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Molecular approaches to assist the screening and monitoring of postharvest biocontrol yeasts

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Abstract

Nineteen yeast isolates obtained from the surface of several fruits and vegetables grown in Southern Italy and Israel were compared by molecular analysis using arbitrarily primed polymerase chain reaction (AP-PCR) and random amplified polymorphic DNA technique (RAPD-PCR). Genetic analysis made it possible to distinguish between closely-related genitically different strains which had the same morphological characteristic, and to discard isolates which were genetically identical. Following PCR characterisation, 6 isolates were selected and tested for their biocontrol activity against major postharvest pathogens (*Penicillium digitatum* on grapefruit, *Botrytis cinerea, Rhizopus stolonifer* and *Aspergillus niger* on table grape and *B. cinerea* and *R. stolonifer* on cherry tomato). All the isolates showed a good biocontrol efficacy on both wounded and non-wounded fruits. Furthermore, the preharvest application of the most effective antagonist (LS15) on table grape resulted in a significant reduction in grey mold ranging from 28.3% to 38.2% compared to the untreated control. The RAPD-PCR technique was also useful for identifying and monitoring the survival of the antagonist after field application.

Introduction

Due to the development of fungicide-resistant strain of postharvest pathogens and the public concern about fungicide residues considerable efforts have been made to find microorganisms capable of controlling postharvest decay. Despite the success demonstrated with microbial antagonists in laboratory and small scale tests, the level of control achieved under commercial conditions falls short of that of conventional fungicides (Wilson and Wisniewski, 1994; Droby et al., 1998a). New molecular technologies improving the preliminary selection of biocontrol agents (Bevivino et al., 1998) and the monitoring of field applied antagonists may greatly facilitate the selection and screening of yeast isolates having a superior antagonistic activity. Morphological and cultural characteristics alone are not sufficient to distinguish between strains of the same species and do not allow the exploitation of any possible naturally available genetic variability. Random amplified polymorphic DNA (RAPD-PCR) and arbitrary primed polymerase chain reaction (AP-PCR) techniques make it possible to distinguish between closely related strains accurately and efficiently (Wong et al., 1996). DNA fingerprinting by means of RAPD-PCR and AP-PCR may also provide a valid means for characterising yeast populations (Welsh and McClelland, 1990; Williams et al., 1990; Droby et al., 1998;1999) and may significantly improve the preliminary selection of biocontrol agents. The identification and the exclusion of genetically-similar isolates can reduce the number of isolates needed to be biologically screened and may thus facilitate the search for biocontrol agents.

Since infection of fruits by postharvest pathogens often occurs in the field prior to harvest (Biggs, 1995), it may be advantageous to apply antagonists before harvest (Ippolito and Nigro, 2000). Effective antagonists must proliferate on the plant surface and remain active against target pathogens during periods favourable for infection (Lo et al., 1998); therefore, in field applications it is necessary to use microrganisms that can tolerate low nutrient availability, UV radiation, temperature changes, and dry conditions. To develop an effective biocontrol strategy, the survival of biocontrol agents needs to be monitored. The typical method used for detection and quantification of microbes in ecosystems is the viable-cell count, typically reported as colony forming units (cfu) per unit area or per weight of sample (Li et al., 1996). However, using this method, it is difficult to distinguish introduced strains from indigenous ones. RAPD-PCR markers may allow recognition of the selected antagonistic strains after their introduction into phyllosphere (Gullino et al., 1995; Schena et al., 1999).

Among the microrganisms considered for biological control, yeasts are particularly promising. They can colonise plant surfaces or wounds for long periods under dry conditions and scientists suggest that their mechanisms of antagonism are mainly based on competition for space and nutrients (Mercier and Wilson, 1994; Janisiewicz et al., 1994; Robert, 1990; Filonow et al., 1996; Ippolito et al., 2000). *Pichia guilliermondii* Wickerham, *Metschnikowia* spp., *Debaryomyces hansenii* Zopf and *Candida oleophila* Montrocher, are effective biocontrol agents and are commonly isolated from the carposphere (Droby et al., 1989; Mercier and Wilson, 1994; Filonow et al., 1996; Piano et al., 1997; Lima et al., 1997; Nigro et al., 1999).

The aims of this research were: (i) to genetically characterise epiphytic yeasts in order to distinguish between closely related strains, (ii) to evaluate the effectiveness of selected yeasts in the control of postharvest pathogens, (iii) to study the survival of a promising isolate (LS15) of *Metschnikowia* spp., when applied in the field prior to harvest.

Materials and methods

Isolates

Nineteen isolate of yeasts were selectively isolated from the surface of fruits and vegetables from Southern Italy and Israel (Wilson et al., 1993). Yeasts belonged to the species *D. hansenii* (301, 303, 304, 305, 307, 456, I34), *P. guilliermondii* (243, A42, 475, 490, 495, 498, 506), *C. oleophila* (182) and *Metschnikowia* spp. (LS15, 291, 311 and 320). Isolates 243, LS15, 291, 311 and 320 were isolated in Southern Italy from fig (243), tomato (LS15), pear (291), quince fruits (311) and table grape (320). The other yeasts were isolated in Israel from grapefruit. For long-term storage, yeast isolates were suspended in 10% glycerol at $-80\,^{\circ}$C. For short-term storage isolates were kept at $4\,^{\circ}$C on potato dextrose agar (PDA).

