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Selection and evaluation of phyllosphere yeasts as biocontrol agents against grey mould of tomato

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Abstract

Phyllosphere yeasts antagonistic to the infective activity of *Botrytis cinerea* were isolated from leaves of greenhouse-grown tomatoes and evaluated in a detached leaf assay for their ability to suppress grey mould. Nine of 30 recovered yeast isolates were found to reduce a disease index by >90% when compared to an untreated control. In greenhouse experiments, the yeast isolate *Rhodotorula glutinis* Y-44 was the most efficient in controlling grey mould of tomato plants. In further experiments in greenhouse-grown tomato plants the effectiveness of *R. glutinis* Y-44 was compared with two commercial fungicides. It was demonstrated that *R. glutinis* Y-44 was as effective as fungicides in controlling the pathogen. Moreover, the population of *R. glutinis* Y-44 was monitored for 8 weeks after application on tomato plants. The isolate successfully colonized the plant surface, although the population decreased by 10-fold 8 weeks after application. Since *B. cinerea* is also a major post-harvest pathogen for tomato fruits, the ability of *R. glutinis* Y-44, to protect artificially infected wounded tomato fruits was also tested. It was shown that *R. glutinis* Y-44 was able to reduce by 50% the percentage of infected wounds compared to the untreated controls.

Introduction

Botrytis cinerea is a destructive pathogen of greenhouse-grown tomatoes (*Lycopersicon esculentum*) and a major post-harvest pathogen of tomato fruits which are highly susceptible to fungal rot during storage. Control of grey mould caused by *B. cinerea*, is often difficult and costly. In heated greenhouses development of grey mould on foliage and fruits can be reduced by combined heating and ventilation to reduce relative humidity (Winspear et al., 1970; Yunis et al., 1990); however, this needs a high energy input and in general terms it is ineffective against direct infection from pruning and harvesting wounds on the stem (Dik and Elad, 1999). There are several fungicides against grey mould; fludioxonil + cyprodinil and fenhexamid are among the most recent, but a characteristic feature of *B. cinerea* is that it frequently becomes resistant to chemical fungicides (Katan, 1982; Katan et al., 1989; Ziogas et al., 2003; 2005). Moreover, there is no registered post-harvest fungicide for control of grey mould in tomatoes and pressure of the public is increasing to limit the use of fungicides. Thus, there is a need to develop alternative control methods.

Biological control is an alternative option to reduce *Botrytis* infection and has been shown to be

effective in many crops in greenhouse and storage conditions (Karabulut and Baykal, 2003). Dik et al. (1999) reported that application of yeast isolates belonging to the species *Aureobasidium pullulans*, *Cryptococcus luteus*, *C. laurentii*, and *C. albidus* reduced the percentage of disease incidence and sporulation of *B. cinerea* in greenhouse-grown tomato plants. Moreover, certain isolates of *Trichoderma harzianum* and *Cladosporium cladosporioides* were effective in reducing the percentage of grey mould infection on stem wounds of tomato plants (Eden et al., 1996a). In addition, the potential of several yeast isolates has been evaluated as biocontrol agents (BCAs) against grey mould infection of stored tomatoes. Schena et al. (1999) investigated the efficacy of *Aureobasidium pullulans* isolates against *Botrytis* infection on stored cherry tomato fruits. They demonstrated the reduction in the percentage of decay on the yeast treated tomato fruits compared to the untreated control fruits.

The main objectives of this work were to (i) isolate yeasts from the phyllosphere of tomato leaves and create a collection of antagonistic yeasts against *B. cinerea*, using a novel detached leaf test, (ii) investigate their effectiveness against *B. cinerea* in tomato plants under controlled growth chamber conditions, (iii) evaluate the biocontrol activity of the most efficient isolate, *Rhodotorula glutinis* Y-44, against *B. cinerea* under greenhouse conditions in comparison with commercial fungicides, along with its efficiency in colonizing the phylloplane of tomato plants and (iv) determine the ability of *R. glutinis* Y-44 to control post-harvest grey mould on wounded tomato fruits.

Materials and methods

Isolation of antagonistic yeasts

Yeasts were isolated from leaves of 8–10 week-old greenhouse-grown tomatoes originating from three different tomato-producing regions of Greece. In two of these regions (Tirintha and Salamina) tomato plants are organically grown, while in the third region (Preveza County), chemical treatments are extensively used.

