Food Microbiology 83 (2019) 1–8

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Role of biocontrol yeasts *Debaryomyces hansenii* and *Wickerhamomyces anomalus* in plants' defence mechanisms against *Monilinia fructicola* in apple fruits

Monika Czarnecka[a], Barbara Żarowska[a], Xymena Połomska[a], Cristina Restuccia[b,*], Gabriella Cirvilleri[b]

[a] Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences, ul. Chełmońskiego 37/41, 51-630 Wrocław, Poland
[b] Department of Agriculture, Food and Environment (Di3A), University of Catania, via Santa Sofia 100, 95123 Catania, Italy

ARTICLE INFO

Keywords:
Biocontrol yeasts
Brown rot
Apple
Host-defence
POD
CAT

ABSTRACT

The role of killer yeasts of the species *Debaryomyces hansenii* and *Wickerhamomyces anomalus* in biocontrol of *Monilinia fructicola,* and their involvement in plant defence mechanisms against brown rot in apple fruits, were investigated. *D. hansenii* KI2a and *W. anomalus* BS91 strains showed the highest *in vitro* biocontrol activity, inhibiting mycelium growth by 69.53% and 66.08% respectively, as compared to control fungal cultures. Brown rot on apple fruits was significantly reduced by BS91 and two strains of *D. hansenii* KI2a and AII4b by 92.46%, 85.10% and 70.02%, respectively, in comparison to infected fruits, which did not receive any pre-treatment. In enzymatic tests, the most significant changes in peroxidase (POD) and catalase (CAT) activities were observed in fruits inoculated either with BS91 followed by *M. fructicola* infection or with AII4b yeast strain alone, where POD activities were significantly higher by 67% and 54%, respectively, and CAT activities were significantly lower by 65% and 68%, respectively, than in untreated control fruits. These results confirmed the ability of killer yeasts to influence host-defence related enzyme activities in apple fruit tissue and may suggest that AII4b killer strain has a potential as biocontrol agent prior to infection by triggering immune response mechanisms in plant tissue, whereas BS91 strain is the most effective during pathogen infection and could be used as biocontrol agent in postharvest disease onset. Accordingly, the antagonistic strains of *W. anomalus* BS91 and *D. hansenii* KI2a and AII4b could be proposed as active ingredients for the development of biofungicide against *M. fructicola*.

1. Introduction

The use of killer yeasts as biological control agents (BCAs) in crop protection is considered a promising alternative to chemical fungicides, due to their effectiveness in controlling pre- and post-harvest fungal diseases and low environmental impact. As mechanisms involved in the biological control phenomenon, the following are mentioned: competition for space and nutrients, synthesis and secretion of antifungal substances, i.e. antibiotics, killer toxins, enzymes and volatile compounds, as well as the induction of plants' immune response to pathogens (Grzegorczyk et al., 2015; Parafati et al., 2015, 2017a; 2017b; Pretscher et al., 2018). However, the specificity and spectrum of biocontrol activity differ among killer yeast species (Liu et al., 2013b; Spadaro and Droby, 2016).

Debaryomyces hansenii and *Wickerhamomyces anomalus* are tolerant to extreme environmental stress conditions, such as low pH and high osmotic pressure. Moreover, *D. hansenii* yeasts are able to grow at low temperatures below 14 °C and they also exhibit killer phenotype in the range 10–20 °C (Van den Tempel and Jackobsen, 2000; Aggarwal and Mondal, 2009 Żarowska, 2012; Banjara et al., 2016; Prista et al., 2016). In turn, optimum temperature conditions for *W. anomalus* growth and the activity of its killer toxin vary among strains and is in the range of 16–30 °C (Passoth et al., 2006; Guo et al., 2013; Muccilli et al., 2013). Killer strains of *D. hansenii* and *W. anomalus* species exhibit antagonistic activity against other yeasts and filamentous fungi (Parafati et al., 2016; Żarowska, 2012). Several studies have demonstrated the efficacy of *D. hansenii* and *W. anomalus* as biological control agents against various postharvest phytopathogenic fungi, such as: *Colletotrichum gloeosporioides,* causing anthracnose on papaya fruits (Hernández-Montiel et al., 2018; Lima et al., 2013), *Penicillium italicum* – pathogen of citrus fruits (Hernández-Montiel et al., 2010; Parafati et al., 2016), *P. digitatum*, causing postharvest decay of grapefruits (Droby et al., 1989; Platania

* Corresponding author.
E-mail address: crestu@unict.it (C. Restuccia).

https://doi.org/10.1016/j.fm.2019.04.004
Received 20 November 2018; Received in revised form 19 February 2019; Accepted 6 April 2019
Available online 08 April 2019
0740-0020/

et al., 2012) and *Botrytis cinerea* – pathogen of various plant species (Parafati et al., 2015; Santos et al., 2004). In addition, *D. hansenii* was proven to effectively inhibit the growth of pathogenic *Candida* species (Banjara et al., 2016). Furthermore, *D. hansenii* and *W. anomalus* killer strains have been reported to exhibit antagonistic activity against *Monilinia fructigena* and *M. fructicola* (Grzegorczyk et al., 2017), from which the latest one is considered the most destructive pathogen of pome and stone fruits (EFSA, 2011).

M. fructicola may infect fruits and remain latent in unfavourable environmental conditions. The infection occurs during ripening phase and the symptoms are visible on blossoms, buds, branches, twigs and fruits, where *M. fructicola* spreads, germinates and eventually leads to the formation of so-called mummy fruits (Di Francesco and Mari, 2018). Current management of brown rot relies on the use of chemical treatments, such as benzimidazoles, sterol biosynthesis inhibitors, succinate dehydrogenase inhibitors, dicarboximides and quinone outer binding site inhibitors (Miessner and Stammler, 2010). However, due to the current trends related to healthy food, produced without the use of chemical agents, alternative methods are sought, ensuring a sustainable agricultural and food economy with the use of measures with less impact on the environment and human health (Spadaro and Droby, 2016; Sisquella et al., 2014). Recently, the effectiveness of plant-derived isoquinoline alkaloid – berberine was reported to effectively inhibit brown rot on peach (Fu et al., 2017). Also, yeast species of *Pichia membranaefaciens*, *Kloeckera apiculata* (Zhang et al., 2017) and *Aureobasidium pullulans* (Mari et al., 2012) demonstrated their effectiveness in the control of *M. fructicola* on stone fruit. In Europe, *M. fructicola* is considered a quarantine pathogen by the European and Mediterranean Plant Protection Organization (EPPO) that mainly affects nectarines, peaches and plums (De Cal et al., 2009; Martini and Mari, 2014); however, recently, there were reports on its infection on apple fruit (Pereira and Larissa May De Mio, 2018; Beckerman et al., 2016, Peter et al., 2015; Grabke et al., 2011), which indicates the spread of this pathogen to other plant species, and necessitates to widen research on effective brown rot control also to apple fruits.

The evaluation of BCAs effectiveness, carried out in plant-pathogen-antagonist models, is often based on the examination of disease reduction and symptoms inhibition, in the presence of beneficial microorganisms, during various storage conditions. However, little is known about the phenomena occurring in plants in early stages of the phytopathogen's attack, in the presence of killer yeasts. Early defence mechanisms in plants, triggered by biotic or abiotic stress factors, are associated with the production of reactive oxygen species (ROS) that can be formed in the cell wall, cytoplasmic membrane and in individual cell organelles (Wojtaszek, 1997). In high concentrations, ROS act non-specifically and negatively affect the functions of all encountered cells, damaging proteins, lipids and nucleic acids which consequently leads to cells death (Jones and Dangl, 2006). However, plant cells use detoxifying enzymes, such as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD), as well as their natural antioxidants for the protection from extensive spread of oxidative damage in their own cells. Elevated ROS levels, caused by changes in CAT, POD and SOD activities, promote hypersensitive response (HR) in plants, which provides resistance to biotrophic pathogens, that obtain energy from living cells; however, on the other hand, facilitate invasion of necrotrophs, which can grow over the dead cells (Thakur and Sohal, 2013). One of the factors that causes oxidative burst in plants is the attack of pathogenic filamentous fungi, where fungal elicitors trigger HR response in plants.

