Minufiya J. Agric. Res. Vol.37 No. 4(1): 749 - 758 (2012) "http://www.mujar.net"

تطبيق استخدام خميرة البيكيا أنومالا للمقاومة الحيوية للعفن الأزرق فى الموالح

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الملخص العربى

تم عزل ١٠ عزلات من فطر البنسيليوم المسبب للعفن الأزرق في الموالح ثم تنميتهم على بيئة(أجار بطاطس دكستروز) لتنقيتهم وتوصيفهم. وأيضا تم عزل سلالة خميرة من حواف الفاكهة وتنميتها وجمعها لاستخدامها كمضاد حيوي ضد فطر العفن الأزرق(تستخدم بديلا عن المبيدات).

تم تسجيل ومتابعة التفاعل بين الخميرة والفطر الممرض في المعمل وكذا على الثمرة. تم تسجيل طبيعة العمل والتفاعل بين الفطر والخميرة باستخدام الميكروسكوب الالكتروني وتقدير الإنزيمات التي تنتجها الخميرة مثل إنزيم الكايتينيز وبيتا ٣,١جلوكانيز. فأظهرت النتائج أن سلالة الخميرة المستخدمة لها قدرة مرتفعة ضد الفطر. وأوضحت الصور الالكترونية التصاق كلا من هيفات الفطر مع خلايا الخميرة وانتهت بدخولها تماما للهيفا وتحطيمها وهذا يدل على الانخفاض الملحوظ في أعراض الإصابة عند استخدامها على الثمار.

APPLICATION OF *PICHIA ANOMALA* FOR THE BIOCONTROL OF CITRUS BLUE MOLD

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(Received: May 2, 2012)

ABSTRACT: *Ten samples of blue mold Pen.italicum were isolated from naturally infected citrus fruits (Lemon, orange and grape fruit). They were cultured on (PDA)for purification and identification. Pichia anomala (Yeast strain)was isolated from the surface of fruits. They were grown and harvested to be used as biocontrol agent against blue mold, i.e. to be used as a replacement of pesticides to control blue mold.*

The interaction between yeast and pathogen hyphae was deteced or monitored in vitro and in planta. This mode of action was recorded using Scanning Electron Microscope (SEM) and evaluating β-1,3-glucanase and chitinase enzymes. Our data showed that our selected yeast strain has high and reliable antagonistic activity against Pen.italicum. Furthermore,SEM test showed adherence of Pen.italicum hyphae and yeast cells. Eventually, fungus hyphae were totally penetrated and destroyed. Furthermore, in planta antagonism with P.anomala resulted in notable reduction in symptoms of infection. Production of β-1,3-glucanase and chitinase enzymes showed values that indicate potential biocontrol of yeast against Pen.italicum

Key words: *Biocontrol; Antagonist, Penicillium italicum; Citrus*

INTRODUCTION

The role of citrus fruits in providing nutrients and medicinal value has been recognized since ancient times. Citrus fruits, belonging to the genus *Citrus* of the family Rutaceae, are well known for their refreshing fragrance, thirst-quenching ability, and providing adequate vitamin C and has been used for the treatment of scurvy since the 17th century, FAO (1998). Citrus is the most widely produced fruit, as a group of several species and it is grown in more than 80 countries, Chang and Lai (1992). Although citrus fruits have a relatively long postharvest life compared with other tropical and subtropical fruits, the losses of these fruits are higher in most develo*pin*g countries because the fruits are handled, marketed, and stored under ambient conditions with insufficient refrigeration. In developed countries, after harvesting and postharvest treatments, fruits enter into cool-chain and remain at lower temperature until they are consumed. Pathogenic infection is a serious problem for fruit packers, because it results in off flavous and decay, rendering fresh fruit unsuitable for consumption and causing heavy economic losses. Of the most important pathogens affecting the fruit are *Penicillium digitatum* and *Penicillium italicum* the cause of citrus green- and blue mold respectively. These pathogens can cause enormous economic losses, particularly in fruits fated for export. Blue mold alone for instance is reported to cause annual losses of up to $50 million in California, Eckert and Eaks (1989). Currently, all pesticides must be re-registered in the USA and European and this is mainly due to a build up of pathogen resistance, particularly with *Pen. italicum*, Eckert (1988). Due to these recent developments, it is becoming important to discover alternative, environmentally safer, and where possible, cheaper alternative control actions. One of such options is

