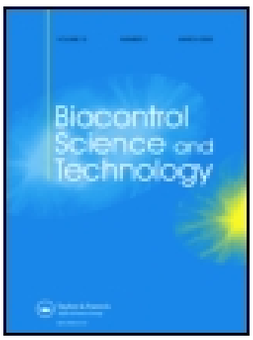

Biocontrol Science and Technology

ISSN: 0958-3157 (Print) 1360-0478 (Online) Journal homepage: http://www.tandfonline.com/loi/cbst20

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To cite this article: Carlos Alberto Tuão Gava, Ana Paula Carvalho de Castro, Carliana Araújo Pereira, Josélia Santana Gonçalves, Ludmilla Ferreira Cajuhi Araújo & Cristiane Domingos da Paz (2018): Photoprotector adjuvants to enhance UV tolerance of yeast strains for controlling mango decay using pre-harvest spraying, Biocontrol Science and Technology

To link to this article: https://doi.org/10.1080/09583157.2018.1499869

Published online: 14 Jul 2018.

BIOCONTROL SCIENCE AND TECHNOLOGY
https://doi.org/10.1080/09583157.2018.1499869

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RESEARCH ARTICLE

Photoprotector adjuvants to enhance UV tolerance of yeast strains for controlling mango decay using pre-harvest spraying

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ABSTRACT

Post-harvest mango decay is caused by multiple pathogens in tropical conditions but concerns regarding the risk of food contamination by fungicides established biocontrol as a promising alternative. However, occurrence of quiescent infections requires pre-harvest applications of biocontrol agents (BCA), exposing them to harmful UV radiation effects. The objective of this work was to evaluate UV sensitivity of yeast BCA strains previously selected against multiple pathogens that cause mango decay and evaluate suitable UV protectants. In a first bioassay conducted exposing yeast suspensions sprayed on glass plates, it was verified that *Saccharomyces* sp. ESA47 and *Pichia kudriavzevii* CMIAT171 were highly sensitive to UV, while Saccharomyces *cerevisiae* ESA45 had a slightly lower mortality. A bioassay using fragments of mango peels evaluated UV protection from increasing concentrations of starch, dextrin, casein, benzophenone, and cinnamic acid derivative compounds. Results showed that starch and isoamyl p-methoxycinnamate (NHE-1000) resulted in higher survival for yeast strains in doses of 10.0 and 1.0 g kg^{-1}, respectively. Application of the yeast BCA in a semi-commercial mango orchard resulted in a significant reduction of post-harvest disease incidence and severity. Field application of the yeasts in technical grade preparations containing both UV protectants enhanced the control efficiency by 52.5, 31.9, and 37.7% for ESA45, ESA47, and CMIAT171, respectively.

ARTICLE HISTORY
Received 23 November 2017
Returned 30 June 2018
Accepted 10 July 2018

KEYWORDS
UV protectants; *Mangifera indica*; biological control; post-harvest rot

Introduction

The main strategy to control fruits and vegetables post-harvest decay has been pre- and post-harvest application of fungicides. However, there is a large concern regarding their harmful effects towards the producer and consumer health, and the environment contamination, as well as the risk of development of resistant pathogen populations. Among a

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variety of potential biocontrol agents (BCA), yeasts have some advantages since they are natural fruit surface inhabitants. Yeasts are largely applied in food industries and have become familiar to consumers, posing little technological constraints for industrial production. Controlling the storage environment (temperature, humidity, and gas concentration) gives an advantage to biocontrol of post-harvest diseases (Droby, Wisniewski, Macarisin, & Wilson, 2009). However, most post-harvest mango diseases are caused by multiple pathogens that have a quiescent stage, infecting immature fruits in the field (Prusky, Alkan, Mengiste, & Fluhr, 2013). These characteristics of the pathosystems require that application of biopesticide in the integrated management of post-harvest pathogens also be done in the pre-harvest phase.