Further development of immune response in plants is called systematic acquired resistance (SAR) and is often associated with phytoalexins biosynthesis, reinforcement of cell walls, accumulation of pathogenesis-related proteins, possessing antimicrobial properties and synthesis of other defence enzymes (Ryals et al., 1996; Van Loon and Van Strien, 1999; Van Breusegem et al., 2001). Therefore, pathogens may also reduce the chance of reactive oxygen signalling molecules to reach more distant parts of the plant by scavenging them, and thus delay or inhibit SAR development. Nevertheless, the role of ROS and the activity of ROS generating and scavenging enzymes in fungal virulence differ among different plant-pathogen models and even conflicting findings have been reported in this matter.

In several studies it was proven that antagonistic yeasts, such as *Candida oleophila* (Droby et al., 2002; Chan et al., 2007), *Pichia membranefaciens* (Chan et al., 2007), *Rhodosporidium paludigenum* and *Metschnikowia fructicola* (Lu et al., 2013) may play an important role in the induction of the plants' immune response. However, the mechanisms involved in inducing immunity in plants attacked by pathogens in the presence of beneficial yeasts are still unclear and have not been clearly defined so far.

In present work, we conducted study on the efficacy of killer yeasts of the species *D. hansenii* and *W. anomalus* in the control of the pathogen *M. fructicola* on apples. The encouraging results of our previous studies on the efficacy of *D. hansenii* and *W. anomalus* killer strains in controlling fruit decay caused by *Monilinia* sp. on peach and plum fruits (Grzegorczyk et al., 2017) prompted us to conduct further research aimed mainly at the evaluation of the role of killer yeasts in plants' defence mechanisms against *M. fructicola* in apple fruits. Therefore, we examined enzymatic activity of peroxidase and catalase, as well as the total antioxidant capacity in the host-pathogen-antagonist experimental models, where models lacking one or two of these elements served as control. This approach mimics the phenomena, taking place in non-laboratory conditions, and also allows to gain insight in the overall effect of the interaction between plant, fungal pathogen and beneficial microorganism with the use of uncomplicated techniques and tools. An additional reason for the use of selected yeast strains in this study was that both yeast species have been granted Qualified Presumption of Safety status by European Food Safety Authority, which may support their use as a potential biocontrol agents (BIOHAZ, 2012).

2. Materials and methods

2.1. Plant material

Apple fruits *Malus domestica* 'Ligol' cultivar used for *in vivo* assays were obtained from commercial orchards in Poland and have not received any pre-harvest fungicide treatment. Healthy and homogenous fruits were selected and randomly assigned to different treatments. Prior to any treatment, fruits were washed with tap water, surface-disinfected by dipping for 3 min in 70% ethanol solution, rinsed with sterile distilled water and then air-dried under the laminar chamber.

2.2. Killer yeasts and fungal pathogen

D. hansenii AII4b, MI1a and KI2a killer strains were isolated from blue-veined *Rokpol* cheese (Wojtatowicz et al., 2001), whereas *W. anomalus* BS91 yeast strain was isolated from naturally fermented olive brine (Muccilli et al., 2011). Yeasts were cultured on yeast peptone dextrose agar plates (YPDA) at 25 °C for 48 h, then single colony biomass was subjected to *in vitro* biocontrol assay or inoculated to 50 mL of yeast peptone dextrose broth (YPDB) adjusted to pH 4.5 with McIlvaine buffer and incubated either: at 14 °C (*D. hansenii* strains) or 25 °C (*W. anomalus*) for 24 h on a rotary shaker at 160 rpm. Yeast cells from liquid culture were pelleted at 8000 g for 10 min at room temperature (RT). Subsequently, supernatant was discarded and yeast pellet was re-suspended in McIlvaine buffer of the pH 4.5 and centrifuged again at 8000 g for 10 min at RT. This procedure was repeated twice before re-suspending the resulting yeast cell pellet in McIlvaine buffer (pH 4.5) to the final concentration of 1×10^8 yeast cells/mL, used as inoculum in *in vivo* assays. Fungal pathogen *M. fructicola* was recovered from decayed peach fruit and cultivated on potato dextrose agar plates (PDA) of pH 6.0 for 7 days, prior to use.

2.3. In vitro biocontrol assay

In order to evaluate the antagonistic activity of *D. hansenii* AII4b, MI1a, KI2a and *W. anomalus* BS91 against *M. fructicola*, a loopful of each yeast strain was streaked orthogonally near the edge of Petri dish, containing YPD medium of a pH 4.5. At the same time, mycelial discs (5-mm square plugs) of actively growing mycelium of *M. fructicola* were individually placed 3 cm away from yeast inoculum. Control plates, inoculated with pathogen only, were also prepared. Dual cultures were incubated for 7 days at 23 °C. At the end of the incubation period, radial growth reduction was calculated in relation to growth of the control as follows: %I = (C-T/C) x 100, where %I represented the inhibition of radial mycelial growth, C was radial growth measurement in control and T was the radial growth of the pathogen in the presence of yeast strains. Three replicates for each yeast strain were used and the experiments were repeated twice.

2.4. In vivo biocontrol assays

2.4.1. Treatment

In order to assess the efficacy of killer yeasts as biocontrol agents, the method described by Grzegorczyk et al. (2017) and Parafati et al. (2015), with modifications, was used. Artificial wounds were performed using a sterile needle to make 2-mm deep and 5-mm wide wounds along the equatorial areas (two wounds per each fruit). Each wound was inoculated with 20 µL of 1×10^8 yeast cells/mL. Fruits were then placed onto perforated aluminium packaging and into plastic boxes lined with wet paper towels and incubated at 23 °C and 95% RH for 12 h prior to pathogen inoculation, in order to allow wound site colonization by yeasts. Yeast-treated wounds were then inoculated with mycelial disks (5-mm square plugs) of actively growing mycelium of *M. fructicola* and fruits were further incubated for 72 h in boxes placed into plastic bags and sealed tightly to prevent air leakage and to provide favourable conditions for the postharvest onset of the disease. Unwounded fruits and wounded fruits inoculated with fungal mycelium only, or applied with 20 µL of McIlvaine buffer of pH 4.5 served as control. Nine fruits were used for each treatment and the experiment was repeated twice.

2.4.2. Antagonistic activity of killer strains against M. fructicola

Data concerning disease severity (DS) was evaluated using an empirical 1-to-5 scale: 1 = no visible symptoms (0%); 2 = soft rot (25%); 3 = early stage of disease (limited mycelium) (50%); 4 = middle stage of disease (mycelium/sporulation) (75%); 5 = advanced stage of disease (abundant mycelium/sporulation) (100%). Average disease severity was calculated by the following formula:

$$DS = \frac{\sum (Ci)}{N} \times 100$$

where DS is the average severity index, C the number of fruits in each class, i (1-to-5) the numerical values of classes, N the total number of fruits examined. Lesion diameter (LD) was also assessed by measuring the average diameter of the damaged area. Ratios between individual treatment and control (i.e. fruits inoculated with pathogen only) for DS and LD parameters were calculated, the data was presented in percentage and expressed as disease severity reduction (DSR) and disease development inhibition (DDI), respectively.

