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Evaluation of the effectiveness of potential biocontrol yeasts against black sur rot and ochratoxin A occurring under greenhouse and field grape production conditions

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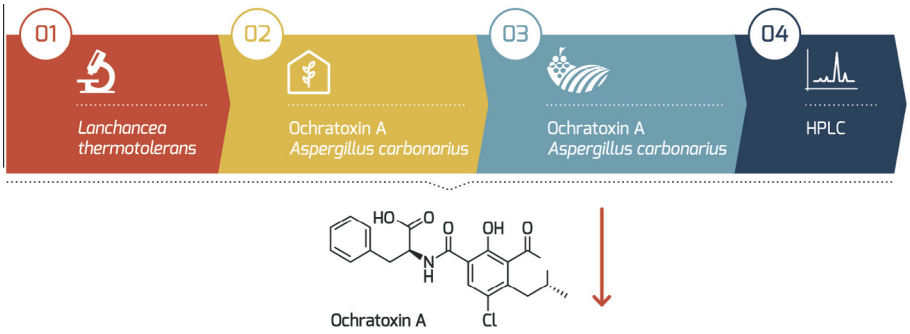
HIGHLIGHTS

- Two *Lanchancea thermotolerans* strains were evaluated as biological control agents.
- The antagonistic yeasts were effective to control ochratoxin A accumulation.
- The antagonistic activity was observed under greenhouse and field conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

The efficacy of two strains of *Lanchancea thermotolerans* in preventing the growth and ochratoxin A (OTA) accumulation of ochratoxigenic fungi under greenhouse and field conditions were evaluated during three consecutive year trials. The data from this study showed that both yeast strains were able to control *Aspergillus* section *Nigri* species ochratoxin A accumulation in wine grapes at harvest stage. The inhibitory effects were dependent on the ochratoxigenic species, yeast strains, and year analyzed. Over all conditions assayed, ochratoxin A accumulation was reduced from 27% to 100%, depending on the conditions evaluated. These results are promising for future development of a bio-pesticide.

1. Introduction

Vitis vinifera L. or commonly grape vine is a perennial plant grown in temperate regions of the world. Grapes are susceptible to fungal diseases that can reduce the quality of the harvested grapes and affect the organoleptic features of wines. Aspergillus

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bunch rot is a preharvest mouldy disease caused by species belonging to *Aspergillus* section *Nigri* (Battilani et al., 2006; Bejaoui et al., 2006; Perrone et al., 2006; Serra et al., 2003; Tjamos et al., 2006; Varga and Kozakiewicz, 2005). *Aspergillus* section *Nigri* are commonly isolated from vineyards and includes species such as *A. carbonarius* and *A. niger* aggregate, responsible for ochratoxin A (OTA) contamination of grapes and wines (Visconti et al., 2008). The presence of this toxin in wines results in a health risk to consumers since OTA is classified as a possible carcinogen to humans (group 2B) by the International Agency for Research on Cancer (IARC, 1993). Based on the available scientific toxicological and exposure data, the European Union has established 2 μg/kg maximum level permitted for OTA in wines, musts, grape juice and 10 μg/kg for raisins (European Commission, 2006).

Aspergillus section *Nigri* species are frequently isolated in Argentinian vineyards showing different incidence and potential of OTA production (Chulze et al., 2006; Magnoli et al., 2004; Ponsone et al., 2007). It was also found that the geographic region was relevant in the isolation frequency of a particular species within *Aspergillus* section *Nigri*, and this frequency was intrinsically related to temperature and humidity conditions recorded in each area evaluated (Chiotta et al., 2009, 2010, 2013). The highest incidence of *A. carbonarius* was observed in the Northern regions of Argentina, such as La Rioja and San Juan, while species within *A. niger* aggregate were isolated in high frequency in all grape growing regions (Chiotta et al., 2013).

The control of bunch rot relay on viticultural management practices, which are frequently ineffective during veraison when rainfall occurs close to harvest. The public concern and the recent legislation over pesticide residues in food, the environmental impact and the resistance to fungicides of plant pathogens has increased the interest in alternative methods for disease control. In addition, prevention of the OTA-producing fungi growth is the most effective strategy for controlling the entry of this mycotoxin in the food and feed chains (Zhang et al., 2007). Among the alternative strategies available, biological control has been proposed as one alternative to reduce the impact of ochratoxigenic species. Among the microorganisms considered as potential biological control agents there are bacteria, yeasts and filamentous fungi. Yeasts are very interesting because they have the ability to colonize plant surfaces or wounds for long periods under dry conditions, (Bleve et al., 2006; Dimakopoulou et al., 2008) their simple nutritional requirements, the capacity to grow in fermenters on inexpensive media, and the ability to survive in a wide range of environmental conditions (Wilson and Wisniewski, 1989).

