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Yeast biocontrol against green mold in pear orange postharvest

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Abstract

The objective of this work was to evaluate the efficiency of the β enzyme and the mechanisms of action by means of a post-biological control solution of the orange-harvest type of the pear variety. The experiments were carried out at the Laboratory Center for Support to the Academic Unit of Garanhuns (CENLAG), belonging to the Federal University of Agreste of Pernambuco (UFAPE). The Completely Randomized Design (DIC) was used. The results were selected for analysis of variance, in triplicate (ANO), with a 5% probability level test ($p < 0.05$), using the statistical software Sisvar. See that killer activity was observed in 85.7% of the lovely yeasts. *Candida peltata* URM4681 showed the best production of β -1,3-glucanase between 1,071 and 5,422 U mL $^{-1}$. In a design 23 in protein medium, it was a very large 4-fold, 3351-fold increase in their enzyme. trials in sit8, selected, 5%, reduction, in the number of infected treated with the when compared with the extractor. Regarding the evolution and severity of the disease, it is not known that the fruits treated with enzymatic extract did not differ statistically from those treated with the fungicides IMZ and TBZ both preventively. It was concluded that C. peltata URM4681 is a good biocontrol agent against green mold in citrus.

Keywords: activity, citriculture, toxin killer

Introduction

Citriculture is an important global economic activity and, for decades, this sector has been responsible for generating job opportunities. Currently, Brazil is the largest orange producer in the world, which contributes to the country's economy. However, citrus production has been facing several problems that compromise fruit quality (Diering et al., 2022).

Decomposition is common by Penici one of the main problems during storage and post-harvest storage in citrus fruits worldwide. A deeper understanding of fruit mechanism and developmental responses by specific microorganisms for the development of defense solution to this problem according to Caserta et al. (2020).

Several cultural practices are recommended for the control of green mold, such as elimination of infected fruits, care in harvesting and transport to avoid injuries and constant cleaning of equipment. However, chemical treatment, with fungicides from the benzimidazole and imidazole groups in pre and/or post-harvest, is the most used method (Ghooshkhaneh et al., 2018).

Biological control agents represent an alternative for the protection of fruits during harvest and post-harvest, as they generally present low cytotoxicity and high biodegradability, without cytotoxic effect even at high concentrations. In general, these combined characteristics make these products suitable for inhibiting the growth of pathogens and for a more sustainable agriculture (Zhang et al., 2018). In this sense, the use of yeasts is highlighted, as they present several biochemical mechanisms, including the secretion of hydrolytic enzymes such as β-1,3-glucanases and chitinases, capable of hydrolyzing the cell wall components of some pathogenic fungi (Hong et al., 2017; Sun et al., 2019).

In addition, yeasts can produce antimicrobial substances, inducing host resistance, secrete killer

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proteins, important in the process of biological control of filamentous fungi, this process being called killer activity (Grzegorczyk et al., 2017) and have the ability to form biofilms on the inner surface of the fungus. In this sense, the present work aimed to evaluate the β-1,3-glucanase production and killer activity of seven yeasts, as well as to evaluate their efficiency in the biocontrol of green mold in pear orange fruits.

Material and Methods

Two experiments were carried out, both at the Research Support Laboratory Center of the Academic Unit of Garanhuns (CENLAG) belonging to the Universidade Federal do Agreste de Pernambuco-UFAPE. The first experiment consisted of bioprospecting of β-1,3-glucanase-producing fungal isolates. For this, seven yeasts belonging to the fungal bank of biotechnological interest at Micoteca URM, Department of Mycology, Center for Biosciences of the Federal University of Pernambuco, were used, five of them from the genus *Candida (C. buinensis* URM4674, *C. diversa* URM4680, *C. peltata* URM4681, *C. glabrata* URM4682, *C. sake* URM4489), in addition to the yeasts *Clavispora opuntiae* URM7229 and *Debaryomyces hansenii* URM688, from the Brazilian Agricultural Research Corporation-EMBRAPA, grape and wine.

To verify the ability to produce killer activity by the mentioned microorganisms, the methodology proposed by Ceccato-Antonini et al. (2004), using as control the yeast Saccharomyces cerevisiae BRM025642, sensitive to the killer factor, and the yeast *Issachenkia terricola* BRM044692, neutral to the killer factor (positive control), after cultivation in YEPD medium for 24 hours at 28 °C.

