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Research note

Biocontrol of postharvest brown rot of sweet cherries by *Saccharomyces cerevisiae* Disva 599, *Metschnikowia pulcherrima* Disva 267 and *Wickerhamomyces anomalus* Disva 2 strains

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ABSTRACT

In this work, *Metschnikowia pulcherrima* Disva 267, *Wickerhamomyces anomalus* Disva 2, and *Saccharomyces cerevisiae* Disva 599 yeast strains were evaluated for their biocontrol activity on postharvest decay brown rot, mainly caused by *Monilinia laxa* on sweet cherries, using three increasing concentrations (10^6, 10^7 and 10^8 CFU/mL). *M. pulcherrima* significantly reduced brown rot incidence, severity and McKinney index at all three concentrations, *W. anomalus* was effective at the concentration of 10^7 CFU/mL, and *S. cerevisiae* reduced brown rot only at 10^8 CFU/mL. *M. pulcherrima* and *W. anomalus* survived on the surface of sweet cherries during 2 weeks cold storage. When the three yeasts were sprayed on the canopy of sweet cherry trees at 10^7 CFU/mL, *M. pulcherrima* and *W. anomalus* showed good survival and colonization. In contrast, under the same conditions, *S. cerevisiae* strain did not survive. None of the yeasts produced phytotoxic substances, both on intact and on wound-inoculated fruit. Therefore, *M. pulcherrima* Disva 267 and *W. anomalus* Disva 2 could be promising biocontrol agents, able to survive in field and storage environments, providing a clear decrease in postharvest decay. However, further investigations with large scale trials are needed to lead to a possible formulation and commercial use.
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1. Introduction

In recent years, the increasing worldwide demand for agricultural products, together with the low tolerance to decay by the market, has required more effective postharvest control practices. Currently, the control of postharvest decay of fruit relies on the use of synthetic fungicides. However, the application of fungicides to fruit to reduce postharvest decay has been discouraged by normative restrictions and by the negative public perception regarding the safety of pesticides (Janisiewicz and Korsten, 2002). In this context, considerable efforts of researchers and farmers are being made toward production of fruit and vegetables with high quality traits together with minimal pesticide residues (Schirra et al., 2011). A list of alternative treatments reported to be effective in postharvest disease management has been given by Feliziani and Romanazzi (2013). Among these alternative treatments, the use of antagonistic microorganisms, applied on the fruit surface at pre- and/or postharvest stages, can be profitably used to control the development of postharvest decay. Biocontrol agents may act against postharvest decay fungi directly, by parasitizing the pathogens or secreting antibiotics, or indirectly by competing with the pathogen for nutrients and/or space, or inducing resistance in the host tissue (Sharma et al., 2009). The application of biocontrol agents was reported to be effective in reducing pathogen development, and increasing the fruit shelf-life (Fiori et al., 2008; Nunes, 2012; Cao et al., 2013). Thirty years ago, Wilson and Pusey (1985) showed the potential of biocontrol agents in the postharvest environment using a strain of *Bacillus subtilis* to control brown rot on peach, caused by *Monilinia fructicola*. After this pioneering work, over the years many other researchers have explored the possibility of using microbial strains for biological control of fruit and vegetables against plant pathogens. The pathogens that cause the most important postharvest diseases belong to the genera of *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Geotrichum*, *Gloeosporium*, *Mucor*, *Monilinia*, *Penicillium* or *Rhizopus* (Barkai-Golan, 2001), while antagonistic microorganisms used in pre- and postharvest control are usually yeasts or bacteria. Some examples of antagonistic microorganisms that are

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the base of registered and commercially available products and that are currently applied are: *Pseudomonas syringae*, *Cryptococcus albidus*, *Bacillus subtilis*, *Candida sake*, *Pantoea agglomerans*, *Aureobasidium pullulans*, *Candida oleophila*, *Bacillus amyloliquefaciens*, and *Metschnikowia fructicola* (Sharma et al., 2009; Nunes, 2012).

The main objectives of this work were to: (i) evaluate potential phytotoxic effects of *S. cerevisiae*, *W. anomalus* and *M. pulcherrima* on wounded sweet cherries; (ii) study the biocontrol activity of *S. cerevisiae*, *W. anomalus* and *M. pulcherrima*, applied at three concentrations, on postharvest decay of sweet cherries; (iii) evaluate their population dynamics on sweet cherry fruit.

2. Materials and methods

2.1. Yeast strains and media

Three yeast strains, *Metschnikowia pulcherrima* Disva 267, *Wickerhamomyces anomalus* Disva 2 and *Saccharomyces cerevisiae* Disva 599, provided by the Department of Life and Environmental Sciences, Marche Polytechnic University, were used for treatments on sweet cherry fruit. *M. pulcherrima* Disva 267 was isolated from a grape berry surface, *W. anomalus* Disva 2 was isolated from a baker matrix, and *S. cerevisiae* Disva 599 was characterized as a killer yeast (K1 strain) (American Type Culture Collection 60782). Yeast strains were grown in YPD medium (10 g/L bacto Yeast extract, 10 g/L bacto Peptone and 20 g/L D-glucose). All of the strains were subcultured at 3-month intervals on YPD agar medium and maintained at 4 °C.

