Biological Control 79 (2014) 24–35

Contents lists available at ScienceDirect

Biological Control

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

Biological control of *Botrytis* bunch rot in Atlantic climate vineyards with *Candida sake* CPA-1 and its survival under limiting conditions of temperature and humidity

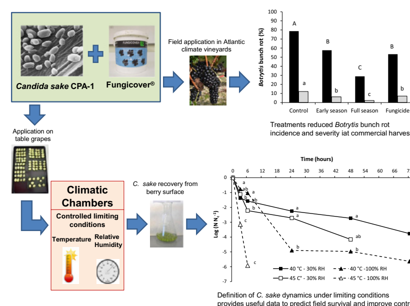
C. Calvo-Garrido [a], N. Teixidó [b], J. Roudet [c], I. Viñas [a], J. Usall [b], M. Fermaud [c,*]

[a] Food Technology Department, Lleida University, XaRTA-Postharvest, Agrotecnio Center, Rovira Roure 191, 25198 Lleida, Catalonia, Spain
[b] IRTA, XaRTA-Postharvest, Scientific and Technologic Parc of Gardeny, FRUITCENTRE building, 25003 Lleida, Catalonia, Spain
[c] INRA, UMR1065 Santé & Agroécologie du VignoblE, ISVV, Université de Bordeaux, CS 20032, 33882 Villenave d'Ornon cédèx, France

HIGHLIGHTS

- *C. sake* field treatments reduced *Botrytis* bunch rot in Atlantic climate vineyards.
- *C. sake* maintain high population density on berries after field applications.
- Survival pattern of *C. sake* under different T and RH limiting conditions is described.
- An incubation period, prior to exposure to limiting conditions, increased BCA survival.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 March 2014
Accepted 30 May 2014
Available online 16 June 2014

Keywords:
Abiotic factors
Extreme conditions
Fatty acids
Fungal disease
Grapevine
Vitis vinifera

ABSTRACT

Candida sake CPA-1 is an antagonistic yeast that has previously been shown to effectively control *Botrytis* bunch rot in grapes. The efficacy of biological control agents is dependent on their survival, which may also depend on climatic conditions. However, few studies have evaluated the effect of abiotic factors affecting the survival of biological control agents, such as temperature (T) or relative humidity (RH). In this study, efficacy of *C. sake* (5×10^7 CFU mL^{-1}), which was applied with the additive Fungicover (FC; 50 g L^{-1}), was tested against BBR in the laboratory and in field trials under the Atlantic climate conditions of the Bordeaux region (France). The study also evaluated the survival of *C. sake* under T and RH regimes simulated in climatic chambers. Two or five applications of *C. sake* plus FC during the growing season significantly reduced BBR severity at harvest by 48% and 82%, respectively, when compared to the control. Similar reductions were achieved after inoculation with selected virulent *Botrytis cinerea* strains (75% compared to control) in laboratory experiments. *C. sake* populations showed minimal decreases between field applications and were favored by simulated Atlantic climate conditions. The survival pattern of *C. sake* exposed to 40 and 45 °C combined with 30% and 100% of RH was described, demonstrating a sharp decrease during the first 24 h. Allowing 48 h for *C. sake* to incubate and become established on fruits prior to the exposure to 40 °C and 30% RH increased survival ($P < 0.05$). These results confirm the efficacy of treatment with *C. sake* plus FC under favorable climatic conditions for BBR development, while survival studies may help to improve the survival and efficacy of yeast BCAs, such as *C. sake* CPA-1.
© 2014 Elsevier Inc. All rights reserved.

Abbreviations: T, temperature; RH, relative humidity; FC, Fungicover; BCA, biological control agent; BBR, *Botrytis* bunch rot; Rf, accumulated rainfall.
* Corresponding author.
E-mail address: fermaud@bordeaux.inra.fr (M. Fermaud).

http://dx.doi.org/10.1016/j.biocontrol.2014.05.011
1049-9644/© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Biological control by antagonistic microorganisms has been extensively studied in recent decades and is regarded as a promising alternative to the use of synthetic fungicides to control fruit pathogens (Droby et al., 2009; Nicot, 2011). Among these pathogens, *Botrytis cinerea* Pers.:Fr., the causative agent of *Botrytis* bunch rot of grapes (*Vitis vinifera* L.), is an important disease in vineyards, causing substantial economic losses in wine and table grapes. In the Bordeaux winegrowing region (France), for example, BBR quantitatively reduces the yield if meteorological conditions are conducive to epidemic development. Moreover, it has recently been shown to cause significant loss of wine sensory quality, perceptible from a disease threshold as low as 5% of rotted berries at harvest (Ky et al., 2012). The epidemiological development of BBR is complex because it is dependent on multiple infection pathways (Elmer and Michailides, 2004). Among the key factors determining disease development are the following: (i) the genetic structure of the *B. cinerea* population (Martinez et al., 2008, 2005); (ii) the grape berry ontogenic resistance associated with the fruit developmental stage (Deytieux-Belleau et al., 2009); (iii) the grapevine susceptibility related to plant vegetative vigor (Valdés-Gómez et al., 2008) and important interactions between the pathogen and insect vectors such as *Lobesia botrana* (Denis & Schiffermüller), the European Grape Berry Moth ''EGBM'' (Fermaud and Lemenn, 1992) and *Thrips obscuratus* (Crawford) (Fermaud and Gaunt, 1995).

During the past few decades, promising studies have reported effective preharvest disease control by commercially available biological control agents and other microorganisms at various developmental stages (Elmer and Reglinski, 2006; Nally et al., 2012; Parry et al., 2011). Furthermore, postharvest applications of several BCAs have also been demonstrated to be effective during storage of table grapes (Romanazzi et al., 2012). The antagonistic yeast *Candida sake* CPA-1, applied in combination with the additive Fungicover®, significantly reduced BBR incidence and severity at harvest in field experiments in dry Mediterranean climate conditions (Calvo-Garrido et al., 2013; Cañamás et al., 2011). It effectiveness was also reported against postharvest diseases of pome fruit through pre- and post-harvest applications (Teixidó et al., 1998b, 1999). Spatial and nutrient competition on the fruit surface was considered to be the main mechanism by which *C. sake* CPA-1 mediates disease suppression, as described for other antagonistic yeast and yeast-like fungi (Droby et al., 2009; Filonow, 1998; Jijakli, 2011; Lima et al., 1997). This mode of action requires the presence and persistence of BCA cells at high concentrations on fruit tissues, and a minimal concentration of 10^4 CFU cm^{-2} is needed to maintain consistency of control, making the survival of BCA populations a crucial factor (Andrews, 1992).

BCA survival is affected by abiotic factors such as temperature, relative humidity and UV radiation (Lahlali et al., 2011; Magan, 2001; Teixido et al., 2010). The influence of these factors can be reduced during controlled postharvest storage. However, under field conditions, populations are subjected to daily fluctuations of temperature and RH, as well as periods of limiting conditions interfering with BCA survival. Especially in hot and dry summer climates, days with maximum T over 35 °C are frequent and RH can drop below 30% during the day. These conditions may affect the survival of a yeast BCA like *C. sake*, which has an optimal growth temperature of 25 °C in culture medium (Teixidó et al., 1998c).

In addition, differences in T and RH during the growing season are known to be key factors determining BBR epidemic development (Elmer and Michailides, 2004). Performance of BCAs may also be modified by environmental conditions, as suggested by studies investigating the effects of RH and T on the relationship between a pathogen and a BCA (Agra et al., 2012).

