https://doi.org/10.17221/103/2017-PPS

New Epiphytic Yeasts Able to Reduce Grey Mold Disease on Apples

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Abstract

Kheireddine A., Essghaier B., Hedi A., Dhieb C., Sadfi-Zouaoui N. (2018): New epiphytic yeasts able to reduce grey mold disease on apples. Plant Protect. Sci., 54: 248–257.

Botrytis cinerea, the causal agent of grey mould, is a predominant agent causing extensive postharvest and quality losses of apples in Tunisia and worldwide. Efforts to manage this disease have met with limited success. For this reason, the use of microorganism preparations to control fungal diseases as an alternative to fungicides became an urgent need. From a total of 60 epiphytic yeasts, 10 were assessed *in vitro* against *B. cinerea* and selected isolates showing antagonism were evaluated for their ability to suppress the grey mould *in vivo*. On Petri plates, the most promising strains (three strains of *Aureobasidium pullulans*, one *Cryptococcus flavescens*, and one *Citeromyces matritensis*) showed a zone of inhibition against the pathogen fungus not exceeding 10 mm. *In vivo*, these isolates showed a remarkable antifungal activity since they significantly reduced disease severity on apples from 63% to 95% compared to the control. In conclusion, the work has demonstrated that the three strains, L7 of *Aureobasidium pullulans*, L2 of *Citeromyces matritensis*, and L10 of *Cryptococcus flavescens*, were highly effective and can be used as potential biocontrol agents in controlling the post-harvest decay of apples caused by *B. cinerea*.

Keyword: *Botrytis cinerea;* apples; antagonistic yeasts; biocontrol

During the last two decades, the horticulture sector based on apples and pears has undergone a remarkable expansion in Tunisia. In fact, Tunisia is the third producer of apples on the African continent with 11% of the production. Despite its vital role in national economic development, this culture is exposed to many challenges such as phytosanitary problems which cause significant losses. The grey mould caused by *Botrytis cinerea* is one of the most destructive diseases causing minor rot throughout the growing season and significant rot within packinghouses. It is the first post-harvest pathogen in damaging pears and the second, after blue mould, in affecting apples (Rosenberger 1990).

Chemical control was the only means used to combat these pathogens (Rojas-Grau *et al*. 2008; Salomao *et al*. 2008; Liu *et al*. 2013). However, the emergence of resistant fungal strains to commonly used chemicals, the detection of residues on food and the deleterious effect on the plant and its environment imposed the need to develop safer and more eco-friendly alternatives for the control of postharvest decays (Romnazzi *et al*. 2016; Liu *et al*. 2017). Biological control of pathogens in various storage conditions using the natural microflora existing on fruits (bacteria, yeasts, and/or fungi) has emerged as one of the most promising alternatives to chemicals (Bull *et al*. 1997).

Supported by the Ministry of Higher Education and Scientific Research of Tunisia, Grant No. LR16ES05.