Pre-harvest spraying of microbial biopesticides exposes them to UV radiation, which is probably the most important factor affecting their persistence. In the studies by Santos, Silva, Monteiro, and Gava (2011), almost 90% of the conidia of *Beauveria bassiana* strain LCB63 were killed by short exposition to natural sunlight. Cañamás et al. (2008) also observed that the number of viable cells of *Pantoea agglomerans* CPA-2 applied to orange fruits drastically decreased after 4 h of exposure to solar radiation, while it decreased only slightly on fruits exposed to shade conditions. Carotenoid pigments and antioxidant compounds are associated with tolerance to oxidative stress produced by UV in yeasts (Sui, Wisniewski, Droby, & Liu, 2015). A significant example of the production of such compounds is the production of coenzyme Q10 and mycosporine, a hydrosoluble aminocyclohexenone, by *Xanthophyllomyces dendrorhous* in response to UV exposition (Libkind, Moline, & Van Broock, 2011).

Yeast tolerance to UV is species-dependent and isolation from the environment exposed to sunlight should generate tolerant strains. *Pichia kudriavzevii*, for example, presents an interesting ability to shift to a biofilm form that is more resistant to environmental stresses and shows enhanced biocontrol efficiency for pears decay (Chi et al., 2015). Preconditioning by heat-shock and osmotic stress have been pointed out as strategies to improve UV tolerance of yeast BCA (Estruch, 2000; Liu et al., 2011). In the studies by Cheng et al. (2016), heat-shock pre-treatment activated ROS detoxification enzymes on *Rhodotorula mucilaginosa* and increased its tolerance to salt, as well as oxidative and pH stresses.

Microorganism formulation technologies achieved great advances in recent years (Arora, Balestrini, & Mehnaz, 2016). Their main functions are the stabilisation of the BCA during storage, improvement of handling and application, enhancement of the biopesticide effect, as well as protection of the agent from harmful environmental factors (Burges, 1998; Jijakli & Lahlali, 2016). Low field persistence of microbial BCA has been attributed to sunlight and attempts to increase its survival have relied on UV protectant adjuvants (Fernandes, Rangel, Braga, & Roberts, 2015). UV protectants prevent photodamage by two mechanisms for: (i) absorbing, blocking or reflecting damaging wavelengths, preventing them from reaching the cell surface and (ii)scavenging reactive oxygen species (ROS) by antioxidant compounds (Burges, 1998). This author lists a large number of UV protectants that can be applied in spray tank mix or commercial formulations. However, selection of a UV protectant for a microbial BCA is a case-to-case study. Besides this, a cost-effective commercial formulation would be more acceptable by users if it incorporates a sunscreen, independent from tank mixing of a second or third product.

Findings from a previous study showed that *Saccharomyces cerevisiae* ESA45, *Saccharomyces* sp. ESA47, and *Pichia kudriavzevii* CMIAT171 are potential biocontrol agents (BCA) of mango decay caused by multiple pathogens (Gava, de Castro, Pereira, & Fernandes-Júnior, 2017). The objective of this research study was to confirm the intrinsic tolerance of these potential yeast BCAs to UV and to select UV protectant adjuvants to be applied in a biopesticide formulation.

Material and methods

Microorganism preservation and inoculum production

The yeast strains *S. cerevisiae* ESA45, *Saccharomyces* sp. ESA47, and *P. kudriavzevii* CMIAT171 were isolated from ripe fruits in the Brazilian semi-arid region and previously screeened against multiple pathogen infections that cause mango fruit post-harvest decay (Gava et al., 2017). The strains were kept in a 15% glycerol solution and stored at −80°C. In order to produce the inoculum used in different experiments, isolates were cultivated in Sabouraud Dextrose Agar plus yeast extract (SDA + Y) medium (peptone 20 g, dextrose 40 g, yeast extract 20 g, distilled water 1.0 L, agar 20.0 g L^{-1}) and cells were scraped from medium surface and suspended in NaCl (0.8%). Suspensions were standardised to OD 590 nm = 0.2 (approximately 10^6 CFU mL^{-1}, data not shown) and then applied directly in the experiments. Larger volumes of cell suspensions were cultivated in flasks containing SD + Y liquid medium incubated in an orbital shaker at 120 rpm, during 72 h at 27°C.