During the selection of yeasts as biocontrol agents, one aspect to consider is that the selected yeasts must be generally recognized as safe (GRAS) (Banerjee, 2009). There are numerous cases of clinical infections caused by yeasts belonging to *Candida*, *Saccharomyces*, *Rhodotorula*, *Pichia*, *Lachancea*, *Hanseniaspora* and *Yarrowia* genera particularly in immune compromised individuals (García-Martos et al., 1999; Lherm et al., 2002; Diekema et al., 2005; de Llanos Frutos, 2007). Therefore, it is important to evaluate the possible presence of pathogenicity factors of the selected biocontrol yeasts for humans and animals. Among fungal properties frequently associated with pathogenesis, are the ability to grow at high temperatures, pseudohyphal formation, to adhere and invade host cells and to secrete degradative enzymes such as proteinases and phospholipases (Nally et al., 2012, 2013).

In a previous study we have demonstrated that two indigenous yeast strains of *Lanchancea thermotolerans* isolated from grapes were able to control growth and ochratoxin A accumulation by *Aspergillus* section *Nigri* (Ponsone et al., 2011). In addition, we showed that the presence of *L. thermotolerans* RCKT4 and RCKT5 strains can reduce ochratoxin A accumulation by *Penicillium* and *Aspergillus* strains. In addition, the efficacy of *L. thermotolerans* RCKT4 and RCKT5 to diminish OTA accumulation by *Aspergillus* and *Penicillium* strains in culture media has been promising (Ponsone et al., 2013). At present there are few reports using yeast as biocontrol agents to reduce both, growth and ochratoxin A accumulation by *A.* section *Nigri* under greenhouse conditions (Dimakopoulou et al., 2008) and there is a lack of information about *L. thermotolerans* used as biocontrol agent on *A.* section *Nigri* under field trials.

The aims of the present study were -to evaluate the pathogenicity traits of the selected *L. thermotolerans* strains (RCKT4 and RCKT5) proposed as potential biocontrol agents on grapes, -to determine the biocontrol activity of the selected strains to reduce growth of ochratoxigenic *Aspergillus* section *Nigri* and OTA accumulation under greenhouse and field conditions.

2. Materials and methods

2.1. Strains

2.1.1. Antagonist yeast strains

Two antagonistic yeast strains of *L. thermotolerans* RCKT4 and RCKT5 previously isolated from wine grapes in Argentina that showed *in vitro* biocontrol activity reducing growth and ochratoxin A accumulation by *A.* carbonarius and *A. niger* aggregate (Ponsone et al., 2011) were evaluated.

The yeast strains were maintained onto yeast extract peptone dextrose medium (YEPD: yeast extract, 5 g/L; peptone, 5 g/L; dextrose, 40 g/L; agar, 20 g/L). Pure cultures are kept as a glycerol stock at -80% in the culture collection of the Department of Microbiology and Immunology at the Universidad Nacional de Río Cuarto.

2.1.2. Phenotypic traits associated with pathogenicity of *L. thermotolerans* strains

The two selected yeast strains were evaluated for growth at 37 °C and 42 °C, phospholipase and protease production, invasive growth and pseudohyphal formation to assess yeast pathogenicity (Nally et al., 2012, 2013). A human pathogenic yeast *Candida albicans* ATCC10231 was used as a positive control in all pathogenicity assays. All yeast strains were handled according to biosecurity standards of the World Health Organization (WHO, 2004) and National Committee for Clinical Laboratory Standards (NCCLS, 1997). All experiments were done in triplicate.

2.1.3. Aspergillus section Nigri strains

For the greenhouse experiments, *A. carbonarius* strain ASNRC 66, (former RC13I, Ponsone et al., 2011), ochratoxin A producer, isolated from grapes in Argentina was evaluated (Chiotta et al., 2009, 2010). The strain was grown for 7 days on malt extract agar (MEA), which composition included: malt extract, 20 g/L; glucose, 20 g/L; peptone, 1 g/L; agar 20 g/L) at 25 °C. The strain was stored as a glycerol stock at −80 °C in the culture collection of the Department of Microbiology and Immunology at the Universidad Nacional de Río Cuarto.

2.2. Inocula preparation

2.2.1. Yeats biomass production

For the greenhouse assays the yeast strain inocula were obtained by growing the yeast in a bioreactor with a capacity of 5 Lt, using the following culture medium containing (g/L): fermentable sugars, 150.8 (FS, provided by sugar cane molasses); $(NH_4)_2HPO_4$ (DAP), 6.9; yeast extract (YE), 1.0; these medium provide maximum yield and productivity values (Pelinski et al., 2012).

For the field trials, biomass was produced and formulated by BIOFERM® (Austria). The formulated yeasts were used as wettable

powder, from which cell suspensions was prepared at the different inoculum levels (1×10^4 or 1×10^6 cells/mL) for the experiments. The inocula were prepared with sterile distilled water containing a fatty ethoxylate alcohol as adjuvant (0.01%).

