual spores, remove mycelial parts and assure uniform mixing. Spore concentration was determined with a hemacytometer slide and adjusted to the desired concentration. Suspensions were used for inoculation within 1 hour.

### Isolation of biocontrol agents

Potential yeasts were isolated from fruits (apple, Orange, tomato fruits and grape berries) that were not exposed to chemical sprays for several weeks prior to picking from different geographical locations in Egypt i.e. Giza, Cairo, Qalyubia, Fayoum, Menoufia and Gharbia Governorates. Isolates of yeasts present on the surface of the fruits were obtained by submerging individual fruits in a 600 -1000 ml beaker containing sterile phosphate buffer (pH 6.5) and 0.03% Tween-80. Beakers containing the fruits were covered with polyethylene sheets and rubber banded. Beakers were then shaken on a rotary shaker at 120 rpm for 30 min. Serial 0.1 dilution's were plated on various media, acidified nutrient yeast dextrose agar (NYDA) medium (per litre: nutrient broth 8 g, yeast extract 5 g, dextrose 10 g and 20 g of agar), acidified malt yeast glucose peptone agar (MYGP) medium (per litre: malt extract 3 g, yeast extract 3g, peptone 5g, glucose 10 g, and 20g of agar) and pH was adjusted with (N) HCl to 4.5 for griping the growth of the other microorganisms. Plates were incubated at  $21\pm 0.5^\circ\text{C}$  for 48 hrs. After appearance of colonies, isolates selected at random based on the visual characteristics (colour and shape), in addition to microscopic examination to distinguish the yeasts from the bacteria. Then, purification of isolated yeasts was made by triple re-streaking. If all colonies on the plate at the final streaking appeared uniform, they were assumed to be pure; if not, they were streaked an additional three times. Pure selected isolates were maintained on malt extract agar (malt extract 20 g; peptone 3 g and 18 g of agar) slants and stored under a phosphate buffer at  $4\pm 0.5^\circ\text{C}$  for further use (Janisiewicz, 1987 and Janisiewicz, 1991).

### Preparation of bioagents

The cultures of the yeasts were activated on fresh slants for 24 hrs and transferred to 250 ml Erlenmeyer flasks with 50 ml of nutrient yeast dextrose broth (NYDB) medium. The flasks were placed on a rotary shaker at 120 rpm for 48 hrs at  $22\pm 2^\circ\text{C}$ . A droplet, 30  $\mu\text{l}$ , of liquid culture of the yeast was used in primary screening. For secondary screening, the liquid culture medium was then centrifuged at 10000 rpm under cooling  $4^\circ\text{C}$  for 10 min, cells were re-suspended in 30 ml sterile distilled water (SDW), and vortexed for 1 min, re-centrifuged and re-suspended in 10 ml SDW. Serial desired concentrations in secondary screening were obtained by adjusting the suspension after cell yeast concentration was determined with a hemacytometer slide and confirmation were made by plate dilution method on the basis of colony forming unit (cfu/ml) (Janisiewicz, 1991).

### Fruit inoculation

Navel orange fruits were washed with chlorinated water (250 ppm NaOCl), then air-dried. Two wounds were done per fruit between the calyx and stem end axis by the removal of a tissue block measuring 4 mm wide and 2mm deep. A droplet, 30  $\mu\text{l}$ , of liquid culture of the yeast isolate in primary screening or 30  $\mu\text{l}$  of washed cells from each tested concentration was applied into each wound in secondary screening. Wounds were then inoculated with 25  $\mu\text{l}$  of the pathogen spore suspension ( $1\times 10^4$  or  $1\times 10^5$  conidia/ml) of *P. digitatum* to each wound within 60-90 minutes. Wounds treated with sterile fresh NYDB for primary screening, or SDW, for secondary screening and pathogen spore suspensions were used as control "check" (Janisiewicz, 1991).

### Fruit incubation

Each treated fruit was put on glass dish (11 cm in diameter) and placed in cylindrical plastic box (16 cm in diameter by 12 cm in height) lined with good moistened filter paper on the bottom, then the boxes were covered and incubated for 5 days at  $21\pm 1^\circ\text{C}$  for primary screening and secondary screening phase one or at  $7\pm 1^\circ\text{C}$  for 21 days for secondary screening phase two.

### Disease assessment

Fruits were evaluated for rot development after incubation period. Mean of lesion diameter (mm) was measured = A. Infected area (mm<sup>2</sup>) was calculated as  $(A/2)^2 \times 3.14$ .

Percentage of infected area as compared with control (check) was calculated as  $\frac{\text{infected area (mm}^2\text{) for treatment}}{\text{infected area (mm}^2\text{) for control (check)}} \times 100$ .

### Activity of yeast culture filtrates

Yeast cultures were centrifuged for separation of yeast cells for secondary screening then the supernatant was filtered through a 0.22 µm pore size nitrocellulose membrane. Wounded navel orange fruits were prepared as described previously. Thirty µl of the supernatant was applied into each wound. This was followed by applying 25 µl of *P. digitatum* suspension ( $1 \times 10^5$  conidia/ml) within 60-90 minutes. Wounds treated with sterile fresh NYDB and pathogen spore suspension were used as control (check). The fruits were incubated as previously described for 5 days at  $21 \pm 1^\circ\text{C}$ . Lesion diameter (mm) was measured and infected area (mm<sup>2</sup>) as well as percentage of infected area as compared with control (check) was calculated as mentioned before.

### Identification of yeast isolates

The four promising yeast isolates were kindly identified at Unit of Microorganisms Identification and Biological Control, Agricultural Research Center, Giza using YT Biolog microplates of the Biolog system (Biolog Inc., Hayward, CA) according to the recommended procedure. The plates were inoculated with the yeast suspensions made from cultures grown in NYDB medium, which were washed twice in sterile distilled water before application to the plates. The data from the YT plates was analyzed with the MLCLUST program (Biolog Inc.).

### Induction of disease resistance against *P. digitatum* by promising yeast isolates

Navel orange fruits were washed with chlorinated water (250 ppm NaOCl), then air-dried. Two wounds were done per fruit between the calyx and stem end axis by the removal of a tissue block measuring 5 mm wide and 2 mm deep. Each wound was treated with 50 µl of sterile distilled water as control or with 50 µl of  $1 \times 10^8$  washed cells of each of promising yeast isolates. After incubation, in cylindrical plastic boxes as mentioned

before, at  $23 \pm 1^\circ\text{C}$  for 48 hrs, a neighbouring wound (4x 4x 2 mm) was made approximately 6 mm away from the initial wound and inoculated with 25 µl of a spore suspension of *P. digitatum* ( $1 \times 10^4$  conidia/ml). The fruits were re-incubated, in cylindrical plastic boxes to maintain high RH (more than 90%) at  $21 \pm 1^\circ\text{C}$ . The average lesion diameters were determined 66 hrs after inoculation with spore suspension of the pathogen.