Natural tolerance of yeast isolates to UV-C in vitro

Yeast cell suspensions (10^6 cells mL^{-1}) were prepared as described above, and 500 µL pulverised onto dry sterile plastic Petri dishes and exposed to airflow in a sterile chamber until there was no sign of visible water. Then, the plates were exposed to an artificial UV source using a 15 W UV bulb at 254 nm wavelength (G15T8, Osram) with 480 h of use. The UV fluence dose upon the plate surface was measured using a portable UV radiometer (UV-35, Huatec Inc.) equipped with a photosensor containing a spectral UV-C filter. Plates were exposed at 40 cm from the lamp and treated for a period from 1 to 12 min, with an effective irradiance of 14.1 W. UV doses applied were 0.0; 0.42; 0.86; 1.66; 3.10; and 4.20 kJ m^2.

Yeast cells were removed from the plates by adding 3 mL of 0.05% Triton X-100 solution and scraping the dish surface with a bent glass rod. According to a method adapted from Santos et al. (2011), 100 µL of the resulting suspension was then collected with a micropipette and spread with a bent glass rod in Petri dishes containing SDA + Y medium. Experiments were conducted twice with three replicates per treatment. Control plates were exposed to the same doses but enclosed in aluminum paper.

Applying UV protectants to yeast BCAs in mango epidermis

This assay evaluated the effect of different UV protectants on the survival of yeast BCAs applied on fragments of mango peel. Technical grade preparations (TGP) of yeast BCAs

containing increasing concentrations (0; 250; 500; 1,000; 1,500; and 2,000 mg. kg^{-1}) of hydrophobic and hydrophilic UV protectants described in Table 1. Yeast cell suspensions (10^6 cells mL^{-1}) were prepared as described above and mixed with UV protectants dissolved in a 0.1% solution of Triton X-100.

Mango fruits cv. 'Tommy Atkins' collected from a local farm in the maturation stage 2, according to Kienzle et al. (2012), and selected for absence of apparent injuries. Fruits were sterilised using the ethanol 70% (30s)/sodium hypochlorite 0.5% (2 min) protocol and peel fragments were removed using a sterilised stainless-steel blade. Twelve peel fragments of 8 cm^2 (4 × 2 cm) were transferred to sterile Petri dishes and sprayed with 1 mL of TGPs using a Potter tower (Burkhard Scientific – UK) with a pressure of 15 bars.cm^2. The zero-dose treatment only received the yeast BCA suspended in 0.1% Triton X-100. Fragments without any applications were used as a control in reference to the sterilisation process.

Thirty minutes after TGP application, when fragments were apparently dry, they were exposed to 3.2 kJ m^2 of UV-C radiation (average LD_{90} for the isolates) in a sterilised UV chamber as described above. A control treatment, without UV protectants, was exposed to the same UV doses, but enclosed in aluminum paper, eliminating the effect of intrinsic strain growth rate. Yeast cells were removed from peel fragments by agitation in flasks containing 50 mL of 0.05% Triton X-100 solution for 30 min at 200 rpm. Obtained suspensions were plated in PDA medium after 1:10 serial dilution and CFU counted two days after inoculation. Treatment results are presented as the percentage proportion of the number of colonies forming unities, or viable cell recovery rate (CR), of the exposed treatment and the protected triplicates.

Field experiment applying yeast BCA TGPs to control mango decay

The experiment was performed in a 7-year old mango orchard cv 'Kent' on September/October 2016 at the Embrapa Tropical Semi-Arid experimental station, located in Petrolina (Pernambuco State, Brazil). Disease management in the mango orchard was accomplished through two applications of copper hydroxide during vegetative growth; two spraying of micronized sulphur alternated with one spraying of azoxystrobin during flowering; two applications of azoxystrobin from fruit set until the initial fruit

Table 1. Physicochemical description of UV protectants used in the bioassays.