The survival of the yeast cells formulated was checked before each application using plate count methodology.

2.2.2. Aspergillus carbonarius inoculum

Aspergillus carbonarius ASNRC66, was grown for 7 days on malt extract agar (MEA) at 25 °C. Conidia (10^4 conidia/mL) were washed from the agar with peptone water (peptone 0.1%) containing 0.01% Tween-20 (Sigma) and the conidia concentration was determined using a Neubauer chamber. This strain was used to perform artificial contamination of the grapes during the greenhouse trial.

2.3. Biocontrol activity of Lanchancea thermotolerans

2.3.1. Greenhouse experiments

The greenhouse evaluation was done using vine plants of Cabernet Sauvignon *cv.* This grape plant variety was chosen because showed hight sensitivity to *Aspergillus carbonarius* infection (Chiotta et al., 2013). The evaluation was done during two vintages: 2010, and 2011.

2.3.2. Field trials

The field trials were carried out in a commercial vineyard, managed under organic conditions, planted with Cabernet Sauvignon *cv.* grapes and with natural occurrence of *A. carbonarius*. The vineyard was located in Chilecito, La Rioja province, Argentina. The trials were done during the 2011, 2012 and 2013 vintages. Data on climatic conditions (mean daily air temperature, relative humidity and rainfall) were collected from a station close to the vineyards.

2.3.3. Treatments and antagonist applications

2.3.3.1. Aspergillus carbonarius artificial contamination. For the greenhouse trial, in order to encourage *Apergillus carbonarius* colonization and ochratoxin A accumulation in the grapes, the grape buches were artificially contaminated with a spore suspension (10^4 conidia mL^{-1}) of ASNRC66 at veraison.

2.3.3.2. Antagonist applications. For both evaluations, greenhouse and field trials, the treatments were as follows: *L. thermotolerans* RCKT4 and RCKT5 strains at two inoculum levels (10^4 and 10^6 cells/ml), applied to healthy and artificially damaged grapes. Each inoculum tested was guided and applied to the grape bunches. For application of antagonistic yeast agents, backpacks were used, the spray was applied in each plant until all the grape bunches were fully wet. The experiments were done in a complete randomized block design including three plants for each treatment, with 4 replicates.

The biocontrol yeasts were applied to the grapes, at veraison stage, in healthy grapes. One month after veraison, the grapes were mechanical damaged with a metal brush in order to favor grapes colonization by *Aspergillus* section *Nigri* along with a second application of the potential biocontrol agents. Control treatments included grapes sprayed with the diluent.

2.4. Aspergillus section Nigri evaluation

Untreated grapes were sampled at ripening stage and the *A.* section *Nigri* frequency was determined. The second and third sampling dates of treated and untreated vine plants were done at one month after veraison and at harvest stage during 2010, 2011, vintages for the greenhouse experiment and 2011, 2012 and 2013 vintages for the field trials.

2.5. Fungal isolation and frequency of A. section Nigri

At each sampling date, from each bunch, ten berries were randomly selected (100 berries per sample), surface disinfected for 1 min in sodium hypochlorite solution (1%), rinsed in sterile distilled water (three times), and 100 berries placed on the surface of a dichloran 18% glycerol agar (DG18) and dichloran-rose bengal-chloramphenicol (DRBC) media. The plates were incubated at 25 °C for 7 days. After the incubation period, the percentage of infected grapes by *Aspergillus* section *Nigri* was determined. (Pitt and Hocking, 1997). *Aspergillus* identification was done according to Klich (2002). To determine the yeast populations, plate count methodology was used. The grape samples were manually blended and diluted 10-1 to 10-3 in peptone water 0.1% (w/v). The dilutions were plated onto WL® (Oxoid) medium supplemented with chloramphenicol 0,0001% (w/v) according to the methodology proposed by Combina et al. (2005). During 2013 vintage plate count methodology was also applied to determine *A.* section *Nigri* frequency.

2.6. OTA accumulation

The OTA content was determined in grapes collected from the greenhouse and field trials at harvest stage The methodology proposed by Visconti et al. (2001) was used. In brief, grapes were diluted with water solution containing PEG (1%) and $NaHCO_3$ (5%), mixed, and filtered to remove particulate matter. Ten mL portion was taken and added to an immunoaffinity column (OchraTest™; Vicam, Digen Ltd, Oxford, UK). The column was washed with 10 ml PBS containing 1% Tween 20 and then with 10 ml double distilled water. OTA was eluted from the column with 1.5 mL of methanol (HPLC grade), at a flow rate of 1–2 drops per second.