2.4.3. Assay of defence-related mechanisms

2.4.3.1. Enzyme extraction. One cm deep apple fruit plugs were cut out with cork borer around wounds of each treatment, and randomly along the equatorial area in unwounded control fruits. Each sample was weighted and manually crushed on ice in a pre-chilled mortar with the addition of acid-treated sand and 3 mL of cold McIlvaine buffer containing 1 mM EDTA. The homogenates of individual samples were centrifuged for 5 min at 8000 g at 4 °C. The supernatant was collected and subjected to the enzymatic tests.

2.4.3.2. Determination of total antioxidant capacity. Total antioxidant capacity was determined using method based on the reduction of $Fe3^+$ ions to $Fe2^+$ ions by the antioxidants in tested material, the visual effect of which is the formation of a coloured complex in the solution of 2,4,6-tripyridyl-S-triazine (TPTZ) with $Fe2^+$ ions, according to Benzie and Strain (1996). The increase in absorbance of the TPTZ- $Fe2^+$ complex was proportional to the amount of antioxidants in the tested material. The total antioxidant capacity in tested samples was reported as nmol trolox/g fresh plant tissue.

2.4.3.3. CAT activity assay. Catalase activity was determined based on the kinetic measurement of the decrease in the hydrogen peroxide absorbance in tested sample at 240 nm at 25 °C, according to Andersson et al. (1995). The reaction mixture consisted of 1.9 mL of 50 mM potassium phosphate buffer, 1 mL of 54 mM H_2O_2 and 100 µL of the tested sample. The reaction was detected at 240 nm every minute for 5 min. One unit of CAT activity was defined as the amount of enzyme that produced a decrease of A_{240} by 0.036 per minute at 25 °C. CAT activity in tested samples was then converted to U/g fresh plant tissue.

2.4.3.4. POD activity assay. Peroxidase activity in tested samples was determined using Peroxidase Activity Assay Kit (Sigma-Aldrich, cat. no MAK092). The POD activity test consisted in the colorimetric determination of the substrate oxidation product (quinoline derivative) by POD contained in 50 µL of tested sample in the presence of hydrogen peroxide, the intensity of which was directly proportional to the peroxidase activity. One unit of peroxidase was defined as the amount of enzyme that reduces 1.0 µmol of H_2O_2 per minute at 37 °C. Colorimetric product of peroxidase activity in the samples was measured using Epoch Microplate Spectrophotometer (Think Possible BioTek) at wavelength 570 nm. Peroxidase activity in tested samples was calculated and converted to U/g fresh plant tissue.

2.5. Statistical analysis

Data from *in vitro* and *in vivo* experiments were analysed using Statistica package software (Statistica 13, StatSoft Poland). One-way analysis of variance according to Fisher's least significant differences test ($p = 0.05$) was used to analyse: data from *in vitro* and *in vivo* experiments, concerning antagonistic biocontrol assays, and the determination of enzymes activity and total antioxidant capacity.

3. Results

3.1. Antagonistic activity and inhibitory effect of killer yeasts towards M. fructicola

As shown in Table 1, *W. anomalus* BS91 and *D. hansenii* KI2a were the most efficient in the inhibition of *M. fructicola* mycelium growth on plate, by over 69% and 66%, respectively, comparing to the control. Also AII4b was effective in reducing mycelium growth by over 56% (Table 1). Visual symptoms of the disease were the least abundant in fruits pre-treated with BS91 strain, which was expressed by the highest disease severity reduction of over 92%. However, KI2a most effectively inhibited disease spread on apple fruits by over 70%, comparing to control fruits (Table 2). Rot caused by *M. fructicola* was significantly reduced by KI2a, BS91 and AII4b yeast strains, where lesion diameter was reduced, respectively, by 69%, 61% and 52%, less extensive than in control fruits inoculated with pathogen only (Fig. 1 and Fig. 2).

3.2. Involvement of killer yeasts in defence-related mechanisms of apple fruit in the presence of phytopathogen

Changes in defence-related enzyme activities in the host-pathogen-

Table 1
In vitro antagonistic activity of *Wirckerhamomyces anomalus* BS91, *Debaryomyces hansenii* AII4b, MI1a and KI2a yeast strains referred to mycelial growth inhibition (%) of *Monilinia fructicola* in dual culture assay on PDA medium at the pH 4.5.

	Monilinia fructicola	
	Mycelium diameter (mm)	**Mycelium growth inhibition (%)**
Killer yeast strain		
BS91	17.67 ± 2.51 a	**69.53**
AII4b	25.00 ± 3.00 b	**56.89**
MI1a	45.00 ± 3.00 c	**22.41**
KI2a	19.67 ± 4.16 ab	**66.08**
No treatment	58.00 ± 2.00 c	–

Data presented as mean ± standard error of the mean. Data followed by different letter(s) differs significantly according to Fisher's least significant difference test (p = 0.05).

Table 2
In vivo antagonistic effect of *Wirckerhamomyces anomalus* BS91, *Debaryomyces hansenii* AII4b, MI1a and KI2a yeast strains referred to disease severity reduction DSR (%) and disease development inhibition DDI (%) on apple fruits 72 h after pathogen inoculation.

Killer yeast strain	*Monilinia fructicola* on apple fruit	
	Disease severity reduction DSR [%]	Disease development inhibition DDI [%]
BS91	92.46 a	60.53 a
AII4b	70.02 a	52.43 b
MI1a	10.23 b	19.90 c
KI2a	85.10 a	69.11 a

Ratios between individual samples and control samples for DS (disease severity) and LD (lesion diameter) parameters were calculated and the data were presented as disease severity reduction (DSR) and disease development inhibition (DDI) in percentage. Data representing the same disease parameter followed by different letter are significantly different according to Fisher's least significant difference test (p = 0.05).

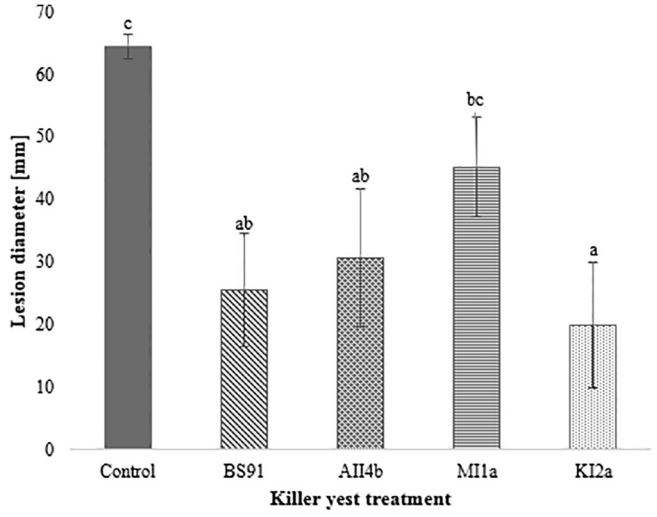

Fig. 1. Lesion caused by *Monilinia fructicola* rot on apple fruit in presence of antagonistic killer yeast strains *Wirckerhamomyces anomalus* BS91 and *Debaryomyces hansenii* AII4b, MI1a and KI2a (mm). Data presented as mean ± standard error of the mean. Data followed by different letter(s) differs significantly according to Fisher's least significant difference test (p = 0.05).