The determination of β-1,3-glucanase (GLU, EC 3.2.1.6) production followed the methodology proposed by Havir & Mchale (1987), with some modifications. Yeasts were grown in triplicate in 25 mL of YPD medium (Yeast Extract, Peptone, Dextrose) containing (in w/v): yeast extract 1%, peptone 2% and glucose 2%, kept at 32º C for 12 h, at 90 rpm, pre-inoculum. Subsequently, 1 mL of the yeast suspension was transferred to 125 mL Erlenmeyer flasks containing 50 mL of YPD medium.

The cultivation was carried out at 32º C, for 96 h, at 120 rpm. In order to obtain a crude enzyme extract, free from yeast cells. The metabolic liquid was filtered through filter paper (Whatman n°4) and Millipore® membrane (0.45 µM), and then centrifuged at 5000 g for 10 min at 4ºC, the supernatant being considered the crude enzyme extract.

The determination of the enzymatic activity was determined according to the methodology of Abeles & Foence, 1970, regarding the colorimetric quantification of glucose released from the laminarin substrate. The reaction mixture was incubated at 37 °C for 1h, containing 50 µL of 0.1 M sodium acetate buffer, pH 5.0, 200 µL of the enzyme extract and 250 µL of laminarin (4.0 mg mL-1). After this period, the reaction was stopped by adding the ADNS reagent (3,5-dinitrosalicylic acid) to determine the content of released reducing sugars. For the analysis of reducing sugars, the methodology described by Miller (1959) was used, where an activity unit (U) was defined as 1 µg of reducing sugar released (measured as glucose) from laminarin per minute. Activity was expressed as U mL^{-1}.

The yeast that presented the best production of β-1,3-glucanase, in addition to the ability to produce the killer toxin, was selected to evaluate the increase in enzyme production in an alternative medium composed of soy extract, yeast extract and meat extract, in a 2-level factorial design 23 (-1 and +1), with four replications at the central point, totaling 12 trials.

The second experiment consisted of evaluating the biocontrol mechanism of postharvest green mold in orange pear. To obtain the pathogen that causes green mold, 20 fruits at ripening point were collected at the supply center of the Municipality of Garanhuns, Pernambuco (08º 53'25" S and 36º 29'34" W). fruits with superficial lesions on the epicarp and that visibly showed fungal colonization.

After collection, the selected fruits were washed in running water, then in distilled water, and epicarp fragments measuring approximately 2 cm2 from the middle region of the fruit and as far away from the lesions as possible were collected from each fruit. The collected epicarp fragments were superficially disinfected by means of serial washing (70% alcohol for 30 sec., 2.5% sodium hypochlorite for 3 min, and three consecutive washes with distilled water for 30 sec. each). Subsequently, one per Petri dish containing Potato, Destroxe and Agar (BDA) + chloramphenicol (antibacterial) medium, the fungal isolation occurred according to Souza et al. (2010). After isolation, the fungi were sent for identification at the Micoteca URM, Department of Mycology, Center for Biosciences of the Federal University of Pernambuco. Getting the identification of the same as *Penicillium citrinum*. Thom.

For the pathogenicity test, suspensions of conidia of *P. citrinum* Thom previously isolated in PDA medium were used for seven days at a temperature of 28 °C in photoperiod. The previously selected and healthy fruits were washed and with the aid of a scalpel, lesions of approximately 3 mm deep were made in the fruit

tissue and 20 mm agar disks containing conidia were transferred and fixed in the lesions. The fruits were kept in humid chamber conditions for 15 days to assess growth and fungal infection capacity (Souza et al., 2010).

For biocontrol analysis, fruits were selected at the city's supply center at a stage of commercial maturation and that presented: uniformity of color, size and shape. In the laboratory, the fruits were superficially disinfected (2.5% sodium hypochlorite for 10 min and washed in distilled water for 4 min), then dried with a paper towel. For pathogen infection, the fruits were injured in the epicarp at two equidistant points, in the median region of the fruits, with sterilized needles at each perforation. The perforations were run to a depth of 3 mm, to facilitate infection according to Cunha (2013). Fruit infection was carried out from the fungal colony.