https://doi.org/10.17221/103/2017-PPS

Moreover, a large number of yeasts and bacteria colonising the surface of fruits and vegetables have been reported as effective agents of biological control against postharvest diseases (Mikani *et al*. 2008; Yu *et al*. 2008). These various biological agents showed a high ability to control several postharvest pathogens in different crops such as apples (Janisiewicz 1987), grapes (Senthil *et al*. 2011), peaches (Di Francesco *et al*. 2017), strawberries (Lima *et al*. 1997), citruses (Nunes *et al*. 2009), and tomatoes (Sadfi-Zouaoui *et al*. 2008a). The main inhibitory action of antagonistic bacteria is to produce antibiotics or toxic compounds which could be harmful to the consumer, which raise many concerns (Platania *et al*. 2012). In contrast, yeasts appear to be the most promising biocontrol agents since their antagonistic activity does not generally include the production of mycotropic spores, mycotoxins or antibiotics, compared to mycelial fungi or bacterial antagonists, which reinforces their safe use essentially for human consumption purposes (Parafati *et al*. 2015; Liu *et al*. 2017). Many species of yeasts act essentially by competition with the pathogen for space and nutrients due to their ability to colonize dry surfaces for long periods because of their simple nutritional needs (Arras *et al*. 2000; Pantelides *et al*. 2015). Their antagonistic activity is also due to their ability to induce positive defence responses in the plant, to secrete antifungal compounds, to induce parasitism with the pathogen, to form biofilm and to involve reactive oxygen species (ROS) in the defence response (Shoresh *et al*. 2010; Liua *et al*. 2013). Several yeast antagonists were effective as biocontrol agents to control apple decay caused by *B. cinerea* such as *Pichia guilliermondii* (Li *et al*. 2016), *Candida oleophila* (Ballet *et al*. 2016), *Candida sake* Saito (Arrarte *et al*. 2017) *Aureobasidium pullulans* (de Bary) Arnaud (Mari *et al*. 2012), *Metschnikowia fructicola* (Pelliccia *et al*. 2011), *Metschnikowia pulcherrima* (Ruiz-Moyano *et al*. 2016; Spadaro *et al*. 2016), *Debaryomyces hansenii* (Pelliccia *et al*. 2011), *Rhodotorula mucilaginosa* (Li *et al*. 2011), *Hanseniaspora opuntiae* (Ruiz-Moyano *et al*. 2016), and *Cryptococcus laurentii* (Calvo *et al*. 2010). These represent a minuscule number compared to known yeast species. In order to select biocontrol agents, different strategies have been used. One approach is to use *in vitro* screening to identify potential antagonists, followed by *in vivo* screening against pathogens in fruit wounds.

The aim of this study was to investigate the possibility of postharvest biological control of *B. cinerea* on apples using epiphytic yeasts.

MATERIAL AND METHODS

Tested fruits. In this study, healthy apples belonging to Delicious and Golden Delicious cultivars were collected from the local market, transported directly to the laboratory and stored at 4°C under dry conditions until use. The fruits free of wounds and rots were selected and as homogeneous in size as possible.

Pathogen inoculums. *Botrytis cinerea* LPV 02 strain had been isolated from Tunisian apple with typical symptoms of grey mould and identified by morphological characteristics. Fungal isolation and identification were taken by the Laboratory of Applied Biotechnology to Agriculture, National Institute of the Agronomic Research in Tunisia (INRAT) in 2008.

The culture was maintained in Potato Dextrose Agar (PDA) medium at 4°C with periodic transfers through apple to maintain its virulence. Spore suspensions were obtained by removing the spores from a 10-day-old culture. The spores were then suspended in 5 ml of sterile distilled water. Suspensions were filtered through four layers of cheesecloth to remove the mycelium and spore concentration was adjusted using a haemocytometer to 1×10^5 spores/ml.

Epiphytic microbial microflora: isolation, purification and conservation. To isolate epiphytic yeasts from the surface of apples, the slightly modified technique described by Janisiewiez (1997) was adopted. Healthy and untreated fruits were washed into beakers containing 200 ml of sterile phosphate buffer (0.05 M, pH 6.5) supplemented with Tween 80 (0.05%). The beakers containing fruits were incubated on a rotary shaker at 25°C for 10 min, and then the fruits were rubbed with a sterile paint brush. Later, serial dilutions of the washing water were made in sterile distilled water and were used as a source of possible antagonists. An aliquot of 0.1 ml of each dilution was plated on Nutrient Yeast Dextrose Agar (NYDA) containing 8 g/l of nutrient broth, 5 g/l yeast extract, 10 g/l glucose, and 20 g/l of agar (Hong *et al*. 2007). The cultured plates were incubated at 28°C for 48 hours. Then, the colonies were purified following successive subculturing and were examined under the microscope. The purified strains were stored at 4°C on NYDA medium and in 50% glycerol

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at –80°C. Liquid yeast cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of NYDB. These flasks were incubated on a rotary shaker at 28°C for 48 hours. After that, cells were centrifuged at 6000 rpm for 10 min and washed twice with sterile distilled water to remove the growth medium water. The cell pellet was resuspended in 50 ml of sterile distilled water. Desired concentrations were obtained by adjusting the volume of suspension to 10^8 CFU/ml (Wisniewski *et al*. 1995).

