137
Arab Univ.
J. Agric. Sci.,
Ain Shams Univ.,
Cairo, 23(1), 137 - 155, 2015

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

[12]

Shehata[1], S.T.

1- Department of Plant Pathology, Faculty of Agriculture, Ain Shams University, Shobra El-Kheima, Cairo, Egypt

Keywords: Postharvest diseases; Navel orange; Biological control; *Penicillium degitatum*; *Candida edax; Debaryomyces hansenii; Endomycopsella vivi*

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±1°C. Among twelve yeast isolates were passed to the secondary screening (phase two) at 7±1°C, washed cells of yeast isolates CT 503, CT 507, CT 508, CT 512 and CT 550 at $1x10^{9}$, $2x10^{8}$ and $1x10^{8}$ CFU/ml produced complete protection for 21 days to wounded navel orange fruits inoculated with spore suspension of *Penicillium degitatum* ($1x10^{4}$ 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 $6.6x10^{7}$ 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 inhibitory 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 mar-

(Received 21 January, 2015)
(Accepted 10 February, 2015)

kets. 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 **(Macarisin 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);** *Rhodosporidium 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±1°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±0.5°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± 0.5°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±2°C. A droplet, 30 µl, 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°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 µl, of liquid culture of the yeast isolate in primary screening or 30 µl of washed cells from each tested concentration was applied into each wound in secondary screening. Wounds were then inoculated with 25 µl of the pathogen spore suspension ($1x10^4$ or $1x\ 10^5$ 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±1°C for primary screening and secondary screening phase one or at 7±1°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^2) was calculated as $(\mathbf{A}/2)^2 \times 3.14$.

Percentage of infected area as compared with control (check) was calculated as infected area (mm^2) for treatment **/** infected area (mm^2) for control (check) x 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 ($1x10^5$ 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±1°C. Lesion diameter (mm) was measured and infected area (mm^2) 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 $1X10^8$ washed cells of each of promising yeast isolates. After incubation, in cylindrical plastic boxes as mentioned before, at 23±1°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* (1×10^4 conidia/ml). The fruits were re-incubated, in cylindrical plastic boxes to maintain high RH (more than 90%) at 21 ±1°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. dgitatum* ($1x10^4$ conidia /ml) during 5 days of storage at 21±1°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.

Arab Univ.
J. Agric. Sci.,
Ain Shams Univ.,
Cairo, 23(1), 137 - 155, 2015

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

Yeast isolates(3)	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(4)	100.0

(1) PIACC = Infected area (mm^2) for treatment/ Infected area (mm^2) 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 $1x10^4$ 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. $1x10^9$, $2x10^8$, $1x10^8$, $6.6x10^7$ and $5x10^7$ on the base of CFU/ml of washed yeast cells water suspensions of 22 yeast isolates were applied to study their biocontrol efficacy against $1x10^4$ or $1x10^5$ conidia /ml of *P. dgitatum.* 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 $2x10^8$ 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. $5x10^7$ CFU/ml against *P. dgitatum* $1x10^4$ conidia /ml.

However, results of using the four higher concentrations of the yeast cell water suspension against the fungus *P. dgitatum* at $1x10^5$ 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 concentra-

(Received 21 January, 2015)
(Accepted 10 February, 2015)

tion of the antagonist, i.e. $1x10^9$ CFU/ml, with the exception of CT 534 and CT 535 isolates. Meantime, at $1x10^8$ 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. $6.6x10^7$ CFU/ml, showed higher values of percentage of infected area as compared

Table 2. Percent of infected area as compared with control (PIACC)(1) of wounded navel orange fruits, treated(2) with different concentrations of washed yeast cells of twenty two isolates, and inoculated with *Penicillium digitatum* $1x10^4$ conidia/ml, then stored at 21±1°C for 5 days

Concentration (cfu/ml) / Isolates (4)	$2x10^8$	$1x10^8$	$6.6x10^7$	$5 x10^7$	Control(3)
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 (mm^2) for treatment/ Infected area (mm^2) for control x 100