The population dynamics of antagonistic yeast and yeast-like BCAs have been assessed during storage, following postharvest applications on a variety of fruit commodities: mainly in pome fruit (Jijakli, 2011; Lima et al., 2003; Manso and Nunes, 2011; Tian et al., 2004; Viñas et al., 1998) but also in sweet cherries (Ippolito et al., 2005; Tian et al., 2004) and citrus (Teixidó et al., 2001). Other studies have also evaluated the field survival of these BCAs for controlling diseases during the growing season (Guetsky et al., 2002; Lima et al., 2002) or following pre-harvest applications to control fruit pathogens during storage (Benbow and Sugar, 1999; Ippolito and Nigro, 2000; Lahlali et al., 2009; Teixidó et al., 1998b). On grapes, BCA population studies include the evaluation of field survival after postharvest applications of *Aureobasidium pullulans* and *Candida oleophila* (Lima et al., 1997), *Candida guillermondii* and *Acremonium cephalosporium* (Zahavi et al., 2000), *Metschnikowia fructicola* (Karabulut et al., 2003), *Metschnikowia pulcherrima* and *Pichia guilliermondii* (Kinay and Yildiz, 2008), *Cryptococus laurentii* (Meng and Tian, 2009), the developing yeast-like fungi BCA-L1 (Parry et al., 2011) and *C. sake*, evaluated in both conventional and organic-managed vineyards (Calvo-Garrido et al., 2013; Cañamás et al., 2011).

However, few reports have studied the direct effects of key abiotic factors such as T and RH on BCA survival. The *in vitro* response of *C. sake* to water, temperature and pH stress has been studied (Teixidó et al., 1998c). Lahlali et al. (2008) established a model for the survival of *Pichia anomala* (strain K) and *C. oleophila* (strain O), exposing populations on treated apples to different temperatures (5, 15 and 25 °C) and RH (75% and 98%). Furthermore, the efficacy and survival of *C. oleophila* (strain O) was also tested in extreme conditions of water activity and RH (Lahlali and Jijakli, 2009). However, there are no similar studies on grapes, and none has evaluated the *in vivo* survival of a BCA under selected T and RH regimes in controlled conditions. This information could be valuable for predicting survival and hence improving the efficacy of BCA treatments in the future.

The aims of this work were (1) to test the efficacy of *C. sake* CPA-1 plus Fungicover applications against *B. cinerea*, in the laboratory and in the field under the climatic conditions of Bordeaux vineyards, (2) to evaluate populations of the BCA under these field conditions, and (3) to investigate the effects of different regimes of simulated climatic conditions and limiting conditions of T and RH on *C. sake* CPA-1 survival on grape berries.

2. Materials and methods

2.1. Yeast and fungal material

Three pathogenic *B. cinerea* strains (213, 344 and 351) were selected from the collection of INRA (UMR 1065 SAVE), Bordeaux. The strains have been characterized as II-*vacuma* for strain 351 and II-*transposa* for strains 213 and 344. They were selected based on their marked aggressiveness, ranging from high virulence (213) to intermediate-high virulence (351 and 344), compared to other *B. cinerea* strains from the same collection (Martinez et al., 2003). Stock cultures were maintained on solid malt agar (MA) medium (15 g L^{-1} Cristomalt, Materne, France and 20 g L^{-1} of agar) and then subcultured on MA at 21 °C (±1 °C) in the dark.

The strain CPA-1 of *C. sake* was deposited in the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. *C. sake* was used for experiments as a

formulated product developed in the IRTA research center located in Lleida (Catalonia, Spain), following cell production and formulation methods described by Cañamás et al. (2011), and then stored at 5 °C (±1 °C) in INRA facilities prior to application of treatments.

For all field and laboratory experiments, *C. sake* was applied together with the additive Fungicover (Biodúrcal S.L., Granada, Spain). Its composition is a combination of various fatty acids (mainly lauric, palmitic and stearic acids) in an aqueous-alcoholic solution, and it is used as coating agent for *C. sake* field applications because it has been demonstrated to improve survival of the BCA (Cañamás et al., 2011) and also to reduce BBR by itself (Calvo-Garrido et al., 2013).

2.2. Efficacy of C. sake and Fungicover treatments against B. cinerea infection in controlled laboratory conditions

The combination of *C. sake* CPA-1 plus the additive FC was tested in its capacity to control berry infections following *B. cinerea* artificial inoculations using mycelial plugs. Infection severity was compared to an untreated control and a treatment with Fungicover alone.

Table grapes (cv. Italia) were washed for 15 min in continuous tap-water flow to remove particles and synthetic fungicide residues. Then, single apparently sound berries were cut from bunches, using scissors, keeping the pedicel attached (average berry maturity = 13.1 °Brix, measured by refractometry).

The experimental design included three replicates per treatment, with each replicate unit consisting of 15 berries placed on a grid. The treatments were (i) untreated berries (Control), (ii) berries sprayed with *C. sake* at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1} (CS + FC) and (iii) berries sprayed with Fungicover at 50 g L^{-1} alone (FC). Treatment application was carried out by spraying over the 15 berries of each replicate until runoff, using a pump hand sprayer (F2 Plus model, Berthoud Ltd., Villefranche, France). To favor *C. sake* establishment prior to inoculation with *B. cinerea*, after fruit drying, each replicate unit was placed in a high humidity chamber (HHC) consisting of a plastic box (22 × 13 × 4 cm) containing a sterile absorbent paper in the base with 50 ml of sterile deionized water and was incubated for 18 h at 21 °C (±1 °C) and 100% RH.

Mycelial plugs (4 mm in diameter) were cut from the edge of 7-day-old colonies of *B. cinerea* and placed on the berries with the mycelium facing the fruit surface, using a sterile scalpel. Once inoculation was performed and the boxes were closed again to create HHC, the 27 replicate units (three replicates by three treatments by three *B. cinerea* isolates) were placed in an incubator (EX-111; TABAI ESPEC CORP, Osaka, Japan) at constant temperature of 21 °C (±0.5 °C) and 100% RH in the dark until symptom assessments. After 5, 7, 11 and 14 days of incubation, rot severity was visually scored in each berry as the percentage of berry surface showing the typical brown color following *B. cinerea* infection. Average rot severity of each replicate unit was calculated as the mean severity of the 15 berries in each HHC. Based on the average rot severity in each replicate unit plotted over the time, the area under the disease progress curve was calculated for subsequent statistical analysis.

2.3. Efficacy of field applications of C. sake and Fungicover in Bordeaux vineyards

2.3.1. Experimental field site

In 2012, a field efficacy assay was conducted in an INRA experimental vineyard (*V. vinifera* L.) located near Bordeaux ("Grande Ferrade", Villenave d'Ornon, France). The vineyard of Merlot cultivar was planted in 1991 on typical gravelly soil and was grafted onto '101-14' rootstock. The planting density was approximately 5350 vines ha^{-1}, with a row by vine spacing of 1.70 × 1.10 m and a north-south row orientation. Plants were fertilized by applying 1600 kg ha^{-1} of commercial compost (Vegethumus®, Frayssinet Ltd., Rouairoux, France) every two years from 1992 to 2008. The experimental vineyard was treated against downy and powdery mildew every 2 weeks from 26 Apr. 2012 to 26 Jul. 2012, following the dosages recommended by the manufacturers (six applications of cymoxanil 4% w w^{-1} + mancozeb 46.5% w w^{-1}, two applications of trifloxystrobine 50% w w^{-1}, two applications of tebuconazol 25.8% w w^{-1}, two applications of quinoxyfene 22.58% w w^{-1} and one application of spiroxamine 50% w w^{-1}). No treatments were applied against BBR or *L. botrana* in the experimental plots.

2.3.2. Anti-Botrytis treatments and experimental design

The growing season was divided into five key phenological stages from flowering to harvest: 50% flowering (04 Jun. 2012), 50% flowering + 15 days (18 Jun. 2012), Pre-bunch closure (06 Jul. 2012), Veraison (13 Aug. 2012) and 21 days before harvest (06 Sep. 2012). Applications of *C. sake* plus the additive Fungicover were performed at those stages during the whole season or focused only on the early part of the growing season ("Full season" and "Early season" treatments). Efficacy of the treatments was compared to an untreated control and to a synthetic fungicide program. Details of concentrations, active ingredients applied and timetable of spray applications are summarized in Table 1.