Pathogens were isolated from decayed citrus fruits (*Penicillium digitatum* Pers.) or grape berries (*Botrytis cinerea* Pers., *Aspergillus niger* Tiegh. and *Rhizopus stolonifer* Ehrenb) and kept on PDA at $5\,^{\circ}$C for short-term storage.

Characterisation of yeast antagonists

DNA polymorphism was evaluated using RAPD-PCR and AP-PCR techniques. For RAPD-PCR reactions, the following primers, from Operon Technologies, Inc. USA were used: GATGACCGCC (OPC-05) and TGGACCGGTG (OPC-08). For AP-PCR analysis the following primers derived from minisatellite or repeat sequences were used: GACAGGACAGGACAG $(GACAG)_3$, GACAGACAGACAGACA $(GACA)_4$, and CAGCAGCAGCAGCAG $(CAG)_5$ (Freeman and Shabi, 1996). The primers used for AP-PCR and RAPD-PCR were selected as they produced consistently reproducible patterns that were less sensitive to variations under assay conditions (Droby et al., 1999).

Primer GGAGGCTGTT (OPB-15) from Operon Technologies, in previous trials, (unpublished data) was very effective in differentiating isolate LS15 from other *Metschnikowia* spp. For this reason this primer was used to identify and monitor the population of isolate LS15 on the surface of table grape berries. In these studies, a pure culture of isolate LS15 was used as a control.

Genomic DNA was extracted using the method of Hoffman and Winston (1987) with some modifications. One loop of each antagonist taken from a colony actively growing on a PDA plate, was suspended in an Eppendorf tube containing 100 µl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA). To make a final concentration of 10^7–10^8 cells ml^{-1}, 0.3 g of acid-washed glass beads (∅ of 425–600 µm, Sigma-Aldrich,

Milano, Italy), phenol (50 μl), chloroform (48 μl) and isoamyl alcohol (2 μl) were added to the solution. Eppendorf tubes were vortexed at high speed for 5 min, and then spun in a centrifuge at room temperature for 5 min. The supernatant was used for the PCR reactions.

For AP-PCR, amplification reactions were performed in a total volume of 20 μl, containing 10–100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 1 unit of Taq polymerase (Promega, Madison, WI, USA) and 1 μM of primer. The reactions were incubated in a programmable thermal controller (PTC-100^{tm}, Peltier-Effect Cycling, MJ Research, Inc., USA), starting with 5 min of denaturation at 95 °C followed by 30 cycles consisting of 30 s at 95 °C, 30 s at either 60 °C for $(CAG)_5$ or 48 °C for $(GACA)_4$, and $(GACAC)_3$ and 1.5 min at 72 °C.

For RAPD-PCR, reactions were performed in a total volume of 25 μl, containing 10–100 ng of genomic DNA, 50 mM KCl, 10 mM Tris–HCl pH 8.8, 0.1% Triton X-100, 100 μM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 0.5 unit of Taq polymerase and 5 pmol of a single 10-base primer. The reactions were incubated in a PTC starting with 5 min of denaturation at 94 °C. This was followed by 45 cycles consisting of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. Negative controls (no template DNA) were used in every experiment to test for the presence of contamination in reagents. The amplification products were analysed by electrophoresis in 2% agarose gels in TAE buffer at 70 V for 1.5 h, and detected by staining for 10 min with ethidium bromide (2 μg ml^{-1}).

Screening of antagonists on individual wounded fruit

Six isolates showing different band patterns (304, 495, 243, LS15, 291, and 320), plus *Candida oleophila*, isolate 182 (Droby et al., 1993), chosen among 19 isolates, were selected and assayed for their biocontrol activity. The isolates genetically similar were discarded. Tests were conducted on individual wounded fruits to evaluate the biocontrol activity against *P. digitatum* on grapefruit, *B. cinerea*, *A. niger* and *R. stolonifer* on table grape and *B. cinerea* and *R. stolonifer* on cherry tomato. Fruits were surface-sterilised with Na-hypochlorite (2 min in a 2% solution), rinsed with tap water and allowed to dry before wounding. On grapefruit, wounds were made to a uniform depth of 3 mm at three sites around the stem-end using a dissecting needle. On table grape berries and cherry tomatoes, wounds were made to a uniform depth of 2 mm at one site in the equatorial zone. Isolates were grown in 25 ml of nutrient yeast-extract dextrose broth (NYDB) in 100 ml Erlenmeyer flasks on a rotary shaker at 150 rpm for 48 h at 25 °C. Cells were harvested by centrifugation (1100 g for 15 min), rinsed twice with sterile deionized water, resuspended in distilled water to the initial concentration of about 5×10^8 cells ml^{-1} and diluted to obtain the final concentrations of 10^8, 10^7, and 10^6 cells ml^{-1}. Pathogens were grown on PDA plate for 7–10 days at 22 °C. A spore suspension was made in sterile distilled water containing 0.05% tween 80, filtered through a double layer of sterile muslin cloth, vortexed for 1 min to assure uniform mixing, and diluted to the final concentration of 5×10^4 spore ml^{-1}. Twenty μl of yeast cell suspension were pipetted into each wound, and 2 h later wounds were inoculated with 20 μl of pathogen spore suspension. Wounds treated with water served as a control.