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

(3) Wounds of navel orange fruits treated with 30 µl sterile distilled water and inoculated by 25 µl $1x10^4$ 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)(1) of wounded navel orange fruits, treated(2) with different concentrations of washed yeast cells of twenty two isolates, and inoculated with *Penicillium digitatum* 1x10^5 conidia/ml, then stored at 21±1°C for 5 days

Concentration (cfu/ml) / Isolates (4)	1x10^9	2x10^8	1x10^8	6.6x10^7	Control(3)
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 (mm^2) for treatment/ Infected area (mm^2) for control x 100

(2) Navel orange fruits inoculated by 30 µl of different concentrations of washed cells of yeast tested isolates and 60-90 minutes later challenged with 25 µl 1x10^5 conidia/ml of *P. digitatum*

(3) Wounds of navel orange fruits treated with 30 µl sterile distilled water and inoculated by 25 µl 1x10^5 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 (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 (1x10^5 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 µl per wound) with different concentrations of washed yeast cells of isolate CT 550 and challenged with (25 µl per wound) spore suspension of *Penicillium digitatum* 1x10^{4} conidia/ml, then stored at 21±1°C for 5 days

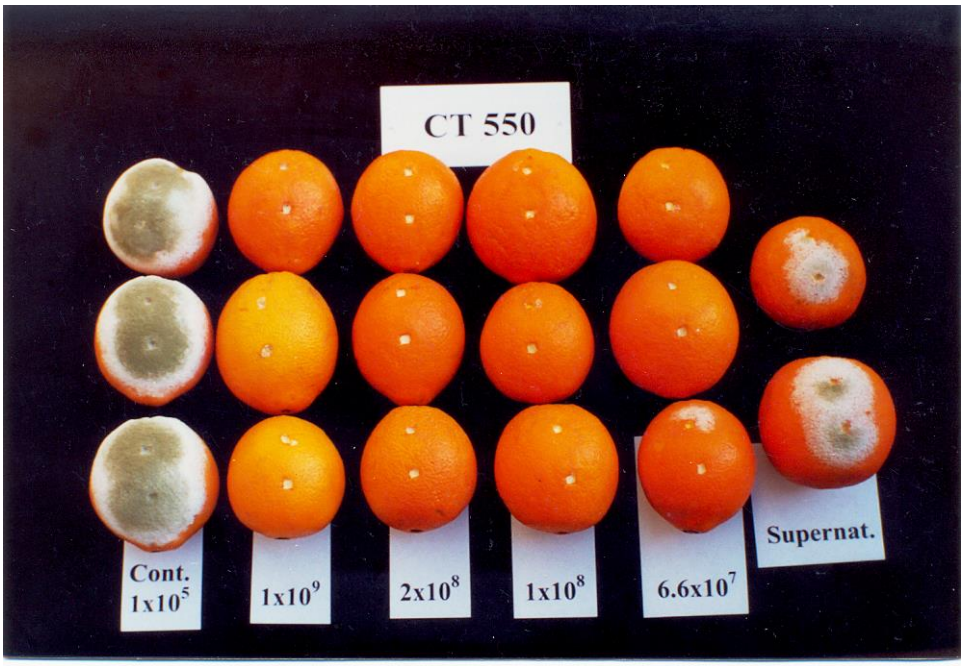

Photo 2. Development of green mould lesions on wounded navel orange fruits treated (30 µl per wound) with different concentrations of washed yeast cells of isolate CT 550 and challenged with (25 µl per wound) spore suspension of *Penicillium digitatum* 1x10^{5} conidia/ml, then stored at 21±1°C for 5 days

Table 4. Infected area (mm^2) and Percentage of infected area as compared with control (PIACC)(1) of wounded navel orange fruits, treated (2) 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 (3)	Area mm^2	PIACC	Isolates	Area mm^2	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 l	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 (4)	6789.4 a	100	Control	6789.4 a	100