In vitro screening of antagonists. Yeasts isolated from apple samples were screened for their ability to inhibit fungal growth on PDA plates using a slightly modified dual culture technique. The possible interaction of yeasts with pathogen hyphae was assessed in Petri dishes (9 cm in diameter). A plug (5 mm diameter) of agar containing 5-day-old mycelium of *B. cinerea* was placed on the agar surface on one side of the plate. The inoculums of yeasts, prepared from cultures grown on NYDA for 48 h, were placed on the other side of the plate as a strip. The plates were then sealed and incubated for 7 days at room temperature (28 ± 2°C) and the zone of inhibition (mm) was measured. The percentage of mycelial growth inhibition (GI)was calculated by the Equation (1):

$$GI\ (\%) = [(R1 - R2)/R1] \times 100 \quad (1)$$

where: R1 – distance (in mm) between the point of implant of the fungal disc and the side of the Petri plate; R2 – distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist

All *in vitro* tests were performed in triplicate and the test was repeated 3 times (Sadfi-Zouaoui *et al*. 2008b).

In vivo screening of yeasts on wounded apples cv. Golden Delicious. Five strains among the isolated yeasts were selected based on their high abilities to inhibit the pathogen *in vitro*. The effectiveness of these strains was also tested *in vivo*. For each experiment, fresh aliquots of the pathogen and the antagonist were adjusted to 10^5 conidia/ml and 10^8 CFU/ml, respectively. Untreated and healthy apples (no wounds or scars on the surface) were selected for the experiments. They were disinfected with 2% of sodium hypochlorite for 2 min and were air-dried. Each apple was wounded on 3 sides (3 mm deep × 3 mm wide) at the equator with a cork-borer. 20 µl of cell suspension at 10^8 cells/ml was placed into each wound 2 h prior to the inoculation with the pathogen spore suspension (10^5 spores/ml).

Wounds were inoculated with the pathogen alone or with sterile distilled water as positive and negative controls, respectively. To evaluate the effect of yeasts on fruit quality, fruit wounds were inoculated with 40 µl of cell suspension at 10^8 cells/ml alone and compared with the control fruit wounds. The fruits were kept at 23°C for 6 days in the dark (in covered plastic containers). High humidity (95%) was maintained by adding water to the bottom of the tray.

After the incubation period, wounds were examined and the lesion diameters were measured regularly until the end of the test (De Capdeville *et al*. 2007; Sadfi-Zouaoui *et al*. 2008b). The disease reduction percentage of grey mould on apples was calculated using the Equation (2):

$$\text{Disease reduction}\ (\%) = [(D - d)/D] \times 100 \quad (2)$$

where: D – lesion diameter recorded in an untreated apple inoculated by the pathogen alone; d – lesion diameter in an infected apple treated with both the pathogen and the antagonist

For each treatment, three apples were assayed and the experiment was repeated at least three times.

The disease incidence percentage of grey mould on apples was calculated acording to Equation (3):

$$\text{Disease incidence}\ (\%) = (A/B) \times 100 \quad (3)$$

where: A – number of infected apples in a yeast-treated tray; B – number of total fruits per tray

Three independent experiments were performed with three fruits per treatment (strain) and each experiment was used as a replication for the statistical analysis using *LSD* test.

Molecular identification of yeast antagonists. For DNA isolation, the strains were grown on NYDA for 48 hours. DNA was extracted using the rapid mini-preparation described by Liu *et al*. (2000). Amplifications of the ITS1-5.8S-ITS2 region of these isolates were achieved using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*. 1990). The DNA amplifications were carried out in a 25 µl reaction volume containing 2.5 µl PCR buffer, 1 µl $MgCl_2$, 0.5 µl dNTP, 0.75 µl ITS1, 0.75 µl ITS4, and 0.5 µl *taq* DNA polymerase was used in a PCR procedure (Lott *et al*. 1993; Martorell *et al*. 2005).