Tomato leaves were transferred shortly after collection to the laboratory and were processed on the same day. They were placed in 100 ml sterile distilled water (SDW) containing 0.02% Tween-20 and were shaken for 10 min at 150 rpm at room temperature.

Then, 10-fold dilutions were prepared and 500 μl of each dilution were plated in Petri dishes containing yeast malt agar (YMA, containing 3 g yeast extract, 3 g malt extract, 5 g bactopeptone, 10 g glucose and 15 g agar (Oxoid) l^{-1}). Petri dishes were incubated for 3 days at 25 °C. The isolated yeast colonies were transferred to YMA and stored at 4 °C.

Preparation of inoculum

Yeasts were grown in liquid culture of yeast and malt extract (YM, containing 3 g yeast extract (Difco), 3 g malt extract (Difco), 5 g bactopeptone (Difco) and 10 g glucose (Fluka) l^{-1}) in an orbital incubator at 150 rpm for 2 days at 25 °C. Yeast suspensions were centrifuged at 9000 rpm for 10 min, and resuspended in SDW containing 0.01% Agral 90 (Syngenta).

Botrytis cinerea was grown for 7 days on potato dextrose agar (PDA) at 25 °C, with a 12/12 h day/night regime. Conidia were washed from the agar with SDW containing 0.02% Tween-20 and filtered through a 70 μm filter.

Plant material

Unless otherwise stated, the tomato plants were grown in $9 \times 9 \times 10$ cm^3 pots containing sterile soil. The tomato plants were held at 20 °C, with a 12/12 h day/night regime and approximately 80% relative humidity (RH). The plants were fertilized with 12–4–6% N–P–K (10 ml per plant) every second week. No pesticides were applied.

Evaluation of antagonistic activity of the isolated yeasts against Botrytis cinerea *in a detached leaf test*

A collection of 30 yeast isolates, 10 from each sampling region, was used for the evaluation of their antagonistic activity against *B. cinerea* in a detached leaf test. Lateral tomato leaflets from young leaves of 8–10 week-old (fourth truss) plants cv. Early Pack (selected because of the flat shape of leaflets suitable for Petri dish experiments) were detached, surface-sterilised with 0.5%

sodium hypochlorite for 5 min, rinsed three times in SDW and placed in Petri dishes (one leaflet per Petri dish) containing sucrose, mannitol and agar (SMA, containing 10 g sucrose, 20 g D-mannitol and 5 g agar (Oxoid) l^{-1}) with tetracycline at 25 μg ml^{-1}. Petri dishes were incubated at 20 °C, with a 12/12 h day/night regime. After 1 day, 500 μl at a concentration of 10^7 cfu ml^{-1} of the selected yeast isolates containing 0.01% Agral 90 were pipetted to the leaves. Control leaflets were inoculated with SDW containing 0.01% Agral 90. One day later, each leaflet was wounded at three locations. Wounds were spot-inoculated with 25 μl conidial suspension (10^5 conidia ml^{-1}) of *B. cinerea*. The experiment was repeated three times with 15 replicates per treatment.

Disease development on each leaflet was rated 8 days after *B. cinerea* inoculation by using the following classes: 4, >15 mm diam of rot (20 mm maximum diam of rot); 3, 11–15 mm diam of rot; 2, 6–10 mm diam of rot; 1, 1–5 mm diam of rot; and 0 = no rot. Disease index was calculated as the mean from the disease ratings by the formula:

$$\text{Disease index} = \sum(\text{class no.} \times \text{no. of leaflets in the class})/\text{total no. of leaflets.}$$

Whole plant evaluation of disease suppression under controlled growth chamber conditions

Further evaluation of the yeast isolates that showed disease index <1 was performed with tomato plants cv. Noa (selected because it is a commercial variety) under controlled growth chamber conditions. For this purpose, 8–10 week-old (fourth truss) tomato plants were wounded by detaching two leaves. Subsequently, the tomato plants were sprayed to run-off with a suspension of the selected yeast isolates (10^7 cfu ml^{-1}) containing 0.01% Agral 90. Control plants were sprayed with SDW containing 0.01% Agral 90. After 1 day, the wounds were inoculated with 5 μl of *B. cinerea* spore suspension of 10^5 spores ml^{-1}.

The tomato plants were held in a growth chamber at 15 °C (Eden et al., 1996b reported that maximum infection at terminal stem wounds is observed at 15 °C), 95% RH, with a 12/12 h day/night regime. The percentage of infected wounds was recorded 15 days after *B. cinerea* inoculation. The experiment was repeated three times with ten replicates per treatment.