Each replicate unit in the field experiment consisted of seven adjacent vines, of which the first and last vines were buffer vines, the second and third plants were used for *C. sake* population sampling, and the remaining three vines were used to assess BBR at the end of the season. Replicate units were sorted in a completely randomized block design with five replicates per treatment.

C. sake was applied as the formulated product previously described for the laboratory experiment. Field applications were conducted with an electric backpack sprayer (F200 model, INFACO s.a.s., Cahuzac sur Vère, France) by applying treatments until runoff, focusing on the inflorescences or grape bunches only.

2.3.3. Bunch rot assessment

At the end of the season, two assessments of BBR were conducted: (1) at commercial harvest time (26 Sept. 2012) and (2) 1 week later, when grape bunches were over-ripe (04 Oct. 2012). Bunch rot was visually assessed on 50 bunches per replicate unit, evaluating disease Incidence (percentage of bunches with *B. cinerea*

Table 1
Field treatments with *Candida sake* CPA-1 and Fungicover applied to control *Botrytis* bunch rot in Merlot wine grapes.

Treatment[a]	50% flowering	50% flowering + 15 days	Pre bunch closure	Veraison	21 days before harvest
Control	–	–	–	–	–
Full season	CS + FC	CS + FC	CS + FC	CS + FC	CS + FC
Early season	CS + FC	–	CS + FC	–	–
Fungicide	Fenhexamid	–	Cyprodinil + Fludioxonil	–	–

[a] CS + FC: *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}; Fenhexamid: Fenhexamid 750 g ha^{-1}; Cyprodinil + Fludioxonil: 1200 g ha^{-1} of Cyprodinil (37.5%) plus Fludioxonil (25%).

rot symptoms) and Severity (percentage of *B. cinerea*-rotted berries per bunch).

2.3.4. Evaluation of C. sake populations on floral and fruit organs

C. sake populations on vine tissues were monitored during the growing season in treated plots. Flowers or berries were collected from the second and third vines of each replicate unit, using sterile clippers. The four replicates corresponded with the first 4 blocks of the original five-block experimental design. "Full season"-treated plots were sampled (a) once the tissue surface was dry just after one treatment application and (b) 1 h before the next treatment application. In the "Early season" treatment, populations on berries were assessed at harvest time only. Sampling was conducted twice (27 Sep. and 4 Oct.) to gain consistency and precision of the final average value.

Samples were transported to the laboratory in coolers and then placed in 250-mL Erlenmeyer flasks with phosphate buffer. At flowering, BCA populations were recovered from 2 g of floral organs that were collected from 8 bunches per replicate unit and immersed in 20 mL of phosphate buffer. At pre-bunch closure, 40 pea-sized berries from 20 bunches per unit plot were weighed and then immersed in 50 mL of phosphate buffer. In samplings after veraison, 20 berries from 10 bunches were weighed and then immersed in 50 mL of phosphate buffer. Then, the Erlenmeyer flasks were shaken for 20 min at 150 r.p.m. on a rotary shaker and then sonicated for 10 min in an ultrasonic bath (Branson® 2510, Branson Ultrasonics Corp., Danbury, Connecticut, USA). After serial dilutions of the washing solutions, 100-μL aliquots were plated onto NYDA plates (NYDA: nutrient broth, 8 g L^{-1}; yeast extract, 5 g L^{-1}; dextrose, 10 g L^{-1}; and agar, 15 g L^{-1}) supplemented with streptomycin sulfate (0.5 g L^{-1}). Duplicate plates were incubated in the dark at 25 °C and, after 48 h, colonies were visually recognized and counted, based on their morphological characteristics. Data were collected as CFU mL^{-1} and later expressed as CFU g^{-1} of tissue sample.

2.3.5. Quantification of L. botrana larvae in grape bunches

To evaluate a possible interaction of *C. sake* and FC treatments with *L. botrana* development, incidence of *L. botrana* larvae was quantified at the end of the season on five bunches per replicate plot of the field experiment, with bunches selected randomly, regardless of the *Botrytis* rot level. Samples were collected from all replicate plots of the field experiment, excluding fungicide-treated plots. Next, the five bunches per replicate were immersed and agitated in three liters of brine (NaCl, ca 170 g L^{-1} of water) for 20–30 min in a bucket, as previously described (Fermaud, 1998). The number of *L. botrana* larvae of the third generation per five bunches was counted at the brine surface and then expressed as "Number of *L. botrana* larvae per 100 bunches". The sampling and counting process was carried out twice (04 Oct. 2012 and 09 Oct. 2012) for higher consistency of results.

2.3.6. Meteorological data

A weather station (CIMEL 516i, Cimel Electronique, Paris, France), belonging to the INRA meteorological service network, was placed next to the experimental vineyard to record data for major meteorological variables at hourly intervals. Mean daily values of Temperature (T), Maximal Temperature (T_{max}), Mean Relative Humidity (RH) and accumulated rainfall (Rf) were calculated later.

2.4. Studies on C. sake CPA-1 survival in climatic chambers

2.4.1. General methodology

Five experiments were conducted under controlled conditions to evaluate *C. sake* survival on the grape berry surface according to different temperature and RH regimes.

In these experiments, mature table-grape berries were used after they were washed for 15 min in a continuous tap-water flow to remove particles and possible synthetic fungicide residues. Ten single apparently sound berries were cut, keeping the pedicel attached, and placed onto a grid constituting a replicate unit. Three replicate units per treatment were used in all of the survival experiments.

All of these experiments followed a four-step common methodology. (1) Berry treatment: *C. sake* plus FC treatments were applied until runoff with a pump hand sprayer (F2 Plus model, Berthoud Ltd., Villefranche, France). Samples were allowed to dry for 2 h and then placed in HHCs, as described in Section 2.2. (2) Incubation of treated samples: the treated berries were incubated in the HHCs at 21 °C (±1 °C) and 100% RH in the dark to favor the establishment of *C. sake* cells on the berry surface. (3) Exposure to controlled T and RH regimes: replicate samples placed onto grids were placed in climatic chambers and subjected to different temperature × RH × duration combinations in the dark. (4) Quantification of *C. sake* cells on berry surface: at the indicated times (depending on the experiment), recovery of *C. sake* culturable cells was performed by extracting 10 berries of each replicate unit from the climatic chambers, placing them immediately in 250-mL Erlenmeyer flasks with 50 mL of phosphate buffer and proceeding as described in Section 2.3.4.

For all experiments, incubations at 21 ± 1 °C (step two) were conducted in a climate-controlled storeroom at INRA Bordeaux facilities, with T monitored during storage with an external T datalogger (HOBO® U12, Onset Computer Corp., Cape Cod, Massachusetts, USA). Controlled T and RH regimes (step three) were generated using two climatic chambers (EX-111 model, Tabai Espec Corp., Osaka, Japan and PGR14 model, Conviron Ltd., Winnipeg, Canada). The two climatic chambers were calibrated using external T and RH data loggers (HOBO® U12, Onset Computer Corp., Cape Cod, Massachusetts, USA), to adjust T and RH values of both chambers to the same levels. The same data loggers were also used for monitoring T and RH inside the climatic chambers during the experiments.

Specific conditions of the five studies in climatic chambers were designed as modifications of the described four-step general methodology.