biological control with the use of microbial antagonists, Droby *et al*., (1991). Biological control fits in well with the concept of sustainable agriculture because it mostly exploits natural cycles with reduced environmental impact. Among the biological strategies applicable to postharvest, the induction of resistance in the fruit, the use of plant or animal products with a fungicidal activity, and, above all, the application of antagonistic microorganisms can be considered. Biological control using antagonists, Wilson and Wisniewski (1994) has proved to be one of the most promising alternatives, either alone or as part of an integrated pest management policy to reduce pesticide use. Among these antagonists, many types of yeast such as *Pichia anomala*, McLaughlin *et al*., (1990) which used successfully as biocontrol agent against postharvest citrus rot. This strain was selected due to its high and reliable antagonistic activity against ***Pen. italicum*** on citrus.

MATERIALS AND METHODS

1. Fruits

Interdonato lemons (Citrus lemon) were obtained from local orchards in Egypt and selected by hand. Fruit had not received any preharvest fungicide treatment. Selected lemons were sorted to remove those with apparent injuries or infections and were randomly assigned to different treatments then stored at 4°C for 3–5 days until used.

2. Isolation of the pathogen

From the surface of naturally infected citrus fruits (Lemon, Orange and Grapefruit) with obvious surface blue mold growth, ten samples were isolated and cultured on potato dextrose agar (PDA). Cultures were then incubated for 4 days at 25 °C and single colonies were picked up on slants for the identification. The isolated microorganisms were identified according to their macro-morphological and microscopic features in addition to physiological examinations as stated in the literature, Kiffer and Morele (1997); Barnett and Musiter (1998).

3. Yeast strain

The strain was isolated from the surface of fruits and identified as *Pichia anomala* according to micro and macro morphological characters in Microbiology Department, Faculity of Science, Ain-Shams University. Yeast cells were grown in 250 ml flasks with 50ml yeast malt broth (YMB) on a rotary shaker at 200 rpm at 29 °C for 24h . Cells were harvested by centrifugation at 6000 x g for 5 min, resuspended in sterile saline solution (0.9 % NaCl) and adjusted to the desired concentration with a hemocytometer.

4. Yeast- pathogen direct interaction in *vitro*

The interaction of yeast with pathogen hyphae was assessed in Petri dishes containing yeast malt extract agar media (YMA). On the surface of agar, *Pen. italicum* was inoculated as a single streak in the middle of the plate, then the yeast cells were inoculated as two spots at the margin of the plates and incubated at 28 °C for 3 days until the growth were appeared. The interaction between *P. anomala* and *Pen. italicum* was, also, directly observed under light microscope; a 12 h old culture from *Pen. italicum* in YM broth was inoculated with *P. anomala* cells in a concentration of 2 X 10^5 CFU/ml and incubated at 28 °C. Slides were made from the combined inoculated broth and observed after 1, 3, 5 and 7 h of yeast addition.

5. Yeast-Pathogen-Antagonism Interaction *in Planta*

In Planta tests were carried out on 60 lemon fruits of the cultivar. The fruits were immersed in ethanol 70% for 30sec then leaved until dried and artificially wounded. Wounds, approximately 2 mm in depth and 4 mm long, were made as punctures at two sides around the equator of the fruit with the edge of a sterile stainless cutter tip. Wounds were inoculated at different intervals with 2µl from *P. anomala* and *Pen. italicum* cell suspension or cell-free broth media after yeast growth. The following treatments were performed to determine the *in Planta*

antagonistic activity of *P. anomala* against *Pen. italicum*:

A- Inoculation with the yeast cells only.

B- Inoculation with the cells and after 24h with the pathogen.

C- Inoculation with the cells followed by immediate inoculation with the pathogen.

D- Inoculation with the pathogen and after 24h with the cells.

E- Inoculation with the pathogen only.

F- Inoculation with the yeast filtrate only.

G- Inoculation with the filtrate and after 24h with the pathogen.

H- Inoculation with the filtrate followed immediately by inoculation with the pathogen.

I- Inoculation with the pathogen and after 24h with the filtrate.

J- Wounds inoculated with distilled water (control).