### Statistical analysis

Four replicates per treatment each of 3 fruits were used in all experiments with exception of primary screening that three replicates per treatment each of 2 fruits were used.

Data obtained were subjected to computer statistical software (ASSISTAT) originated by Silva & Azevedo (2009). Data analyzed using analysis of variance (ANOVA), and mean values were compared using Duncan's multiple range test at a significance level of  $P \leq 0.05$ .

## RESULTS

### Primary screening

The primary screening aimed to select isolates which were capable of reducing disease development by more than 95%. The effectiveness of 99 yeast isolates for reducing percentage of infected area as compared with control (untreated) of navel orange fruits was studied. Data in Table (1) show that of 99 yeast isolates, sixteen isolates have completely protected wounded navel orange fruits from infection by *P. digitatum* ( $1 \times 10^4$  conidia/ml) during 5 days of storage at  $21 \pm 1^\circ\text{C}$ . These isolates were AP 617, CT 503, CT 507, CT 512, CT 530, CT 534, CT 535, CT 536, CT 543, CT 548, CT 550, GF 8, GF 11, GF 333, GF 338 and GF 339. However, the isolates AP 612, AP 613, AP 615, AP 620, CT 501, CT 502, CT 504, CT 505, CT 508, CT 510, CT 514, CT 549, CT 551, TG 3 and TR 9 inhibited infection progress more than 99%. Other eleven isolates, namely AP 611, AP 622, CT 511, CT 513, CT 544, GF 15, GF 331, GF 342, TR 3, TR 4 and TY 6 suppressed the percentage of infected area as compared with control more than 95%. Twenty two of such promising isolates were selected based on their efficacy for secondary screening.

**Table 1.** Percentage of infected area as compared with control (PIACC)<sup>(1)</sup> of wounded navel orange fruits, treated<sup>(2)</sup> with different yeast isolates, and inoculated with conidia of *Penicillium digitatum*, then stored at 21±1°C for 5 days

Yeast isolates <sup>(3)</sup>	PIACC	Yeast isolates	PIACC	Yeast isolates	PIACC	Yeast isolates	PIACC	Yeast isolates	PIACC
AP 600	25.93	CT 504	0.52	CT 528	28.66	CT 550	0.00	GF 341	50.52
AP 601	38.25	CT 505	0.31	CT 529	13.29	CT 551	0.03	GF 342	2.71
AP 602	27.68	CT 507	0.00	CT 530	0.00	CT 552	36.74	GF 346	18.56
AP 604	18.16	CT 508	0.13	CT 532	26.40	CT 553	10.24	GF 347	28.99
AP 606	21.73	CT 510	0.06	CT 533	20.74	CT 554	21.73	GF 348	12.52
AP 607	33.64	CT 511	1.26	CT 534	0.00	CT 555	28.34	GF 349	9.56
AP 608	40.96	CT 512	0.00	CT 535	0.00	CT 559	36.56	GF 351	13.29
AP 609	20.18	CT 513	1.63	CT 536	0.00	CT 560	11.46	GF 352	23.04
AP 611	2.32	CT 514	0.29	CT 537	35.45	GF 8	0.00	TG 2	24.69
AP 612	0.36	CT 515	12.30	CT 538	41.55	GF 9	8.82	TG 3	0.14
AP 613	0.21	CT 516	30.85	CT 540	22.02	GF 11	0.00	TG 5	42.55
AP 614	38.82	CT 517	20.74	CT 541	24.69	GF 12	5.61	TR 1	24.39
AP 615	0.29	CT 518	38.44	CT 542	31.36	GF 15	1.19	TR 2	23.49
AP 617	0.00	CT 519	8.01	CT 543	0.00	GF 331	3.18	TR 3	2.14
AP 620	0.94	CT 520	12.30	CT 544	2.66	GF 332	21.87	TR 4	1.55
AP 622	2.32	CT 521	15.03	CT 545	58.23	GF 333	0.00	TR 5	20.18
CT 500	20.60	CT 522	24.39	CT 546	40.18	GF 337	24.69	TR 6	18.29
CT 501	0.74	CT 523	40.57	CT 547	17.13	GF 338	0.00	TR 9	0.40
CT 502	0.04	CT 525	19.09	CT 548	0.00	GF 339	0.00	TY 6	4.25
CT 503	0.00	CT 527	13.63	CT 549	0.77	GF 340	18.56	Control <sup>(4)</sup>	100.0

(1) PIACC = Infected area (mm<sup>2</sup>) for treatment/ Infected area (mm<sup>2</sup>) for control x 100

(2) Navel orange fruits inoculated by 30 µl of liquid culture from each tested isolate and 60-90 minutes later challenged with 25 µl 1x10<sup>4</sup> conidia/ml of *P. digitatum*

(3) Yeast isolates sources were fruits of apple as AP; orange as CT; green tomato as TG; red tomato as TR; yellow tomato as TY and grape berries as GF that did not exposed to chemical sprays for several weeks prior to picking

(4) Navel orange fruits inoculated with sterile fresh NYDB and pathogen spore suspension were used as control

### Secondary screening (phase one)

Secondary screening aimed to determine the effectiveness and usefulness of the potential antagonists selected in primary screening. The effective isolates which were selected from primary screening were used in the secondary screening (Phase one). Serial dilutions i.e. 1x10<sup>9</sup>, 2x10<sup>8</sup>, 1x10<sup>8</sup>, 6.6x10<sup>7</sup> and 5x10<sup>7</sup> on the base of CFU/ml of washed yeast cells water suspensions of 22 yeast isolates were applied to study their biocontrol efficacy against 1x10<sup>4</sup> or 1x10<sup>5</sup> conidia /ml of *P. digitatum*. Data in **Table (2)** & **Photo (1)** show that all yeast isolates in this phase, especially at high doses, highly reduced percentage of infected area as compared with control (check) when inoculated navel orange fruits were stored for 5 days at 21±1°C. No lesions developed on the fruits treated with yeast isolates at 2x10<sup>8</sup> CFU/ml with the exception of isolates CT 510, CT 534, CT 535, GF 8,

and GF 11. Meantime, the percentages of rot reduction were more than 99% for the isolates CT 503, CT 507 and CT 512 at the lowest tested concentration of washed cells of yeast isolates, i.e. 5x10<sup>7</sup> CFU/ml against *P. digitatum* 1x10<sup>4</sup> conidia /ml.