antagonist experimental model were studied on BS91 and AII4b killer strains. As shown in Fig. 3, the most significant peroxidase activity increase was observed in fruits pre-treated with BS91 and subsequently infected with fungal pathogen, where POD activity was three-fold higher than that in unwounded control fruits. The wounding of the plant surface resulted in a significant increase in the activity of peroxidase in relation to the unwounded control fruits. However, the inoculation of fruit tissue with antagonistic or pathogenic microorganism resulted in further increase in the activity of this enzyme, especially for AII4b, where POD activity was by 54% higher than that in unwounded control fruits (Fig. 3). CAT activity decreased in response to wounding alone, as well as after the inoculation of wounded tissue with *M. fructicola*, where the activities of this enzyme were respectively by 27% and 30% lower, as compared to unwounded control fruits (Fig. 4). However, the lowest level of CAT activity were observed in fruits treated with AII4b alone, followed by fruits inoculated with BS91 and further infected with *M. fructicola*, values of which were, respectively, 68% and 65% lower than that in unwounded control fruits (Fig. 4). Total antioxidant capacity in fresh plant tissue was of the highest level in unwounded fruits. Inflicting an injury on fruit surface caused a decrease in this parameter by 20%, comparing to unwounded control, as shown in Fig. 5. In fruits pre-treated with BS91 and AII4b, and further inoculated with *M. fructicola*, a significant decrease in total antioxidant capacity was recorded, which was, respectively, up to 53% and 47% lower, as compared to unwounded control fruits (Fig. 5).

4. Discussion

Injury or disruption of plant tissue may be classified as physical elicitor stimulating plant immune response (Thakur and Sohal, 2013). Following the perception and recognition of elicitor by the plant, various host defence pathways may be activated; however wounding alone may trigger mainly unspecific immune response, based on generation of ROS by NADPH oxidases and cell wall peroxidases (Bolwell and Wojtaszek, 1997). In our experiment, inflicting an injury on the fruit surface affected catalase and peroxidase in apples tissue. Compared with the control fruits, of intact surface, CAT activity significantly decreased and POD activity significantly increased in wounded fruit tissue. Wounding alone also caused the significant decrease in total antioxidant capacity. CAT is responsible for detoxifying hydrogen peroxide, and its lower activity at the site of the injury might have enhanced oxidative burst development. In turn, lowering of total antioxidant capacity might have been due to the activity of peroxidase, which decompose hydrogen peroxide simultaneously oxidising various organic and inorganic compounds associated with the process of lignification and ethylene biosynthesis in plants (Passardi et al., 2004). Moreover, cell wall peroxidase might have contributed to the increase of total ROS reservoir, destroying host cells at the site of wounding. Also, Ippolito et al. (2000) reported elevated levels of POD activity in wounded apple fruit tissue, comparing to unwounded control fruit over time of incubation. In turn, Xu et al. (2008) observed a decrease of CAT activity during storage of wounded peach fruits. In the present study, AII4b killer strain dramatically affected catalase, which activity in fruit tissue was over two-fold lower than in control fruits (unwounded and wounded), as well as in wounds infected with pathogen only, whereas CAT activity in apple tissue treated with BS91 was significantly higher than in AII4b treated wounds but, at the same time, significantly lower than in fruits infected with *M. fructicola*. Similarly, in cherry tomato treated with *Pichia membranifaciens*, decrease of CAT and superoxide dismutase (SOD) activities were observed, along with the increase in POD activity (Chan and Tian, 2006). However, Hershkovitz et al. (2012) reported down-regulation of POD, SOD and CAT in grapefruit tissue in response to the yeast biocontrol agent *Metschnikowia fructicola*, as well as significantly higher levels of ROS, comparing to untreated fruits.

Fungal elicitors, i.e. mycotoxins and secondary metabolites acting

Fig. 2. Development of brown rot on apple fruits treated with tested killer yeast strains and subsequently infected with *M. fructicola*. The most severe disease development for each treatment is shown; control fruits were applied with McIlvaine buffer (Control) or inoculated with phytopathogen (*M. fructicola*).

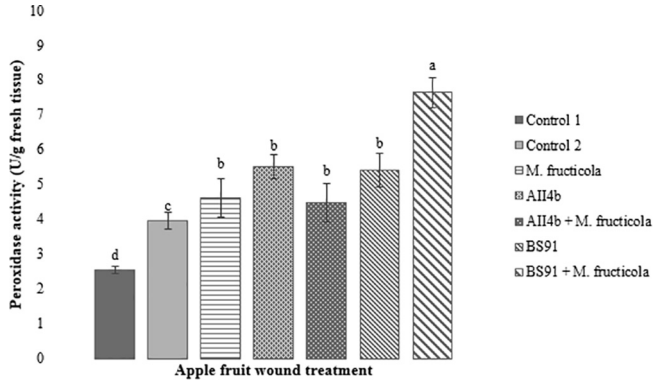

Fig. 3. Peroxidase activity in apple fruit tissue treated with killer yeasts and/or inoculated with *M. fructicola*. Controls: intact fruit surface (1), wounded fruit applied with McIlvain buffer (2). Data presented as mean ± standard error of the mean. Data followed by different letter differs significantly according to Fisher's least significant difference test (p = 0.05).

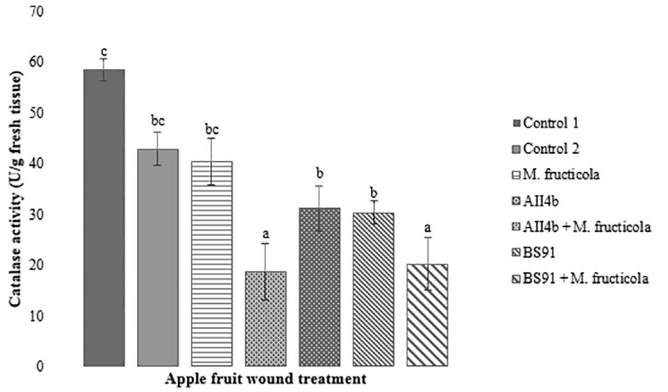

Fig. 4. Catalase activity in apple fruit tissue treated with killer yeasts and/or inoculated with *M. fructicola*. Controls: intact fruit surface (1) and wounded fruit applied with McIlvain buffer (2). Data presented as mean ± standard error of the mean. Data followed by different letter(s) differs significantly according to Fisher's least significant difference test (p = 0.05).

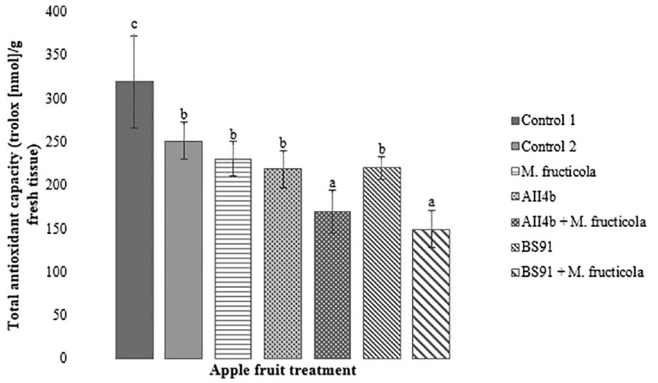

Fig. 5. Total antioxidant capacity in apple fruit tissue treated with killer yeasts and/or inoculated with *M. fructicola*. Controls: intact fruit surface (1) and wounded fruit tissue applied with McIlvain buffer (2). Data presented as mean ± standard error of the mean. Data followed by different letter(s) differs significantly according to Fisher's least significant difference test (p = 0.05).