(1) PIACC = Infected area (mm^2) for treatment/ Infected area (mm^2) 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 $1x10^5$ 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. $1x10^9$, $2x10^8$, $1x10^8$, $6.6x10^7$ and $5x10^7$ on the base of CFU/ml of washed yeast cells water suspensions were used against $1x10^4$ 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. $1x10^9$ and $2x10^8$ 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 $1x10^8$ CFU/ml. However, at $6.6x10^7$ 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. $5x10^7$ 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 $6.6x10^7$ 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^2 and percent of infected area as compared with control (PIACC)(1) of wounded navel orange fruits, treated (2) 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) / Isolates (4)		$1x10^9$	$2x10^8$	$1x10^8$	$6.6x10^7$	$5 x10^7$	Control(3)
AP 617	Area mm^2	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^2	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^2	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^2	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^2	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^2	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^2	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^2	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^2	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^2	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^2	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^2	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^2) for treatment/ Infected area (mm^2) 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 $1x10^4$ conidia/ml of *P.digitatum.*

(3) Wounds of navel orange fruits treated with 30 μl sterile distilled water and inoculated by 25 μl $1x10^4$ 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 identified 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

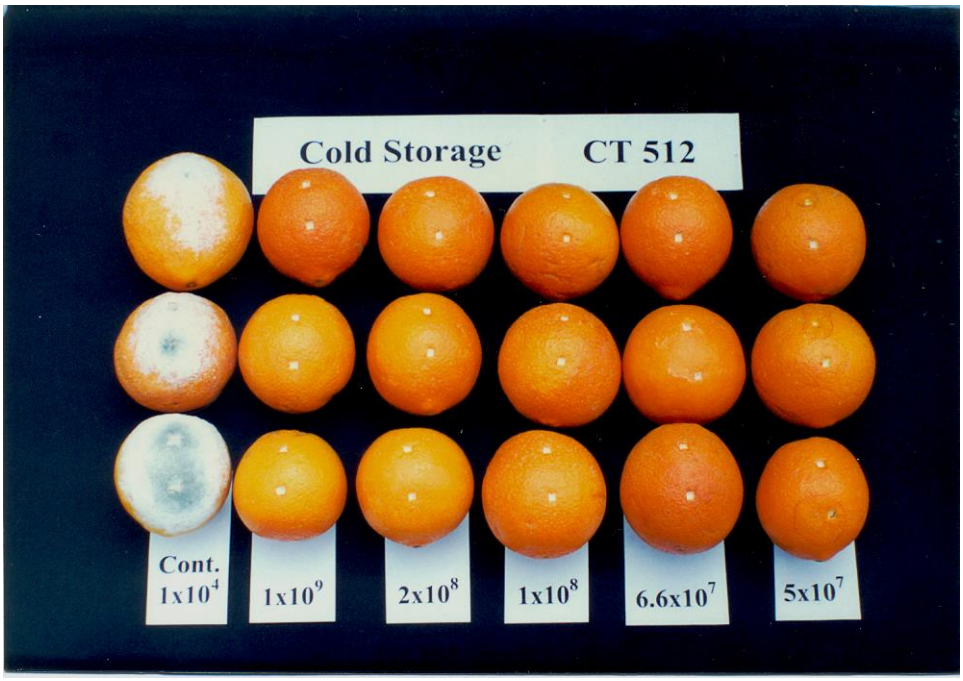

Photo 3. Development of green mould lesions on wounded navel orange fruits treated (30 µl per wound) with different concentrations of washed yeast cells of the best tested isolate (CT 512) and challenged with (25 µl per wound) spore suspension of *Penicillium digitatum* 1x10^4 conidia/ml, then stored at 7±1°C for 21 days