In table grape and cherry tomato tests, each treatment was replicated three times and each replicate consisted of 10 fruits; in grapefruits tests, 3 replications of 6 fruits with 3 wounds per fruit were used. In all the experiments, fruits were mounted on tape strips glued to PVC pads and kept 1–2 cm apart to avoid nesting. Each PVC pad containing a replication, was placed in a proper plastic box covered with a lid and kept at 20±2 °C and 95–98% relative humidity (RH). The percentage of infected wounds was evaluated after 5 days. The entire experiment was repeated twice. An arcsine transformation was applied to the data prior to analyses of variance. Data from the two experiments were combined as statistical analysis determined homogeneity of variances. Treatment means were separated by Duncan's multiple range test.

In vivo *assays on grape bunches*

Yeast isolates were further tested for their biocontrol efficacy against *B. cinerea*, *A. niger* and *R. stolonifer* on table grape (cv. Thompson seedless). Small bunches of 10 berries each were surface-sterilised with Na-hypochlorite as described before and dipped in a suspension of the antagonist (5×10^7 cells ml^{-1}), or water (control). Two hours later, the bunches were inoculated by spraying with pathogen spore suspensions (10^5 spore ml^{-1} for *B. cinerea*, 5×10^4 spore ml^{-1} for *A. niger* and *R. stolonifer*). The treated bunches

were maintained at 20 °C at high RH and after 5 days the percentage of infected berries was assessed. Each treatment was replicated four times and each replicate consisted of eight small bunches placed inside a plastic box covered with a lid. In each box the space among bunches was large enough to avoid nesting. An arcsine transformation was applied to the data prior to analysis of variance. Treatment means were separated by Duncan's multiple range test.

Field tests

Tests were conducted on table grape (cv. Thompson seedless) in a commercial vineyard located in Lachish (Israel) using isolate LS15 of *Metschnikowia* spp. The yeast was grown on NYDB for 48 h at 24 °C, harvested by centrifugation, resuspended in an equal volume of sterile water and diluted to a concentration of approximately 5×10^7 cells ml^{-1}. The antagonist was applied in the field 4 or 5 times, before harvest, at weekly intervals starting on July 15. In the first test (4 sprays), the last spraying was done one week before harvest; in the second test (5 sprays), the last spraying was done 2 h before harvest. A chemical control treatment (Rovral, 25% a.i.) was included and sprays were applied following the same schedule used for the antagonist. In addition, a non-treated control was included. A motor-driven back sprayer was used to apply the yeast suspension and the fungicide. Each treatment was replicated four times and each replicate consisted of three vines arranged in a randomised block design. From each replication 8–10 bunches were harvested, placed in plastic commercial boxes and enclosed with plastic sheet. The incidence of natural infections caused by *B. cinerea* was evaluated after 30 days of storage at 0 °C followed by 9 days of shelf-life at 20 °C. In order to evaluate the diseases intensity, the following empirical scale was used: 0 = bunch without rot; 1 = 0–10% rotted berries; 2 = 10–25% rotted berries; 3 = 25–50% rotted berries; 4 = 50–75% rotted berries; 5 = more than 75% rotted berries. This empirical scale enabled the calculation of the McKinney index (McKinney, 1923) expressing the weighted average of disease severity as percentage of the highest level of disease. The index was calculated by means of the formula: $Mi = \left[\sum(d \times f)/Tn \times D\right] \times 100$ where *d* is the degree of disease intensity assessed on the bunch and *f* is the frequency of each class of disease intensity; *Tn* is the total number of the bunches examined (healthy and diseased) and *D* the highest degree of disease intensity occurring on the empirical scale. An arcsine transformation was applied to the data prior to analysis of variance. Treatment means were separated by Duncan's multiple range test.

Population dynamics of isolate LS15 in the field and during storage

To assess survival of isolate LS15 on fruit, the antagonist was applied once at a concentration of 5×10^7 cells ml^{-1} and the epiphytic population was evaluated on grape berries at weekly intervals. In the first sampling, grape berries were picked 2 h after the application of the antagonist (July 14) and then after 7, 14 and 21 days. The epiphytic population was also assessed at the beginning (August 11) and at the end of the storage period (September 10). Each replication consisted of 5 berries which were shaken in 100 ml of sterile distilled water on a rotatory shaker at 150 rpm for 30 min. The rinse water was serially diluted and plated on PDA plates (0.1 ml/plate). Plates were incubated at 25 °C, and after 3–4 days the colonies of the antagonist showing typical morphological characteristics of *Metschnikowia* spp. (margin aspect, size, colour, etc.), were recorded. At the end of storage, to assess if the recorded colonies belonged to the introduced antagonist or to other natural occurring *Metschnikowia*-like strains, some colonies were randomly selected in plates seeded with washing water of treated or untreated berries and were genetically analysed by RAPD-PCR with primer OPB-15. A pure culture of isolate LS15 was used as a control.