6. Mode of Action of Antagonistic Yeast

6.1. Enzyme Assay

Yeast strain was cultured in (YMB) which contains glucose as the sole carbon source. A 250ml flask containing 100ml culture media was incubated on a rotary shaker at 200 rpm at 28°C for 3 days. Culture filtrate was harvested by centrifuging at 6000 x g for 5 min, and the supernatant was used for enzyme assays.

β-1,3-glucanase activity assay was performed by measuring the amount of reducing sugar by using glucan as standard. A reaction mixture was prepared by adding 1gm of glucan (Sigma) + 2gm of agar in 100ml distilled water and shaken well until completely melting. Then pour the media in petri dishes and left to solidify. After solidification, pores were made on each plate by cork poorer and inoculated each pore by stable amount of filtrate and left the plates for a time of 20-30 min. After that staining with congored (1%) (Which stain the polysaccharides with red color and does not react with monosaccharide) and left for 10 sec, then wash thoroughly with a solution of Nacl (15gm of Nacl+500ml distilled water) until developing clear zone.

For the chitinase assay a reaction mixture was prepared by the same method of β -1,3-glucanase assay, but instead of glucan we used chitin, (knowing that chitin is not soluble in water even after boiling).

6.2. Scanning Electron Microscopy

A uniform 3mm deep x 2mm wide wound was made at the equator of fruit using a sterile nails. Then, 2µl aliquot of *P.anomala* was pipetted into each wound site. After 2h, 2µl suspension of *Pen. italicum* was inoculated into each wound. After air-drying, fruit were put into a plastic tray wrapped with a high density polyethylene sleeve in order to retain high humidity. Wounded tissue was excised from treated fruit 24h after the treatment. After that, samples were dehydrated in a graded ethanol series, critical-point dried with CO2 and coated with gold-palladium for cell interaction assays. The tissues were then viewed using a Hitachi S-800 SEM.This method made to see the antagonism between fungi and yeast on planta. Also made antagonism in broth YMB by inoculated the broth firstly with yeast, then inoculated with fungi at different time intervales (immediately, 2h, 4h, and 6h).After incubation at 28°C for 3days, take touch of the broth on magnetic slides and coated with gold-palladium for cell interaction assays.

RESULTS

1. Yeast- pathogen direct interaction in *vitro*

The influence of direct interaction between *P. anomala* and *Pen. italicum* after their combined growth in YMA for 3 days at 28°C is shown in Fig (1). The grown yeast spots hindered the growth of fungal pathogen and prevent the progress of fungal spreading on the surface of agar media.

The yeast strain, which was detected as strong antagonist, was examined for yeast-pathogen direct interaction in vitro using a light microscope. The hypha of the pathogen was heavily colonized by *P. anomala*. Antagonist adhered to almost all

the hyphae of the pathogen and concentrated over the fungal mycelia (Fig 2). The yeast cells began to attach onto fungal mycelia after 1h (Fig 2 a) and their adhesion increased over the mycelia after 3 h (Fig 2 b). A lysed mycelial mass was observed after 5 h from yeast treatment (Fig 2 c) and after 7 h, only minute residues from *Pen. italicum* mycelia could be observed (Fig 2 d).

Fig. 1. Direct interaction between *Pichia anomala* and *Penicillium italicum* after their combined growth in YMA for 3 days