on damaged plant tissue, may induce its oxidative burst, which subsequently leads to the enhancement of the plant's immune response against pathogen attack (Wojtaszek, 1997). For instance, *Fusarium oxysporum* toxin activates peroxidases, oxalate oxidases and amine oxidases, enzymes involved in the development of oxidative burst, in *Arabidopsis thaliana* cell walls (Bindschedler et al., 2006; Davies et al., 2006), whereas *A. thaliana* cells treated with spores or fungal elicitor of *Botrytis cinerea*, accumulate hydrogen peroxide and superoxide anion radical (Govrin et al., 2006). However, the generation of ROS by the plant is not always sufficient in control the pathogen, for example *Sclerotinia sclerotiorum* secretes oxalic acid, which inhibits oxidative burst in plant tissue (Hegedus and Rimmer, 2005; Walz et al., 2008). Similar effect was observed in citrus fruits infected by *P. digitatum*, where the pathogen inhibited the secretion of hydrogen peroxide by plant cells (Macarisin et al., 2007). Although these fungal pathogens are all necrotrophic, and therefore it could be expected they would benefit from oxidative burst, yet in certain cases they delimit it by scavenging reactive oxygen species. This may be due to the fact that, ROS play dual role in plant-pathogen interaction, for they also act as mobile signalling molecules, inducing number of molecular, biochemical and physiological responses in infected plant cells (Neill et al., 2002; Torres, 2006). In our studies, *M. fructicola*, inoculated into untreated wounds, affected POD activity, which was higher as compared to control fruits, whereas CAT level has not changed significantly. This may indicate that *M. fructicola*, as necrotrophic fungus, enhanced HR-response by increasing cell wall peroxidase activity in order to overproduce ROS. This is in accordance with the studies conducted by Liu et al. (2013a), where the application of *M. fructicola* spores onto the surface of intact peach flower petals caused the increase of the activity of NADPH oxidase and cell wall peroxidase, resulting in enhanced production of hydrogen peroxide in host cells. In turn, application of exogenous antioxidants significantly reduced brown rot disease comparing to control, where no ROS scavenging compounds were applied. Also, Lee et al. (2010) reported that exposure of *M. fructicola* to hydrogen peroxide increased the expression of cutinase transcripts, enzyme involved in pectin decomposition, which suggested the role of ROS in pathogen virulence. Additionally, Lee and Bostoc (2006) reported that the presence of antioxidants in conidial suspension of *M. fructicola* inhibited appressorium formation from germinated conidia and subsequently reduced brown rot lesion development, which also support the hypothesis that *M. fructicola* benefits from HR response in plant tissue. However, Xu et al. (2008) claimed that *M. fructicola* stimulated the activity and expression levels of POD and CAT in peach fruit, which may support opposite hypothesis of negative role of ROS in *M. fructicola* infections, since both of these enzymes are involved in scavenging ROS.

According to Navarrete et al. (2009) *D. hansenii* has low basal levels of enzymes involved in metabolism of ROS and, therefore, does not tolerate elevated levels of ROS in its environment. Very low activity of CAT in wounds treated with AII4b might have contributed to the spread of oxidative burst and caused death of host cells and yeast cells at the wound site. This can be indirectly supported by our previous studies, were the wound site colonization of *D. hansenii* was weaker than that of *W. anomalus* BS91 in peach fruits (Grzegorczyk et al., 2017). In present study, POD activity was enhanced, after 3 days of incubation, in fruits

inoculated either with yeast or with fungal pathogen; however the most significant changes occurred in fruits treated with BS91 and further infected with *M. fructicola*, where POD activity increased significantly as compared to other experimental models and, at the same time, CAT activity decreased. Treatment with AII4b and subsequent infection with *M. fructicola* caused significant increase in CAT activity and decrease in POD activity. What is also worth noting, total antioxidant capacity in fruit tissue decreased significantly in presence of both microorganisms, antagonist and pathogen, as compared to the controls and to samples where only one microorganism was applied into the wound. These findings may indicate that BS91 was able to modulate oxidative burst while colonizing fruit tissue, in its own favour, however after encountering opportunistic fungal pathogen, it enhanced HR response of the plant contributing to unspecific oxidative damage of cells at the site of infection. In this model, the antioxidant capacity was of the lowest level detected, which also may indicate strong oxidation of various biomolecules, including reduced precursors of the compounds involved in cell wall enhancement and of antimicrobial activity (Passardi et al., 2004). Similar conclusions were made by Castoria et al. (2003), where they assessed the relationship between wound site colonization, resistance to oxidative stress and antagonism of biocontrol yeasts *Cryptococcus laurentii* LS-28 (of higher antagonistic activity) and *Rhodotorula glutinis* LS-11 (of lower antagonistic activity) against *B. cinerea* and *P. expansum* in apple fruit tissue. According to their studies, LS-28 was more resistant to ROS-generated oxidative stress *in vitro* than LS-11, which positively correlated with more abundant wound site colonization. Furthermore, the combined application of both biocontrol yeasts and ROS deactivating enzymes in apple wounds prevented the decrease in number of LS-11 cells and enhanced the wound site colonization and antagonistic activity against tested pathogens of both yeast strains. In our previous studies, *D. hansenii* killer strains KI2a and MI2a exhibited better performance *in vitro* in dual culture assays with fungal pathogens of *Monilinia* sp. than in *in vivo* experiments, where they were inoculated into fruit tissue of peach and plum fruit and further infected with pathogenic fungus (Grzegorczyk et al., 2017). This might have been due to the fact that *D. hansenii* strains were susceptible to ROS generated in early immune response of host cells, which in turn reduced the number of viable cells and thus hindered their antagonistic potential evidenced in *in vitro* trials. From these reports and present study, it may be concluded that the ability to tolerate high levels of ROS produced by fruit tissue in response to wounding is an essential characteristic of effective yeast antagonist. However, Lu et al. (2014) managed to enhance SOD and CAT activities in *Rhodosporidium paludigenum* and therefore improved its antagonistic activity against *Penicillium expansum* in apple fruit, by pre-exposing yeast cells to chitin, added to cultivation medium. Based on their study authors suggested that reactive oxygen metabolism of *R. paludigenum* might be related to its biocontrol ability.

Dual cultures of antagonistic yeasts and pathogenic fungus on YPDA plates of pH 4.5 were carried out at 23 °C for 7 days. Our previous studies showed that fungal growth of *M. fructicola* was inhibited by BS91 and KI2a with higher efficacy in medium of a pH 4.5 than pH 6.0 (Grzegorczyk et al., 2017). The observation that acidic conditions emphasized the biocontrol efficacy of *W. anomalus* and *D. hansenii* antagonistic strains towards various pathogenic fungi were reported by many researchers (Platania et al., 2012; Parafati et al., 2015, 2016; Żarowska, 2012; Muccilli et al., 2013). Also, the pH of fruits infected by this pathogen is acidic. The use of YPDA medium was dictated by the fact that in these conditions measurable results of direct antagonistic activity of tested yeasts were obtained already after 7 days of culturing, instead of 11 days, as in our previous studies (Grzegorczyk et al., 2017). Prolonged cultivation of both microorganisms, from which yeasts exhibit faster growth rate than pathogenic fungi, may lead to depletion of nutrients and space in medium; this behaviour has a significant impact on overall antifungal performance of yeast antagonists and may mask the parasitism effect, which in our view is of significant importance for its selectivity and specificity. For instance, in our previous studies MI1a strain inhibited *M. fructicola* and *M. fructigena* in *in vitro* assays on PDA medium of pH 6.0, after 11 days of culturing, with comparable efficacy as BS91 and KI2a yeast strains, whereas in *in vivo* trials only the latest two strains were effective in controlling these pathogens. In present study, the mycelium growth inhibition in presence of antagonistic yeasts varied among tested killer strains. BS91 and KI2a were able to inhibit growth of *M. fructicola* by over 65% and AII4b by over 55%, whereas MI1a was in turn not effective, which positively corresponded to *in vivo* trial results. In Zhang et al. (2017) studies, yeast strains belonging to the species *Kloeckera apiculata* and *Pichia membranaefaciens* inhibited the growth of *M. fructicola* by over 85% in PDA dual cultures after 10 days of incubation and were also effective in control of this pathogen in *in vivo* assay on plums.