Photoprotector	Denomination	Appearance	Solubility	Maximum λ (nm)
Starch	Soluble Starch	Powder	Hydrosoluble	ND
Dextrin	Dextrin	White to beige; powder	Hydrosoluble	ND
Casein	Casein hydrolisate	White to beige; powder	Hydrosoluble	ND
Neo Heliopan AV®	Octyl Methoxycinnamate (2-ethylhexyl-p-methoxycinna mate)	Slightly yellow; liquid	Liposoluble	290–320
Neo Heliopan E1000®	Isoamyl p-methoxycinnamate	Colorless; liquid	Liposoluble	290–320
Oxybenzone®	2-hidroxi-4-metoxibenzo-phenone (Benzophenone)	White to crystalline; powder	Liposoluble	288–325

Note: ND – maximum λ not known.

development. Spraying was done during the morning using an airblast sprayer (Arbus 400, Jacto Inc.)

The experiment consisted of weekly spraying of TGPs containing only 10^7 cells mL^{-1} of ESA45, ESA47, and CMIAT171, and a second group where TGPs were previously prepared by adding starch 1.0% plus NHE-1000 0.1%. All six TGPs received 0.5% of a commercial formulation of partially esterified soybean oil (SO) in the tank mix. Control treatments received only SO 0.5% or TGP. Treatments started at 21 days after fruit set, the end for safe application of conventional fungicide and maximum fruit growth. Six sprayings were applied in the period. Spraying was performed in the early morning, using a backpack sprayer with a standard solid cone nozzle directed to the fruits. The experiment was conducted in a randomised block design in a factorial arrangement: 2 formulations × 4 preparations (yeast BCA + control); and four replications, with each replicate formed by five mango trees. The control treatment was not sprayed.

Harvest was performed during the morning period, through selection of fruits in stages 2–3, according to the maturity scale by Kienzle et al. (2012). One hundred fruits with no apparent damage were harvested from each plot and temporally packed in plastic containers previously lined with bubble wrap and carefully transported to the laboratory. Post-harvest processing was similar to the procedure adopted in commercial packing houses. Fruits were initially washed with detergent under tap water and their peduncles were standardised at 20 mm. They were selected for the absence of mechanical injuries, size uniformity, and maturation uniformity, then dried with forced air provided by an industrial blower. After processing, fruits were placed in a standard paper box containing corrugated paper at the bottom, reducing damage risk by compression, and covered with a waxed paper sheet (hygroscopic internal surface and hydrophobic external surface). Fruit boxes were stored under controlled temperature (25°C and 70% RH) during 10 days. Mangoes were evaluated daily for incidence and severity of fruit decay. At the end of the experiment, symptomatic fruits were picked and the etiological agent was isolated and morphologically identified.

Data treatment and statistical analysis

Data were evaluated using Lilliefors and Levene tests for homoscedasticity and homogeneity of variance, respectively. Percentage data were transformed by applying the equation:

$$X'_{ij} = \text{arcsen}\sqrt{X_{ij}/100} \tag{1}$$

In which X_{ij} and X'_{ij} are the observed and transformed data, respectively. Data from the two experiments regarding natural UV tolerance were pooled for variance analysis (ANOVA) and logistic regression (logit analysis) using the model:

$$Y = a + (b-a)/1^{[(LD_{50}-X_{ij})\text{slope}]} \tag{2}$$

Thus, obtaining the lethal UV dose (LD_{50}) for each isolate. Transformation of CR data into mortality rate to apply in logit analysis was performed through the equation:

$$M = 1 - \frac{CR}{100} \tag{3}$$

Data from experiments with UV protectants dose/response were used in ANOVA and linear regression. Linear models obtained were compared using a partial least-square analysis and applied to determine the dose of maximum protection (Geladi & Kowalski, 1986).