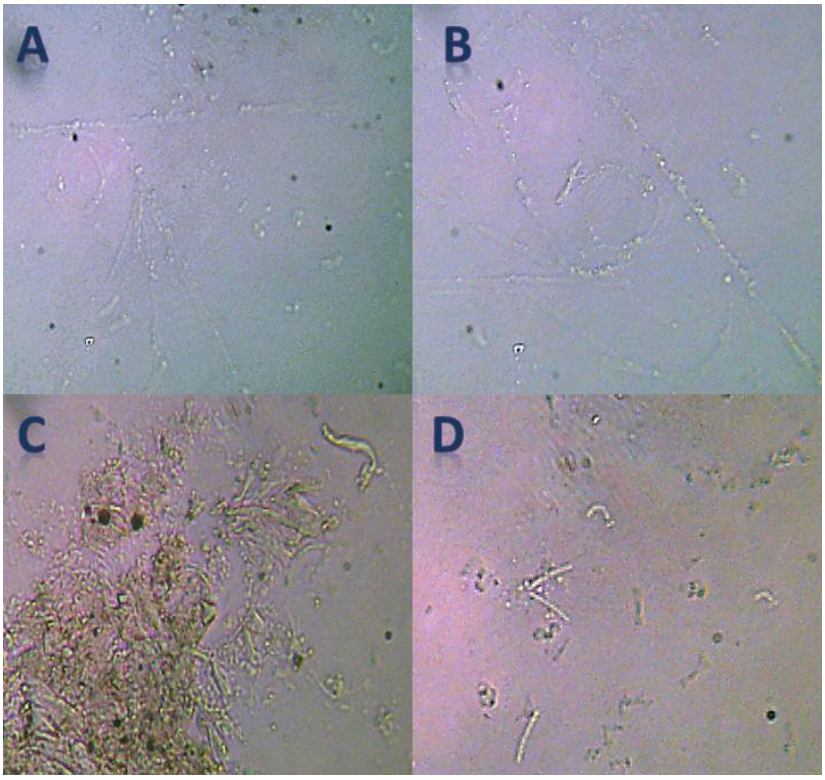


Fig. 2. Microscopic observation of direct interaction between *Pichia anomala* and *Penicillium italicum* during their combined growth in YM broth for 1 h (A), 3 h (B), 5 h (C) and 7 h (D).

2. Yeast- pathogen direct interaction *in Planta*

Table (1) showed the result of different treatments with *P.anomala* cells or filtrate along with fungal pathogen *Pen.italicum*. It illustrated, that inoculated lemon with pathogen only, had a heavy fungal growth compared to the lemon inoculated with pathogen only, and after 24h inoculated with cells of yeast, lemon showed no apparent fungal growth. Also, when lemon inoculated with yeast cells or filtrate only, no growth or decay was appeared as shown in Fig.3.

Table (1): The result of yeast- pathogen direct interaction *in planta*

Treatment	Observations on treated fruits
A: Inoculation with cells only	-Normal appearance
B: Cells→ pathogen	-No decay -Almost Normal appearance
C: Cells followed by pathogen	-No growth - No decay
D: Pathogen → cells	-No apparent Fungal growth - decay of 10 mm
E: Pathogen only	-Decaying half of fruit. -Heavy fungal growth.
F: Filtrate only	-No decay -Normal
G: Filtrate → pathogen	- decay of 18 mm - Faint growth
H: Filtrate followed by pathogen.	- decay of 14 mm

	-Faint fungal growth
I: Pathogen → filtrate	-Decay of 20 mm - Faint fungal growth
J: With water	-No growth. -No soft