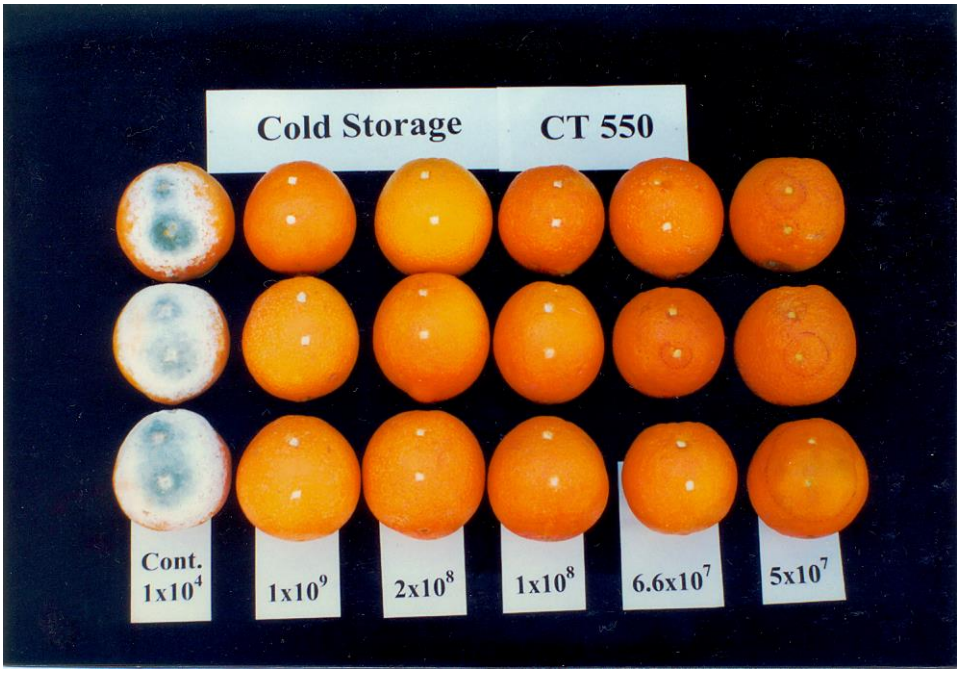

Photo 4. Development of green mould lesions on wounded navel orange fruits treated (30 µl per wound) with different concentrations of washed yeast cells of isolate CT 550 and challenged with (25 µl per wound) spore suspension of *Penicillium digitatum* 1x10^4 conidia/ml, then stored at 7±1°C for 21 days

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

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

(1) PIACC = Infected area (mm^2) for treatment/ Infected area (mm^2) for control x 100

(2) Navel orange fruits treated by 50 µl of sterile distilled water as control or with 50 µl of 10^8 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* (1×10^4 conidia/ml)

(3) 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 1×10^5 conidia/ml. Such results were nearly the same when inoculum of the pathogen concentration was lowered to 1×10^4 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 10^8 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 postharvest 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±1°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 appear 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)-β-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 guilliermondii* 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 (β-1,3-glucanase and chi-

tinase). 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 β-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 postharvest 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 µg/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 biocontrol 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.

REFERENCE

Abano, E.E. and Sam-Amoah, L.K. 2012. Application of antagonistic microorganisms for the control of postharvest decays in fruits and vegetables**. International Journal of Advanced Biological Research (I.J.A.B.R.) 2:1-8.**

Abraham, A.O., Laing, M.D. and Bower, J.P. 2010. Isolation and *in vivo* screening of yeast and *Bacillus* antagonists for the control of *Penicillium digitatum* of citrus fruit. **Biological Control 53: 32–38.**

Arras, G. 1996. Mode of action of an isolate *of Candida famata* in biological control *of Penicillium digitatum* in orange fruits. Postharvest. **Biol. Technol. 8:191-198.**

Arras, G. and Arru, S. 1999. Integrated control of postharvest citrus decay and induction of phytoalexins by *Debaryomyces hansenii*. **Advances in Horticultural Science 13: 76-81.**

Boeswinkel, H.J. 1976. Storage of fungal cultures in water. **Trans. Br. Mycol. Soc. 66: 183-185.**

Bouzerda, L., Boubaker, H., Boudyach, E.H., Akhayat, O. and Aoumar, A.A.B. 2003. Selection of antagonistic yeasts to green mold disease of citrus in Morocco. **Journal of Food, Agriculture and Environment 1: 215–218.**

Buzzini, P. and Martini, A. 2001. Large-scale screening of selected *Candida maltosa, Debaryomyces hansenii* and *Pichia anomala* killer toxin activity against pathogenic yeasts. **Medical Mycology 39: 479-482.**

Breuer, U. and Harms, H. 2006. *Debaryomyces hansenii*- an extremophilic yeast with biotechnological potential. **Yeast 23: 415–437.**

Castoria, R. and Wright, S.A.I. 2010. Host responses to biological control agents. In: Prusky, D., Gullino, M.L. (Eds.), Postharvest Pathology, 2. Springer, Dordrecht, The Netherlands, **pp. 171–181.**