However, results of using the four higher concentrations of the yeast cell water suspension against the fungus *P. digitatum* at 1x10<sup>5</sup> conidia /ml, presented in **Table (3)** & **Photo (2)**, indicate that no lesions developed on the fruits treated with all yeast isolates at the highest tested concentration of the antagonist, i.e. 1x10<sup>9</sup> CFU/ml, with the exception of CT 534 and CT 535 isolates. Meantime, at 1x10<sup>8</sup> CFU/ml, the percentages of rot reduction ranged from 67.36% for isolate GF 8 to 99.28 for isolate CT 512. The percentage of rot at the lowest tested dose of washed cells of yeast isolates, i.e. 6.6x10<sup>7</sup> CFU/ml, showed higher values of percentage of infected area as compared

**Table 2.** Percent of infected area as compared with control (PIACC)<sup>(1)</sup> of wounded navel orange fruits, treated<sup>(2)</sup> with different concentrations of washed yeast cells of twenty two isolates, and inoculated with *Penicillium digitatum*  $1 \times 10^4$  conidia/ml, then stored at  $21 \pm 1^\circ\text{C}$  for 5 days

Concentration (cfu/ml) Isolates <sup>(4)</sup>	$2 \times 10^8$	$1 \times 10^8$	$6.6 \times 10^7$	$5 \times 10^7$	Control <sup>(3)</sup>
AP 613	0.00	2.03	10.54	43.7	100
AP 617	0.00	0.00	0.13	7.49	100
CT 502	0.00	0.00	2.92	8.22	100
CT 503	0.00	0.00	0.00	0.09	100
CT 507	0.00	0.00	0.00	0.06	100
CT 508	0.00	0.00	1.78	7.23	100
CT 510	0.25	4.32	28.45	32.34	100
CT 512	0.00	0.00	0.00	0.04	100
CT 530	0.00	0.00	2.67	10.9	100
CT 534	0.13	1.65	4.45	25.8	100
CT 535	0.13	1.52	4.95	21.8	100
CT 536	0.00	0.00	1.14	10.29	100
CT 543	0.00	0.00	0.76	11.46	100
CT 548	0.00	0.13	1.65	9.30	100
CT 550	0.00	0.00	0.13	4.42	100
CT 551	0.00	0.00	0.13	10.9	100
GF 8	0.25	3.05	14.35	42.4	100
GF11	0.13	2.92	28.32	39.1	100
GF 333	0.00	1.3	5.21	23.79	100
GF 338	0.00	0.25	2.41	13.71	100
GF 339	0.00	0.13	1.65	12.81	100
TG 3	0.00	0.13	3.56	21.2	100

(1) PIACC = Infected area ( $\text{mm}^2$ ) for treatment/ Infected area ( $\text{mm}^2$ ) for control x 100

(2) Navel orange fruits inoculated by  $30 \mu\text{l}$  of different concentrations of washed cells of yeast tested isolates and 60-90 minutes later challenged with  $25 \mu\text{l}$   $1 \times 10^4$  conidia/ml of *P. digitatum*

(3) Wounds of navel orange fruits treated with  $30 \mu\text{l}$  sterile distilled water and inoculated by  $25 \mu\text{l}$   $1 \times 10^4$  conidia/ml of *P. digitatum* were used as control (check)

(4) Yeast isolates sources were fruits of apple as AP; orange as CT; green tomato as TG and grape berries as GF that did not exposed to chemical sprays for several weeks prior to picking

**Table 3.** Percent of infected area as compared with control (PIACC)<sup>(1)</sup> of wounded navel orange fruits, treated<sup>(2)</sup> with different concentrations of washed yeast cells of twenty two isolates, and inoculated with *Penicillium digitatum*  $1 \times 10^5$  conidia/ml, then stored at  $21 \pm 1^\circ\text{C}$  for 5 days

Concentration (cfu/ml) Isolates <sup>(4)</sup>	$1 \times 10^9$	$2 \times 10^8$	$1 \times 10^8$	$6.6 \times 10^7$	Control <sup>(3)</sup>
AP 613	0.00	0.36	8.64	29.73	100
AP 617	0.00	0.00	2.16	12.34	100
CT 502	0.00	0.00	1.68	9.39	100
CT 503	0.00	0.00	0.84	7.32	100
CT 507	0.00	0.00	0.84	6.69	100
CT 508	0.00	0.00	2.16	12.90	100
CT 510	0.00	4.20	26.04	35.67	100
CT 512	0.00	0.00	0.72	5.95	100
CT 530	0.00	0.00	2.76	12.43	100
CT 534	0.24	1.32	5.52	22.42	100
CT 535	0.24	3.24	16.44	24.65	100
CT 536	0.00	0.00	2.52	10.47	100
CT 543	0.00	0.12	2.28	9.64	100
CT 548	0.00	0.12	1.96	13.61	100
CT 550	0.00	0.00	0.84	3.60	100
CT 551	0.00	0.48	1.68	13.33	100
Gf 8	0.00	8.28	32.64	40.24	100
Gf 11	0.00	6.84	30.24	40.50	100
Gf 333	0.00	0.48	6.84	19.01	100
Gf 338	0.00	0.48	3.60	14.61	100
Gf 339	0.00	0.84	8.40	19.29	100
TG 3	0.00	0.57	3.12	14.32	100

(1) PIACC = Infected area ( $\text{mm}^2$ ) for treatment/ Infected area ( $\text{mm}^2$ ) for control  $\times 100$

(2) Navel orange fruits inoculated by  $30 \mu\text{l}$  of different concentrations of washed cells of yeast tested isolates and 60-90 minutes later challenged with  $25 \mu\text{l}$   $1 \times 10^5$  conidia/ml of *P. digitatum*

(3) Wounds of navel orange fruits treated with  $30 \mu\text{l}$  sterile distilled water and inoculated by  $25 \mu\text{l}$   $1 \times 10^5$  conidia/ml of *P. digitatum* were used as control (check)

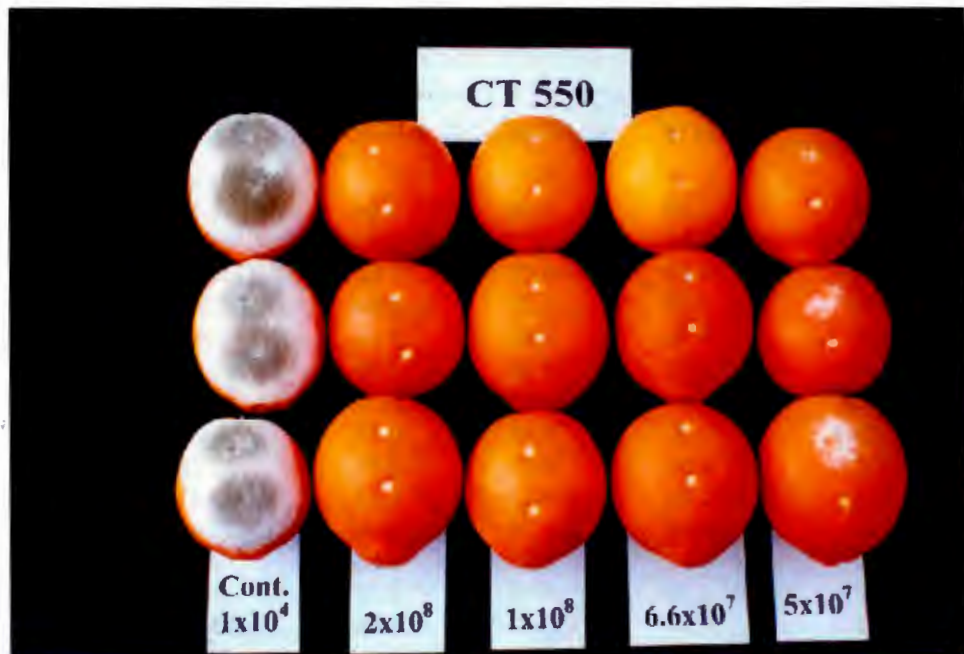
(4) Yeast isolates sources were fruits of apple as AP; orange as CT; green tomato as TG and grape berries as GF that did not exposed to chemical sprays for several weeks prior to picking