Results

Characterisation of yeast antagonists

Genetic variability among nineteen yeasts isolated from the surface of several fruits in southern Italy and Israel was assessed by AP-PCR and RAPD-PCR (Figure 1). The patterns were complex, showing between one and five bands of various intensity ranging from approximately 0.3–2 kb in size. Among the different genera, considerable differences were observed. On the contrary, little intraspecific polymorphism was observed among both *D. hansenii* isolates and the Israeli isolates of *P. guilliermondii*; in particular, with the primer $(GAC)_5$, the absence of one band enabled us to distinguish isolate I34 from the other isolates of *D. hansenii*, whereas with primer OPC-05, the presence of two more bands, enabled

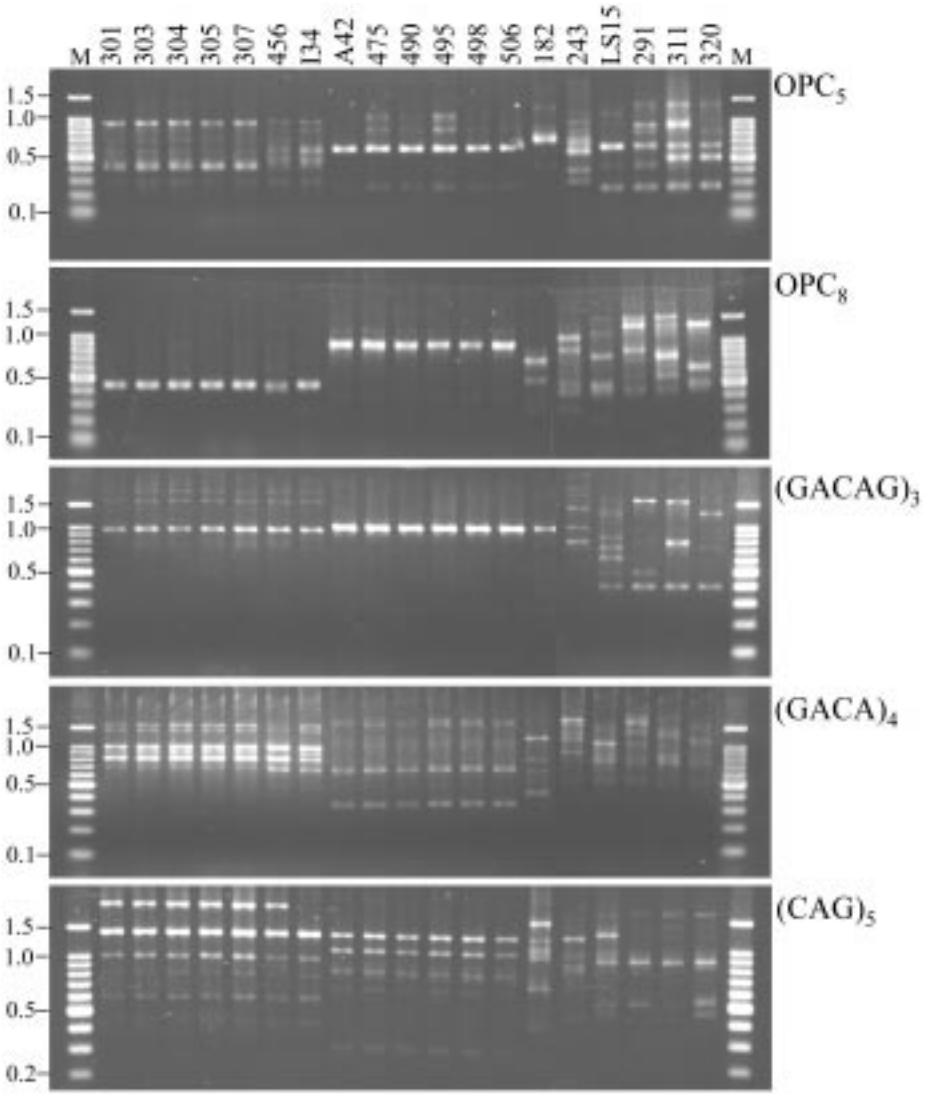

Figure 1. Gels stained with ethidium bromide showing amplification products generated from 19 yeast isolates with primers OPC-05, OPC-08, $(GACAG)_3$, $(GACA)_4$ and $(CAG)_5$. The first lane on the left and the last one on the right contain a DNA marker (M) consisting of 11 double-stranded DNA fragments (100 bp DNA ladder, Promega, Madison, WI, USA).

us to distinguish isolates 475 and 495 from the other isolates of *P. guilliermondii*. Considerable differences with the five primers were observed between isolate 243 of *P. guilliermondii* obtained in Italy and the seven isolates of *P. guilliermondii* obtained in Israel. High polymorphism was observed among the four isolates of *Metschnikowia* spp., suggesting that these four isolates were genetically different.

All the colonies showing the typical morphology of *Metschnikowia* spp., reisolated from grape sprayed in the field with the antagonist, had the same RAPD-PCR patterns as the pure culture of isolate LS15 (Figure 2A). In contrast, eleven colonies reisolated from untreated table grape were genetically different from isolate LS15 (Figure 2B).