The PCR reactions were run in a thermal cycler at an initial step of 95°C for 5 min, 35 cycles of denaturation at 94°C for 2 min, annealing at 56°C for 2 min, extension at 72°C for 2 min, and a final extension at

https://doi.org/10.17221/103/2017-PPS

72°C for 10 min (Esteve-Zarzoso *et al*. 1999). The PCR products were visualised after electrophoresis, purified by using the Promega kit and sent for sequencing. Data outputs were analysed by the Chromas program v1.45 and sequences were identified by a database similarity search in the GENBANK Collection using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were aligned with those of related species using CLUSTAL W (Thompson *et al*. 1994) and checked visually. A neighbour-joining phylogenetic tree was produced by MEGA v7.0 software package using *Schizosaccharomyces octosporus* as an outgroup (Tamura *et al*. 2007). The nucleotide sequences reported in this paper have been deposited in the GenBank Database.

Morphological, physiological, and biochemical characteristics. According to the methods described by Kurtzman *et al.* (1998), morphological characteristics of yeast antagonists were examined. Yeast cells were cultured in a liquid medium for 2–3 days at 28°C in order to observe their shape and different processes of vegetative reproduction. Colony morphologies of each yeast isolate were examined in cultures grown on a solid medium (NYDA) at 28°C for 48 hours. The features of yeast cultures on the plates, i.e. colour and texture, were recorded 3–7 days after incubation. The assimilation of a variety of carbon compounds and the growth of yeasts at different temperatures (4, 25, 28, and 37°C) were tested on NYDA. Each test was repeated at least three times. Yeast isolates were tested for enzyme production. Urea broth filtered using a 0.2 µm filter was inoculated with fresh yeast suspension before being incubated at 28°C for 48 hours.

Statistical analyses. The trial was conducted in a factorial arrangement with three replications. All data were subjected to the statistical analysis of variance (ANOVA) using STATISTICA v5.0 software (STATSOFT France 1997). The comparison of means was done using the *LSD* test at 5%.

RESULTS

Isolation and in vitro screening of yeast antagonists. From a collection of 60 yeasts, only 10 yeasts named from L1 to L10 were selected to test their ability to reduce grey mould *in vitro* and *in vivo*. These strains were chosen because they were the only genetically identified ones. They were characterised and identified by the analysis of their ITS regions.

Table 1. *In vivo* screening of the yeast antagonists able to reduce grey mold disease in infected apple fruits (mean ± SE in %)

Yeast isolate	Disease reduction	Disease incidence
B. cinerea	0.00 ± 0.00	99.00 ± 0.58
H_2O	100.00 ± 0.00	0.00 ± 0.00
L1	63.20 ± 1.61	16.00 ± 1.20
L2	92.90 ± 2.57	11.00 ± 0.58
L3	21.33 ± 4.98	66.60 ± 0.88
L4	20.00 ± 6.93	77.00 ± 1.53
L5	70.30 ± 2.60	14.00 ± 1.73
L6	13.33 ± 7.06	83.33 ± 0.88
L7	95.00 ± 0.58	10.00 ± 1.00
L10	82.00 ± 4.58	33.00 ± 1.53

Five of them (L1, L2, L5, L7, and L10) were the most effective. The *in vitro* screening results from the dual culture technique showed the capacity of the five yeasts to inhibit the growth of *B. cinerea* on PDA by giving rise to a surrounding zone into which no pathogen growth occurred (Figure 1A).

The highest *in vitro* activity was described by the strain L2 with a percentage of mycelial growth inhibition exceeding 14% as shown in Figure 1B. The five yeast strains were tested further to confirm their potential in controlling grey mould caused by *B. cinerea* on fruit.

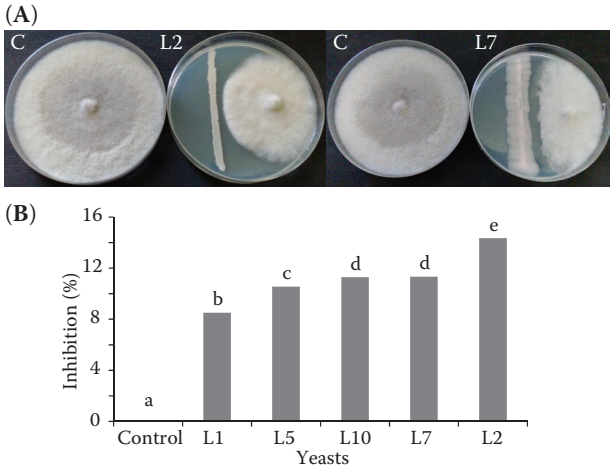

Figure 1. *In vitro* effect of yeasts on *Botrytis cinerea* growth inhibition on PDA plates using the dual culture technique (control – pathogen alone): (**A**) dual culture between the pathogen and the best antagonists L2 and L7 compared to the control (C – pathogen alone) and (**B**) the percentage of mycelial growth inhibition is calculated and the same letter signifies that there is no difference between values according to *LSD*