Comparison of the yeast isolate R. glutinis *Y-44 with fungicides under greenhouse conditions*

The activity of the most efficient yeast isolate in the previous screening experiments yeast isolate *R. glutinis* Y-44 (the identification of the isolate was done by the test system BCCM/Allev as it is described in Robert et al. (1994; 1997), carried out by The Belgian Coordinated Collections of Microorganisms BCCM/MUCL) was compared with the activity of two commercial fungicides: fludioxonil + cyprodinil and fenhexamid. A yeast suspension at a concentration of 10^7 cfu ml^{-1} and the fungicides at label application rate were sprayed onto 4–6 week old (third truss) tomato plants cv. Noa. Control plants were sprayed with SDW. After 1 day, the plants were sprayed with a suspension of *B. cinerea* at a concentration of 10^5 spores ml^{-1} to run-off.

Ten days after the first application, the yeast isolate *R. glutinis* Y-44 and the fungicides were applied again at the same concentration and 1 day later the plants were reinoculated by the pathogen as in the first application. Reinoculation ensured availability of the pathogen during the experimental period. Disease severity, expressed as percentage of diseased leaves over the total number of leaves per plant was recorded at 10, 20 and 30 days after *B. cinerea* reinoculation. The experiment was repeated three times with 10 replicates per treatment.

Population dynamics

The yeast isolate *R. glutinis* Y-44 was evaluated as epiphytic colonizer of tomato leaves under greenhouse conditions. Spontaneous mutants resistant to 100 μg ml^{-1} benomyl were selected for *R. glutinis* Y-44. Yeast suspension of benomyl-resistant mutants of the strain *R. glutinis* Y-44 was applied to 8–10 week-old (fourth truss) tomato plants cv. Noa at a concentration of 10^7 cfu ml^{-1} to run-off.

Epiphytic population was recorded weekly for 8 weeks after application. To estimate epiphytic populations, five pieces of 1 cm^2 of leaf area were collected per plant and shaken for 30 min in SDW

containing 0.02% Tween-20 and the suspension was plated onto YMA medium containing 100 μg ml^{-1} benomyl. The plates were incubated at 25 °C for 3 days. The experiment was repeated three times with ten replicates per experiment.

Evaluation of disease suppression under commercial greenhouse conditions

The activity of the yeast isolate *R. glutinis* Y-44 was compared with the activity of the two commercial fungicides: fludioxonil + cyprodinil and fenhexamid under commercial greenhouse conditions. Yeast suspension at a concentration of 10^7 cfu ml^{-1} and the fungicides at label application rate were sprayed on to 4–6 week-old (third truss) tomato plants cv. Noa grown in sandy loam soil. Control plants were sprayed with H_2O. Each treatment sprayed to run-off. After 2 weeks, *R. glutinis* Y-44 and the fungicides were applied again at the same concentration. Disease incidence, expressed as percentage of infected plants over the total number of plants per treatment was recorded at 15, 30 and 45 days after the second application of the fungicides and the yeast isolate.

The experiment was established in three experimental plots. Each plot consisted of eight *R. glutinis* Y-44, Switch, Teldor and untreated control rows with 50 plants per row, a total of 1,600 plants. The plants were fertilized with 20–20–20% N–P–K (500 ml per plant) every week.

Postharvest biocontrol efficacy of the yeast isolate R. glutinis *Y-44*

Tomato fruits were surface-sterilised in 0.5% sodium hypochlorite for 5 min, rinsed three times in SDW and wounded at two sites with a dissecting needle (3 mm deep, 3 mm diam). The fruits were dipped for 30 s in a suspension of 10^7 cfu ml^{-1} of the yeast isolate *R. glutinis* Y-44 containing 0.01% Agral 90. Control fruits were dipped in SDW containing 0.01% Agral 90. After 1 day, the wounds were inoculated with 25 μl of 10^5 conidia ml^{-1} *B. cinerea*. The percentage of infected wounds was recorded for 11 days after inoculation. The fruits were held at 15 °C, 95% RH, with a 12/12 h day/night regime. The experiment was repeated three times with 10 replicates per treatment.