Fruit rot incidence data (%) from the field experiment was transformed using the equation above, while colony counting data were transformed using the following equation:

$$X_{ij}^{'} = \log_{10} X_{ij} \tag{4}$$

X_{ij} and $X_{ij}^{'}$ from both equations are also the observed and transformed data, respectively. The significance of the presence and absence of UV protectants in the TGPs was evaluated by contrast analysis in the ANOVA procedure. Incidence and severity average were compared using the Tukey test ($p < 0.05$) and were presented as non-transformed averages (± standard deviation).

Results and discussion

Natural tolerance of yeast isolates to UV-C in vitro

Recovery of viable cells strongly declined after exposure to initial doses of UV-C (Figure 1) and mortality data was adjusted to the logit regression model ($p > 0.05$). There was no significant difference between ESA47 and CMIAT171 mortality curves, but ESA45 was slightly more tolerant to intermediate UV doses as shown by the LD_{50} and slope differences (Table 2). This result corroborates previous studies performed during the selection of these strains (Gava et al., 2017). Besides DNA and membrane repair apparatus (Sui et al., 2015), yeast resistance mechanism to UV damage comprise the production of

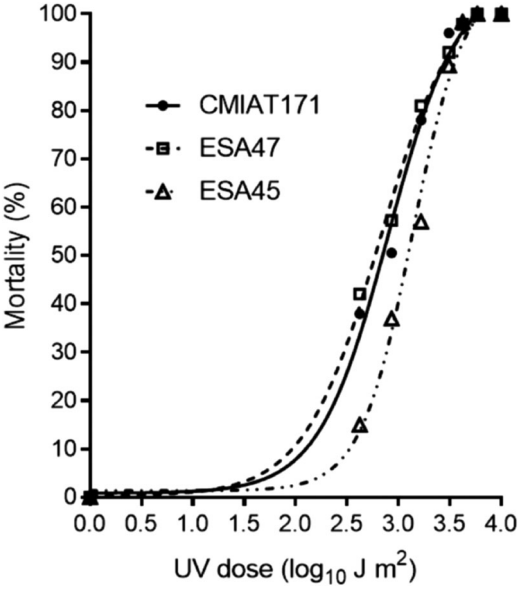

Figure 1. Mortality of *S. cerevisiae* ESA45, *Saccharomyces* sp. ESA47, and *P. kudriavzevii* CMIAT171 exposed to increasing doses of UV radiation.

Table 2. Lethal dose [LC50 (± CI) kJ m^2] of UV to yeast strains in dose response assays to evaluate natural intrinsic tolerance of yeast strain cells.

	CMIAT171	ESA47	ESA45
Intercept (a)	0.836	0.2751	1.326
Top (b)	107.6	107	107.4
Slope	1.283	1.167	1.719
LD_{50} (J m^{-2})	441.25	739,21	1,363.07
Conf. Interval	263.03–616.60	501.18–997.24	794.33–1737.80
Sig. (p)	0.4916	0.3076	0.4809

pigments and ROS detoxifying mechanisms (Dimitrova, Pavlova, Lukanov, & Zagorchev, 2010; Libkind et al., 2011; Moliné et al., 2010). ESA45 was obtained from the mesocarp of grape berries, but it unexpectedly showed a large UV tolerance than the other strains. Strains from this research study did not produce pigments *in vitro*, which may explain their sensitivity to UV.

Applying UV protectants to yeast BCAs in mango epidermis

The effect of six UV protectants on the survival of the yeast strains was tested applying yeast TGPs to mango peel fragments. After application on the peel fragments, yeast TGPs were exposed to a standard UV dose equivalent to 3.2 kJ m^{-2} using a UV-C lamp, which caused around 90% mortality to all strains in the natural tolerance assay. The rate of viable cell recovery (CR) from mango peel fragments was dependent on yeast strain and UV protectant dose ($F_{4;27} = 23.791$; $p < 0.01$). In general, yeast exposure to UV without protectant resulted in CFU counts close to zero.