2.7. OTA detection and quantification

The HPLC apparatus system was a Hewlett-Packard (Waldbronn, Germany) chromatograph with a loop of 50 µl, equipped with a spectrofluorescence detector (excitation, 333 nm; emission, 460 nm) and a C18 column (150×4.6 mm, 5 µm particle size; Supelcosil LCABZ, Supelco, Bellefonte, PA, USA), connected to a precolumn (20×4.6 mm, 5 µm particle size; Supelguard LC-ABZ, Supelco). The mobile phase was pumped at 1.0 ml min^{-1}, and consisted of an isocratic system as follows: 99:99:2 acetonitrile, water and acetic acid respectively. OTA was quantified on the basis of HPLC fluorometric response compared with OTA standard (purity >99%; Sigma Aldrich Co., St Louis, MO, USA). The lowest limit of detection was 0.05 ng g^{-1} and the quantification limit was 0.1 ng g^{-1} respectively.

2.8. Statistical analysis

Data on fungal and OTA contamination in grapes were analyzed by ANOVA test, followed by Tukey mean separation test ($p < 0.05$) All statistical analyses were carried out using the Software SigmaStat for windows version 3.5 (SPSS, Chicago, USA).

3. Results and discussion

3.1. Phenotypic traits associated with pathogenicity of L. thermotolerans strains

The major objective of the present work was to test two *Lanchancea thermotolerans* strains under greenhouse and field conditions in order to assess their potential ability for biological

Table 1
Phenotypical assay for yeast pathogenicity factors in humans.

Yeasts strains	37 °C	42 °C	Phospholipase	Protease	Invasive growth	Pseudohyphal formation
L. thermotolerans RCKT4	+	–	–	–	–	–
L. thermotolerans RCKT5	+	–	–	–	–	–
C. albicans ATCC10231 (positive control)	+	+	+	+	+	+

Growth at 37 °C and 42 °C: (+) growth; (–) no growth. Enzymatic activity (phospholipases, proteases): (+) activity; (–) no activity. Pseudohyphal formation: (+) present; (–) absent. Invasive growth: (+) present; (–) absent.

control of Aspergillus section Nigri and ochratoxin A production. *Lachancea thermotolerans* RCKT4 and RCKT5 showed good antagonistic activity against *A.* section *Nigri* in a previous study under *in vitro* conditions (Ponsone et al., 2011).

In order to test these strains under greenhouse or field conditions, the first step is to test pathogenicity factors regarding yeast pathogenicity in humans, being growth at temperatures above 37 °C only one of them (Mc Cusker et al., 1994). The possible pathogenicity factors to humans of the selected yeasts were evaluated and the data are showed in Table 1. According to the results obtained, the selected yeast strains showed no pathogenic traits that can be harmful to humans. Both strains were able to grow only at 37 °C but not at 42 °C. Also, non-phospholipase, neither protease activity nor pseudohypal formation were associated to anyone of the *L. thermotolerans* strains evaluated. Phospholipases have only been detected in opportunistic *C. albicans, C. neoformans, C. glabrata, S. cerevisiae, Malassezia furfur* and *Rhodotorula rubra* (Chen et al., 1997; de Llanos et al., 2006; Kantarcioglu and Yücel, 2002). *Saccharomyces cerevisiae* strains isolated from a systemic infection have been reported to produce more pseudohyphal and invasive growth than the food and industrial strains (de Llanos et al., 2006). The switching from normal yeast to hyphal growth has been associated with pathogenesis and virulence in species such as *C. albicans* and in clinical isolates of *S. cerevisiae* (Gognies and Belarbi, 2002). Studies showed that laboratory and industrial *S. cerevisiae* strains, growth range was within 37–42 °C, but only pathogenic isolates were able to grow at 42 °C (de Llanos et al., 2006; Murphy and Kavanagh, 1999). Proteinase secretion is another important pathogenic factor in many *Candida* species and *S. cerevisiae* (de Llanos et al., 2006).

The selected biocontrol yeasts showed one phenotypical traits of pathogenicity to humans, the ability to grow at 37 °C, similar to those found in previous reports, but none of the strains showed more than one of the assayed pathogenic traits. Although the capacity to grow at body temperature seems to be associated with virulence, it does not appear to be the sole determining factor (de Llanos et al., 2006). So that the yeasts were evaluated under greenhouse and field assays.