with other treatments. The percentages of rot reduction ranged from 59.5% for isolate GF 11 to 96.4% for isolate CT 550 regarding the twenty two isolates under study. Largely the efficacy of the most isolates in reduction of lesion diameter of green mould of navel orange was decreased by increasing the concentration of pathogen spore suspension.

#### Activity of yeast culture filtrates

The relative abilities of different crude culture filtrates of yeast isolates under study to interfere with mould development by *P. digitatum* were investigated. Green mould lesion area ( $\text{mm}^2$ ) and

percentage of infected area as compared with control (PIACC) were recorded after navel orange fruit were treated by cell free culture filtrates of twenty two yeast isolates and inoculated with *P. digitatum* spore suspension ( $1 \times 10^5$  conidia/ml). Data in **Table (4)** Show that all cell free culture filtrates of the twenty two yeast isolates did not prevent mould development but significantly reduced rot infected area and percentage of rot as compared with control (PIACC). The percentages of rot reduction ranged between 93% for isolate CT 512 and 54.9% for Isolate CT 535. However, there were no significant differences among isolates CT 535, AP 613 and CT 548, as well as among isolates CT 548, CT 502, CT 543, CT 530 and CT 536.



**Photo 1.** Development of green mould lesions on wounded navel orange fruits treated (30  $\mu$ l per wound) with different concentrations of washed yeast cells of isolate CT 550 and challenged with (25  $\mu$ l per wound) spore suspension of *Penicillium digitatum*  $1 \times 10^4$  conidia/ml, then stored at  $21 \pm 1^\circ\text{C}$  for 5 days



**Photo 2.** Development of green mould lesions on wounded navel orange fruits treated (30  $\mu$ l per wound) with different concentrations of washed yeast cells of isolate CT 550 and challenged with (25  $\mu$ l per wound) spore suspension of *Penicillium digitatum*  $1 \times 10^5$  conidia/ml, then stored at  $21 \pm 1^\circ\text{C}$  for 5 days



**Table 4.** Infected area (mm<sup>2</sup>) and Percentage of infected area as compared with control (PIACC)<sup>(1)</sup> of wounded navel orange fruits, treated <sup>(2)</sup> with supernatant (culture filtrate) of twenty two different yeast isolates, and inoculated with *Penicillium digitatum*, then stored for 5 days at 21±1°C

Isolates <sup>(3)</sup>	Area mm <sup>2</sup>	PIACC	Isolates	Area mm <sup>2</sup>	PIACC
AP 613	2969.1 b	43.7	CT 536	2483.8 cd	36.5
AP 617	1401.3 g	20.7	CT 543	2595.4 c	38.2
CT 502	2606.7 c	38.4	CT 548	2837.8 bc	41.7
CT 503	1024.4 hi	15.1	CT 550	2184.3 de	32.2
CT 507	861.4 ij	12.7	CT 551	1962.5 ef	28.9
CT 508	1186.3 gh	17.4	GF 8	1256.0 gh	18.5
CT 510	1232.6 gh	18.2	GF 11	1724.9 f	25.4
CT 512	476.0 i	7.0	GF 333	2021.8 ef	29.8
CT 530	2517.0 c	37.1	GF 338	1201.7 gh	17.6
CT 534	1393.0 g	20.5	GF 339	724.3 j	10.6
CT 535	3066.4 b	45.1	TG 3	1982.2 ef	29.1
Control <sup>(4)</sup>	6789.4 a	100	Control	6789.4 a	100

(1) PIACC = Infected area (mm<sup>2</sup>) for treatment/ Infected area (mm<sup>2</sup>) for control x 100

(2) Navel orange fruits treated by 30 µl of the supernatant of different tested yeast isolates and 60-90 minutes later inoculated with 25 µl 1x10<sup>5</sup> conidia/ml of *P. digitatum*

(3) Yeast isolates sources were fruits of apple as AP; orange as CT; green tomato as TG and grape berries as GF that did not exposed to chemical sprays for several weeks prior to picking

(4) Wounds treated with sterile fresh NYDB and pathogen spore suspension were used as control

(5) Means followed by the same letter are not significantly different according to Duncan's multiple range test, (p = 0.05)

### Secondary screening (phase two)

According to secondary screening (phase one), the most effective twelve yeast isolates were selected for more investigation in phase two. In this phase of screening, five concentrations, i.e. 1x10<sup>9</sup>, 2x10<sup>8</sup>, 1x10<sup>8</sup>, 6.6x10<sup>7</sup> and 5x10<sup>7</sup> on the base of CFU/ml of washed yeast cells water suspensions were used against 1x10<sup>4</sup> conidia /ml of *P. digitatum* under cold storage. Data in **Table (5)** and **Photo (3 & 4)** indicate that all yeast isolates under study in this phase clearly reduced percentage of rot as compared with control when inoculated navel orange fruits were stored at 7±1°C for 21 days. No lesions developed on the fruits treated with yeast isolates under study at the highest tested concentrations of the antagonist, i.e. 1x10<sup>9</sup> and 2x10<sup>8</sup> CFU/ml with the exception of isolates CT536 and CT502. Meantime, of twelve yeast isolates under test, only five isolates i.e. CT 503, CT 507, CT 508, CT 512 and CT 550 have completely inhibited rot formation at 1x10<sup>8</sup> CFU/ml. However, at 6.6x10<sup>7</sup> CFU/ml, the percentages of rot reduction were 100% for the isolates CT 503, CT 507 and CT 512. The percentages of rot reduction of the remaining isolates ranged between 87.6 for isolate CT 536 and 99.81 for Isolate CT 550. On the other side, at

the lowest tested concentration of washed cells of yeast isolates, i.e. 5x10<sup>7</sup> CFU/ml, no isolate had potential to prevent rot development and the percentages of rot reduction ranged between 69.03 for isolate CT 548 and 95.02 for Isolate CT 512. At all tested concentrations of the tested yeast isolates with exception of the lowest one, there were no significant differences among four promising different isolates i.e. CT 503, CT 507, CT 512 and CT 550. Meantime, at 6.6x10<sup>7</sup> CFU/ml, there were significant differences between the four promising isolates and the remaining isolates. Also, at cold storage the efficacy of the yeast isolates for controlling mould development depended on the concentration of the yeast isolate under test as this efficacy decreased by decreasing the population density of the yeast. Generally, from these results can be concluded that isolates CT 512, CT 507, CT 550 and CT 503 significantly had highest efficiency in controlling green mould of navel orange under cold storage and normal temperature.