Biocontrol activity of yeast isolates

In the assays on wounded grapefruit, cherry tomato, and table grape, yeast isolates showed biocontrol

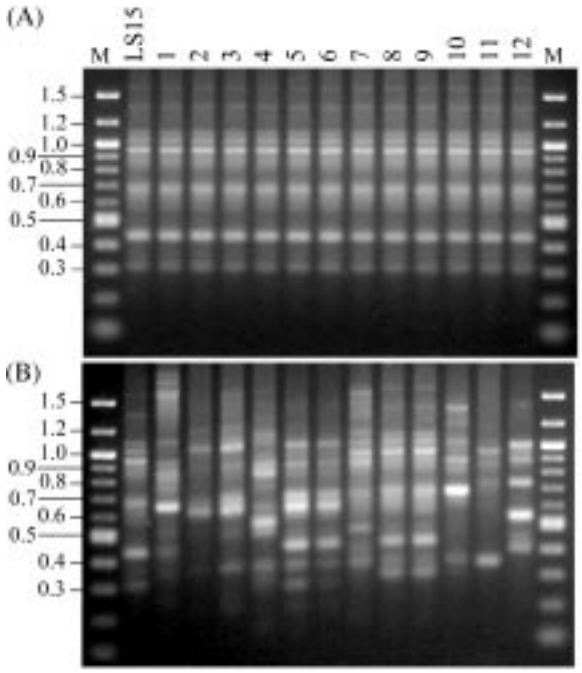

Figure 2. DNA amplification products with primer OPB-15 of 12 isolates of *Metschnikowia* spp., isolated from table grape berries treated with isolate LS15 in the field (A) or from one untreated (B). In both the pictures (A and B) the second line on the left, contains amplification products generated from a pure culture of isolate LS15, while the first one on the left and the last one on the right contain DNA markers (M) consisting of 14 discrete fragments (100 bp DNA Ladder, New England Biolabs GmbH, Germany).

activity which depended on the concentration used (Figures 3–5). At high concentrations (10^8 and 10^7 cells ml^{-1}), almost all antagonists provided a significant ($P \leq 0.01$) reduction in decay compared to the water control. No significant differences were found between the new isolates and *C. oleophila* isolate 182. In particular, at the highest concentration (10^8 cells ml^{-1}) a complete inhibition of decay was achieved for *P. digitatum* on grapefruit (isolates 182, 495, and LS15) (Figure 5), *B. cinerea* on table grape (isolates 182, 304, 243, LS15, and 320) (Figure 3A), *A. niger* on table grape (isolate LS15) (Figure 3B), *R. stolonifer* on table grape (isolates 243, 291, and 320) (Figure 3B), *B. cinerea* on cherry tomato (isolates 304, and LS15) (Figure 4A), and *R. stolonifer* on cherry tomato (isolates 182, 304, 495, LS15 and 320) (Figure 4B). Isolate LS15 was particularly effective, providing a significant reduction in decay caused by *R. stolonifer* on table grape (89%) and a complete inhibition of the other

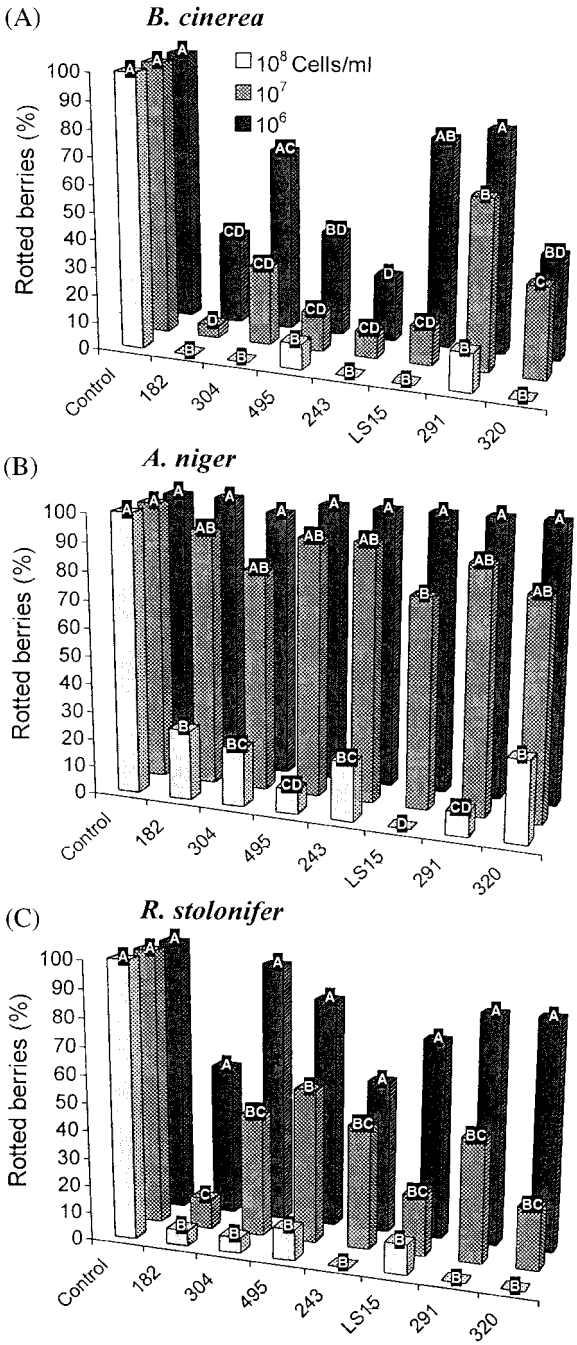

Figure 3. Biocontrol activity of 7 different yeast isolates (182, 304, 495, 243, LS15, 291, and 320) against *B. cinerea* (A), *A. niger* (B), and *R. stolonifer* (C), on single berries of table grape. Wounded sterile berries were treated with a cell suspension of the antagonists at three different concentrations (10^8, 10^7 and 10^6 cells ml^{-1}) or water (control) and after 2 h, with a spore suspension of the pathogens. The percentage of infected wounds was

pathogens on the 3 fruits tested. At the concentration of 10^7 cells ml^{-1} all the antagonists provided a significant reduction of the rots caused by *B. cinerea*, *R. stolonifer* and *P. digitatum* but, with the exception