2.4.2. Effect of constant limiting T and RH conditions on C. sake survival

Thompson seedless table grapes were sprayed with *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} together with Fungicover at 50 g L^{-1} and then incubated in HHCs at 21 °C (±1 °C) for 12–18 h. Then, treated berries were exposed to constant regimes of T and RH in climatic chambers for 72 h. Four treatments were tested: (1) 40 °C and 30% RH, (2) 40 °C and 100% RH, (3) 45 °C and 30% RH and (4) 45 °C and 100% RH. During the same 72-hour period, another set of samples was incubated at 21 °C (±1 °C) and 100% RH as a Control treatment. For each treatment, evaluation of *C. sake* populations on berries was performed after 0, 3, 6, 24, 48 and 72 h of exposure. The results of the *C. sake* population reductions were finally expressed, for each sample time, as $\text{Log}(N\,N_c^{-1})$, where N = populations in treated sample (CFU g^{-1}) and N_c = mean value of the population in the three replicates of the Control treatment (CFU g^{-1}). The experiment was repeated at 40 °C and 30% RH to increase the consistency of data for this treatment, which was considered a representative standard of hot and dry limiting field conditions.

2.4.3. Effect of the additive Fungicover on C. sake survival under constant limiting conditions

In this study, *C. sake* was applied alone or associated with different concentrations of FC to evaluate whether *C. sake* survival under

constant limiting conditions of temperature and RH was affected by the FC dose.

Three treatments were evaluated based on application of *C. sake* at 5×10^7 CFU mL^{-1} on table grapes (cv. Sugraone): *C. sake* alone (CS), *C. sake* plus Fungicover at 25 g L^{-1} (CS + FC25) and *C. sake* plus Fungicover at 50 g L^{-1} (CS + FC50). Once treated, samples were incubated in HHCs at 21 °C (±1 °C) for 12–18 h and then exposed to 40 °C and 30% RH for 72 h. During the same period, as a Control treatment, a similar set of samples was incubated at 22 °C (±1 °C) and 100% RH. Evaluation of *C. sake* populations on berries was performed after 0, 6, 24, 48 and 72 h. The results of the *C. sake* population counts are expressed for each sample time as Log(CFU g^{-1}).

2.4.4. Effect of the incubation time prior to constant limiting conditions on C. sake survival

The effect on *C. sake* survival capacity of different periods of incubation under optimal conditions before a period of constant limiting conditions was tested. For that purpose, grape berries (cv. Sugraone) were treated with *C. sake* CPA-1 at 5×10^7 CFU L^{-1} plus Fungicover at 50 g L^{-1} and then incubated at 21 °C (±1 °C) and 100% RH for 0, 24 or 48 h (0, 24 and 48 h treatments). Just after the incubation period, samples were exposed to a 48-hour period of limiting conditions at 40 °C and 30% RH. Evaluation of *C. sake* populations on berries was performed at 0 and 48 h after the beginning of the period of limiting conditions. The results of the *C. sake* populations before the limiting conditions period are expressed as Log(CFU g^{-1}). Population reductions are expressed as Log(N N_0^{-1}), where N = CFU g^{-1} of *C. sake* after the exposure to limiting conditions and N_0 = average value of CFU g^{-1} of *C. sake* before the period of exposure to limiting conditions.

2.4.5. C. sake survival under Atlantic and Mediterranean simulated climatic regimes

To evaluate the survival of *C. sake* populations on grape berries under Atlantic or dry Mediterranean climatic conditions, two simulated night/day regimes were designed using meteorological data from representative weather stations from each climatic region (Merignac Airport in the Bordeaux region and IRTA weather station placed in an experimental vineyard in the Lleida area, respectively). Average values of T and RH variables during the middle of the growing season were calculated using meteorological data from July 1st to August 31st (2006 to 2011) at the two weather stations.

Treatments consisted of two simulated climatic regimes (BDX and LDA treatments for Bordeaux and Lleida simulated conditions, respectively), which included simulated day and night conditions. Day conditions consisted of a combination of the Average Maximal daily temperature and the Average Minimal daily RH in each region. Simulated night conditions consisted of a combination of the Average Minimal daily temperature and the Average Maximal daily RH in each region. Temperature and RH values of the simulated night/day regimes are described in Table 2. Day and night durations were 15 and 9 h, respectively, based on the approximate durations of day and night in both regions on August 1st.

Sugraone table grapes were treated with *C. sake* CPA-1 at 5×10^7 CFU L^{-1} plus Fungicover at 50 g L^{-1} and incubated in HHCs at 21 °C (±1 °C) for 12–18 h. Then, samples were placed in the climatic chambers programmed with the simulated climatic regimes (BDX and LDA), while another set of samples was maintained in the HHC at 21 °C (±1 °C) and 100% RH for the whole experiment, considered as the Control treatment. *C. sake* populations were recovered at 0, 1, 4, 7 and 15 days after the start of the simulated conditions. The results of the *C. sake* population counts at each sample time are expressed as Log(CFU g^{-1}).

Table 2
Temperature and RH conditions of the Atlantic and Mediterranean simulated climatic regimes applied to *C. sake* populations in survival studies.

Simulated climatic regime	Day (15 h)	Night (9 h)
Atlantic climate Bordeaux, France (BDX)	27 °C – 43% RH	16 °C – 93% RH
Dry Mediterranean climate Lleida, Catalonia, Spain (LDA)	31 °C – 39% RH	16.5 °C – 82% RH

2.4.6. Effect of the adaptation to simulated climatic regimes on C. sake survival under constant limiting conditions

The objective of this experiment was to test whether *C. sake* populations that have been exposed to simulated climatic conditions (LDA or BDX, as described above) survive differently when exposed to a subsequent period of constant limiting conditions. After the application of *C. sake* CPA-1 at 5×10^7 CFU L^{-1} plus Fungicover at 50 g L^{-1} on table grapes (cv. Sugraone) and the incubation in HHCs at 21 °C (±1 °C) for 12–18 h, samples were placed in climatic chambers programmed with the simulated climatic regimes (BDX and LDA treatments, Table 2) for 5 days. Then, samples exposed to both Atlantic and dry Mediterranean simulated conditions were put together under limiting conditions (40 °C and 30% RH) in a climatic chamber for 72 h. Populations of *C. sake* were measured at 0, 6, 24, 48 and 72 h after the start of the exposure to limiting conditions. Population reductions are expressed for each sample time as Log(N N_0^{-1}), as previously described in Section 2.4.3.

2.5. Statistical analysis

Data were analyzed by multiple-factor ANOVA, and significant treatment differences ($P < 0.05$) were determined using Tukey's test. LSD Student's *t*-test was used in the analysis of the efficacy experiment, comparing treatments to the untreated control. To improve homogeneity of variances, CFU data of *C. sake* CPA-1 population counts were log-transformed, and *L. botrana* larvae counts were also transformed [Square root ($x + 1$)] prior to ANOVA. Data analysis was performed using JMP8 software (SAS Institute Inc., NC, U.S.A.).

3. Results

3.1. Efficacy of C. sake and Fungicover treatments against B. cinerea infection in controlled laboratory conditions

The symptom-development curve of the berry infection produced by *B. cinerea* mycelial plugs in the three tested treatments is presented in Fig. 1. The data include the mean severity results of the three *B. cinerea* strains tested (351, 344, 213) because the interactions between treatment effect and strains were not significant and the data could thus be analyzed together. However, there were significant differences ($P < 0.05$) in mean severity produced by strain 213 (II-*transposa*) compared to strains 344 and 351 (II-*transposa* and II-*vacuma*, respectively). The area under the disease progress curve produced by strain 213% was 33% larger than the corresponding area for strains 344 or 351, indicating elevated aggressiveness of 213 compared to the other two (data not shown).

Mean severity in untreated berries increased steadily, reaching 94% at 14 days after inoculation. Severity in treated berries was significantly lower ($P < 0.05$), and differences between the two treatments were also significant ($P < 0.05$). The CS + FC and FC treatments reduced the area under the curve by 75% and 47%, respectively (Fig. 1), when compared to the untreated control. Nonetheless, maximal severity reduction by CS + FC and FC treatments was achieved at five (92% and 60% reduction, respectively) and seven (85% and 56%, respectively) days after inoculation.