The hydrophobic protectants oxybenzone, NHE-1000, and NHAV significantly increased CFU count (Figure 2). The usage of NHE-1000 resulted in the highest CR for ESA45 and ESA47 (Figure 2(a,b)), with an optimal dose of 1.0 and 1.2 g kg^{-1}, respectively.

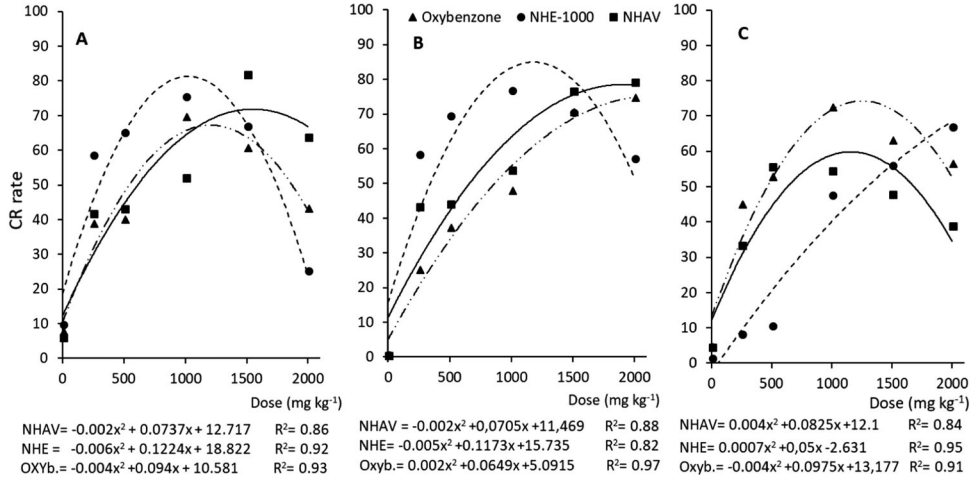

Figure 2. Proportional cell recovery rate (CR) of *S. cerevisiae* ESA45 (a), *Saccharomyces* sp. ESA47 (b), and *P. kudriavzevii* CMIAT171 (c), which were submitted to different hydrophobic UV protectants in mango peel fragments using technical grade preparations and exposed to 3.2 kJ m^2 of artificial UV-C radiation.

Oxybenzone was the most efficient protectant to ESA45, with an optimal dose of 1.3 g kg^{-1} (Figure 2(c)). However, concentrations higher than 1.5 g kg^{-1} of both compounds showed deleterious effects for yeasts. A related effect was observed by Santos et al. (2011), while testing the compatibility of these compounds for *B. bassiana* conidia.

Results for hydrophilic protectants were also dependent on the yeast isolate ($F_{4;27}$ = 12.091; $p < 0.05$). The organic polymers dextrin, casein, and starch significantly reduced ESA45 mortality for doses of 5.4 g kg^{-1} for the two first adjuvants, and 8.2 g kg^{-1} for the last (Figure 3(a)). ESA47 and CMIAT171 had a different pattern and only starch resulted in a significant protection with optimal doses of 5.8 and 10 g kg^{-1}, respectively (Figure 3(a,b)). For both ESA47 and CMIAT171 strains, the applied casein and dextrin doses did not achieve the inflection point in their regression curves. Adjuvants improved CMIAT171 survival, but maximum CR was lower than 50%. Jijakli and Lahlali (2016) reported similar results, even though these authors did not evaluate yeast survival. They recorded a strong negative impact of UV radiation on biocontrol activity of *Pichia anomala* and *Candida oleiphila,* which was attenuated by the addition of lignin and folic acid to the formulation. While lignin blocks UV on cell surfaces, such as the compounds evaluated in this study, folic acid and other compounds, like riboflavin and tyrosine, have antioxidant properties (Lahlali, Brostaux, & Jijakli, 2011), reducing the deleterious effect of reactive oxygen species (ROS) on macromolecules and organelle membranes.