In present study, fruits were inoculated either with *D. hansenii* killer strains KI2a, AII4b and MI1a or *W. anomalus* BS91 and 12 h later inoculated with *M. fructicola*. The measurements of disease development and severity were taken after 3 days of incubation. Biocontrol assays are often performed in advantageous condition for the prior growth and development of antagonistic yeast before the onset of the fungal disease. For instance, *W. anomalus*, inoculated into oranges 3 days before infection with *P. digitatum* was able to completely reduce the occurrence of disease 5 days after infection and up to 88% after 10 days (Platania et al., 2012). However, when *W. anomalus* was inoculated into grapes simultaneously with *B. cinerea*, it was able to reduce disease progression up to 50% after 5 days of incubation (Parafati et al., 2015), and when inoculated 12 and 24 h after *Colletotrichum gloeosporioides* infection in papaya, *W. anomalus* was able to limit the disease only up to 10% and 4%, respectively (Lima et al., 2013). However, according to our previous studies, the application of *D. hansenii* KI2a and *W. anomalus* BS91 killer strains 24 h prior to *Monilinia* spp. infection, resulted in higher efficacy in biocontrol of this pathogen, than when yeast inoculation occurred 48 h before pathogen infection (Grzegorczyk et al., 2017). Data obtained from the present study showed that all of the tested strains exerted various levels of antifungal activity towards *M. fructicola*. Although it is likely that the overall performance of *D. hansenii* killer strains was hindered by its susceptibility to ROS, in plant-antagonist model before applying fungal mycelium, yeasts that survived oxidative burst and managed to colonized fruit tissue were still effective in controlling *M. fructicola*, which was especially visible for KI2a and AII4b strains. BS91, which was probably more resistant to oxidative burst, could have colonized fruit tissue to higher extent than *D. hansenii* strains, and exerted strong antagonistic effect against *M. fructicola*. Although the highest disease development reduction, of over 69%, was observed for KI2a, the most efficient in disease symptom reduction was BS91 killer strain (by over 92%). AII4b showed a lower efficacy in control of *M. fructicola*, when compared to BS91 and KI2a, however it was able to reduce disease severity by 70% and mycosis spread by over 52%. MI1a, in turn, proved to be ineffective in controlling fungal infection in apple wounds. In their research, Mari et al. (2012) applied *A. pullulans* as biocontrol agent for *M. fructicola* in the storage of nectarines and observed complete inhibition of the disease; however, fruits were stored for 21 days at 0 °C and subsequently for 7 days at 20 °C. In turn, in Zhang et al. (2017) studies, two antagonistic yeast strains of the species *P. membranaefaciens* and *K. apiculata* showed significant reduction of rot incidence, caused by *M. fructicola* on plums, by 76% and 65.8% after 6 days of incubation at 20 °C. Killer toxin activity and direct parasitism were observed in *D. hansenii* at lower temperatures of 11–23 °C, whereas in *W. anomalus* at temperatures above 22 °C (Chalutz and Wilson, 1990; Żarowska et al., 2004; Muccilli et al., 2013). The temperature applied for *in vivo* test in this study was 23 °C, which could have made more favourable conditions for killer activity of *W. anomalus* than *D. hansenii*. In addition, there are several other differences between these two killer yeast species. For instance, in *W. anomalus*, killer phenomenon may be correlated with the activity of β-1,3-D-glucanase (Muccilli et al., 2013; Parafati et al., 2016, 2017a), whereas in *D. hansenii* may be correlated with the activity of β-1,6-D-glucanase

(Żarowska et al., 2004; Santos et al., 2002). Moreover, *W. anomalus* is able to form biofilm and produce VOCs, exhibiting strong antifungal activity (Parafati et al., 2015, 2016, 2017b). *D. hansenii* in turn was proven to inhibit ochratoxigenic moulds in dry-cured meat (Andrade et al., 2014) and several moulds in dairy products (Liu and Tsao, 2009), however it may be sensitive to ROS (Prista et al., 2016; Navarrete et al., 2009). Low level of detoxification enzymes in *D. hansenii* can promote oxidative burst and cell death at wound site before pathogen infection, whereas *W. anomalus* exhibits such mechanisms after encountering the pathogen.

Based on the conducted studies, it can be concluded that tested killer yeasts are able to modulate the activities of CAT and POD in apple fruit tissue; however the nature of changes in enzymes activities depends on the killer yeast species and co-presence of *M. fructicola* at the wound site. The phenomena, associated with yeasts' involvement in plant's defence response, may have an impact on the overall performance of killer yeasts in biocontrol of *M. fructicola*. However, further studies, including measurements of ROS generation by plant's tissue exhibited to yeast cells and fungal mycelium, should be carried out in the future. Understanding the complex array of mechanisms in biocontrol will facilitate the selection of an appropriate biological control agent (BCA) suitable for the given application. Also, it should be considered that the subsequent or simultaneous application of two antagonists bearing complementary features, as biocontrol agents, may improve the overall effect of fungal phytopathogen biocontrol and contribute to sustainable agriculture.

Acknowledgements

This work was supported by research grant POIG.01.03.01-02-080/12 “The use of yeast *Y. lipolytica* and *D. hansenii*, their enzymes and toxins for application in industry and agriculture” co-financed by the European Union from the European Regional Development Fund.