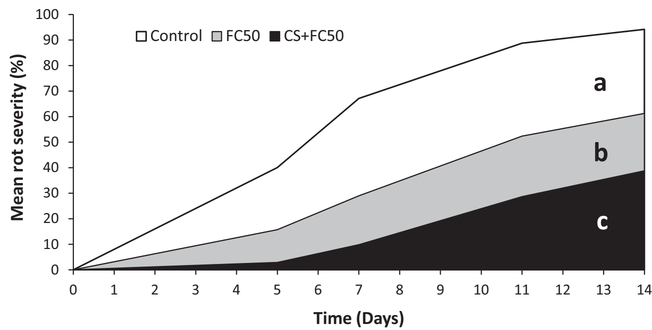


Fig. 1. Mean severity of rot in table grapes treated with *C. sake* and Fungicover before challenge inoculation with *Botrytis cinerea* mycelial plugs (strains 351, 213 and 344). Grape berries (cv. Italia) were not treated (Control), treated with *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1} (CS + FC) or treated with Fungicover alone at 50 g L^{-1} (FC). After incubation at 21 (±1 °C) at 100% RH, berries were inoculated and then incubated at 21 °C and 100% RH for 14 days. All values are means of three replicate units per treatment × strain. Mean values of the calculated area under disease progress curve connected by the same letter are not significantly different ($P < 0.05$) according to LSD Student's *t*-test.

3.2. Efficacy of field applications of *C. sake* and Fungicover in Bordeaux vineyards

3.2.1. Control of BBR at harvest

Fig. 2 summarizes the incidence and severity results of *Botrytis* bunch rot in the field experiment conducted on an experimental Merlot vineyard. In the rot assessment at commercial harvest (Fig. 2a), incidence and severity were high in untreated plots, reaching 78% and 12%, respectively. All treatments significantly ($P < 0.05$) reduced the incidence and severity of BBR. Two applications of *C. sake* and Fungicover at key phenological stages ("Early season" treatment) reduced the incidence by 26% and the severity by 48% compared to the untreated control, and these reductions were not significantly different from those achieved by the Fungicide treatment (32% and 41% reductions of incidence and severity, respectively). The "Full season" treatment was the most effective, reducing bunch rot incidence by 63% and severity by 82%, compared to control.

One week after commercial harvest maturity (Fig. 2b), *Botrytis* bunch rot incidence and severity in untreated plots increased up to 99% and 39%, respectively. Only the "Full season" and Fungicide treatments significantly ($P < 0.05$) reduced incidence, by 13% and 18%, respectively. However, all treatments were able to reduce BBR severity, by 34% (Early season), 77% (Full season) or 74% (Fungicide). The "Early season" treatment showed significantly ($P < 0.05$) lower efficacy than the "Full season" and Fungicide treatments.

3.2.2. Population dynamics of *C. sake* during field efficacy experiment

Populations of the antagonistic yeast *C. sake* were very high on flowers, recovering more than 7 Log(CFU g^{-1}) after the spray application at 50% flowering (Fig. 3). Then, *C. sake* decreased to 5.7 Log(CFU g^{-1}) prior to the next application. At flowering plus 15 days, populations were restored, up to 6.7 Log(CFU g^{-1}), and the decrease rate was low until the pre-bunch-closure application.

When the *C. sake* plus Fungicover mixture was applied on berries, from pre-bunch-closure onwards, populations always stayed over 5 Log(CFU g^{-1}) after the spray applications and did not significantly ($P > 0.05$) decrease between applications. The decrease rate between these samples was very low, and the sprays also did not significantly ($P > 0.05$) increase the *C. sake* numbers on the berry surface compared to the populations present just before the application.

In the "Early season" plots, in which the last spray application was carried out at pre-bunch-closure (6 Jul. 2012), the *C. sake* population recovered at harvest was 4.9 (Fig. 3). This value was not different ($P > 0.05$) from that observed on "Full season" berries (5.1 Log CFU g^{-1}).

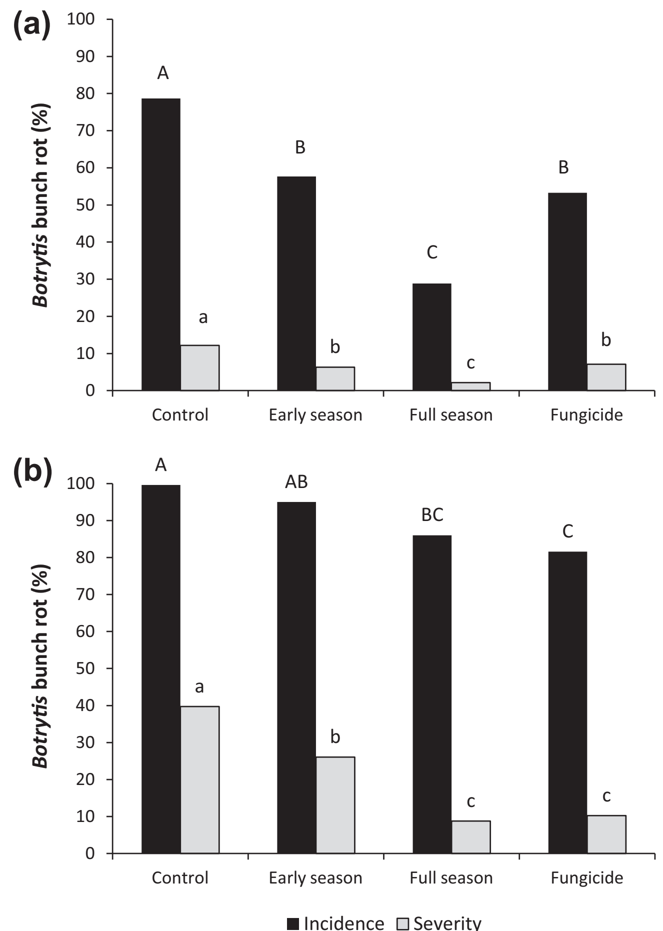


Fig. 2. Incidence and severity of *Botrytis* bunch rot at harvest in an experimental vineyard cv. Merlot in Bordeaux. Bunch rot was assessed over 50 bunches per replicate plot at (a) commercial harvest date (26/09/2012) and (b) 1 week after commercial harvest (04/10/2012). *C. sake* CPA-1 at 5×10^7 CFU/g plus Fungicover at 50 g/L was applied two times (Early season) or five times (Full season) at key phenological stages. Control: untreated; Fungicide: One application of Fenhexamid at 50% flowering (Dose: 750 g ha^{-1}) and one application of Cyprodinil (75%) + Fludioxonil (25%) at pre bunch closure (Dose: 1200 g ha^{-1}). All values are means of five replicate units. Mean values of incidence or severity linked by the same letter are not significantly different ($P < 0.05$) according to LSD Student's *t*-test.

3.2.3. Quantification of *L. botrana* larvae in grape bunches

Statistical analysis showed there was no interaction ($P > 0.05$) between the sample date (04 Oct. 2012 and 09 Oct. 2012) and the treatment effects. Therefore, data were pooled for the analysis of treatment efficacy. The results of the *L. botrana* counts showed elevated incidence in untreated Merlot bunches at harvest, reaching 123 larvae per 100 bunches. However, incidence of *L. botrana* was 72% lower (34 larvae per 100 grape bunches; $P < 0.05$) in the "Full season" treatment plots compared to control. The "Early season" treatment showed a similar incidence to the untreated control (124 larvae per 100 grape bunches.

3.2.4. Meteorological data

Meteorological conditions before veraison (1 Jun. 2012 to 13 Aug. 2012) were characterized by mean T = 20.2 °C, mean T_{max} = 25.9 °C, mean RH = 65.9% and Rf = 120.0 mm. During the late-season period, after veraison (14 Aug. 2012 to 10 Oct. 2012), mean T = 20.0 °C, mean T_{max} = 26.3 °C, mean RH = 66.3% and Rf = 73.5 mm.