Chalutz, E. and Wilson, C.L. 1990. Postharvest biocontrol of green and blue mold and sour rot of citrus fruit by *Debaryomyces hansenii*. **Plant Dis. 74: 134 -137.**

Chand-Goyal, T. and Spotts, R.A. 1997. Biological control of postharvest diseases of apple and pear under semi commercial and commercial conditions using three saprophytic yeasts. **Biol. Control 10: 199-206.**

Chester, R. and Cooper, Jr. 2011. Yeasts Pathogenic to Humans. In: Kurtzman, C. P.; Fell, J. W. and **Boekhout, T.** (eds.), **The Yeasts, a Taxonomic Study, Fifth Edition.** ELSEVIER, Amsterdam, **pp. 9-19.**

Desnos-Ollivier, M., Ragon, M., Robert, V., Raoux, D., Gantier, J.C. and Dromer, F. 2008. *Debaryomyces hansenii* (*Candida famata*), a rare human fungal pathogen often misidentified as *Pichia guilliermondii* (*Candida guilliermondii*). **Journal of Clinical Microbiology 46: 3237-3242.**

Droby, S. and Chalutz, E. 1994. Mode of action of biocontrol agents for postharvest diseases. In: Biological Control of Postharvest Diseases of Fruits and Vegetables—Theory and Practice. Wilson, C. L. and Wisniewski, M. E. (eds). CRC Press, Boca Raton, FL. **pp. 63-75.**

Droby, S., Chalutz, E., Wilson, C.L. and Wisniewski, M. 1989. Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. **Can. J. Microbiol. 35: 794-800.**

Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E.E. and Porat, R. 2002. Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila.* **Phytopathology 92: 393-399.**

Eckert, J.W. and Eaks, I.L. 1989. Postharvest disorders and diseases of citrus fruits. In: Reuther, W.; E.C. Calavan and G.E. Carman (Eds.), The Citrus Industry, 4. University of California Press, Berkeley, CA, USA, **pp. 179–260.**

El-Ghaouth, A., Wilson, C.L., Wisniewski, M.E., Droby, S., Smilanick, J.L. and Korsten, L. 2002. Biological control of postharvest diseases of citrus fruits. In: Gnanamanickam, S.S. (ed.). Biological Control of Crop Diseases. Marcel Dekker, New York, pp. 288-312.

FAOSTAT. 2012. Data base results 2011, Food and Agricultural Organization of the United Nations, http;//fao.org.

Filonow, A.B. 1998. Role of competition for sugars by yeasts in the biocontrol of grey mould of apple. **Biocontrol Sci. Technol. 8: 243-256.**

Fravel, D.R. 2005. Commercialization and implementation of biocontrol. **Annual Review of Plant Biology 43: 337–359.**

Geng, P., Chen, S., Hu, M., Haq, M., Lai, K., Qu, F. and Zhang, Y. 2011. Combination of *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mold of citrus fruit**. Int. J. Food Microbiol. 151: 190–194.**

Gimenez-Jurado, G., Cidadao, A.J. and Beijnvan der Waaij, A. 1994. A novel heterothallic ascomycetous yeast species: *Stephanoascus smithiae,* teleomorph of *Candida edax.* **Syst. Appl. Microbiol. 17: 237-246.**

Gullino, M.L. and Kuijpers, L.A.M. 1994. Social and Political Implications of Managing Plant Diseases with Restricted Fungicides in Europe. **Annu. Rev. Phytopathol. 32: 559-579.**

Hector, R.F. 1993. Compounds active against cell walls of medically important fungi. **Clin. Microbiol. Rev., 6: 1-21.**

Holmes, G.J. and Eckert, J.W. 1999. Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. **Phytopathology 89: 716–721.**

Hutchins, K. and Bussey, H. 1983. Cell wall receptor for yeast killer toxin: Involvement of (1 leads to 6)-beta-D-glucan. **Journal of Bacteriology, 154: 161-169.**

Ippolito, A., El-Ghaouth, A., Wisniewski, M. and Wilson, C. 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. **Postharv. Biol. 19: 265-272.**