To evaluate the biocontrol mechanism of green mold caused by *P. citrinum* Thom, 60 fruits were used per treatments, divided into three replications of 20 fruits. Seven treatments were evaluated, which consisted of immersion of the fruit in the enzymatic extract of β-1,3-glucanase produced by the yeast *Candida peltata* URM4681 by experiment 1, at a concentration of 50 mg mL-1, in addition, two synthetic fungicides: Imazalil (IMZ) and Thiabendazole (TBZ) were used as treatments at the dosage recommended by the manufacturer and distilled water was used as a control. In addition to three application periods, 24 hours before fruit infection by the pathogen, 24 hours after fruit infection by the pathogen and 24 hours before and after fruit infection by the pathogen. After application of the treatments, the fruits were conditioned at 28° C for 168 h. After the incubation period, evaluations began on the 8th day after fruit infection, which lasted until the 15th day.

The number of fruits infected by the fungus was evaluated, based on the visual incidence of the pathogen and on the deterioration of the fruits in the inoculation region, fruit epicarp. To assess severity, the empirical scale cited by Platania et al. (2012), where the fruits received scores from 1 to 4, according to the levels of infection, with score 1 corresponding to 0% damage; grade 2, from 1 to 35% damage; grade 3, from 45 to 65% damage, and grade 4, from 66 to 100% damage. Damage was considered to be the presence of visible fungal growth on the epicarp of the fruit. Sporulation was measured by counting with the aid of a Neubauer chamber and counter.

Statistical Analysis

A completely randomized design with three replications was used to quantify the enzyme production and later a 23-factorial design with twelve replications per treatment. In the in-situ test, the severity of the disease was evaluated, with each treatment consisting of twenty fruits with four replications per treatment. Data were submitted to analysis of variance (ANOVA) compared with Tukey's test at a 5% probability level ($p < 0.05$), using the SISVAR statistical software (Ferreira, 2011).

Results and Discussion

Killer activity was observed in 85.7% of the yeasts evaluated (**Table 1**), only *Candida sake* URM 4489 did not show an inhibition halo, indicating that it had a sensitive or neutral character to the activity. In the same sense, Madbouly et al. (2020), analyzing the yeasts *Schwanniomyces vanrijiae, Galactomyces geotrichum*, *Pichia kudriavzevii*, found their efficiency in the production of killer activity, in addition to producing β-1,3-glucanase, as one of its metabolites, which can act in synergistic ways in the biocontrol of *Monilinia fructigena* causal agent of brown rot in fruits.

Our results were also similar to those reported by Cunha et al. (2018), who, analyzing the production of this killer toxin by yeast isolates, confirmed the multiple modes of action of the yeast *Candida stellimalicola* against the fungus *Penicillium italicum*, showing killer activity, enzyme production and inhibition of conidia germination of the fungus in powders. Citrus harvest which may explain the control of the phytopathogen.

Regarding enzyme production, all yeasts were able to produce β-1,3-glucanase at different times analyzed (**Table 2**). However, a reduction in enzyme production can be observed with time, except for the yeasts *C. diversa* URM4680 and *C. peltata* URM4681. With the last one showing prominence in enzymatic production at all times analyzed, in addition to presenting killer activity, which is the reason for its selection for the other tests.

Factorial design data showed variation in *C. peltata* URM4681 β-1,3-glucanase activity from 1.071 to 5.422 U mL^{-1} (**Table 3**), indicating the importance and

Table 1. Yeast isolates and the production of Killer activity according to the sensitivity standard *Saccharomyces cerevisiae* BRM 025642 (presence of the blue zone with or without halo) in YEPD-methylene blue mediumat 28 °C and pH 4.5

Yeast Isolates	Killer Activity
Candida sake URM 4489	Blue Zone / No Halo
Candida buinensis URM 4674	Blue Zone / with Halo
Candida diversa URM 4680	Blue Zone / with Halo
Candida peltata URM 4681	Blue Zone / with Halo
Candida glabrata URM 4682	Blue Zone / with Halo
Debaryomyces hansenii URM 6883	Blue Zone / with Halo
Clavispora opuntiae URM 7229	Blue Zone / with Halo