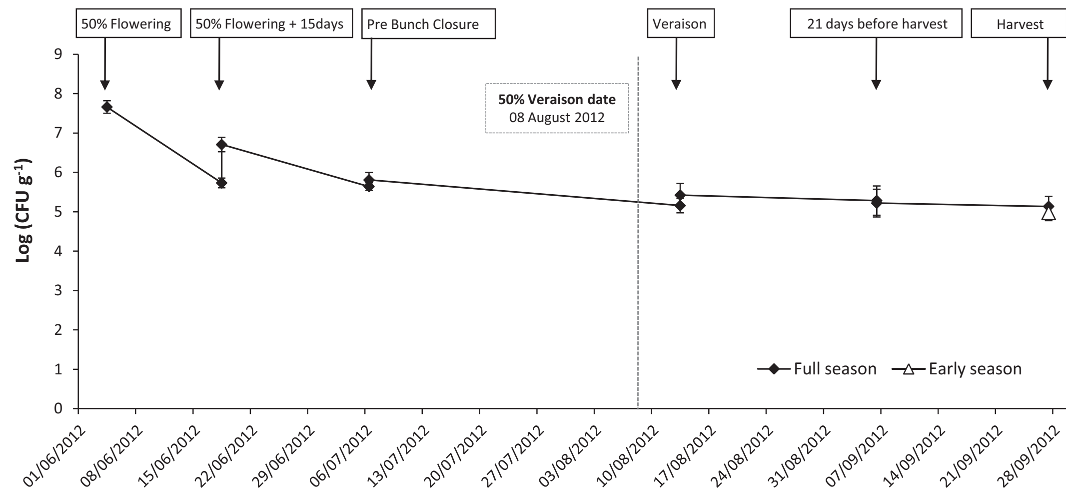


Fig. 3. Population dynamics of *Candida sake* CPA-1 on grapevine tissues from an experimental vineyard cv. Merlot in Bordeaux. *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1} was applied five times at key phenological stages along the season (Full season), or two times (Early season) at 50% flowering and pre bunch closure. Flower or berry samples were taken after spraying and again just prior to the next spray application. Populations in the "Early season" treatment were only assessed at harvest, and the indicated value is the mean of eight replicates. CFU values are per gram of tissue sampled and were log-transformed. Values are means of four replicates, and error bars represent the standard deviation.

3.3. Studies on C. sake CPA-1 survival in climatic chambers

3.3.1. Effect of constant limiting T and RH conditions on C. sake survival

The *C. sake* populations on treated grape berries decreased under the four T and RH regimes tested (Fig. 4). When limiting temperatures were combined with low humidity conditions (40 °C – 30%; 45 °C – 30%), the population decline pattern was similar in both treatments, and no significant differences ($P < 0.05$) were detected at any sampling time. Nonetheless, *C. sake* recovered populations were generally lower in samples maintained at 45 °C. After 48 h of exposure, the population decreased by 2.7 Log units (40 °C – 30%) or 4.1 Log units (45 °C – 30%); after 72 h at 40 °C and 30%, it decreased by 3.7 Log units. The population decrease pattern in samples at 30% RH showed a higher decrease rate during the first 6 h of exposure than the decrease rate observed from 6 to 72 h of exposure.

In samples exposed to high temperature and high RH (40 °C – 100%; 45 °C – 100%), the population decline was higher than observed in the low-RH regimes. At 40 °C and 100% RH, populations decreased by 5.6 Log units after 72 h. After the first 24 h of exposure, the reduction was significantly ($P < 0.05$) higher than the reduction in the 40 °C – 30% treatment. At 45 °C and 100% RH, the decrease was linear and very intense during the first hours of the exposure period, losing 5.9 Log units after 6 h. In the 24-hour samples, *C. sake* populations were below the detection level. Population

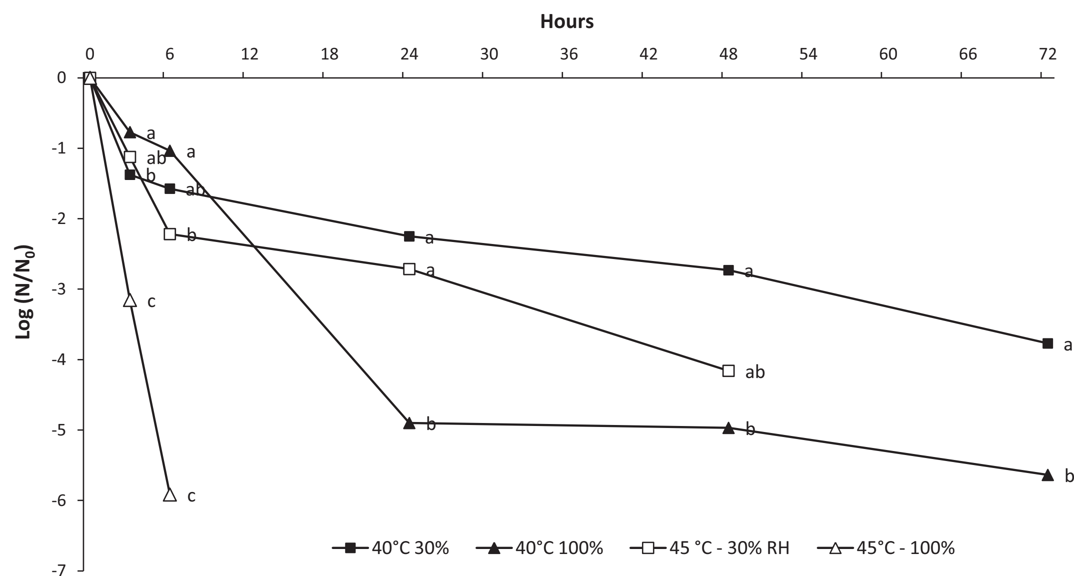


Fig. 4. Reduction of *Candida sake* CPA-1 populations on grape berries exposed to limiting conditions of Temperature and RH. Thompson seedless berries were treated with *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}, then incubated at 21 (±1 °C) and 100% RH before being exposed to regimes of T and RH. N = populations in treatment sample (CFU g^{-1}) and N_c = Mean value of the populations (CFU g^{-1}) on berries incubated at 21 (±1 °C) and 100% RH during the same period. Values shown are means of three replicates except for 40 °C – 30% samples, which are means of six replicates. Mean values linked by the same letter are not significantly different ($P < 0.05$) according to Tukey's test.

reductions were always significantly ($P < 0.05$) different between the 45 °C – 100% and the 45 °C – 30% treatments.

3.3.2. Effect of Fungicover on C. sake survival under constant limiting conditions

Population dynamics of *C. sake* applied with different concentrations of Fungicover and subjected to optimal and limiting conditions of T and RH are shown in Fig. 5. Initial populations at the start of exposure ranged from 5.4 to 5.7 Log(CFU g^{-1}), and no significant differences among samples were detected at that point.

Populations slightly increased in samples incubated in HHCs at 21 °C for 72 h (CS, CS + FC25 and CS + FC50; black symbols), and the final *C. sake* concentration on berry surface ranged from 5.9 to 6.1 Log(CFU g^{-1}). No significant differences were detected among these three treatments at any sampling time.

In all of the samples exposed to 40 °C and 30% RH for 48 h (CS, CS + FC25 and CS + FC50; white symbols), populations decreased in a similar pattern for all of the Fungicover concentrations tested. No significant differences were observed between treatments at any sample time, indicating no effect of the Fungicover dose on *C. sake* survival under the constant conditions evaluated. The decrease pattern was also similar to those observed in Fig. 4, with a remarkable population reduction during the first 6 hours and a second stage with a lower reduction rate. The final *C. sake* population ranged from 1.9 to 2.4 Log(CFU g^{-1}), which means a reduction of approximately 4 Log units compared to populations in the samples incubated under optimal conditions.