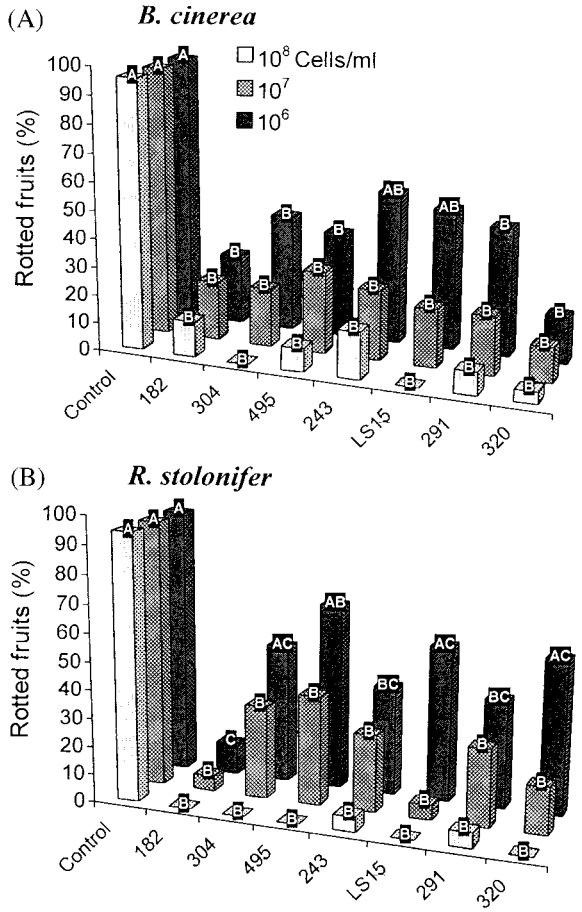

Figure 4. Biocontrol activity of 7 different yeast isolates (182, 304, 495, 243, LS15, 291, and 320) against *B. cinerea* (A) and *R. stolonifer* (B) on individual fruits of cherry tomato. Wounded sterile fruits were treated with a cell suspension of the antagonists at three different concentrations (10^8, 10^7 and 10^6 cells ml^{-1}) or water (control) and after 2 h, with a spore suspension of the pathogens. The percentage of infected wounds was evaluated after 5 days at 20 °C at high RH. For each concentration, values followed by different letters are statistically different according to Duncan's multiple range test ($P \leq 0.01$).

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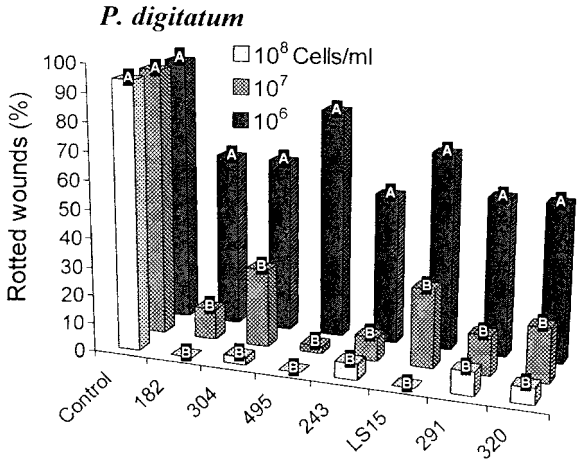

Figure 5. Biocontrol activity of 7 different yeast isolates (182, 304, 495, 243, LS15, 291, and 320) against *P. digitatum* on individual fruits of grapefruit. Wounded sterile fruits were treated with a cell suspension of the antagonists at three different concentrations (10^8, 10^7 and 10^6 cells ml^{-1}) or water (control) and after 2 h, with a spore suspension of the pathogen. The percentage of infected wounds was evaluated after 5 days at 20 °C at high RH. For each concentration, values followed by different letters are statistically different according to Duncan's multiple range test ($P \leq 0.01$).

of isolate LS15, were not significantly effective against *A. niger* (Figures 3–5). At the lowest concentration (10^6 cells ml^{-1}), all the antagonists were less effective in controlling decay; however, some isolates were able to significantly reduce the rots caused by *B. cinerea* on table grape (182, 495, 243, and 320), (Figure 3A), *B. cinerea* on cherry tomato (182, 304, 495, 291, and 320), (Figure 4A) and *R. stolonifer* on cherry tomato (182, 243, and 291), (Figure 4B).

In vivo *assays with small table grape bunches*

Yeast isolates were effective in reducing the incidence of decay caused by *R. stolonifer*, *A. niger* and *B. cinerea* on small table grape bunches (Figure 6A,B and C). All the antagonists used were effective in inhibiting *B. cinerea* and *A. niger* providing a decay reduction ranging from 36.6% to 61.7%, compared to the water control (Figure 6A and B). The three isolates of *Metschnikowia* spp. (LS15, 291, and 320) were particularly effective against *R. stolonifer* with 64.1%, 71.5%, and 77.3% of decay reduction, respectively, compared to the water control (Figure 6C).