References

Aggarwal, M., Mondal, A.K., 2009. *Debaryomyces hansenii*: an osmotolerant and halotolerant yeast. In: Yeast Biotechnology: Diversity and Applications, pp. 65–80.
Andersson, L.A., Johnson, A.K., Simms, M.D., Willingham, T.R., 1995. Comparative analysis of catalases: spectral evidence against heme-bound water for the solution enzymes. FEBS Lett. 370 (1–2), 97–100.
Andrade, M.J., Thorsen, L., Rodríguez, A., Córdoba, J.J., Jespersen, L., 2014. Inhibition of ochratoxigenic moulds by *Debaryomyces hansenii* strains for biopreservation of dry-cured meat products. Int. J. Food Microbiol. 170, 70–77.
Banjara, N., Nickerson, K.W., Suhr, M.J., Hallen-Adams, H.E., 2016. Killer toxin from several food-derived *Debaryomyces hansenii* strains effective against pathogenic *Candida* yeasts. Int. J. Food Microbiol. 222, 23–29.
Beckerman, J., Albright, N., Abbott, C., 2016. First report of Brown rot (*Monilinia fructicola*) on apple (*Malus* × *domestica*). Plant Dis. 100 (9), 1949.
Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. Anal. Biochem. 239 (1), 70–76.
Bindschedler, L.V., Dewdney, J., Blee, K.A., Stone, J.M., Asai, T., Plotnikov, J., Denoux, C., Hayes, T., Gerrish, C., Davies, D.R., Ausubel, F.M., Bolwell, G.P., 2006. Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. Plant J. 47 (6), 851–863.
BIOHAZ (EFSA Panel on Biological Hazards), 2012. Scientific Opinion on the maintenance of the list of QPS biological agents internationally added to food and feed (2012 update). EFSA J. 10 (12), 84.
Bolwell, G.P., Wojtaszek, P., 1997. Mechanisms for the generation of reactive oxygen species in plant defence - a broad perspective. Physiol. Mol. Plant Pathol. 51 (6), 347–366.
Castoria, R., Caputo, L., De Curtis, F., De Cicco, V., 2003. Biological control resistance of postharvest biocontrol yeasts to oxidative stress: a possible new mechanism of action. Phytopathology 93 (5), 564–672.
Chalutz, E., Wilson, C.L., 1990. Postharvest biocontrol of green and blue mold and sour rot of citrus fruit by *Debaryomyces hansenii*. Plant Dis. 74, 134–137.
Chan, Z., Qin, G., Xu, X., Li, B., Tian, S., 2007. Proteome approach to characterize proteins induced by antagonist yeast and salicylic acid in peach fruit. J. Proteome Res. 6 (5), 1677–1688.
Chan, Z., Tian, S., 2006. Induction of H2O2-metabolizing enzymes and total protein synthesis by antagonistic yeast and salicylic acid in harvested sweet cherry fruit. Postharvest Biol. Technol. 39 (3), 314–320.
Davies, D.R., Bindschedler, L.V., Strickland, T.S., Bolwell, G.P., 2006. Production of reactive oxygen species in *Arabidopsis thaliana* cell suspension cultures in response to an elicitor from *Fusarium oxysporum*: implications for basal resistance. J. Exp. Bot. 57 (8), 1817–1827.
De Cal, A., Gell, I., Usall, J., Viñas, I., Melgarejo, P., 2009. First report of Brown rot caused by *Monilinia fructicola* in peach orchards in ebro valley, Spain. Plant Dis. 93, 763.
Di Francesco, A., Mari, M., 2018. *Monilinia* species of fruit decay: a comparison between biological and epidemiological data. Ital. J. Mycol. 47, 13–23.
Droby, S., Chalutz, E., Wilson, C.L., Wisniewski, M., 1989. Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. Can. J. Microbiol. 35, 794–800.
Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E.E., Porat, R., 2002. Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila*. Phytopathology 92, 393–399.
EFSA (European Food Safety Authority), 2011. Pest risk assessment of *Monilinia fructicola* for the EU territory and identification and evaluation of risk management options. EFSA J. 9 (4), 2119.
Fu, W., Tian, G., Pei, Q., Ge, X., Tian, P., 2017. Evaluation of berberine as a natural compound to inhibit peach brown rot pathogen *Monilinia fructicola*. Crop Protect. 91, 20–26.
Govrin, E.M., Rachmilevitch, S., Tiwari, B.S., Solomon, M., Levine, A., 2006. An elicitor from Botrytis cinerea induces the hypersensitive response in *Arabidopsis thaliana* and other plants and promotes the gray mold disease. Phytopathology 96 (3), 299–307.
Grabke, A., Hu, M.J., Luo, C.X., Bryson, P.K., Schnabel, G., 2011. First report of brown rot of apple caused by *Monilinia fructicola* in Germany. Plant Dis. 95 (6), 772.
Grzegorczyk, M., Szalewicz, A., Żarowska, B., Połomska, X., Wątorek, W., Wojtatowicz, M., 2015. Microorganisms in biological control of phytopathogenic fungi. Acta Sci. Pol., Biotechnol. 14 (2), 19–42 ([in polish]).
Grzegorczyk, M., Żarowska, B., Restuccia, C., Cirvilleri, G., 2017. Postharvest biocontrol ability of killer yeasts against *Monilinia fructigena* and *Monilinia fructicola* on stone fruit. Food Microbiol. 61, 93–101.
Guo, F.-J., Ma, Y., Xu, H.-M., Wang, X.-H., Chi, Z.-M., 2013. A novel killer toxin produced by the marine-derived yeast *Wickerhamomyces anomalus* YF07b. Antonie Leeuwenhoek 103, 737–748.
Hegedus, D.D., Rimmer, S.R., 2005. *Sclerotinia sclerotiorum*: when “to be or not to be” a pathogen? FEMS Microbiol. Lett. 251 (2), 177–184.
Hernandez-Montiel, L.G., Gutierrez-Perez, E.D., Murillo-Amador, B., Vero, S., Chiquito-Contreras, R.G., Rincon-Enriquez, G., 2018. Mechanisms employed by *Debaryomyces hansenii* in biological control of anthracnose disease on papaya fruit. Postharvest Biol. Technol. 139, 31–37.
Hernández-Montiel, L.G., Ochoa, J.L., Troyo-Diéguez, E., Larralde-Corona, C.P., 2010. Biocontrol of postharvest blue mold (*Penicillium italicum* Wehmer) on Mexican lime by marine and citrus *Debaryomyces hansenii* isolates. Postharvest Biol. Technol. 56, 181–187.
Hershkovitz, V., Ben-Dayan, C., Raphael, G., Pasmanik-Chor, M., Liu, J., Belausov, E., Aly, R., Wisniewski, M., Droby, S., 2012. Global changes in gene expression of grapefruit peel tissue in response to the yeast biocontrol agent *Metschnikowia fructicola*. Mol. Plant Pathol. 13, 338–349.
Ippolito, A., El Ghaouth, A., Wilson, C.L., Wisniewski, M., 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biol. Technol. 19 (3), 265–272.
Jones, J.D.G., Dangl, J.L., 2006. The plant immune system. Nature 444, 323–329.
Lee, M.H., Bostock, R.M., 2006. Induction, regulation, and role in pathogenesis of appressoria in *Monilinia fructicola*. Phytopathology 96, 1072–1080.
Lee, M.H., Chiu, C.M., Roubtsova, T., Chou, C.M., Bostock, R.M., 2010. Overexpression of a redox-regulated cutinase gene, MfCUT1, increases virulence of the brown rot pathogen *Monilinia fructicola* on *Prunus* spp. Mol. Plant Microbe Interact 23, 176–186.
Lima, J.R., Gondim, D.M.F., Oliveira, J.T.A., Oliveira, F.S.A., Gonçalves, L.R.B., Viana, F.M.P., 2013. Use of killer yeast in the management of postharvest papaya anthracnose. Postharvest Biol. Technol. 83, 58–64.
Liu, J., Macarisin, D., Wisniewski, M., Sui, Y., Droby, S., Norelli, J., Hershkovitz, V., 2013a. Production of hydrogen peroxide and expression of ROS-generating genes in peach flower petals in response to host and non-host fungal pathogens. Plant Pathol. 62, 820–828.
Liu, J., Sui, Y., Wisniewski, M., Droby, S., Liu, Y., 2013b. Review: utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. Int. J. Food Microbiol. 167, 153–160.
Liu, S.Q., Tsao, M., 2009. Biocontrol of dairy moulds by antagonistic dairy yeast *Debaryomyces hansenii* in yoghurt and cheese at elevated temperatures. Food Control 20, 852–855.
Lu, L., Ye, C., Guo, S., Sheng, K., Shao, L., Zhou, T., Yu, T., Zheng, X., 2013. Preharvest application of antagonistic yeast *Rhodosporidium paludigenum* induced resistance against postharvest diseases in Mandarin orange. Biol. Control 67, 130–136.
Lu, H., Lu, L., Zeng, L., Fu, D., Xiang, H., Yu, T., Zheng, X., 2014. Effect of chitin on the antagonistic activity of *Rhodosporidium paludigenum* against *Penicillium expansum* in apple fruit. Postharvest Biol. Technol. 92, 9–15.
Macarisin, D., Cohen, L., Eick, A., Rafael, G., Belausov, E., Wisniewski, M., Droby, S., 2007. Postharvest pathology and mycotoxins *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology 97, 1491.
Mari, M., Martini, C., Guidarelli, M., Neri, F., 2012. Postharvest biocontrol of *Monilinia laxa, Monilinia fructicola* and *Monilinia fructigena* on stone fruit by two *Aureobasidium pullulans* strains. Biol. Control 60, 132–140.
Martini, C., Mari, M., 2014. *Monilinia fructicola, Monilinia laxa* (Monilinia rot, Brown rot). In: Bautista-Banos, S. (Ed.), Postharvest Decay. Control Strategies. Academic Press, Elsevier, Eastbourne, UK, pp. 233–258.
Miessner, S., Stammler, G., 2010. *Monilinia laxa* , *M. fructigena* and *M. fructicola*: risk estimation of resistance to QoI fungicides and identification of species with cytochrome b gene sequences. J. Plant Dis. Prot. 117, 162–167.