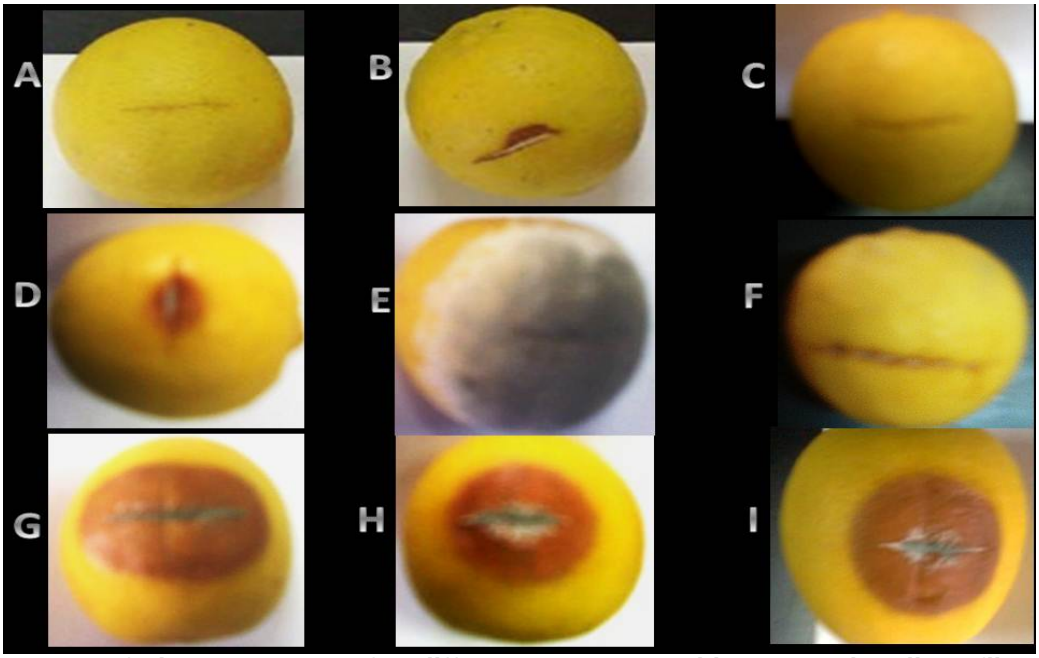


Fig 3: Lemon fruits appearance after different treatments with *P.anomala* cells or filtrate along with fungal pathogen *Pen. italicum*

*** The designation of treatment letters are illustrated in Materials and Methods**

3. Enzyme assays

(i) β-1,3-glucanase production in vitro. *P.anomala* produced β-1,3-glucanase in medium supplemented with β-1,3-glucan as sole carbon source. The appeared clear zone increased with incubation time and when the concentration of stain was very low (1%) (Fig.4a).

(ii) Chitinase production in vitro. Chitinase activity could be detected for *P.anomala* cultured on medium containing chitin (Fig.4b). The clear zone that appear was very light than that appeared with β-1,3-glucanase.

4. Scanning electron microscopy (SEM)

The strongest hyphal colonization produced by ***P.anomala*** was examined using an SEM to obtain their attachment in depth and to understand the possible mode of action of this yeast in suppressing the pathogen. The SEM examination showed adherence between hyphae of *Pen. italicum* and *P. anomala*. Heavy yeast colonization was observed near and around the hyphal tips. In some areas, *Pen. italicum* hyphae were totally surrounded by the yeast cells, and in particular, at the end of the terminal region of the hyphae. The colonized hyphae had undergone some swelling and beading. In other regions, the hyphae of *Pen. italicum* were totally penetrated and destroyed by cells of the antagonistic yeast (Fig.5).

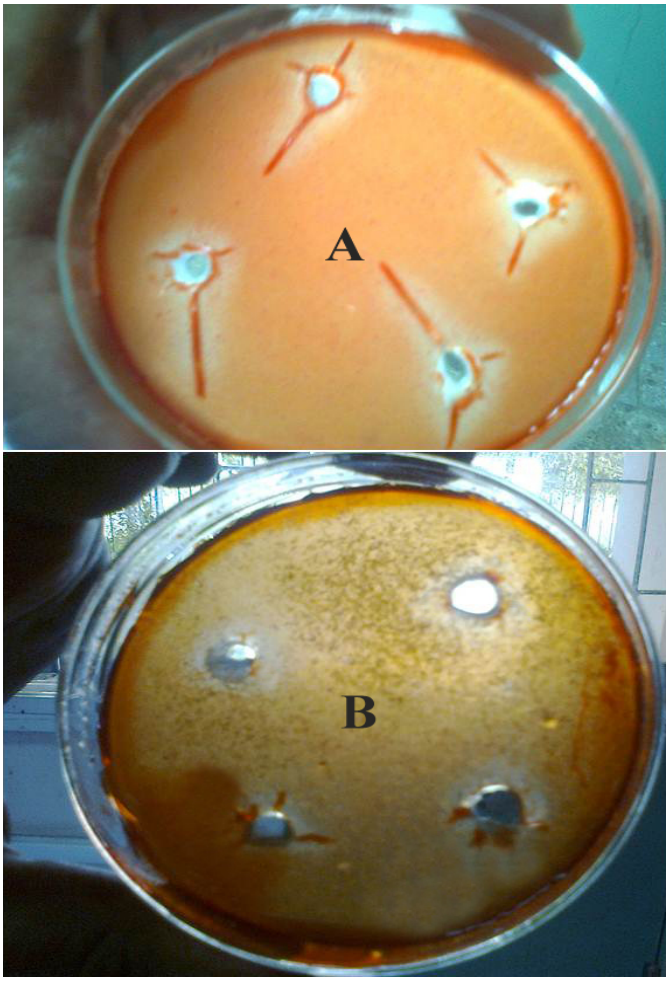
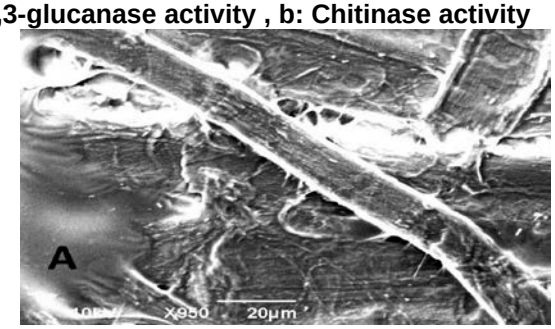