Table 2. Enzymatic activity of β-1,3-glucanase (U mL^{-1} of protein) at different times of evaluation of yeast isolates to the fungal bank of biotechnological interest at Micoteca URM, Department of Mycology, Center for Biosciences of the Federal University of Pernambuco

Yeast Isolates	Time (hours)			
	24 h	48 h	72 h	96 h
	Protein β-1,3-glucanase (U mL^{-1}) enzymatic activity			
Candida sake URM 4489	4.172 ABa	3.663 ABCa	3.608 ABa	1.221 Db
Candida buinensis URM 4674	2.894 BCab	4.012 ABa	2.274 BCb	2.125 CDb
Candida diversa URM 4680	4.118 ABCa	4.741 Aa	4.678 Aa	4.874 Aa
Candida peltata URM 4681	4.387 Aa	4.605 Aa	4.716 Aa	4.906 Aa
Candida glabrata URM 4682	2.779 Cab	3.921 ABa	2.391 BCb	3.796 ABa
Debaryomyces hansenii URM 6883	4.210 ABa	3.071 BCab	2.618 BCb	3.168 BCab
Clavispora opuntiae URM 7229	4.189 ABa	2.427 Cb	2.121 Cb	2.220 CDb

Table 3. Factorial design matrix 23, for β-1,3-glucanase production by *Candida Peltata* URM 4681

Rehearsal	Extract from yeast (g)	Extract of soy (g)	Extract of meat (g)	β-1,3-glucanase (U mL^{-1})
1	0.5	2.8	0.1	3.238
2	0.25	2.8	0.1	5.422
3	0.5	1.4	0.1	2.002
4	0.25	1.4	0.1	1.077
5	0.5	2.8	0.2	3.709
6	0.25	2.8	0.2	3.703
7	0.5	1.4	0.2	2.106
8	0.25	1.4	0.2	2.181
9	0.375	2.1	0.15	2.731
10	0.375	2.1	0.15	4.030
11	0.375	2.1	0.15	3.250

need to employ experimental designs to obtain higher yields in production of the enzyme. The difference between the assay with the lowest activity and the highest activity, assay 4 and 2, respectively, showed a 4.351-fold increase in the production of β-1,3-glucanase. Thus, the composition of assay 2 parameters may provide better stimuli/conditions for the expression and consequently the production of β-1,3-glucanase by *C. Peltata* URM4681, as shown in Table 3.

The results evaluated by analysis of variance (ANOVA), **Table 4**, revealed that regression and lack of adjustment were not statistically significant. The adjusted model was measured by the coefficient of determination (R2) which presented a value of 0.772 and R^{2adj} of 0.499. Indicating that 77.2% of the total variation of residual activity was justified by the adjusted model.

On the other hand, it was found that the soy extract was statistically significant, presenting a positive effect, **Table 5**, indicating that increasing the concentration of soy extract from 1.4g to 2.8g leads to an increase in the production of β-1, 3-glucanase. Thus, *C. Peltata* URM4681 underwent fermentation using 10 g L-1 of yeast extract, 112 g L^{-1} of soy extract and 4 g L^{-1} of meat extract.

The biocontrol of plant diseases consists of the use of functional biomolecules for biological control. These biomolecules are enzymes produced by microorganisms that have an effect on pathogens and can be used as alternative biofungicides to synthetic fungicides since they are multifunctional, biodegradable, non-persistent in the environment and have a low production cost. Liu et al. (2019), like the filtrates produced by yeasts.

In our work, the number of fruits infected by *P. citrinum* Thom was evaluated, based on the visual incidence of the pathogen and on the deterioration of the fruits over the days in the region of inoculation, in the

Table 4. Analysis of variance (ANOVA) for β-1,3-glucanase production by *Candida Peltata* URM 4681 according to a 23-factorial design with four central points

	SQ*	DF*	MS*	F-*value*	*p-value*
Regression	11.775	6	11.77523	27.978	2.556
Residual	3.466	5	1.52267		
Lack of adjustment	2.203	2	1.101802	2.617	0.219
Pure error	1.262	3	0.420872		
Total	15.241	11			

*SQ: sum of squares; GL: degrees of freedom; QM: Mean square.