Statistics

In order to evaluate the data of the experiments, statistical analysis of variance (ANOVA) for each treatment was performed. The results of the multivariate analysis of the data, when a significant ($P \leq 0.05$) F-test was obtained for treatments, were subjected to means separation by Duncan's multiple range test. If necessary to stabilize variance, the data were transformed before analysis (Box Cox Y transformation, Box and Cox, 1964)

Results

Evaluation of antagonistic activity of the isolated yeasts against Botrytis cinerea *in a detached leaf test*

A total of 30 yeast isolates were isolated from the tomato leaves and their antagonistic activity against *B. cinerea* was evaluated in a detached leaf test. The isolates Y-5, -18, -23, -25, -27, -101, -73, -90 originating from organically-grown tomato plants (Salamina and Tirintha regions) and *R. glutinis* Y-44 originating from chemically treated plants (Preveza County), were the most efficient in reducing the disease index (< 1) compared to the untreated control (Table 1, Figure 1).

Whole plant evaluation of disease suppression under controlled growth chamber conditions

All the tested isolates were effective in reducing the percentage of infected wounds on the tomato plants compared to the untreated control (Table 2). The yeast isolate *R. glutinis* Y-44 was the most effective; therefore, it was selected for further experimentation.

Comparison of the yeast isolate R. glutinis *Y-44 with fungicides under greenhouse conditions*

Disease severity was statistically reduced by the two fungicides and the yeast isolate *R. glutinis* Y-44 compared to the untreated control treatment with no statistically significant difference between the fungicides and isolate *R. glutinis* Y-44 (Figure 2).

Table 1. Disease index[a] 8 days after challenge inoculation on detached tomato leaflets treated with antagonistic yeast isolates before wounding and challenging with *Botrytis cinerea*

Region of origin	Yeast isolates	Disease index[a]
Tirintha[b]	Y-64	3.6 ± 0.2 ghi
	Y-67	3.6 ± 0.1 ghi
	Y-73	0.2 ± 0.1 a
	Y-74	3.1 ± 0.2 efg
	Y-76	1.9 ± 0.2 c
	Y-79	3.0 ± 0.1 def
	Y-81	2.7 ± 0.2 d
	Y-90	0.1 ± 0.1 a
	Y-94	3.3 ± 0.1 fg
	Y-101	0.2 ± 0.1 a
Salamina[b]	Y-5	0.3 ± 0.1 a
	Y-12	1.3 ± 0.1 b
	Y-15	3.1 ± 0.3 def
	Y-17	3.8 ± 0.1 hi
	Y-18	0.3 ± 0.1 a
	Y-20	2.8 ± 0.2 def
	Y-23	0.2 ± 0.1 a
	Y-24	2.8 ± 0.2 de
	Y-25	0.2 ± 0.1 a
	Y-27	0.3 ± 0.1 a
Preveza[c]	Y-30	2.8 ± 0.2 def
	Y-37	2.9 ± 0.1 def
	Y-41	3.3 ± 0.2 fgh
	Y-44	0.1 ± 0.1 a
	Y-46	1.1 ± 0.1 b
	Y-50	2.1 ± 0.2 c
	Y-53	3.3 ± 0.2 fg
	Y-56	3.3 ± 0.2 fgh
	Y-59	3.1 ± 0.2 efg
	Y-60	2.8 ± 0.1 def
	Control	4.0 ± 0.0 i

[a]Disease index was calculated as the mean from the disease ratings by the formula: . Values presented are means (±SE) of 15 leaflets per treatment with three replications. Values followed by different letters are statistically different according to Duncan's multiple range test ($P \leq 0.05$).
[b]Yeasts were isolated from tomato plants originating from regions where organic farming is practiced.
[c]Yeasts were isolated from tomato plants originating from a region where chemical treatments are extensively used.

Population dynamics

Rhodotorula glutinis Y-44 cells decreased steadily to about one tenth during the 8 weeks of monitoring, from 1.5×10^5 to 10^4 cfu cm^{-2} (Figure 3).

Evaluation of disease suppression under commercial greenhouse conditions

The two fungicides and the yeast isolate *R. glutinis* Y-44 significantly delayed and eventually reduced symptom expression caused by *B. cinerea* in the commercial greenhouse experiment (Figure 4). At 45 days after the second application of the two fungicides and the isolate Y-44, disease incidence was reduced by 40–55% in treated compared to the untreated control plants.

Postharvest biocontrol efficacy of the yeast isolate R. glutinis *Y-44*

The isolate *R. glutinis* Y-44 significantly reduced ($P \leq 0.05$) the number of infected wounds on fruit compared to the untreated control (Figure 5). At day 11 after pathogen inoculation, over 90% of the wounds on the control tomato fruits were infected while *R. glutinis* Y-44 isolate reduced disease incidence by 52%.