Ismail, M. and Zhang, J. 2004. Post-harvest citrus diseases and their control. **Outlooks Pest Manage. 15: 29–35.**

Jamalizadeh, M., Etebarian, H.R., Aminian, H. and Alizadeh, A. 2008. Biological control of gray mold on apple fruits by *Bacillus licheniformis* (EN74-1). **Phytoparasitica 36: 23–29.**

Janisiewicz, W. 1987. Postharvest biological control of blue mold on apples**. Phytopathology 77: 481-485.**

Janisiewicz, W.J. 1991. Control of postharvest diseases of fruits with biocontrol agents. **Technical Bulletin No. 125, 13 pp., Food and Fertilizer Technology Center, Taipei, Taiwan.**

Janisiewicz, W.J. and Korsten, L. 2002. Biological control of postharvest diseases of fruits. **Annu. Rev. Phytopathol., 40: 411-441.**

Janisiewicz, W.J., Kurtzman, C.P. and Buyer, J.S. 2010. Yeasts associated with nectarines and their potential for biological control of brown rot. **Yeast 27: 389–398.**

Jijakli, M.H. and Lepoivre, P. 1998. Characterization of an exo-β-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. **Phytopathology 88: 335-343.**

Kinay, P., Mansour, M.F., Mlikota-Gabler, F., Margosan, D.A. and Smilanick, J.L. 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. **Crop Prot. 26: 647–656.**

Kurtz, M.B. 1998. New antifungal drug targets: A vision for the future. **ASM News 64: 31-39.**

Lachance, M.A. and Starmer, W.T. 1998. Ecology and yeasts In: *The yeasts, a Taxonomic Study*, 4th edition, (eds. Kurtzman, C.P. and Fell, J.W.), Elsevier, Amsterdam. **pp. 21-30.**

Ladaniya, M. 2008. Citrus fruit: biology, technology and evaluation. Academic Press is an imprint of Elsevier, San Diego, USA, **pp. 417-499.**

Lahlali, R., Hamadi, Y., Guilli, M.E. and Jijakli, M.H. 2011. Efficacy assessment of *Pichia guilliermondii* strain Z1, a new biocontrol agent, against citrus blue mould in Morocco under the influence of temperature and relative humidity. **Biological Control 56: 217–224.**

Lahlali, R., Serrhini, M.N. and Jijakli, M.H. 2004. Efficacy assessment of *Candida oleophila* (strain O) and *Pichia anomala* (strain K) against major postharvest diseases of citrus fruits in Morocco. **Communications in Agricultural and Applied Biological Sciences 69: 601-609.**

Lahlali, R., Serrhini, M.N. and Jijakli, M.H. 2005. Development of a biological control method against postharvest diseases of citrus fruits. **Communications in Agricultural and Applied Biological Sciences 70: 47-58.**

Lima, G., De Curtis, F., Castoria, R. and De Cicco, V. 1998. Activity of the yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis* against postharvest rots on different fruits. **Biocontrol Sci. Technol. 8: 257-267.**

Lu, L., Lu, H., Wu, C., Fang, W., Yu, C., Ye, C., Shi, Y., Yu, T. and Zheng, X. 2013. *Rhodosporidium paludigenum* induces resistance and defense-related responses against *Penicillium digitatum* in citrus fruit. **Postharvest Biol. Technol. 85: 196–202.**

Luo, Y., Zhou, Y. and Zeng, K. 2013. Effect of *Pichia membranefaciens* on ROS metabolism and postharvest disease control in citrus fruit. **Crop Prot. 53: 96–102.**

Macarisin, D., Cohen, L., Eick, A., Rafael, G., Belausov, E., Wisniewski, M. and Droby, S. 2007. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. **Phytopathology 97: 1491–1500.**

Manso, T. and Nunes, C. 2011. *Metschnikowia andauensis* as a new biocontrol agent of fruit postharvest diseases. **Postharvest Biol. Technol. 61, 64–71.**

Mari, M., Neri, F. and Bertolini, P. 2007. Novel Approaches to Prevent and Control Postharvest Diseases of Fruit. **Stewart Postharvest Review, 3(6): 1-7.**