https://doi.org/10.17221/103/2017-PPS

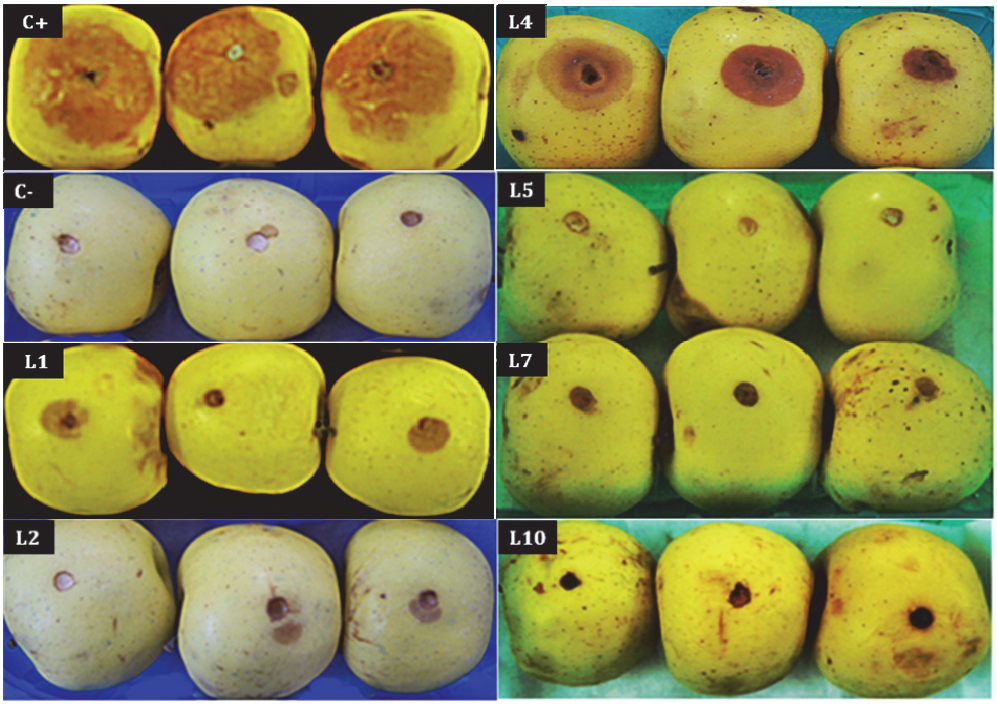

Figure 2. *In vivo* screening of epiphytic yeasts highly antagonistic to *Botrytis cinerea* compared to the strain L4 (less effective), by observing the development of grey mould on apples co-inoculated with yeast isolates and the pathogen (in controls: (C+) apples were inoculated only with the pathogen while in (C–) they were inoculated with sterilised distilled water)

In vivo screening of yeast antagonists. The biocontrol efficacy of the five selected yeasts was confirmed by using the *in vivo* test on apple fruits (Table 1 and Figure 2). Wounded apples treated with *B. cinerea* developed signs of disease in two days and the diameter of the lesions induced in the fruit was 25 mm. The treatments of fruits with the five yeasts at the concentrations of 1×10^8 cells/ml reduce the lesion diameter of the grey mould disease on infected apples by more than 63%. Moreover, results showed that the strain L7 significantly reduced the development of the pathogen compared to controls (Table 1). In fact, it had the highest *in vivo* biocontrol efficacy by about 95% of disease reduction. The disease incidence with this strain did not exceed 10%. Yeast strains L1, L2, L5, and L10 reduced grey mould on apple by 63.2, 92.9, 70.3, and 82.0%, respectively (Figure 3). No signs of disease were observed in negative controls or in wounds inoculated solely by the yeast cell suspensions.