### Identification of promising yeast isolates

According to screening results, the yeast isolates CT 503, CT 507, CT 512 and CT 550 were the most effective bioagents to control of green

**Table 5.** Infected area mm<sup>2</sup> and percent of infected area as compared with control (PIACC)<sup>(1)</sup> of wounded navel orange fruits, treated <sup>(2)</sup> with different concentrations of yeast cells of twelve isolates, and inoculated with *Penicillium digitatum*, then stored at 7±1°C for 21 days

Concentration (cfu/ml)		1x10 <sup>9</sup>	2x10 <sup>8</sup>	1x10 <sup>8</sup>	6.6x10 <sup>7</sup>	5 x10 <sup>7</sup>	Control <sup>(3)</sup>
Isolates <sup>(4)</sup>							
AP 617	Area mm <sup>2</sup>	0.00 n	0.00 n	4.58 mn	78.50 jl	984.65 e	5538.96 a
	PIACC	0.00	0.00	0.08	1.42	17.78	100
CT 502	Area mm <sup>2</sup>	0.00 n	3.69 mn	37.55 lm	201.0 hi	1026.8 e	5538.96 a
	PIACC	0.00	0.07	0.68	3.63	18.54	100
CT 503	Area mm <sup>2</sup>	0.00 n	0.00 n	0.00 n	0.00 n	582.91 f	5538.96 a
	PIACC	0.00	0.00	0.00	0.00	10.52	100
CT 507	Area mm <sup>2</sup>	0.00 n	0.00 n	0.00 n	0.00 n	343.44 gh	5538.96 a
	PIACC	0.00	0.00	0.00	0.00	6.21	100
CT 508	Area mm <sup>2</sup>	0.00 n	0.00 n	0.00 n	220.24 hi	1103.91 de	5538.96 a
	PIACC	0.00	0.00	0.00	3.97	19.92	100
CT 512	Area mm <sup>2</sup>	0.00 n	0.00 n	0.00 n	0.00 n	275.97 gh	5538.96 a
	PIACC	0.00	0.00	0.00	0.00	4.98	100
CT 530	Area mm <sup>2</sup>	0.00 n	0.00 n	23.74 lm	306.19 gh	1324.95 cd	5538.96 a
	PIACC	0.00	0.00	0.43	5.52	23.92	100
CT 536	Area mm <sup>2</sup>	3.14 mn	29.85 lm	351.70 gh	687.01 f	1577.87 bc	5538.96 a
	PIACC	0.06	0.54	6.35	12.40	28.49	100
CT 543	Area mm <sup>2</sup>	0.00 n	0.00 n	12.04 lm	166.95 ij	1423.47 bc	5538.96 a
	PIACC	0.00	0.00	0.22	3.01	25.70	100
CT 548	Area mm <sup>2</sup>	0.00 n	0.00 n	3.69 mn	340.71 gh	1715.67 b	5538.96 a
	PIACC	0.00	0.00	0.07	6.15	30.97	100
CT 550	Area mm <sup>2</sup>	0.00 n	0.00 n	0.00 n	9.61 mn	484.11 fg	5538.96 a
	PIACC	0.00	0.00	0.00	0.19	8.74	100
CT 551	Area mm <sup>2</sup>	0.00 n	0.00 n	6.68 mn	268.67 hi	1012.66 e	5538.96 a
	PIACC	0.00	0.00	0.12	4.85	18.28	100

(1) PIACC = Infected area (mm<sup>2</sup>) for treatment/ Infected area (mm<sup>2</sup>) for control x 100.

(2) Navel orange fruits inoculated by 30 µl of different concentrations of washed cells of tested yeast isolates and 60-90 minutes later challenged with 25 µl 1x10<sup>4</sup> conidia/ml of *P. digitatum*.

(3) Wounds of navel orange fruits treated with 30 µl sterile distilled water and inoculated by 25 µl 1x10<sup>4</sup> conidia/ml of *P. digitatum* were used as control (check).

(4) Yeast isolates sources were apple fruit as AP and orange fruit as CT that did not exposed to chemical sprays for several weeks prior to picking.

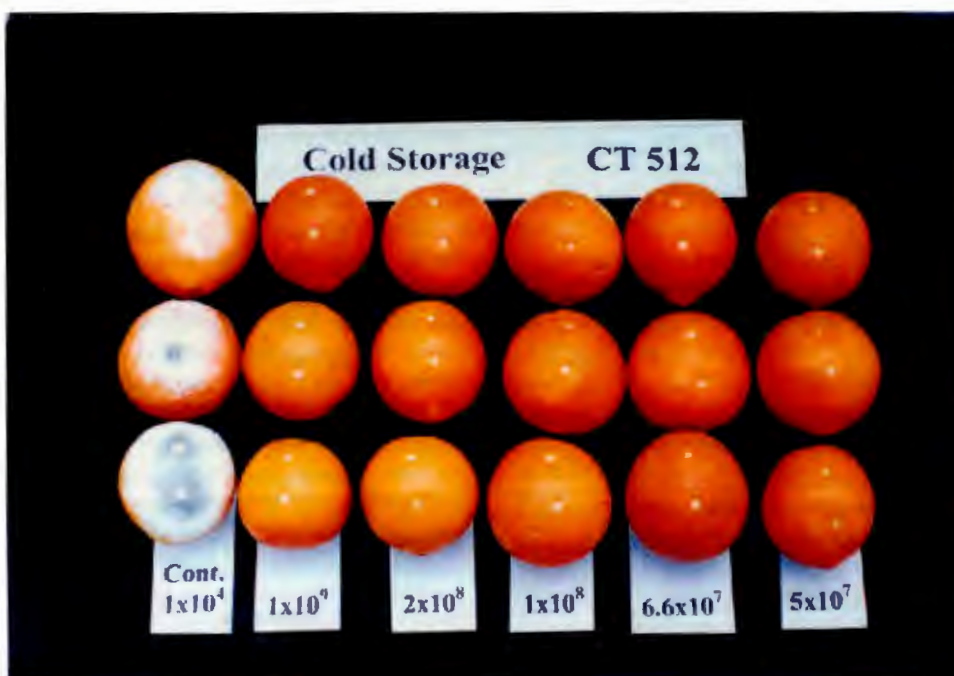
(5) Means followed by the same letter are not significantly different according to Duncan's multiple range test, (p = 0.05)

mould of navel orange fruits in this study. After the analysis of data from the YT plates with the MLCLUST programme, yeast isolate CT 503, CT 507 were identified to be *Debaryomyces hansenii* var. *hansenii* strain C. The probabilities were 94 and 92 while similarities were 0.66 and 0.68 for the two isolates, respectively. However, the yeast isolate CT 512 was identified as *Endomycopsella vivi*, its probability and similarity were 98 and 0.98 respectively. Meantime, the isolate CT 550 was iden-