Table 5. Estimation of the effects of variables on β-1,3-glucanase production by *Candida Peltata* URM 4681 according to a 23 factorial design with four central points

Variables	Effects	t	p-Value
(1) Yeast extract	-0.331	-0.72336	0.522 ns
(2) Soy extract	2.176	4.74545	0.018 s
(3) Meat extract	-0.009	-0.02161	0.984 ns
1 for 2	-0.757	-1.64980	0.198 ns
1 for 3	0.297	0.64903	0.563 ns
2 for 3	-0.614	-1.33868	0.273 ns

flavedo of the fruits. The *in situ* tests showed a reduction in the number of infected fruits, treated with the enzymatic extract of β-1,3-glucanase, by the yeast *Candida peltata* URM4681, when applied in a preventive and curative way of the disease, being on average 78.5% when compared to the control treatment. However, when using the IMZ and TBZ fungicides, the control averaged 98.7% when compared to the control treatment, as shown in **Table 6.**

As for sporulation, we observed that the treatment that presented the lowest averages were the fungicides IMZ and TBZ respectively, with an average of 5.4 spores found 24 hours before and 24 hours after inoculation of the pathogen. However, the same did not differ statistically at 5% of significance from the treatment with β-1,3-glucanase enzymatic extract, which showed an average of 8.4 spores.

In the same sense, Wang et al. (2020) found that the yeast *Metschnikowia citriensis* efficiently controlled the development of citrus acid rot caused by *Geotrichum citri-aurantii* and significantly inhibited mycelial growth and spore germination, suggesting that the yeast could be used as a potential alternative to fungicides. synthetic agents in the control of pathogens in postharvest of citrus fruits.

In our work, the greatest statistical differences were found when the treatments were compared with the control, which presented the highest spore means, as shown in **Table 7**.

Regarding the severity of the disease, we observed that the fruits treated with the enzymatic extract of β-1,3-glucanase, constituted by the yeast *Candida peltata* URM4681, presented damage of an average of 7.57% when we evaluated the applications 24 hours before and 24 hours after inoculation of the pathogen, which represents the scale of Platania et al. (2012) a level 2 damage (from 1 to 37% of damage), with a reduction in the incidence of the disease and in the diameter of the lesions, not differing statistically from the IMZ and TBZ fungicides, whose treatments also showed fruits with level damage. 2 based on the same scale, both preventively and curatively (**Table 8**).

As in our studies, Ferraz et al. (2016), when

Table 6. Number of fruits infected by green mold, after preventive and curative treatment consisting of β-1,3-glucanase, Imazalil fungicides, Thiabendazole and the control

Treatments	Days after inoculation of the pathogen inoculation							
	24 hours before pathogen inoculation							
	8°	9°	10°	11°	12°	13°	14°	15°
β-1,3-glucanase	0	0	1.6	8.3	13.3	20	23.3	26.6
Imazalil	0	0	0	5	8.3	13.3	15	16.6
Tiabendazol	0	0	0	1.6	5	8.3	11.6	11.6
Control	5	21.6	43	66.6	66.6	80	86.6	90
	24 hours after pathogen inoculation							
β-1,3-glucanase	0	0	1.6	6.6	13.3	20	23.3	26.6
Imazalil	0	0	0	3.3	6.6	11.6	15	15
Tiabendazol	0	0	1.6	5	8.3	11.6	13.3	15
Control	1.6	16.6	31.6	45	55	70	80	83.3
	24 hours before and 24 hours after inoculation of the pathogen							
β-1,3-glucanase	0	0	0	3.3	6.6	11.6	13.3	13.3
Imazalil	0	0	1.6	3.3	6.6	8.3	8.3	8.3
Tiabendazol	0	0	0	3.3	6.6	10	10	10
Control	8.3	23.3	28.3	48.3	63.3	76.6	85	90

24 hours before pathogen inoculation

Table 7. Green mold sporulation in Pera orange fruits, after preventive and curative treatment consisting of β-1,3-glucanase, Imazalil, Thiabendazole and control