Mehrotra, N.K., Sharma, N., Ghosh R. and Nigam, M. 1996. Biological control of green and blue mould disease of citrus fruit by yeast**. Indian Phytopath, 49: 350-354.**

Nakase T, and Suzuki, M. 1985. Taxonomic studies on *Debaryomyces hansenii* (Zopf) Lodder et Kreger-Van Rij and related species. II. Practical discrimination and nomenclature. **J Gen Appl Microbiol 31: 71–86.**

Naqvi, S.A.M.H. 2004. Diagnosis and management of pre and post-harvest diseases of citrus fruit. In: Naqvi, S.A.M.H. (ed.), Diseases of Fruits and Vegetables, Volume I. Kluwer Academic Publishers, **pp. 339-359.**

Nunes, C.A. 2012. Biological control of postharvest diseases of fruit. **Eur. J. Plant Pathol. 133: 181–196.**

Oro, L., Feliziani, E., Ciani, M., Romanazzi, G. and Comitini, F. 2014. Biocontrol of postharvest brown rot of sweet cherries by *Saccharomyces cerevisiae* Disva 599, *Metschnikowia pulcherrima* Disva 267 and *Wickerhamomyces anomalus* Disva 2 strains. **Postharvest Biology and Technology 96: 64–68.**

Pelser, P. du T. 1977. Postharvest handling of South African citrus fruit. **Proc. Int. Soc. Citriculture Florida, Vol. 1, pp. 244–249.**

Pérez, E., Blanco, O., Berreta, C., Dol, I. and Lado, J. 2011. Imazalil concentration for *in vitro* monitoring of imazalil resistant isolates of *Penicillium digitatum* in citrus packinghouses. **Postharvest Biol. Technol. 60: 258–262.**

Pimenta, R.S., Morais, P.B., Rosa, C.A. and Correa Jr., A. 2009. Utilization of Yeasts in Biological Control Programs. In: Satyanarayana, T. and Kunze, G. (eds.), Yeast Biotechnology: Diversity and Applications, Springer Science, pp. 199-214.

PITT J.I. 2000. A Laboratory Guide to Common Penicillium Species, Third Edition. Csiro Division of Food Processing. North Ryde, N.S.W, **197 p.**

Platania, C., Restuccia, C., Muccilli, S. and Cirvilleri, G. 2012. Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on Tarocco orange fruits (*Citrus sinensis*). **Food Microbiol. 30: 219–225.**

Raacke, I.C., von Rad, U., Mueller, M.J. and Berger, S. 2006. Yeast increases resistance in Arabidopsis against *Pseudomonas syringae* and *Botrytis cinerea* by salicylic acid dependent as well as independent mechanisms**. Mol. Plant–Microbe Interact.19: 1138–1146.**

Ragsdale, N.N. and Sisler, H.D. 1994. Social and Political Implications of Managing Plant Diseases with Decreased Availability of Fungicides in the United States. **Annu. Rev. Phytopathol. 32: 545-557.**

Richard, W.J. and Prusky, D. 2002. Expression of an antifungal peptide in *Saccharomyces*: a new approach for biological control of the postharvest disease caused by *Colletotrichum coccodes*. **Phytopathology 92: 33–37.**

Rodov, V., Ben-Yehoshua, S., Fang, D.Q., D'Hallewin, G. and Castia, T. 1994. Accumulation of phytoalexins scoparone and scopoletin in citrus fruits subjected to various Postharvest treatments. In: Geibel, M.; Treutter, D. and Feucht, W. (Editors), **Int. Scient. Symp. on Natural Phenols in Plant Resistance, Weihenstephan. Acta Hortic., 381: 517-523.**

Sánchez-Torres, P. and Tuset, J. 2011. Molecular insights into fungicide resistance in sensitive and resistant *Penicillium digitatum* strains infecting citrus. **Postharvest Biol. Technol. 5: 159–165.**

Santos, A., Marquina, D., Barroso, J. and Peinado, J.M. 2002. (1-6)-β-D-glucan as cell wall binding site for *Debaryomyces hansenii* Killer Toxin. **Lett. Appl. Microbiol. 34: 95–99.**

Santos, A., Sanchez, A. and Marquina, D. 2004. Yeasts as biological agents to control *Botrytis cinerea*. **Microbiological Research 159: 331-338.**