Characterisation and identification of selected antagonists. Yeast isolates have been identified on the basis of their morphological, physiological, and biochemical properties. No mycelium was observed for L2 and L10 strains. Pseudomycelium was observed for strains L1, L5, and L7. Different characteristics of the colony and the cell are shown in Table 2. The round to ovoid shape was predominant among yeast cells. The physiological and biochemical profiles including assimilation of sugars, hydrolysis of urea, and growth at various temperatures are summarised in Table 2.

From the Internal Transcribed Spacers (ITS) region analysis (Figure 4), the active yeast isolates were identified as belonging to *A. pullulans* (L5, L7,

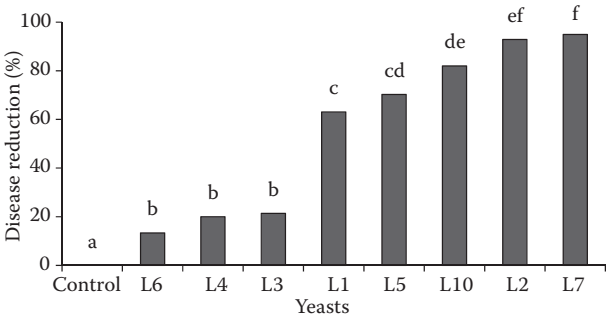

Figure 3. *In vivo* effect of yeasts on the percentage of disease reduction after co-inoculating the apple wounds by the suspension of yeasts at 1×10^8 cells and the conidial suspension of *B. cinerea* at 1×10^5 spores/ml (Control: apple inoculated by the pathogen alone). Data were pooled from three experiments; *LSD* value = 12.087 at 5% level; individuals with different letter are significantly different; those with the same letter belong to the same group)

https://doi.org/10.17221/103/2017-PPS

Table 2. Comparison of morphological, physiological and biochemical characteristics of the five effective yeasts (L1, L2, L5, L7, and L10) with *Aureobasidium pullulans*, *Citeromyces matritensis*, and *Cryptococcus flavescens* as listed by Kurtzman and Fell (1998)

Characteristics	L1, L5, L7	*Aureobasidium pullulans*	L2	*Citeromyces matritensis*	L10	*Cryptococcus flavescens*
Colony's morphology	circular, slimy and smooth		circular butyrous, smooth and glistening		circular with the surface irregular and lowly postulate	
Pigmentation	pinkish		white		zellowish-cream, dull	
Pellicule formation on broth medium	+	+	–	–	+	+
Cell's morphology	ovoidal, singles very variable in shape and size.		round, singles, small groups, no pseudohyphae		round, singles, pairs, short chains	
Assimilation						
Glucose	+ + +	+	+	+	+	+
Saccharose	+ + +	+	+	+	+	+
Maltose	+ + +	+	+	+	+	+
Lactose	+ – +	–	–	–	+	+
D-Xylose	+ + +	+	+	–/d/w	+	+
Galactose	+ ± +	+	–	+/–	+	+
Urea hydrolysis	+ + +	+	–	–	±	+
Growth temperature (°C)						
4	+ – –	+	+	+	+	+
25	++ + +	+	++	++	++	++
28	++ + ++	+++	++	++	++	++
37	+ + +	+	–	v	+	v

(+) positive reaction; (–) negative reaction; (±) moderate; d – delayed growth; w – weak growth; v – variable

and L1) with 98% similarity, to *C. matritensis* (L2) and to *C. flavescens* (L10) with 100% similarity. The ITS sequences of these isolates have been deposited in the GenBank database, under the accession numbers KY967606 for L1, KY967605 for L5, KY967607 for L7, KY967604 for L2, and KY967603 for L10.