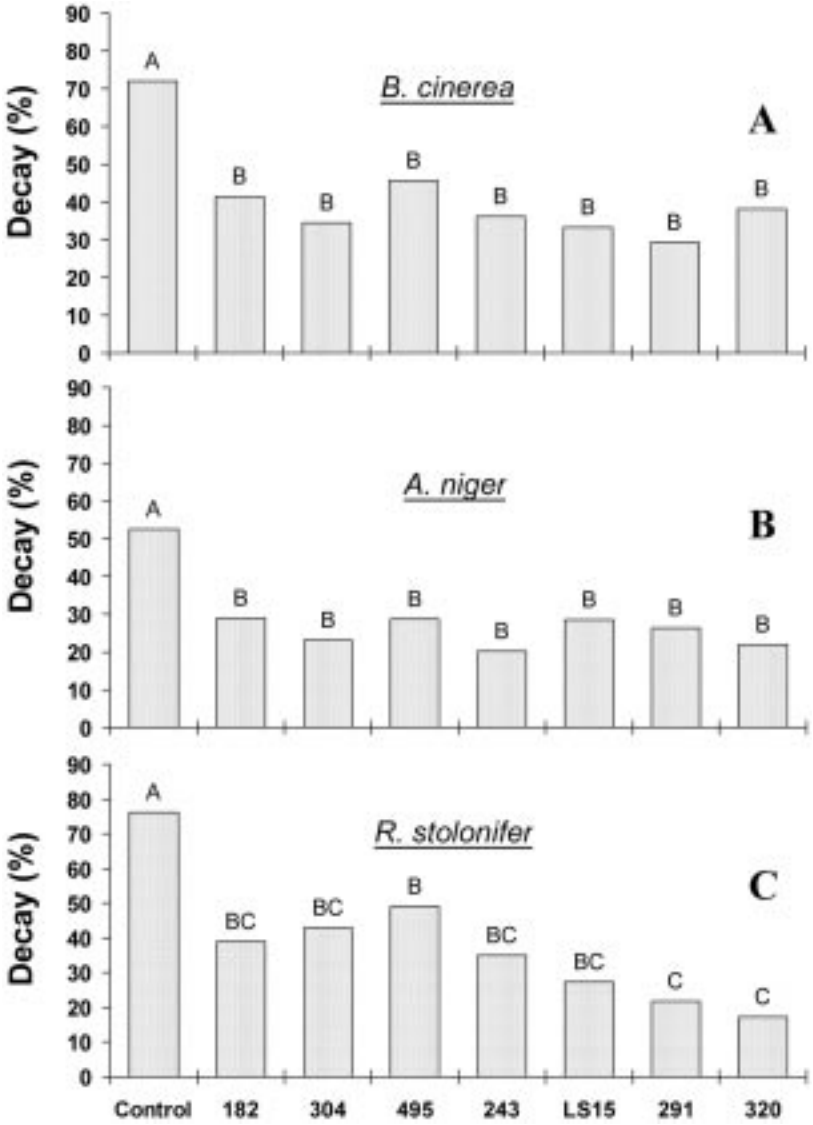

Figure 6. Biocontrol activity of 7 different yeast isolates (182, 304, 495, 243, LS15, 291, and 320) on small table grape bunches against *B. cinerea* (A), *A. niger* (B) and *R. stolonifer* (C). Surface-sterilised bunches were dipped in a suspension of the antagonists (5×10^7 cells ml^{-1}), or water (control) and 2 h later, were inoculated with a spore suspension of the pathogens. The treated bunches were kept at 20 °C, high RH, and after 5 days the percentage of infected berries was assessed. For each assessment, columns with different letters are statistically different according to Duncan's multiple range test ($P \leq 0.01$).

No significant differences in biocontrol activity were observed between the new isolates and the antagonist 182.

Biocontrol activity under field conditions

Further tests to control grey mold on table grape under field conditions were conducted with LS15 (Figure 7). A preharvest application of LS15 (4 and 5 sprayings) resulted in a significant ($P \leq 0.01$) reduction of the disease index as compared to the untreated control. On grapes sprayed 4 times, the decay was reduced by 28.3%, but a greater reduction was achieved when the antagonist was applied 5 times (38.2%). Four applications of the chemical treatment (iprodione) were the most effective in reducing disease index.

The population of *Metschnikowia* spp. rapidly decreased on grape berries sprayed with the antagonist under field conditions (Figure 8). From the time of application up until the harvest, populations decreased from 7×10^5 to 5×10^1 cfu/berry. During cold storage, the concentration of *Metschnikowia* spp. increased reaching 6×10^5 cfu/berry. On untreated table grapes the population of *Metschnikowia* spp. was very low both in the field and during the storage.

Discussion

The selected antagonists belonging to the species *D. hansenii* (isolate 304), *P. guilliermondii* (isolates 495 and 243) *Metschnikowia* spp. (isolates LS15, 291, and 320) and *C. oleophila* (isolate 182) were highly effective against several postharvest pathogens that cannot be controlled with a single chemical fungicide. These yeasts are commonly isolated from the carposphere (Wilson et al., 1993) and have been considered as possible biocontrol agents able to reduce the development of postharvest diseases of fruits and vegetables (Droby et al., 1993; Mercier and Wilson, 1994; Ippolito et al., 1994; Filonow et al., 1996; Piano et al., 1997; Lima et al., 1997; Droby et al., 1999). In all the experiment on wounded fruits, the biocontrol activity of the antagonists was related to the cell concentration. These data indirectly suggest that competition for space and nutrient have an important role in the mode of action of these yeasts. As reported by Wilson and Wisniewski (1994) a quantitative balance exists at the wound site between the numbers of antagonist and pathogen propagules, which affects the outcome of the interaction and determines whether the wound becomes the site of infection.