Fig.4. Extracellular Enzyme activity in *P. anomala* filtrate measured using congo red staining. a: β-1,3-glucanase activity , b: Chitinase activity



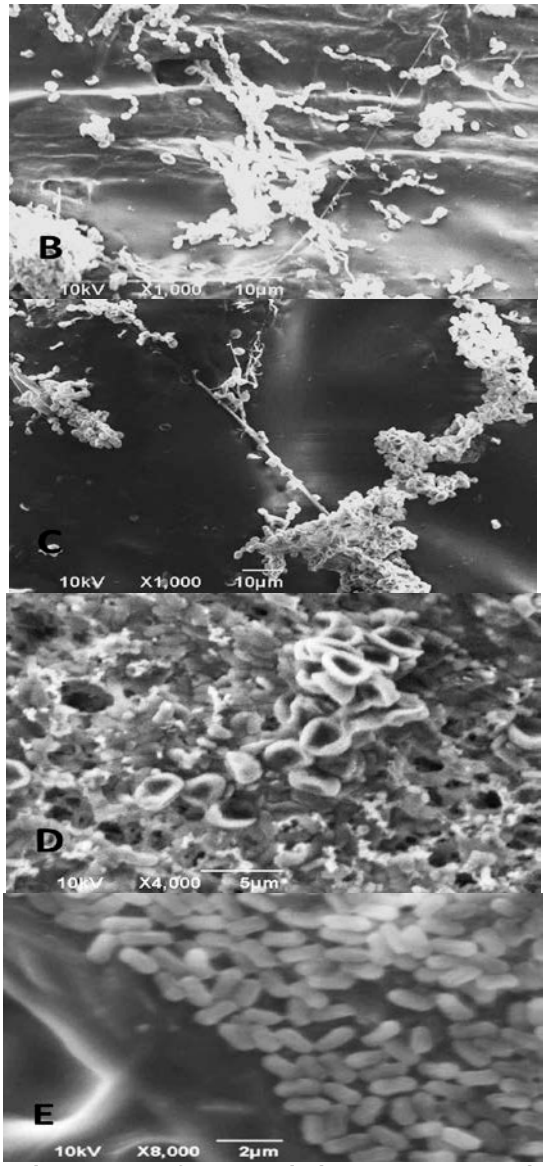


Fig.5. Scanning electron microscopy of antagonistic yeast *P.anomala* interacting with hyphae of *Pen. italicum* and showing a healthy fungal hyphae (Fig5 a) and the accumulation of extracellular matrix around the hyphae (Fig5 b), then heavy yeast colonization around the hyphal tips was appeared (Fig5 c) and that lead to the formation of swelling and beads in fungal hyphae (Fig5 d). Finally, fungal hyphae were totally lysis and penetrated by cells of antagonistic yeast as appeared in Fig5 e.

DISCUSSION

The results clearly demonstrated that citrus fruit naturally infected with *Pen. italicum* and showed symptoms in the form of spores that are typically cylindrical at first then vary in shape and dimensions, Howard (1936). Initial fruit symptoms include a water-socked, soft area on the peel, which is easily punctured on impact and converted to a white mycelium appears on the surface, followed by the formation of blue powdery spore masses, which forms a cloud when disturbed. Screening of antagonistic yeast strain against *Pen. italicum* was tested in *vitro*. The results showed that *P. anomala* had strongly resisted the hyphal development during the incubation period.

Observation of the antagonist-pathogen interaction in *planta* revealed that when the yeast strain was inoculated into the artificial wounds, resulted in a significant reduction in the disease activity of *Pen. italicum* on citrus fruit. This yeast strain was involved in a significant reduction of rot lesion diameter, decay and weight loss compared to the infected control.