3.3.3. Effect of the incubation time prior to constant limiting conditions on C. sake survival

Populations of *C. sake* significantly increased on the berry surface when they were incubated at 21 °C and 100% RH for 24 or 48 h after BCA application (Fig. 6a). During the first 24 h, populations significantly increased ($P < 0.05$) from 4.7 to 5.7 Log(CFU g^{-1}). Between 24 and 48 h of incubation, *C. sake* populations also increased ($P < 0.05$) from 5.7 to 5.9 Log(CFU g^{-1}).

The decrease in *C. sake* populations on berries after 48 h of exposure to limiting conditions (40 °C – 30% RH), when berries had been previously incubated in optimal conditions (21 °C – 100% RH) for 0, 24 and 48 h, is shown in Fig. 6b. The populations decreased by 3.4 and 3.0 Log units in samples previously incubated for 0 and 24 h, respectively. This reduction was significantly ($P < 0.05$) higher than

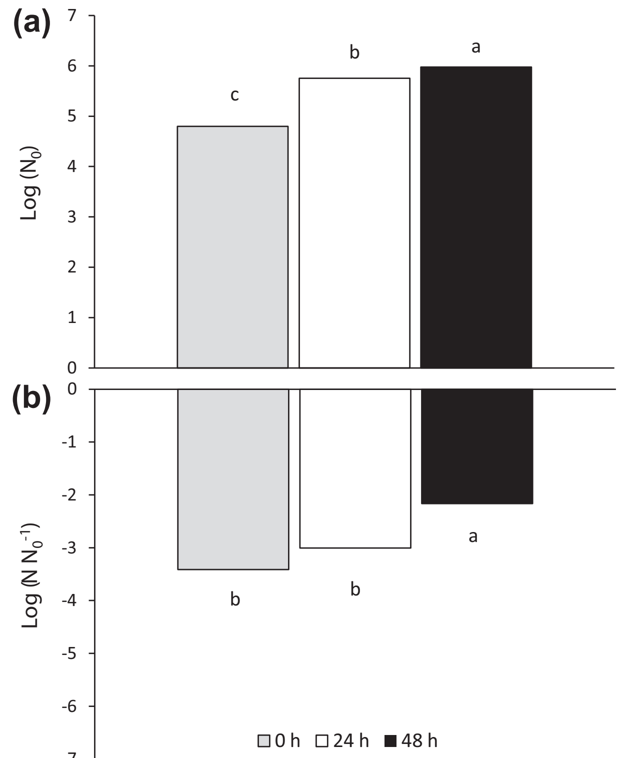


Fig. 6. Effect of incubation time prior to exposure to limiting conditions of T and RH on *Candida sake* survival. (a) Populations on berry surface after incubation at 21 (±1 °C) and 100% RH for 0, 24 or 48 h (0, 24, 48 h). (b) Reduction of populations on berry surface at the end of a 48-hour period of limiting conditions (40 °C – 30%), when populations had been previously incubated at 21 (±1 °C) and 100% RH for 0, 24 or 48 h (0, 24, 48 h). Treatment mixture applied to grape berries (cv. Sugraone) consisted of *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}. N = CFU g^{-1} of *C. sake* after exposure to limiting conditions period, N_0 = average value of CFU g^{-1} of *C. sake* before the start of the limiting conditions period. Values are means of three replicates. Mean values connected by the same letter are not significantly different ($P < 0.05$) according to Tukey's test.

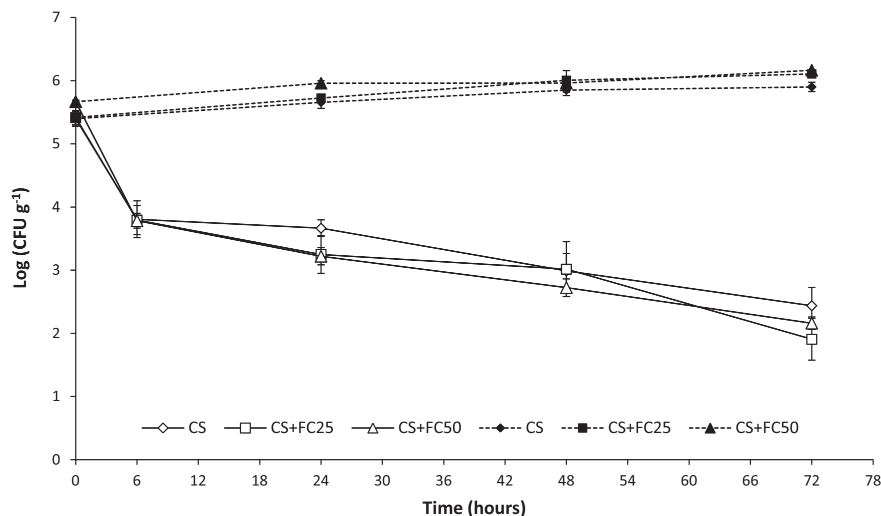


Fig. 5. *Candida sake* population dynamics on grape berries treated with combinations of the yeast and different concentrations of Fungicover. Values represent recovered *C. sake* populations from the grape berry surface during a 72-hour period in optimal conditions (21 ± 1 °C and 100% RH; dotted lines and black symbols) or limiting conditions (40 °C and 30% RH; solid lines and white symbols). Berries (cv. Sugraone) were treated with *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} alone (CS), combined with Fungicover at 25 g L^{-1} (CS + FC25), or combined with Fungicover at 50 g L^{-1} (CS + FC50). Values are means of three replicates, and error bars represent the standard deviation.

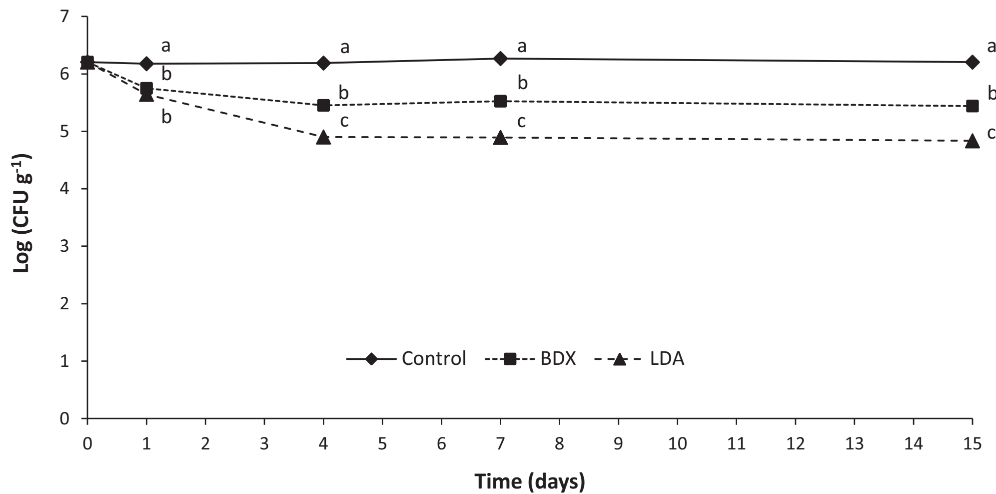


Fig. 7. Population dynamics of *Candida sake* CPA-1 on grape berries exposed to simulated Atlantic and Mediterranean climatic regimes. Sugraone grape berries were treated with *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}, incubated at 21 (±1 °C) and 100% RH and then exposed for 15 days to: constant 21 (±1 °C) and 100% RH conditions (Control), simulated Atlantic conditions (BDX) or simulated Mediterranean conditions (LDA). Values are means of three replicates. Mean values connected by the same letter are not significantly different ($P < 0.05$) according to Tukey's test.