3.2. Greenhouse trials

Under greenhouse condition both *L. thermotolerans* strains were not entirely able to control *A* section *Nigri* growth in both vintages evaluated, mostly when mechanical damage and artificial inoculation of *A. carbonarius* ASNRC66 where performed. For example, during 2011 vintage, there is significate difference between the control ASNR66 inoculated grapes and the treated with *Lanchancea* strains, but when the damaged was performed, the treatments with RCKT5 10^4 and 10^6 and RCKT4 10^4 (plus ASNRC66 inoculation and mechanical damage) showed significate higher *Aspergillus* section *Nigri* percentage of infection ($p < 0.05$) (Fig. 1). During 2012 vintage the results where similar, even when there was higher percentage of infection of *Aspergillus* section *Nigri* as a general tendency, the treatments with RCKT5 10^6 and RCKT4 10^6 plus ASNRC66 artificial inoculation showed significant higher contamination percentage of *Aspergillus* section *Nigri* than the ASNR66

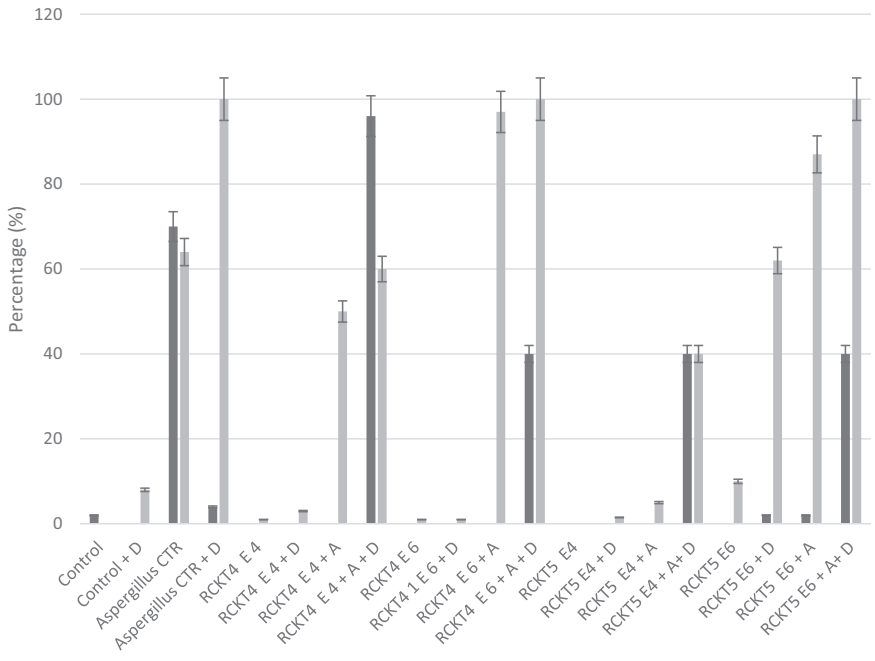

Fig. 1. Dynamic of *Aspergillus* section *Nigri* in a greenhouse trial. Grapes cv Cabernet Sauvignon at harvest stage under different treatments. (D: artificial damage; CTR: control; RCKT4 E4: RCKT4 10^4 cells/mL; RCKT4 E6 RCKT4 10^6 cells/mL; RCKT5 E4: RCKT4 10^4 cells/mL; RCKT5 E6 RCKT4 10^6 cells/mL).

Table 2
Percentage of ochratoxin A reduction under greenhouse conditions, in healthy and artificially damaged grapes.

Vintage Year	Strain	Treatment	Inoculum level	
			10^4	10^6
2011	RCKT4	Healthy	79,5	14,5
		Damaged	ND	ND
		Healthy[a]	51	ND
		Damaged[a]	16	ND
	RCKT5	Healthy	32,5	ND
		Damaged	27,7	41
		Healthy[a]	20	ND
		Damaged[a]	20	10
2012	RCKT4	Healthy	100	100
		Damaged	100	100
		Healthy[a]	97	100
		Damaged[a]	100	96
	RCKT5	Healthy	100	100
		Damaged	100	100
		Healthy[a]	100	98
		Damaged[a]	97	95

[a] Blocks where the grapes were inoculated with *A. carbonarius* ASNRC66. ND ochratoxin A Not Detected, detection limit LD $\leqslant$0.1 ng g^{-1}.

artificially contaminated grapes control ($p < 0.05$). In this case, when mechanical damage was applied, all the yeast treatments where significant effective compared with the artificially ASNRC66 contaminated and damaged control ($p < 0.05$), this result suggest that the yeast strains colonize the wounds preventing in this way *Aspergillus* colonization. Regarding to ochratoxin A accumulation, during 2011 vintage, it was observed that both strains of *L. thermotolerans* were significantly effective to control OTA production by *A. carbonarious* ASNRC66. Both *L. thermotolerans* strains where effective at an inoculum level of 1×10^6 cells/mL ($p < 0.05$). During 2012 vintage both yeast strains at both inoculum levels evaluated were highly effective reducing OTA accumulation in grapes (Table 2) ($p < 0.05$). Even when there is artificial contamination with an ochratoxigenic strain (ASNRC66), there is a significant reduction of ochratoxin A contamination in the biocontrol treated grapes ($p < 0.05$) This result support the theory of a post-transcriptional control mechanism proposed by Ponsone et al., 2013.