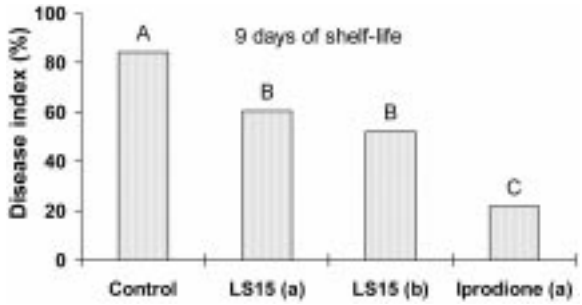

Figure 7. Incidence of grey mold on table grape treated in the field with isolate LS15, Rovral (iprodione) or untreated (control). The grapes were sprayed four (a) or five (b) times at weekly intervals and the incidence of natural infections caused by *B. cinerea* was evaluated after 30 days of storage at 0 °C, followed by 9 days of shelf-life at 20 °C. Columns with different letters are statistically different according to Duncan's multiple range test ($P \leq 0.01$).

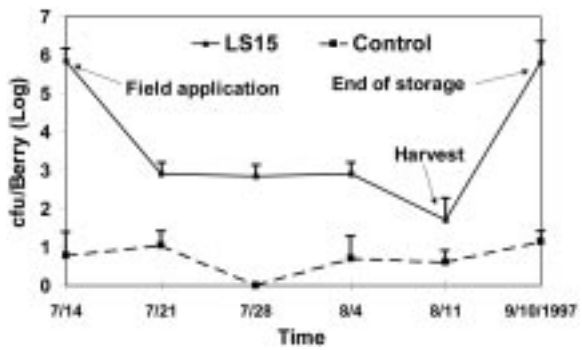

Figure 8. Population dynamics of *Metschnikowia* spp. on table grape berries treated with isolate LS15 or untreated (control). The antagonist was sprayed just once at a concentration of 10^7 cells ml^{-1} and the epiphytic population was evaluated in the field at weekly intervals (5 times), and at the end of storage.

A molecular approach enabled the selection of effective antagonists in a relatively short time. Since isolates with the same phenotype can differ significantly in their genotype and in their biocontrol activity (Leibinger et al., 1997), the genetic characterisation of yeasts is a useful procedure for screening and exploiting all the genetic variability available on the carposphere. RAPD-PCR and AP-PCR techniques, allowed isolates that were genetically similar to each other to be eliminated and isolates having the same morphology but being genetically different to be recognised. Thus, the selection of new biocontrol agents was more effective and less time consuming. RAPD-PCR and AP-PCR appear promising for practical applications; very little amount of template DNA is required and, as shown in this work, with a fast protocol for DNA extraction, a large number of isolates can be analysed at the same time. Furthermore, RAPD-PCR and AP-PCR do not require any prior knowledge of target DNA; thus, it is possible to study any novel microorganism. Investigation of microbial diversity greatly benefited from the development of techniques based on genotype analysis; DNA fingerprints generated by these techniques made it possible to differentiate between individuals within the same species and even mutants derived from the same parental strains (Wong et al., 1996).

The ability to identify a specific isolate is important, especially once a biocontrol agent is applied in the field and there is a need to determine its population

dynamic. This information may allow a better understanding of the antagonist ecology and the optimisation of formulations and application times providing a higher degree of protection (Wilson and Wisniewski, 1994). The integration of RAPD-PCR analysis with conventional dilution plating permitted us to effectively follow the population dynamics of isolate LS15 both in the field and during cold storage. The methods traditionally used to monitor the survival of a biocontrol agent under field conditions were ineffective in identifying a specific isolate of the same species (Li et al., 1996). RAPD-PCR allowed us to distinguish the introduced isolate from the other natural yeasts present on untreated grape berries. In this way, it was possible to confirm that the yeasts reisolated from fruits treated in the field and showing the characteristic morphology of *Metschnikowia* spp., belonged to isolate LS15 and not to other related isolates with a similar morphology.

Isolate LS15 significantly reduced grey mold when applied 4 or 5 times in the vineyard. *Metschnikowia* spp. is known to be an effective antagonist of *B. cinerea* (Piano et al., 1997; Nigro et al., 1999). The best protection against postharvest rots was achieved by iprodione, but we must consider that its effectiveness probably was the result of a heavy treatment schedule. In fact, as planned for the biocontrol agent, the chemicals were sprayed in the field four times, including one just one week before harvest.

Data on the epiphytic microbial population of table grape berries indicate that isolate LS15 does not tolerate the environmental conditions typical of Israel, characterised by high temperature and low RH. These results may be explained considering that the antagonist was isolated in a different environment (Italy) and from a different culture (tomato). However, it is worth noting that isolate LS15 was able to survive in the field although at low population level, and to increase its population during cold storage. The ability of a biocontrol agent to be effective in different crop/pathogen/environment system and in a wide range of temperatures and storage conditions, are some of the most important characteristics required for a practical application of biocontrol agent against postharvest diseases of fruits and vegetables (Wilson and Wisniewski, 1994; Ippolito and Nigro, 2000).

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