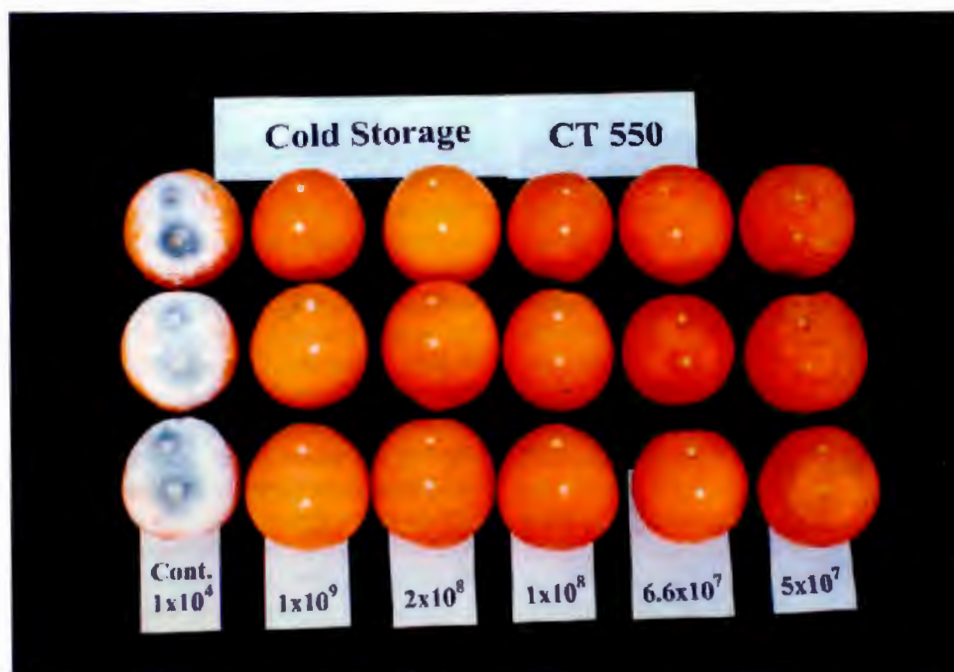
tified as *Candida edax*, its probability and similarity were 94 and 0.74, respectively.

#### Induction of disease resistance against *P. digitatum* by promising yeast isolates

The relative abilities of the promising yeast isolates under study to induce disease resistance against *P. digitatum* on navel orange fruits were studied. Data in Table (6) indicate that inoculation



**Photo 3.** Development of green mould lesions on wounded navel orange fruits treated (30  $\mu$ l per wound) with different concentrations of washed yeast cells of the best tested isolate (CT 512) and challenged with (25  $\mu$ l per wound) spore suspension of *Penicillium digitatum*  $1 \times 10^4$  conidia/ml, then stored at  $7 \pm 1^\circ\text{C}$  for 21 days



**Photo 4.** Development of green mould lesions on wounded navel orange fruits treated (30  $\mu$ l per wound) with different concentrations of washed yeast cells of isolate CT 550 and challenged with (25  $\mu$ l per wound) spore suspension of *Penicillium digitatum*  $1 \times 10^4$  conidia/ml, then stored at  $7 \pm 1^\circ\text{C}$  for 21 days

**Table 6.** Induced resistance against *Penicillium digitatum* on navel orange fruits, Lesion diameter (mm), infected area (mm<sup>2</sup>) and Percentage of infected area as compared with control (PIACC)<sup>(1)</sup> of wounded fruits treated <sup>(2)</sup> with promising yeast isolates and 48 hour later, neighbouring wounds<sup>(3)</sup> were inoculated with *P. digitatum*, then stored at 21°C for 66 hours

Isolates <sup>(4)</sup>	Lesion diameter mm	Area mm <sup>2</sup>	PIACC
CT 503 <i>Debaryomyces hansenii</i> C	29.2	667.8 b	73.6
CT 507 <i>Debaryomyces hansenii</i> C	28.3	626.5 b	69.0
CT 512 <i>Endomycopsella vivi</i>	25.3	503.8 c	55.5
CT 550 <i>Candida edax</i>	27.0	572.3 bc	63.1
Control	34.0	907.5 a	100.0

(1) PIACC = Infected area (mm<sup>2</sup>) for treatment/ Infected area (mm<sup>2</sup>) for control x 100

(2) Navel orange fruits treated by 50 µl of sterile distilled water as control or with 50 µl of 10<sup>8</sup> washed cells of each promising yeast isolate, then stored at 23 ± 1°C for 48 hours

(3) After incubation at 23 ± 1°C for 48 h, a neighbouring wound was made approximately 6 mm away from the initial wound and inoculated with 25 µl of spore suspension of *P. digitatum* (1 × 10<sup>4</sup> conidia/ml)

(4) Yeast isolates sources were fruit of orange that did not exposed to chemical sprays for several weeks prior to picking

(5) Means followed by the same letter are not significantly different according to Duncan's multiple range test. (p = 0.05)

of bioagents CT 512 (*E. vivi*), CT 550 (*C. edax*) and CT 507 & CT 503 (*D. hansenii* strain C) significantly enhanced the induction of resistance in navel orange fruit. The lesion diameters of green mould 66 hours later after inoculated by spore suspension of *P. digitatum* in the second wound that was made approximately 6 mm away from the initial wound which inoculated with the isolates CT 512, CT 550, CT 507 and CT 503 were reduced by 25.5%, 20.5%, 16.7% and 14.1% respectively. Meantime, the reduction of lesion area ranged from 44.5% for isolate CT 512 to 26.4% for isolate CT 503. However, there were no significant differences among three different isolates i.e. CT 503, CT 507 and CT 550.