Discussion

The experimental data presented in this paper demonstrate the occurrence of phyllosphere yeast isolates able to control grey mould on tomato plants. Nine out of a total of 30 yeasts showed significant biocontrol activity against *B. cinerea*, reducing the disease index to <1 in a detached leaf test. Although 8 out of the 9 most efficient yeast isolates originated from organically-grown tomatoes suggesting that organically-grown plants could be an attractive pool for selecting BCAs, the most efficient strain was *R. glutinis* Y-44 isolated from tomato plants treated with fungicides. Several studies have reported the activity of *R. glutinis* isolates against grey mould infection in pears, apples, strawberries, kiwifruits and table grapes (Lima et al., 1998; Benbow and Sugar, 1999).

In further experiments the effectiveness of the isolate *R. glutinis* Y-44 was compared with the commercial fungicides fludioxonil + cyprodinil and fenhexamid. It was demonstrated that isolate *R. glutinis* Y-44 was as effective as the fungicides in controlling grey mould in commercial greenhouse-grown tomato plants. Therefore, *R. glutinis* Y-44 isolate has the potential to substitute for the chemical fungicides, providing an alternative biological control method against *B. cinerea*.

BCAs differ from chemicals in that, in order to be effective, they must survive on the plant surface and consequently remain active against target

Figure 1. The antagonistic activity of 30 yeast isolates against *B. cinerea* was tested on wound-inoculated tomato leaflets. Arrows point to *B. cinerea* hyphae on control and Y-56 treated tomato leaflets, while on the Y-23, -18, -25 and -44 treated leaflets there is no visible hyphal growth of the pathogen 5 days after inoculation.

pathogens during periods favourable to plant infection (Lo et al., 1998). Monitoring of the yeast isolate *R. glutinis* Y-44 on greenhouse-grown tomato plants showed that *R. glutinis* Y-44 survived on the surface of tomato plants during the 8 weeks recording period (8 weeks after application the population density was 10^4 cfu cm^{-2}). Even if the population was steadily declining, it could be suggested, considering the results of the greenhouse experiments, that 8 weeks is a sufficient period of time for the yeast isolate to control grey mould infection. Dik and Elad (1999) have also reported that yeast populations on cucumber stems ranging from 5×10^3 to 1×10^6 cfu cm^{-2} were effective against *B. cinerea*, irrespective of the decline in the population density over time.

Furthermore, it is known that *B. cinerea* belongs to the post-harvest pathogens of tomato fruits. Most post-harvest diseases are initiated at wounds that occur during harvest or packing. In several

Table 2. Whole plant evaluation of the most effective yeast isolates from the detached leaflet test, in controlling rot caused by *Botrytis cinerea* on wounded tomato plants

Yeast isolates	% of infected wounds[a]
Y-44	1.5 ± 1.5 a
Y-18	4.5 ± 2.5 ab
Y-101	5.8 ± 3.8 ab
Y-23	5.8 ± 3.8 ab
Y-25	5.8 ± 3.8 ab
Y-27	7.3 ± 3.8 ab
Y-73	7.5 ± 5.4 ab
Y-5	13.6 ± 2.6 bc
Y-90	22.6 ± 2.6 c
Control	53.6 ± 3.1 d

[a]Values presented are means (±SE) of 10 plants per treatment with three replications. Values followed by different letters are statistically different according to Duncan's multiple range test ($P \leq 0.05$).

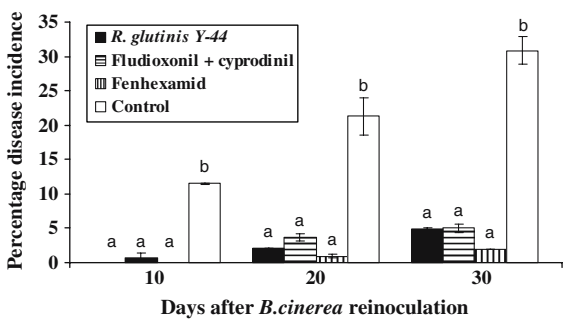

Figure 2. Activity of the yeast isolate *R. glutinis* Y-44 against *B. cinerea* on tomato plants compared with two commercial fungicides: fludioxonil + cyprodinil, fenhexamid and H_2O as control under greenhouse conditions. Means of ten plants per treatment with three replications. Vertical bars indicate standard errors. For each day, columns with different letters are statistically different according to Duncan's multiple range test at $P \leq 0.05$.