2.2. Evaluation of phytotoxic effects on sweet cherry fruit

To evaluate the potential phytotoxic effects of *M. pulcherrima*, *W. anomalus* and *S. cerevisiae* strains on sweet cherries, wounded fruit were inoculated with cell suspensions (10 μL per fruit) at three concentrations (10^6, 10^7 and 10^8 CFU/mL) of each yeast strain. Yeast suspensions were prepared separately from cells grown on YPD for 24 h at 25 °C. Each yeast culture was centrifuged at 5000 × *g* for 10 min and re-suspended in sterile water; the concentrations were adjusted to the three different concentrations by using a Thoma-Zeiss chamber. Sweet cherry fruit cv Giorgia were randomly assigned to the different treatments, in three replicates of eight cherries each. The fruit were punctured with a sterile needle in the equatorial area to make a 3 mm deep and 3 mm wide wound on their surface (one wound per each fruit). On the wound, after 5 min, each suspension of 10 μL was deposited. Fruit treated with 10 μL of sterile distilled water were used as a control. After inoculation, the fruit were covered with plastic bags and incubated at 25 ± 1 °C, 95%–98% RH, for 25 d. The size of the wound was measured daily.

2.3. Postharvest treatments

Sweet cherry cv Sweet Heart were harvested from an organic orchard in Ancona, central-eastern Italy. The fruit were selected for uniformity in size and color, and absence of defects. The cherries were randomized and immersed for 15 min in 1 L of each yeast suspension of *M. pulcherrima*, *W. anomalus* and *S. cerevisiae*, at 10^6 CFU/mL, 10^7 CFU/mL and 10^8 CFU/mL. Distilled water was used as a control. Fruit were air-dried for 30 min, placed in small plastic boxes (10 cherries each) that were placed into large boxes. The boxes were stored for 14 d at 0 ± 1 °C, and then exposed to 6 d of shelf-life at 20 ± 1 °C, 95%–98% RH. Thirteen replicates of 10 cherries per treatment were used.

2.4. Evaluation of postharvest decay

After 14 d storage at 0 ± 1 °C, 95%-–98% RH, and following 6 d of shelf-life at 20 ± 1 °C, the levels of decay due to each of the pathogens were assessed separately according to the symptoms. In case of doubt, isolations from rotted tissues were carried out on potato dextrose-agar, and the mold was identified according to the micro-macro morphological characteristics (Feliziani et al., 2013). The main postharvest pathogen for sweet cherry is *Monilinia* spp., however other pathogens such as *Botrytis cinerea*, *Rhizopus stolonifer*, *Alternaria alternata*, *Penicillium expansum*, and *Cladosporium* spp. occasionally infect sweet cherry fruit after the harvest. The disease incidence was expressed as the percentage of infected fruit. Disease severity was assessed using the following scale according to the percentage of cherry surface covered by fungal mycelia: 0, uninfected cherry; 1, surface mycelia just visible to 25% of the cherry surface; 2, 26%–50% of the cherry surface covered with mycelia; 3, 51%–75% of the cherry surface covered with mycelia; 4, more than 75% of the cherry surface covered with mycelia (Romanazzi et al., 2001). The infection index (or McKinney index), which incorporates both the incidence and severity of the disease, was expressed as the weighted means of the disease as a percentage of the maximum possible level (McKinney, 1923). Specifically, it was calculated by the formula: $I = [\Sigma(d \times f)/(N \times D)] \times 100$, where *d* is the category of rot intensity scored on the sweet cherry and *f* its frequency, *N* the total number of sweet cherries examined (healthy and rotted) and *D* is the highest category of disease intensity occurring on the empirical scale (Romanazzi et al., 2001).

2.5. Dynamics of population after postharvest treatment

The population dynamics of the inoculated yeasts *M. pulcherrima*, *W. anomalus* and *S. cerevisiae*, on treated cherries were evaluated immediately after the treatments, and after 14 d of storage at 0 ± 1 °C, 95%–98% RH. Three replicates each with 10 cherries were processed. Each sample, suspended in 100 mL of sterile distilled water in 500 mL Erlenmeyer flasks, was placed on a rotary shaker set at 120 rpm for 30 min at 20 ± 1 °C (Romanazzi et al., 2002). Washing water aliquots were plated onto YPD agar and Rose-Bengal supplemented with chloramphenicol (Oxoid, UK) to count yeast and filamentous fungi colonies. Colonies were identified according to their morphological characteristics and recorded separately after 3–7 d of incubation at 25 ± 1 °C.

2.6. Dynamic of population after preharvest treatment

The trials were carried out in an experimental orchard located in a hilly area of the Ancona Province (43° N, 13° E; 203 m a.s.l.) in central-eastern Italy, in June 2013. The 'Sweet Heart' trees were selected for uniformity of production and ripening stage. Suspensions of *M. pulcherrima*, *W. anomalus* and *S. cerevisiae* at the concentrations of 10^7 CFU/mL, 3 d before the harvest were applied to the trees using a back pump sprayer (model WJR2525, Honda, Tokyo, Japan) that delivers the equivalent volume of 1000 L/ha. Trees treated with water were used as controls. The trial was duplicated in the field, applying treatments to two sets of plants at the same time. On the day of the harvest, the cherries were selected for uniformity in size and color, and absence of defects. The fruit were collected in plastic boxes that were then placed into large boxes. The cherries were stored for 14 d at 0 ± 1 °C, 95%–98% RH, and then exposed to 7 d shelf-life at 20 ± 1 °C. The populations of *M. pulcherrima*, *W. anomalus* and *S. cerevisiae* occurring on the treated cherries were evaluated: (i) immediately after the treatment; (ii) at harvest; (iii) at cold storage; (iv) after one week of shelf-life. For each treatment and sampling date, three replicates of 10 cherries

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