Adverse effects of sunlight on biocontrol agents requires that UV protectants are included in formulations, mainly in the tropics (Droby, Wisniewski, Teixidó, Spadaro, & Jijakli, 2016; Jijakli, 2011). Different studies have achieved distinct levels of success with water or oil-based formulations containing yeast cells with different protectants and additives (Droby et al., 2016). In this study, starch and NHE-100 showed the most

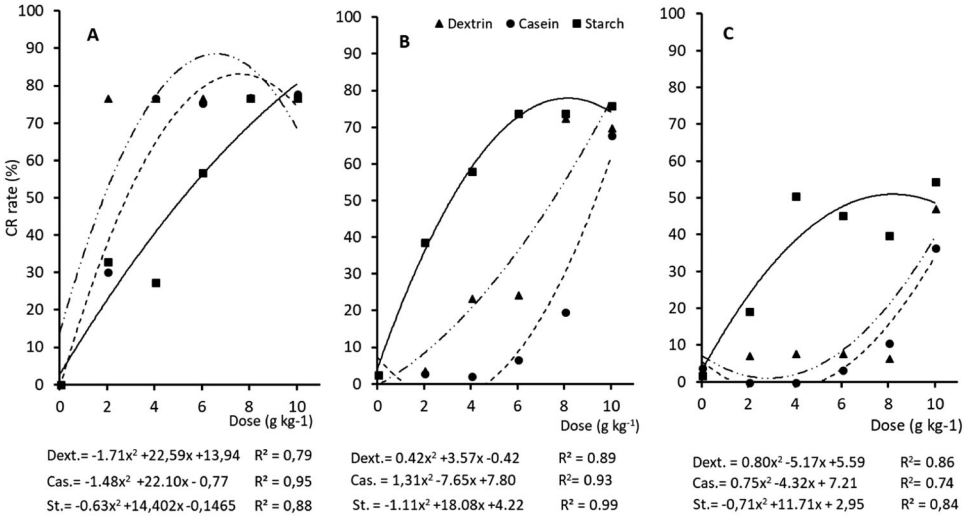

Figure 3. Proportional cell recovery rate (CR) of *S. cerevisiae* ESA45 (a), *Saccharomyces* sp. ESA47 (b), and *P. kudriavzevii* CMIAT171 (c), which were submitted to different hydrophilic UV protectants in mango peel fragments using technical grade preparations and exposed to 3.2 kJ m^2 of artificial UV-C radiation.

promising and cost-effective results. Pre-treatment of yeast BCAs is a promising and cost effective alternative to increase their tolerance to environmental stresses, mainly based on addition of osmolytes (salt or glucose) instead of heat, but also physiological characteristics (Cheng et al., 2016; Chi et al., 2015). Even so, their efficiency will likely be improved by formulation, mainly in field spraying.

In this study, ESA45 showed a slight intrinsic tolerance requiring a lower dose of adjuvant in the TGP. Pre-treatment of yeast strains and a possible biofilm forming by *P. kudriavzevii* CMIAT171 will be explored in future studies and may be applied along with adjuvants, such as starch and NHE-1000. Starch possibly blocks sunlight by producing a thin membrane over yeast cells after desiccation of the TGP. On the other hand, NHE-1000 is a synthetic derivative of ethyl cinnamate originated from *Kaempferia galanga* L (Zingiberaceae). It is a hydrosoluble compound able to link to the cell membrane and absorb UV. Both compounds had a strong effect on the survival of yeast strains on fruit surface and were combined to pre-harvest field sprays in a mango orchard.

Field experiment applying yeast BCA TGPs to control mango decayAll treatments significantly differed from the control for fruit rot incidence and severity through the Tukey test ($p < 0.05$) in the field experiment of mango cv. 'Kent' (Figure 4). Treatments using pre-harvest application of yeast TGPs containing 10 g kg^{-1} starch plus 1.0 g kg^{-1} NHE-1000 were significantly different from those without protectants by orthogonal contrast ($F1;18 = 5.22$; $p < 0.05$). Similarly, fruit rot severity was significantly smaller on fruits treated with protectants containing TGPs (Figure 4). Addition of UV protectants to TGPs increased