3.3. Field trial

On the other hand, in the field trial, during the 2011 vintage there was not natural occurrence of *A.* section *Nigri* species at harvest stage, even more, there was a high yeast colonization of both, treated and untreated grape berries. During this vintage there was not natural occurrence of ochratoxin A in the control grapes, probably due to the absence of *A.* section *Nigri* species. During 2012 vintage, at harvest stage, the isolation frequency of *A.* section *Nigri* was higher than during 2011 vintage, this could be explained due to a rainfall reduction (Figs. 2 and 3). In addition, the total yeast population was similar in treated and untreated grapes. According to Fleet, 2003 the total yeast population in grapes is in the range of 10^4–10^6 UFC/g range, in our study it was observed that the total population was around 10^5 UFC/g in untreated and treated grapes (Fig. 4). It is noticeable that there was a 50–90% reduction in the OTA accumulation both, in healthy and damaged grapes (treated with *L. thermotolerans* RCKT4 10^6 cells/mL) (Table 3). During 2013 vintage, the *A.* section *Nigri* frequency was similar to that observed during 2012 vintage. Although the percentage on OTA content reduction was significant higher than that observed in the previous vintage ($p < 0.05$). The reduction ranged from 60 to 100%, in grapes treated with *L. thermotolerans* RCKT4 at both inoculum levels evaluated (10^4 y 10^6 cells mL^{-1}) and in

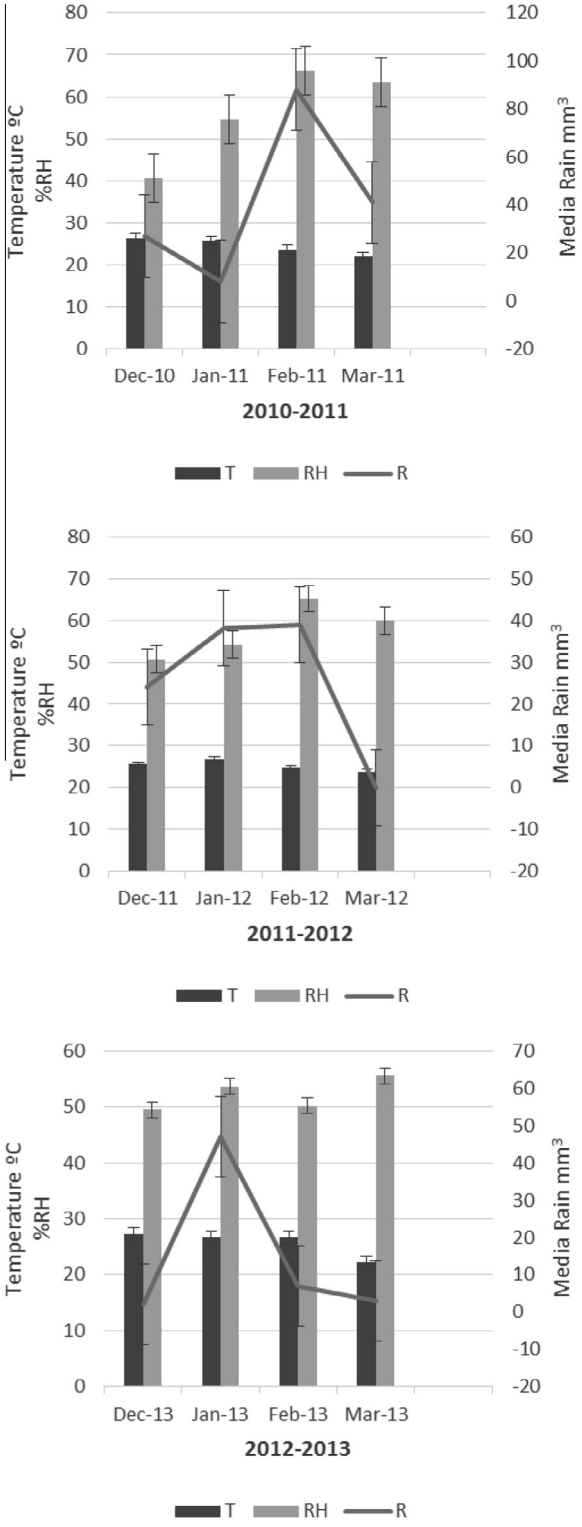

Fig. 2. Temperature (T), relative humidity (RH) and rain (R), computed from December to March in Chilecito during 2010–2011, 2011–2012 and 2012–2013 periods.

grapes treated with *L. thermotolerans* RCKT5 at 10^6 cells mL^{-1} (Table 3). During this vintage, a new variable was introduced; grapes were treated with the biocontrol yeasts at both inoculum levels, with and without adjuvant. Under the treatment without adjuvant OTA accumulation was not reduced but increased.