Schmidt, L.S., Ghosoph, J.M., Margosan, D.A. and Smilanick, J.L. 2006. Mutation at β-tubulin codon 200 indicated thiabendazole resistance in *Penicillium digitatum* collected from California citrus packinghouses. **Plant Dis. 90: 765–770.**

Schmitt, M.J. and Breinig, F. 2002. The viral killer system in yeast: From molecular biology to application. **FEMS Microbiology Reviews 26(3): 257-276.**

Schmitt, M.J. and Breinig, F. 2006. Yeast viral killer toxins: lethality and self protection. **Nat. Rev. Micro. 4: 212-221.**

Schmitt, M. and Radler, F. 1988. Molecular structure of the cell wall receptor for killer toxin KT28 in *Saccharomyces cerevisiae.* **Journal of Bacteriology, 170: 2192-2196.**

Shehata, S.T. 2014. Biological control of Lasiodiplodia rot of mango fruits by yeasts. **Arab Univ. J. Agric. Sci. 22:439-453.**

Shehata, S.T., Atwa, M.A. and Hegazi, M.F. 2006. Biological control of black mould rot of tomato fruits by yeasts. **Annals Agric. Sci. 51: 217-233.**

Silva, F. de A.S.E. and C.A.V. de Azevedo 2009. Principal Components Analysis in the Software Assistat-Statistical Attendance. In: World Congress on Computers In Agriculture, 7, Reno-NV-USA: American Society of Agricultural and Biological Engineers.

Smilanick, J.L., Brown, G.E., Eckert, J.W. 2006. The biology and control of postharvest diseases. In: Wardowski, W.F.; Miller, W. M.; Hall, D. J. and Grierson, W. (Eds.), Fresh Citrus Fruits, second ed. Florida Science Source, LLC, Ocala, FL, USA, **pp. 339–396.**

Singh, D. 2002. Bioefficacy of *Debaryomyces hansenii* on the incidence and growth of *Peni-*

cillium italicum on Kinnow fruit in combination with oil and wax emulsions. **Annals of Plant Protection Science 10: 272–276.**

Smith, M.Th. and de Hoog, G.S. 1998. Stephanoascus M.Th. Smith, van der Walt & E. Johannsen. In: Kurtzman, C. P. and Fell, J. W. (eds.), The Yeasts, a taxonomic study. ELSEVIER, Amsterdam, **pp. 400-403.**

Spadaro D. and Gullino M.L. 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. **Int. J. Food Microbiol. 91: 185–194.**

Spadaro, D., Vola, R., Piano, S. and Gullino, M.L. 2002. Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. **Postharvest Biol. Technol. 24: 123-134.**

Takita, M.A., Castilho-Valavicius, B. 1993. Absence of cell wall chitin in *Saccharomyces cerevisiae* leads to resistance to *Kluyveromyces lactis* killer toxin. **Yeast 9: 589–598.**

Vero, S., Mondino, P., Burgueno, J., Soubes, M. and Wisniewski, M. 2002. Characterization of biocontrol activity of two yeast strains from Uruguay against blue mold of apple. **Postharvest Biol. Technol. 26: 91-98.**

Wilson, C.L. and Chalutz, E. 1989. Postharvest bicontrol of Penicillium rots of citrus with antagonistic yeasts and bacteria. **Scientia Horticulturae 40: 105–112.**

Wisniewski, M.E. and Wilson, C.L. 1992. Biological control of postharvest diseases of fruits and vegetables: recent advances. **HortScience 27: 94-98.**

Wisniewski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C. and Chalutz, E. 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii.* I. Characterization of attachment to *Botrytis cinerea.* **Physiol. Mol. Plant. Pathol. 39: 245-258.**

Young, T.W. and Yagiu, M. 1978. A comparison of the killer character in different yeasts and its classification. **Antonie Van Leeuwenhoek 44(1): 59-77.**

Zhang, D., Spadaroa, D., Garibaldi, A. and Gullino, M. 2011. Potential biocontrol activity of a strain of Pichia guilliermondii against grey mold of apples and its possible modes of action. **Biological Control 57: 193–201.**