Several antagonistic yeasts have been efficaciously used as biocontrol agents on other fruit crops against different postharvest pathogens. Several strains of *P. anomala* have been shown to have biocontrol efficacy against infection by various fungi on citrus fruit, grapefruit, apples, pears and strawberries, Droby *et al.*, (1997); Arras *et al.,* (1999). This study report that *P. anomala* can reduce the incidence of disease caused by *Pen. italicum.* The microscopic examination revealed that hyphae of *Pen. italicum* were heavily colonized by *P. anomala*. An examination by SEM showed adherence between the hyphae of *Pen. italicum* and *P. anomala*. Heavy yeast colonization was observed near and around the hyphal tips. The colonized hyphae had undergone some swelling and beading. Finally, the hyphae of *Pen. italicum* were totally penetrated and destroyed by cells of the antagonistic yeast. Similar results were obtained by Chan and Tian (2005). They indicated that *P. anomala* had a stronger capability for attachment to the fungal hyphae of *Monilinia fructicola, Penicillium expansum* and *Rhizopus stolonifer* than did *Candida albidus*.

Depending on these observations, it can be supposed that the active mechanism of yeast to antagonize *Pen. italicum* is by the production of fungal cell wall degrading enzymes or lethal toxin. This result is supported by finding of the production of killer toxin by *P. anomala*, that have been found to be inhibitory to a wide range of postharvest disease which caused by pathogenic fungi, Buzzini and Martini (2001); Freudlund *et al.,* (2002). Grevesse *et al.* (2003) have suggested that β-1,3-glucanase activity is involved in the lethal action of this *P. anomala* toxin. Izgü *et al.* (2006) have concluded that high β-1,3-glucanase activity of the *P. anomala* lethal toxin highlights for use against human and animal fungal infection and as a biocontrol agent in the food industry. The mode of action of *P. anomala* against some fungal pathogens such as *Pen. digitatum* and *Pen. italicum* has been studied by other researchers who have suggested that they compete for nutrients with secretion of cell wall degrading enzymes, Droby *et al.,* (1990); Wisniewski *et al.,* (1990). Results of this study indicated that *P. anomala* was able to produce significant levels of chitinase in *vitro*. Moreover, it was found that *P. anomala* attached to *Pen. italicum* hyphae and restricted the proliferation of *Pen. italicum*, thus protecting citrus from postharvest fruit rotting fungi *Pen. italicum*. Light microscopy and scanning electron microscopy revealed a general attachment of the isolate to the fungal pathogens. The observations concluded that tenacious attachment, along with secretion of cell wall degrading enzymes, may play a role in the biocontrol activity of this yeast antagonist. Wisniewski *et al.* (1991) indicated that in the presence of the pathogen, the yeast cells produce lytic enzymes that could enhance the attaching ability of yeast to hyphae of pathogens .

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تطبيق استخدام خميرة البيكيا أتومالا للمقاومة الحيوية للعفن الأزرق فى الموالح

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الملخص العربى

تم عزل ١٠ عزلات من فطر البنسيليوم المسبب للعفن الأزرق في الموالح ثم تنميتهم على بيئة(أجار بطاطس دكستروز) لتنقيتهم وتوصيفهم. وأيضا تم عزل سلالة خميرة من حواف الفاكهة وتنميتها وجمعها لاستخدامها كمضاد حيوي ضد فطر العفن الأزرق(تستخدم بديلا عن المبيدات).

تم تسجيل ومتابعة التفاعل بين الخميرة والفطر الممرض في المعمل وكذا على الثمرة. تم تسجيل طبيعة العمل والتفاعل بين الفطر والخميرة باستخدام الميكروسكوب الالكتروني وتقدير الإنزيمات التي تنتجها الخميرة مثل إنزيم الكايتينيز وبيتا١,٣جلوكانيز. فأظهرت النتائج أن سلالة الخميرة المستخدمة لها قدرة مرتفعة ضد الفطر. وأوضحت الصور الالكترونية التصاق كلا من هيفات الفطر مع خلايا الخميرة وانتهت بدخولها تماما للهيفا وتحطيمها وهذا يدل على الانخفاض الملحوظ في أعراض الإصابة عند استخدامها على الثمار.