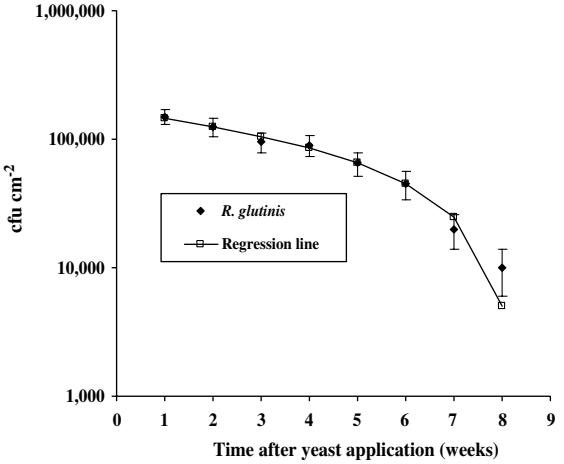

Figure 3. Development of phyllosphere yeast population of *R. glutinis* Y-44, under greenhouse conditions. The relationship of the *R. glutinis* Y-44 population to time is described by the equation: $Y = 16.5 \times 10^4 - 2 \times 10^4(X)$, $r^2 = 0.98$. Means of ten plants with three replications. Vertical bars indicate standard errors.

studies, the ability of *R. glutinis* strains to colonize wound sites and compete with pathogens has been reported (Arras et al., 1996; Chand-Goyal and Spotts, 1997; Leibinger et al., 1997; Sugar and Spotts, 1999). The data from the present study show the ability of the *R. glutinis* Y-44 isolate, to protect wounded tomato fruits against *B. cinerea*,

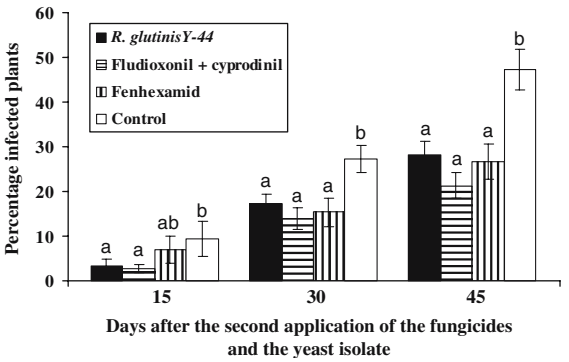

Figure 4. The activity of the yeast isolate *R. glutinis* Y-44 against *B. cinerea* on tomato plants compared with two commercial fungicides: fludioxonil + cyprodinil and fenhexamid under commercial greenhouse conditions. Means of the percentages of disease incidence of 400 plants per treatment and each of three experimental plots. Vertical bars indicate standard errors. For each day, columns with different letters are statistically different according to Duncan's multiple range test at $P \leq 0.05$.

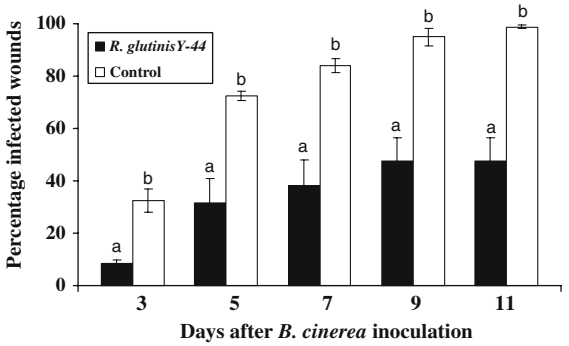

Figure 5. The effectiveness of the yeast isolate *R. glutinis* Y-44 on wounded tomato fruits inoculated by *B. cinerea*. Means of ten tomato fruits per treatment with three replications. Vertical bars indicate standard errors. For each day, columns with different letters are statistically different according to Duncan's multiple range test at $P \leq 0.05$.

up to 50% compared to the untreated control. Thus, this research demonstrates the ability of strain *R. glutinis* Y-44 to prevent pre- and post-harvest grey mould infection on tomatoes.

In conclusion, the development of BCAs as an alternative to fungicides would help to reduce the number of fungicide treatments as part of anti-resistance strategies and in parallel it would provide the farmers with an environmentally-friendly control method. The yeast isolate *R. glutinis* Y-44 could be a helpful tool as it was shown to be as equally effective as commercial fungicides against grey mould infection of greenhouse-grown tomato plants and also decreased the post-harvest disease incidence on tomato fruits. In addition, this research presents a reliable and fast detached leaf test for screening BCAs against *B. cinerea* of tomato plants.

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