Muccilli, S., Caggia, C., Randazzo, C.L., Restuccia, C., 2011. Yeast dynamics during the fermentation of brined green olives treated in the field with kaolin and Bordeaux mixture to control the olive fruit fly. Int. J. Food Microbiol. 148, 15–22.
Muccilli, S., Wemhoff, S., Restuccia, C., Meinhardt, F., 2013. Exoglucanase-encoding genes from three *Wickerhamomyces anomalus* killer strains isolated from olive brine. Yeast 30, 33–43.
Navarrete, C., Siles, A., Martínez, J.L., Calero, F., Ramos, J., 2009. Oxidative stress sensitivity in *Debaryomyces hansenii*. FEMS Yeast Res. 9, 582–590.
Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D., Hancock, J.T., 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. J. Exp. Bot. 53, 1237–1247.
Parafati, L., Cirvilleri, G., Restuccia, C., Wisniewski, M., 2017a. Potential role of exoglucanase genes (*WaEXG1* and *WaEXG2*) in the biocontrol activity of *Wickerhamomyces anomalus*. Microb. Ecol. 73, 876–884.
Parafati, L., Vitale, A., Polizzi, G., Restuccia, C., Cirvilleri, G., 2016. Understanding the mechanism of biological control of postharvest phytopathogenic moulds promoted by food isolated yeasts. Acta Hortic. 1144, 93–100.
Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2015. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. Food Microbiol. 47, 85–92.
Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2017b. Performance evaluation of volatile organic compounds by antagonistic yeasts immobilized oh hydrogel spheres against gray, green and blue postharvest decays. Food Microbiol. 63, 191–198.
Passardi, F., Penel, C., Dunand, C., 2004. Performing the paradoxical: How plant peroxidases modify the cell wall. Trends Plant Sci. 9 (11), 534–540.
Passoth, V., Fredlund, E., Druvefors, U.Ä., Schnürer, J., 2006. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. 6 (1), 3–13.
Pereira, W.V.,, May-De-Mio, L.L., 2018. First report of Brown rot caused by *Monilinia fructicola* on apple in Brazil. Plant Dis. 100 (9), 1949.
Peter, K.A., Gaskins, V.L., Lehman, B., Jurick, W.M., 2015. First report of Brown rot on apple fruit caused by *Monilinia fructicola* in Pennsylvania. Plant Dis. 99 (8), 1179.
Platania, C., Restuccia, C., Muccilli, S., Cirvilleri, G., 2012. Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on Tarocco orange fruits (*Citrus sinensis*). Food Microbiol. 30, 219–225.
Pretscher, J., Fischkal, T., Branscheidt, S., Jäger, L., Kahl, S., Schlander, M., Thines, E., Claus, H., 2018. Yeasts from different habitats and their potential as biocontrol agents. Fermentatio 4, 31.
Prista, C., Michán, C., Miranda, I.M., Ramos, J., 2016. The halotolerant *Debaryomyces hansenii*, the Cinderella of non-conventional yeasts. Yeast 33 (10), 523–533.
Ryals, J., Neuenschwander, U., Willits, M., Molina, A., Steiner, H.-Y., Hunt, M., 1996. Systemic acquired resistance. Plant Cell 8 (10), 1809–1819.
Santos, A., Marquina, D., Barroso, J., Peinado, J.M., 2002. (1→6)-β-D-glucan as the cell wall binding site for *Debaryomyces hansenii* killer toxin. Lett. Appl. Microbiol. 34, 95–99.
Santos, A., Sánchez, A., Marquina, D., 2004. Yeasts as biological agents to control *Botrytis cinerea*. Microbiol. Res. 159, 331–338.
Sisquella, M., Vinas, I., Picouet, P., Torres, R., Usall, J., 2014. Effect of host and *Monilinia* spp. Variables on the efficacy of radio frequency treatment on peaches. Postharvest Biol. Technol. 87, 6–12.
Spadaro, D., Droby, S., 2016. Development of biocontrol products for postharvest diseases of fruit: the importance of elucidating the mechanisms of action of yeast antagonists. Trends Food Sci. Technol. 47, 39–49.
Thakur, M., Sohal, B.S., 2013. Role of elicitors in inducing resistance in plants against pathogen infection: a review. ISRN Biochem., 762412.
Torres, M.A., 2006. Reactive oxygen species signaling in response to pathogens. Plant Physiol. 141 (2), 373–378.
Van Breusegem, F., Vranová, E., Dat, J.F., Inzé, D., 2001. The role of active oxygen species in plant signal transduction. Plant Sci. 161 (3), 405–414.
Van den Tempel, T., Jackobsen, M., 2000. The technological characteristics of *Debaryomyces hansenii* and *Yarrowia lipolytica* and their potential as starter cultures for production of Danablu. Int. Dairy J. 10, 263–270.
Van Loon, L.C., Van Strien, E.A., 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55 (2), 85–97.
Walz, A., Zingen-Sell, I., Theisen, S., Kortekamp, A., 2008. Reactive oxygen intermediates and oxalic acid in the pathogenesis of the necrotrophic fungus *Sclerotinia sclerotiorum*. Eur. J. Plant Pathol. 120 (4), 317–330.
Wojtaszek, P., 1997. Oxidative burst: an early plant response to pathogen infection. Biochem. J. 322 (3), 681–692.
Wojtatowicz, M., Chrzanowska, J., Juszczyk, P., Skiba, A., Gdula, A., 2001. Identification and biochemical characteristics of yeast microflora of Rokpol cheese. Int. J. Food Microbiol. 69, 135–140.
Xu, X., Qin, G., Tian, S., 2008. Effect of microbial biocontrol agents on alleviating oxidative damage of peach fruit subjected to fungal pathogen. Int. J. Food Microbiol. 126 (1–2), 153–158.
Żarowska, B., 2012. Biosynthesis and Characteristics of *Debaryomyces Hansenii* Killer Toxin. University of Environmental and Life Sciences Publishing House Monograph CXLVI 17-105 [in polish].
Żarowska, B., Wojtatowicz, M., Połomska, X., Juszczyk, P., Chrzanowska, J., 2004. Factors affecting killer activity of some yeast species occurring in Rokpol cheese. Folia Microbiol. (Praha) 49 (6), 713–717.
Zhang, J., Xie, J., Zhou, Y., Deng, L., Yao, S., Zeng, K., 2017. Inhibitory effect of *Pichia* membranaefaciens and *Kloeckera apiculata* against *Monilinia fructicola* and their biocontrol ability of brown rot in postharvest plum. Biol. Control 114, 51–58.