Treatments	Days after inoculation of the pathogen inoculation							
	24 hours before pathogen inoculation							
	8°	9°	10°	11°	12°	13°	14°	15°
β-1,3-glucanase	0	0	13.4	15.7	19.7	20.5	22.5	24.8
Imazalil	0	0	0	4.8	6.2	7.8	10.5	12
Tiabendazol	0	0	0	7.8	6.8	8.2	9.4	15.4
Control	63.8	66.8	73.7	76.2	81.8	85.4	90	93.4
	24 hours after pathogen inoculation							
β-1,3-glucanase	0	0	9.2	11.2	13.4	14.6	16.6	18.2
Imazalil	0	0	0	3.8	4.8	6.6	8	10
Tiabendazol	0	0	3.4	5.4	7.2	8.8	11.2	13.2
Control	53.4	55.2	67.8	73.2	78.6	80.8	84.5	87.2
	24 hours before and 24 hours after inoculation of the pathogen							
β-1,3-glucanase	0	0	0	9,4	10,5	12.2	14	14.5
Imazalil	0	0	0.5	2	4	5.4	7.4	8.8
Tiabendazol	0	0	0	2.2	3.8	4.5	6	8.2
Control	66.5	68	69.2	71.2	73.4	76.5	78	99.2

Table 8. Severity of Pera orange fruits with symptoms of green mold, after preventive and curative treatment consisting of β-1,3-glucanase, commercial doses of Imazalil, Thiabendazole and control fungicides

Treatments	Days after inoculation of the pathogen inoculation							
	24 hours before pathogen inoculation							
	8 °	9 °	10 °	11 °	12 °	13 °	14 °	15 °
β-1,3-glucanase	0	0	0.2	0.3	0.4	0.5	0.6	0.7
Imazalil	0	0	0	0,1	0.2	0.25	0.3	0.4
Tiabendazol	0	0	0	0,1	0.15	0.25	0.3	0.35
Control	0.4	0.65	1.4	1.7	2	2.2	2.4	2.6
	24 hours after pathogen inoculation							
β-1,3-glucanase	0	0	0.25	0.3	0.4	0.45	0.55	0.6
Imazalil	0	0	0	0.2	0.3	0.35	0.4	0.45
Tiabendazol	0	0	0.05	0.05	0.05	0.05	0.05	0.1
Control	0.25	0.45	1.2	1.45	1.7	1.85	2.15	2.3
	24 hours before and 24 hours after inoculation of the pathogen							
β-1,3-glucanase	0	0	0	0.5	0.7	0.8	0.95	0.6
Imazalil	0	0	0.1	0.2	0.3	0.4	0,5	0.55
Tiabendazol	0	0	0	0.25	0.35	0.45	0.55	0.55
Control	0.35	0.4	0.95	1.35	1.6	1.9	2.15	2.3

24 hours after pathogen inoculation

studying eight yeast isolates, found that isolates ACB-K1- *Saccharomyces cerevisiae*, ACBL-23- *Rhodotorula minuta*, ACBL-44- *Candida azyma* and ACBL-77- *Aureobasidium pullulans* showed potential for the control of sour rot in citrus fruits, both preventively and curatively. Table 9 shows the application data regarding the number of infected fruits, severity and sporulation of Pera orange fruits during the 15 days of evaluation applied in a preventive and curative way.

In short, our results suggest the development of a bioproduct against the green mold of citrus fruits, with a view to increasing the storage time of the fruits and reducing the doses of pesticides in the culture, which represents a lower risk to human health and of the environment.

Conclusions

Candida peltata URM4681 showed good enzymatic activity of β-1,3-glucanase (U mL^{-1} of protein) and the ability to produce the killer toxin, being efficient in biological control against the green mold caused by P. citrinum Thom and compared to the fungicides Imazalil (IMZ) and Thiabendazole (TBZ), which makes this microorganism a good candidate for the development of innovative products in the biological control of fungal diseases in citrus post-harvest.

This is the first report of *Candida buinensis* URM4674, *Candida buinensis* URM4674, *Candida diverse* URM4680, *Candida peltata* URM4681, *Candida glabrata* URM4682, *Clavispora opuntiae* URM7229 and *Debaryomyces hansenii* URM6883, showed production of killer activity, which represents an important finding within the analysis of biocontrolling microorganisms of fungal pathogens.

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