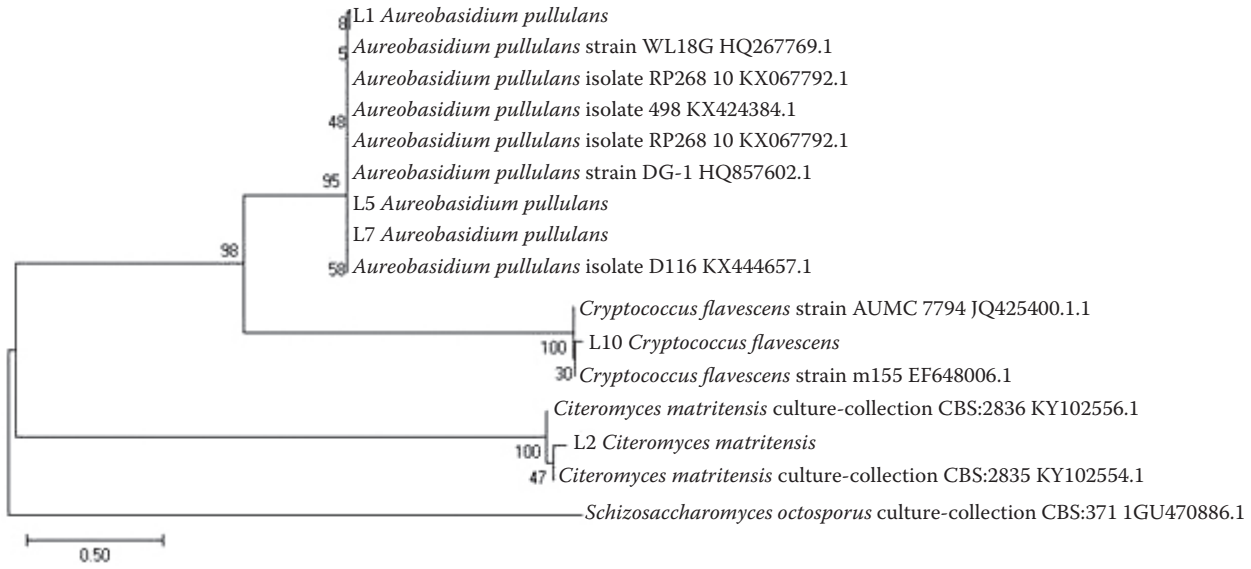

Figure 4. ITS rDNA phylogenetic tree of the yeast isolates L1, L2, L5, L7, and L10 (a neighbour-joining phylogenetic tree was produced using *Schizosaccharomyces octosporus* as an outgroup)

https://doi.org/10.17221/103/2017-PPS

We conclude that there is a correlation between the molecular results and the morphological and physiological characteristics. The results of physiological characteristics of strains L2 and L10 coincide with the data of the rDNA analysis which show their correspondence to *C. matritensis* and *C. flavescens*, respectively.

DISCUSSION

The control of postharvest diseases mainly relies on the application of synthetic fungicides (Xiao & Boal 2009). However, their use has been limited due to several factors such as development of resistant strains of pathogens (Zamani *et al*. 2006) and decrease of public concern about their toxic effect on human health and environment (Pimenta *et al*. 2008). In this respect, microbial biocontrol agents have shown a great potential as an alternative to synthetic fungicides. Several studies have shown the postharvest disease control by using biological agents (Droby *et al.* 2009; Abano & Sam-Amoah 2012; Yu *et al*. 2013; Panebianco *et al*. 2015).

The main objective of this study was to isolate and select epiphytic yeast to prevent or reduce attacks on Golden Delicious apples by *Botrytis cinerea*. The antagonism has been described by several researches (Janisiewicz & Korsten 2002). However, this is the first study in Tunisia which highlights the investigation of epiphytic yeasts from local apples for their potential antagonistic activity against *B. cinerea*.

As the control agent should be adapted to conditions on the fruit surface, the isolation was made from the epiphytic microflora associated with fruit (Fan & Tian 2001). Different yeasts were isolated and purified from the surface of different varieties of healthy apples and pears (Jijakli & Lepoivre 1993; Zhang *et al*. 2005). This method that has often been admitted may have certain limitations. In fact, a microorganism isolated and selected for its antagonistic activity against disease on a specific fruit may not be effective on another fruit. In addition, under practical conditions, microorganisms colonising the fruit surface may encounter problems when inoculated in wounds. Furthermore, this technique allows isolating only the epiphytic microflora but the fruit contains also endophytic microorganisms that may be useful.