#### DISCUSSION

Because a biocontrol agent (microorganism) in postharvest applications besides being affective against postharvest pathogens, should not have phytotoxic effects, or produce secondary metabolites that can be harmful to human health, antagonistic yeasts isolated from the surface of fruits and vegetables have emerged as alternative methods with great potential to control postharvest diseases

(Nunes, 2012). Yeasts from these locations have been consumed by humans for a long period of time and have not shown negative effects on the human body. Yeasts are particularly interesting microorganisms in biological control programs because they are relatively easy to produce and maintain and have several characteristics that can be manipulated in order to improve its use and efficiency (Pimenta et al 2009). Yeasts do not occur randomly throughout the biosphere, and each yeast community may be defined by its habitat (Lachance and Starmer, 1998). Therefore, the present work proposed that the appropriate site to search for a biocontrol yeast strain is at the site of infection by the pathogen i.e. the surface of fruits. Isolation of locally yeast antagonists is more desirable because the antagonists isolated in specific geographic areas may be more effective against the pathogen strains present in that locale (Vero et al 2002; and Bouzerda et al 2003). The finding that yeasts which naturally occur on apples can protect fruit against postharvest diseases (Janisiewicz, 1987) spurred interest in the isolation of yeasts from various fruits in order to find new yeast antagonists against postharvest diseases. Twenty two yeast isolates screened, in the present re-

search, from 99 isolates that prevented development of green mould on orange fruits inoculated with spore suspension of *P. digitatum* at  $1 \times 10^5$  conidia/ml. Such results were nearly the same when inoculum of the pathogen concentration was lowered to  $1 \times 10^4$  conidia/ml. at the same conditions. Obtained results showed that an increase in the yeast cell concentration from  $10^7$  to  $10^9$  cfu/ml provided more effective control of green mould of orange fruit. In this respect, **Droby et al (1989)** reported that an increase of *Debaryomyces hansenii* concentration resulted in more effective biocontrol of *P. digitatum*. **El-Ghaouth et al (2002)** reported that microbial antagonists were more effective in controlling postharvest decay when applied at  $10^9$  cfu/ml; and often no control of decay was observed when antagonistic yeasts were applied at  $10^5$  cfu/ml. **Lahlali et al (2004, 2005)** reported that an increase of antagonist concentration resulted in greater effectiveness against post-harvest fungal pathogens at low pathogen pressure.

In the present research, four yeast antagonist isolates were identified, i.e. *Debaryomyces hansenii* var. *hansenii* strain C for the isolates CT 503 & CT 507 ; *Endomycopsella vivi* for the isolate CT 512 and *Candida edax* for the isolate CT 550 that exhibit good biocontrol efficacy against green mould of navel orange fruits for 21 days storage at  $7 \pm 1^\circ\text{C}$ . The stability of antagonistic effect of the four yeast isolates under variable temperatures is a good indication of their commercial potential and provides great interest to use them as promising biocontrol agents against mould fungi. To select and develop a successful biocontrol agent, it is essential to evaluate its effectiveness under different conditions typically used in practice (**Manso and Nunes, 2011**). From the results of testing the activity of culture filtrate, it was assumed that antibiosis is not important aspect of their mode of action especially for the isolate CT. 550. Meantime, two identified isolates, i.e. CT 512 "*Endomycopsella vivi*" and CT 507 "*Debaryomyces hansenii* var. *hansenii* strain C" proved highly producers of antagonistic and/or toxic exudates against *P. digitatum*, where their culture filtrates localized the fungal infected area with 93% and 87.3% for the two yeast isolates, respectively. According to reported evidence, yeasts can produce toxic proteins or glycoproteins called killer toxins, which can lead to death of sensitive yeast isolates (**Schmitt & Breinig, 2002**). Killer activity has been reported in more than 100 yeast species belonging to more than 20 genera, and killer character does not ap-

pear uniformly among either within a species or in relation to the sources of isolation (**Buzzini & Martini, 2001 and Young & Yagiu, 1978**). In the search for novel and more selective antifungals, yeast and fungal cell wall components represent attractive targets, since these structures are usually restricted to yeasts and higher fungi and do not occur in mammalian cells (**Hector, 1993 and Kurtz, 1998**). In this respect, *D. hansenii* displayed the most important inhibitory effect when using Yeast malt agar medium containing methylene blue (YMA-MB) plates seeded with *Botrytis cinerea* strains (**Santos et al 2004**). Different explanations could be afforded: (i), (1-6)- $\beta$ -D-glucans, chitin and mannoproteins of the sensitive yeast cell walls have been identified as primary receptors for killer toxins (**Hutchins and Bussey, 1983; Schmitt and Radler, 1988; Takita and Castilho-Valavicius, 1993 and Santos et al 2002**). Cell wall polysaccharides of filamentous fungi are mainly composed of chitin, glucans and chitosan. So, the cell wall receptor for a killer toxin could be the same in sensitive yeasts and fungi. (ii), some killer toxin proteins are reported to cause death by blocking calcium channels affecting calcium transport thus leading to cell death (**Schmitt & Breinig 2006**).

Other possible modes of action are: First) competition for space and nutrients, (**Filonow, 1998; Spadaro et al 2002**). **Arras (1996)** reported that Scanning electron microscope observations of the mode of action of the antagonist *Candida famata* against the pathogen *P. digitatum* revealed rapid colonization of the fungal mycelium and the wounds. Meantime, several lines of evidence suggested that competition for nutrients at the wound site could be the main mechanism by which *D. hansenii* inhibits *P. digitatum* and *P. italicum*, because (i), the antagonism could be partially reversed by the addition of nutrients to the wounds during inoculation. (ii), the culturing of antagonist cell with a pathogen on a synthetic medium resulted in marked reduction in the growth rate of pathogen only under limited nutritional condition (**Mehrotra et al 1996**). **Zhang et al (2011)** observed that the competition for sugars and nitrates plays a key role in the interactions of *Pichia guillemontii* strain M8 against *Botrytis cinerea* on apples. Second) Direct interaction with the pathogen (direct parasitism), **Arras (1996)** indicated that the *P. digitatum* hyphae were rapidly colonized by antagonist *Candida famata* and in the space of 24 h a strong attachment was observed, followed by alterations in the hyphal tissue which could be due to the action of lytic enzymes ( $\beta$ -1,3-glucanase and