In addition, the climate conditions could favor the biological control performance of the *L. thermotolerans* strains tested. The

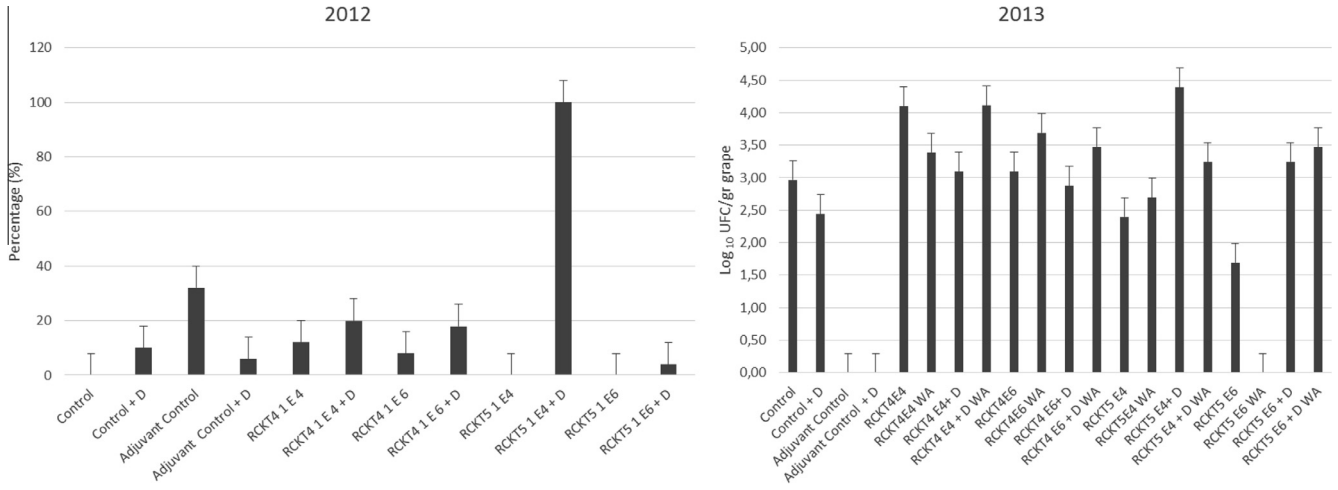

Fig. 3. Dynamic of *Aspergillus* section Nigri in a field trial during two vintages 2011–2012 and 2012–2013. Grapes cv Cabernet Sauvignon at harvest stage under different treatments. (D: artificial damage; A: adjuvant presence; WA: non adjuvant presence; RCKT4 E4: RCKT4 10^4 cells/mL; RCKT4 E6 RCKT4 10^6 cells/mL; RCKT5 E4: RCKT4 10^4 cells/mL; RCKT5 E6 RCKT4 10^6 cells/mL).

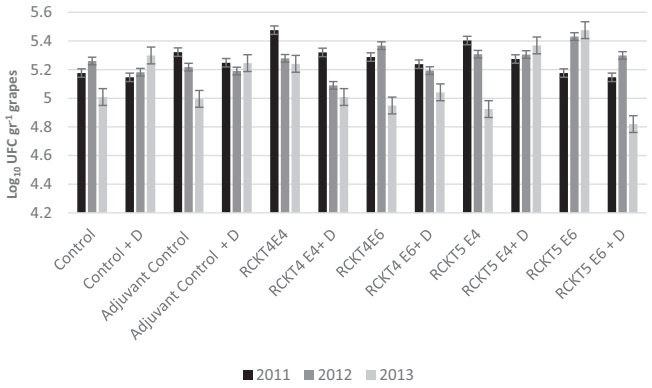

Fig. 4. Total yeasts count at field trial at harvest stage during three vintages. Grapes cv Cabernet Sauvignon at harvest stage under different treatments. (D: artificial damage; RCKT4 E4: RCKT4 10^4 cells/mL; RCKT4 E6 RCKT4 10^6 cells/mL; RCKT5 E4: RCKT4 10^4 cells/mL; RCKT5 E6 RCKT4 10^6 cells/mL).

Table 3
Ochratoxin A reduction in grapes treated with two *Lachancea thermotolerans* strains under field trials.