Totally 10 yeast strains were tested for antifungal activities against *B. cinerea.* All yeast isolates showed more or less antifungal activity against the pathogen compared to the control *in vitro*. The most promising results were obtained by five epiphytic yeasts belonging to the species *Citeromyces matritensis* (L2), *A. pullulans* (L1, L5, L7), and *Cryptococcus flavescens* (L10). These strains showed an inhibition zone not exceeding 10 mm on Petri plate assays. The mycelial growth inhibition of the pathogenic fungus ranged from 8.50% to 14.33% in dual culture. The treatments of wounded apples with the five yeasts significantly reduced the grey mould disease severity by more than 63%. Their application to wounds has no negative effect on fruit quality compared to untreated fruit, which proves their non-toxicity to apples. Instead, 3 isolates of *A. pullulans* L1, L5, and L7 show high efficiency in reducing the disease incidence. The L7 strain had the highest *in vivo* biological efficacy by about 95%.

In an earlier study, Schena *et al.* (1999) found that isolates of *A. pullulans* were able to control *Penicillium digitatum, Botrytis cinerea*, *Rhisopus stolonifer*, and *Aspergillus niger* on grapes and *Rhisopus stolonifer* on cherry tomatoes. The biocontrol activities of *A. pullulans* against blue mould of stored apple caused by *P. expansum* (Bencheqroun 2009), monilinia rot in banana (Wittig *et al*. 1997), and grapes by *Monilinia laxa* (Barkai-Golan 2001) were also revealed. Similar results were obtained in controlling postharvest decay caused by *B. cinerea* of apple fruit by *A. pullulans* (Ippolito *et al*. 2000). Nevertheless in previous researches, the incidence reduction percentage of grey mould by *A. pullulans* did not exceed 19% with an infection rate of 25% (Vero *et al*. 2009).

In addition, the strains L2 of *C. matritensis* and L10 of *C. flavescens* highly reduced the grey mould disease severity on apples by more than 92 and 82%, respectively. Diverse studies evaluated the biological activity of the genus *Citeromyces* previously called the genus *Torulaspora*. In fact, Rosa *et al.* (2010) claimed that *Torulaspora globosa* is able to control the pathogenic fungus *Colletotrichum sublineolum* in sorghum. Ebrahimi *et al.* (2012) showed that *Torulaspora delbrueckii* is beneficial for controlling blue mould caused by *Penicillium expansum* in apple fruits. Nevertheless, as far as we know no report concerning the use of *Citeromyces* species against *B. cinerea* is available, which gave a great importance to this study.

Among the species of the genus *Cryptococcus, C. laurenti*, and *C. albidus* are well-known for their

https://doi.org/10.17221/103/2017-PPS

antagonistic activity against various post-harvest pathogens such as *B. cinerea* (Tian *et al*. 2004). Nevertheless, as far as we know, the use of *C. flavescens* yeast in the *B. cinerea* control in apples has not been surveyed. The already existing studies are only interested in its effectiveness against Fusarium head blight on wheat (Dunlap *et al*. 2007).

Briefly, in this work, three yeast strains L2, L7, and L10 have been selected for their antagonistic properties. The use of such yeasts may constitute an important alternative to synthetic fungicides, which failed to suppress the development of the fungal pathogen. Their potential as biocontrol agents for grey mould is interesting and further investigation is needed to verify the effectiveness of these antagonists under long-term storage on apples.

CONCLUSION

The present study investigated the isolation, identification and screening of potential antagonistic yeasts isolated from the surface of Golden Delicious apples grown in Tunisia. Five yeasts *A. pullulans* (L1, L5, and L7), *C. matritensis* (L2), and *C. flavescens* (L10) reduced the diameter of the grey mould lesion by more than 63%. The L7 strain had the highest *in vivo* biological efficacy by about 95% of the disease reduction followed by *C. matritensis* (L2) and *C. flavescens* (L10) by 92.90 and 82%, respectively. To our knowledge, these latter two yeasts have been newly demonstrated to inhibit the grey mould disease on apples. Further studies are conducted to evaluate their mechanisms of action and exploit them in commercial production and under storage conditions.

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Received: 2017–07–29
Accepted after corrections: 2018–03–19
Published online: 2018–04–04