chitinase). The cell wall protects fungal hyphae and is considered to be the main barrier against cell lysis. Chitin, glucans and chitosan are the principal components of fungal cell walls. The enzymatic hydrolysis of fungal cell wall has been reported and discovered in yeasts. **Wisniewski et al (1991)** and **Zhang et al (2011)** observed a strong *in vitro* adhesion of *Pichia guilliermondii* antagonist cells to *B. cinerea* mycelium, perhaps due to a lectin link. Moreover, *Pichia guilliermondii* shows a high activity of  $\beta$ -1,3- glucanase enzyme and chitinases in minimal salt media with different carbon sources that could result in the degradation of the fungal cell walls (**Jijakli & Lepoivre, 1998** and **Zhang et al 2011**). Third) Induction of host defence mechanisms, in recent years, yeast-induced resistance in plants has become an increasingly attractive option for suppressing plant pathogens (**Raacke et al 2006**). However, induced resistance is not usually a major direct mechanism of post-harvest biocontrol agents, most likely because this event is difficult to monitor since both yeast and pathogen are applied at the same site (**Castoria and Wright, 2010**). In the present study, the antagonistic activity of *D. hansenii* C isolates (CT 503 & CT 507); *E. vivi* isolate (CT 512) and *C. edax* isolate (CT 550) were investigated separately from its ability to induce resistance. The antagonistic yeasts and the pathogen *P. digitatum* were applied in spatially separated wounds on the citrus fruit surface. The results of this study demonstrate that all yeast isolates significantly induced resistance in navel orange fruits but *E. vivi* is more efficient than any other yeast isolate used in this study. In this respect, application of *D. hansenii* or *Candida oleophila* to citrus peel wounds induced production of ethylene, increase phenylalanine ammonia lyase (PAL) activity and accumulation of the phytoalexins scoparone, scopoletin and other phenolic substances in citrus peel strips consequently, it provides evidence of enhancing production of secondary metabolites that are needed to inhibit pathogen infection (**Droby and Chalutz 1994**; **Arras and Arru 1999**; **Droby et al 2002** and **Spadaro & Gullino, 2004**). However, **Arras (1996)** reported that Scoparone and scopoletin had some slight fungistatic action from the second day after inoculation of orange fruits by *Candida famata* which increases progressively over the following days. **Rodov et al (1994)** observed scoparone production of about 890 and 260  $\mu$ g/g fresh peels in Oroblanco grapefruit and Valencia orange fruits, respectively, five days after inoculation with an isolate of *Pichia guilliermondii*.

Concerning the safety of using yeasts as bio-control agents, two promising isolates were identified as *D. hansenii* var. *hansenii* (isolates CT 503 & CT 507). According to the present taxonomy, two varieties of *D. hansenii* are distinguished, *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii*. Differences in the electrophoretic mobility of their glucose-6-phosphate dehydrogenase and their maximum growth temperatures (var. *hansenii* can only grow in temperatures up to 35° C while var. *fabryi* grows up to 39°C) have been used to discriminate between the two varieties of *D. hansenii* (**Nakase & Suzuki 1985** and **Breuer & Harms 2006**). *D. hansenii* is no longer believed to be an important human pathogen as previously thought (**Desnos-Ollivier et al 2008**). Meanwhile, **Gimenez-Jurado et al (1994)** demonstrated *Candida edax* (isolate CT 550) to be the anamorph of *Stephanoascus smithiae* from mating reactions and high nuclear DNA complementarity. According to the present taxonomy and the key characters of species in the genus *Stephanoascus*, it can be observed that *S. smithiae* have no Growth at 37°C (**Smith and de Hoog, 1998**). However, there are no reports of human or animal infection by *Endomycopsella vivi* (isolate CT 512). Also, *E. vivi* is not mentioned in the chapter entitled yeasts pathogenic to humans written by **Chester and Cooper, 2011**.

In conclusion, this research allowed constituting a collection of four yeast isolates that have potential in biological control of postharvest diseases. Other work are needed either in the directions of mode of action and human health or in compatibility with other postharvest control practice to enhance its antagonistic effects as well as studying the effectiveness against a wide range of postharvest pathogens.

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## عزل وإنتقاء وتعريف عزلات من الخمائر الواعدة لإستخدامها فى مكافحة العفن الأخضر فى البرتقال حيويًا

[١٢]

### شحاته طه شحاته<sup>١</sup>

١- قسم أمراض النبات - كلية الزراعة - جامعة عين شمس - شبرا الخيمة - القاهرة

كاملة لمدة ٢١ يوم وذلك لجروح ثمار برتقال أبوسرة المحقونة بمعلق جراثيم فطر *Penicillium degitatum* بتركيز  $10 \times 10^4$  كونيديا / مل. وفى الوقت نفسه، لم يتكون أى عفن على ثمار البرتقال المعاملة بالعزلات *Debaryomyces hansenii* var. ) CT 507 ، CT 503 *(Endomycopsella vivi)* CT 512 و *(hansenii strain C)* عند تركيز  $10 \times 10^6$  (خلية مكونة لمستعمرة / مل) بينما كانت نسبة إنخفاض العفن للعزلة CT 550 (*Candida edax*) ٩٩,٨١%. وجد إن إضافة راشح مزارع الخمائر المختلفة المستخدمة فى الإنتقاء الثانوى ، وعددها إثتان وعشرون عزلة إلى جروح ثمار البرتقال لم يمنع الإصابة ولكن كان له تأثير مثبت على تطور العفن. تم دراسة القدرات النسبية للعزلات الواعدة CT 507، CT 503، CT 512 و CT 550 للحث على المقاومة ضد فطر *P. degitatum*. وجد أن حقن العزلات الواعدة فى ثمار البرتقال تسبب حث للمقاومة بدرجة معنوية، فكان قطر بقعة العفن الأخضر بعد ٦٦ ساعة من حقن معلق جراثيم الفطر فى جروح جديدة تبعد حوالى ٦ ملئميتر عن جروح سابقة حقنت بعزلات الخمائر CT 512، CT 550، CT 507 و CT 503 أقل بنسبة ٢٥,٥ % ، ٢٠,٥ % ، ١٦,٧ % و ١٤,١% على التوالى. وفى هذا الشأن، لم يكن هناك اختلاف معنوى فيما بين العزلات CT 507 ، CT 503 و CT 550.

الكلمات الدالة: أمراض ما بعد الحصاد، البرتقال، مكافحة الحيوية، *Penicillium degitatum*، *Candida edax*، *Debaryomyces hansenii*، *Endomycopsella vivi*

### الموجز

يهدف هذا البحث إلى عزل خمائر من على سطح بعض الثمار وإختبارها كعوامل مكافحة حيوية لأمراض ما بعد الحصاد وذلك فى إطار الحد من استخدام المبيدات الكيميائية. تم اختبار القدرة التضادية لتسعة وتسعون عزلة من الخمائر عزلت من على أسطح ثمار التفاح، العنب، البرتقال والطماطم وذلك فى اختبار تمهيدي ضد مرض العفن الأخضر الذى يصيب ثمار البرتقال أبو سرة. وعلى أساس نتائج الاختبارات التمهيديّة تم اختيار ٢٢ عزلة لاستكمال الإنتقاء الثانوي (المرحلة الأولى) باستخدام تركيزات متدرجة من المحلول المائي لخلايا الخميرة المغسولة لتقييم تأثيرها التضادي على درجة حرارة  $21 \pm 1^\circ \text{C}$ . ومن ١٢ عزلة تم إختبارها فى المرحلة الثانية من الإنتقاء الثانوي على درجة حرارة  $7 \pm 1^\circ \text{C}$  وجد أن استخدام الخلايا المغسولة من عزلات الخميرة CT 503، CT 507، CT 512 و 508، CT 550 بتركيزات  $10 \times 10^4$  ،  $10 \times 10^6$  ،  $10 \times 10^8$  (خلية مكونة لمستعمرة / مل) أعطى حماية

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