Vintage Year	Biocontrol strain	Adjuvant presence	Grape treatment	Inoculum level	
				10^4	10^6
2012	RCKT4	+	Healthy	100	100
		+	Damaged	48,7	99
	RCKT5	+	Healthy	100	100
		+	Damaged	52,64	93,7
2013	RCKT4	+	Healthy	0	0
		+	Damaged	62,4	100
		–	Healthy	+0,2	0
		–	Damaged	+1,1	0
	RCKT5	+	Healthy	0	0
		+	Damaged	67,9	+94,3
		–	Healthy	0	0
		–	Damaged	+4,6	+9,2

The + symbol implies that the percentage value is an OTA increase. Detection limit LD $\leqslant$ 0.1 ng g^{-1}.

field conditions during the three evaluated vintages were consistent with a RH among 40–60% and a range of temperature of 22–26 °C (Fig. 2). De Curtis et al. (2012) showed in previous studies in a lab scale the influence of temperature and humidity (RH) on three biocontrol agents (*Metchnikowia pulcherrima*, and two strains of *Aureobasidium pullulans*), demonstrating that 60% of RH and 20 °C where the most favorable conditions for biocontrol activity, this activity was decreased when the temperature and the RH increase. Our findings suggest a similar behavior of the *L. termotolerans* tested strains. On the other hand, temperature is also an influential factor for OTA production, which peaks at 15–20 °C in *A. carbonarius* (Esteban et al., 2004). Taking this in account, we can say that in the field trial the temperature conditions were unfavorable for OTA production by *A. carbonarius,* while there were favorable to biocontrol activity.

Despite these promising results, as far as we know, there is no other studies reporting on the control of black aspergilli and ochratoxin A contamination on wine grapes by *Lanchancea thermotolerans* in greenhouse or field trials. There are previous on biological control of black aspergilli and ochratoxin A using *Aureobasidium pullulans* strains as control agents (de Curtis et al., 2012; de Felice et al., 2008; Dimakopoulou et al., 2008; Pantelides et al., 2015). Dimakopoulou et al. (2008) demonstrated that in *in vitro* experiments a number of *A. pullulans* strains were highly effective in controlling the rot caused by the ochratoxigenic *A. carbonarius* on wounded wine grape berries in a detached berry test (favorable conditions for fungal infection). In the same study the authors demonstrated that the application of Y-1 strain of *A. pullulans* in field experiments in Greece was as effective as the commercial fungicide mixture fludioxonil + cyprodinil in controlling sour rot, *A. carbonarius* infection and the consequent OTA contamination in must. In another study it was shown that application of three strains of *A. pullulans* on grape bunches caused a significant reduction both in the severity of Aspergillus rots in the vineyard and OTA contamination in yeast treated grape samples. It was also reported that the same three *A. pullulans* strains displayed a good antagonistic activity in laboratory scale experiments on grape wine berries, significantly lowering the levels of infections by *A. carbonarius* (de Felice et al., 2008). It is worth mentioning that these strains were able to degrade *in vitro* OTA to ochratoxin-α which is a less toxic compound. The berries that were treated with the biocontrol agents before the infection by *Aspergillus* sp. showed lower OTA contamination than the infected control berries (de Felice et al., 2008; De Curtis et al., 2012). Pantelides et al. (2015) observed strain-related *Aspergillus pullulans*, *Candida sake, Candida magnus* and *Candida zeylanoides* biocontrol efficacy against *Aspergillus tubingiensis* in a detached berry test. Whatever the mechanism of

OTA reduction, the positive effect of biological control agents on grape berry contamination is remarkable and in line with previous results obtained in vineyard treatments (de Felice et al., 2008). Our results show that biocontrol treatment positively affects the level of OTA contamination, even when protection from infection with *Aspergillus* section *Nigri* is not satisfactory; this is the first report in this kind of behavior. Further studies are necessary to determine and clarify the nature of these interactions in the field. The positive effects of biocontrol are dependent on environmental factors. In particular, temperature appears to be more important than relative humidity for protection efficacy. This emphasizes the need for biological control agents that are able to face up to with the environmental conditions that are more conducive to colonization and infection of grape berries by *A. carbonarius*.

As a preventive treatment to reduce OTA accumulation there are several strategies to consider: 1- to protect wounds that inevitably occur during the phenological cycle of the grapes, because the damage not only directly spoil the fruit but also provide the ways for pathogens, especially wound-invading necrotrophic fungi such as *A.* section *Nigri* to colonize th grape; 2- during the yeast strain as a biocontrol agent application, it is important to add an adjuvant to the formula, in order to favor the yeast adherence, and further colonization of the grape berry surface; 3- to choose the lower inoculum level that guarantees the reduction or inhibition of OTA production from the economical point of view.

The results of the present study are the first report that there are some antagonist native yeast strains among the grapes microbial community associated, which were able to control black aspergilli. The data showed the efficacy of two *L. thermotolerans* strains to reduce both growth and OTA accumulation by *A.* section *Nigri* in grape berries under greenhouse and field conditions. The selected yeast strains were effective at a low inoculum level, being this aspect important from the economic point of view to reduce the cost of the future inoculum production and application in a commercial scale. Furthermore, management of black sur rot and OTA contamination by employing microbial agents has been demonstrated to be a most suitable strategy to diminish the use of chemical fungicides.

These findings are an important input for the future development of biopesticides based on native grape yeasts *L. thermotolerans* to reduce *A.* section *Nigri* species growth and ochratoxin A accumulation.

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