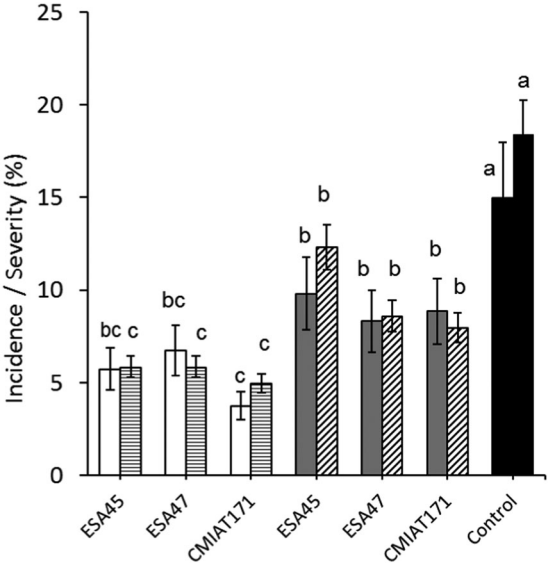

Figure 4. Incidence (solid columns) and severity (columns with patterns) of mango decay (average ± SD) in a commercial mango orchard cv. 'Kent' with application of two TGPs of *S. cerevisiae* ESA45 (a), *Saccharomyces* sp. ESA47 (b), and *P. kudriavzevii* CMIAT171 (c) after an incubation period of 10 days at 25°C. In white and horizontal pattern columns, yeast TGPs contain 1.0% starch plus 0.1% NHE-100, while grey and inclined patterns did not contain such compounds. Pre-harvest treatments were weekly applied during 6 weeks. Columns with different letters indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

the average control efficiency by 52.5%, 32%, and 38% for ESA45, ESA47, and CMIAT171, respectively, in comparison to the same treatments without adjuvants. Similar results were obtained by Jijakli and Lahlali (2016) for the effect of UV protectants on *P. anomala*.

These results were also similar to those obtained using *Bacillus licheniformis* to control mango decay in a semi-commercial scale in South Africa (Govender, Korsten, & Sivakumar, 2005) and for application of *Cryptococcus laurentii* to control blue mould in pear (Yu et al., 2012). However, in those studies the BCA was only applied in post-harvest. In other studies, pre-harvest application of *B. licheniformis* from flowering until harvest alone or in combination with copper sprays, for example, efficiently controlled mango decay with results similar to this study (Silimela & Korsten, 2007). In researches by Meng, Qin, and Tian (2010), pre-harvest spraying of *C. laurentii* significantly decreased post-harvest decay caused by *Botrytis cinerea*. However, while a large number of studies on the use of sunscreens has been done for entomopathogenic fungi (Fernandes et al., 2015), there are few research studies concerning the selection of UV protectants for BCA of plant disease.

Previous studies have shown that a large number of mango decay pathogens in tropical regions are able to produce quiescent infections (Lima et al., 2013; Marques et al., 2013), but application of synthetic fungicides is constrained due to the risks of food contamination. Therefore, effective microbial pesticides could be introduced in the integrated management of those pathogens. This study proposes their application in the mid to final stages of mango fruit development, guaranteeing its protection until harvest. The results from this work showed that formulations containing starch and NHE-1000 significantly increased the survival of UV-sensitive and promising biocontrol yeast strains. Application of yeast BCA in a semi-commercial mango orchard resulted in significant reduction of post-harvest disease incidence and severity, which was enhanced by TGP containing both UV protectants. Future studies will focus on the possibility to substitute pre-harvest fungicide sprays, complete the development of a cost-effective formulation that could also increase the shelf life and field survival of yeast strains, and introduce their use associated to other post-harvest technologies.

Acknowledgments

The authors are grateful to Mr. Herbert Targino for his technical support and significant help in the laboratory studies, and to Mr Genival Ferreira for his meaningful help in the field experiments.

Disclosure statement

No potential conflict of interest was reported by the authors.

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