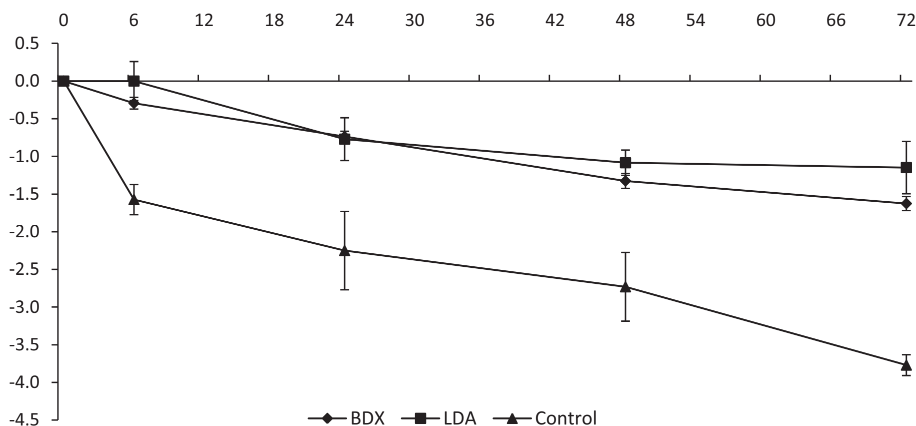


Fig. 8. Reduction of *Candida sake* CPA-1 populations exposed to limiting conditions on grape berries previously exposed to simulated climatic regimes. Berries (cv. Sugraone) were treated with *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}, then incubated at 21 (±1 °C) °C and 100% RH. Prior to the period of limiting conditions, samples were exposed for five days to simulated Atlantic (BDX) or Mediterranean (LDA) climatic regimes. Control samples were not exposed to any simulated regime. The limiting conditions period consisted of 72 h at 40 °C – 30% RH. N = CFU g^{-1} of *C. sake* after exposure to the limiting conditions period, N_0 = average value of CFU g^{-1} of *C. sake* before the start of the limiting conditions period. Values are means of three replicates, and error bars represent the standard deviation.

the reduction observed in samples incubated for 48 h prior to exposure, which decreased by 2.1 Log units.

3.3.4. C. sake survival under Atlantic and Mediterranean simulated climatic regimes

The *C. sake* populations behaved differently under the simulated regimes of T and RH tested, as shown in Fig. 7. At constant 21 °C and 100% RH (Control), the yeast population on berries at the beginning was 6.2 Log(CFU g^{-1}) and remained stable between 6.1 and 6.2 Log(CFU g^{-1}) during the entire assay. When samples were exposed to the simulated climatic regimes, significant decreases were observed during the first 24 h in both treatments (BDX and LDA). Then, populations continued to decrease until 4 days of exposure, to 5.4 and 4.9 Log(CFU g^{-1}), under simulated Atlantic (BDX) and Mediterranean (LDA) simulated climatic regimes, respectively. The *C. sake* concentration on berries at that sampling time differed significantly ($P < 0.05$) among the three treatments. After 4 days, the populations in all conditions tested remained stable until the end of the assay (15 days after the start of exposure), and populations ranged between 5.4–5.5 (BDX), or 4.9–4.8 (LDA), maintaining approximately 0.5 Log unit difference among treatments.

3.3.5. Effect of the adaptation to simulated climatic regimes on C. sake survival under constant limiting conditions

In the samples previously exposed to Mediterranean (LDA) or Atlantic (BDX) simulated regimes, populations decreased between 1.1 and 1.6 Log units, respectively, after 72 h of exposure to limiting conditions (Fig. 8). The decrease in samples not exposed to these simulated regimes was 3.7 Log units (Control).

Differences in the decrease between LDA and BDX treatments were not significant ($P > 0.05$) at any sample time, although the average population in LDA samples at 72 h was 0.5 Log unit higher than in BDX samples.

The *C. sake* populations previously adapted to both climatic regimes survived significantly better than those exposed to limiting conditions immediately after 12 h incubation in optimal conditions (Control).

4. Discussion

An important factor contributing to the success of a BCA in field applications is the ability of the BCA to adapt to different climatic conditions and show efficacy against the target pathogen under a wide range of environmental conditions. For the first time, in this study, *C. sake* CPA-1 treatments were evaluated under Atlantic climate conditions, conducive to BBR, to confirm previous studies in Catalan vineyards with a dry and hot summer climate. In addition, this is the first *in vivo* exhaustive study on the survival of a yeast-BCA under T and RH limiting conditions.

Under laboratory conditions, treatment with *C. sake* plus Fungicover showed high efficacy at controlling *B. cinerea* infection of table grapes (75% severity reduction compared to untreated control). This result was obtained after challenging inoculation with selected virulent *B. cinerea*-II-*transposa* strains, which are usually considered the most aggressive strains on grape berries (Martinez et al., 2005). Although FC alone reduced *B. cinerea* infection severity, *C. sake* showed significant improvements over control compared to the additive alone. This difference remained over time, presenting elevated reduction rates during the first seven days after inoculation.

After five field applications of *C. sake* plus FC during the season (“Full season” treatment), the severity reduction (82% compared to untreated control) was similar to that observed in the laboratory experiment. Such a high efficacy level of *C. sake* and Fungicover field applications was obtained under conducive conditions for BBR. Under these humid Atlantic climate conditions, both the incidence and severity reductions achieved by the “Full season” treatment were similar to those achieved in 2010 in Lleida with a dry and hot summer climate (Calvo-Garrido et al., 2013), although the BCA survival patterns were clearly different, as will be discussed below.

In the 2010 season in Lleida, severity reductions were similar in “Early season” and “Full season” strategies (85% and 89%, respectively), with high disease pressure (21.7% severity in the untreated control; Calvo-Garrido et al., 2013). However, in this experiment in Bordeaux, the “Early season” treatment showed a lower efficacy. Because *C. sake* populations remaining at harvest in “Early season” samples were high (Fig. 3), the difference in severity reduction between the “Full season” and “Early season” treatments is unlikely to be due to the effect of the *C. sake* population level. Thus, the effect of the two extra applications of FC after veraison in the “Full season” treatment may be the key factor accounting for the increased efficacy. A direct effect of FC on *B. cinerea* has been shown clearly in our laboratory experiment (Fig. 1) and in a previously published paper (Calvo-Garrido et al., 2013). The significant reduction of *L. botrana* larvae incidence in the “Full season” plots also indicated that FC interaction with grape berry moth development may also play an important role in BBR control in the vineyard. The mode of action of FC on *L. botrana* requires further investigation, although it may result from the presence of fatty acids (lauric, palmitic and stearic) as major FC ingredients, according to other studies reporting significant reduction of *L. botrana* oviposition in the presence of stearic, palmitic and other long chain fatty acids (Thiery et al., 1995).

Overall, *C. sake* plus Fungicover treatments significantly reduced BBR severity at harvest. Both “Early season” and “Full season” treatments achieved reduction levels similar to a standard fungicide program and may represent an effective alternative to synthetic fungicides under conducive Atlantic climate conditions. In addition, efficacy results were similar to those obtained under dry Mediterranean climate conditions (Calvo-Garrido et al., 2013). This suggests an interesting inter-relationship between climate, *C. sake* survival and BBR: Atlantic climate conditions favor BBR development compared to Mediterranean climate conditions, but they also favor *C. sake* survival, leading to similar final disease reductions at harvest.

In the field experiments in the Lleida region, populations decreased by one to four Log(CFU g^{-1}) between spray applications (Calvo-Garrido et al., 2013; Cañamás et al., 2011). However, *C. sake* populations in our 2012 Bordeaux experiment showed no significant decrease between most of the sprays. High and low field survival of yeast BCAs are reported in the literature, linked in some cases to the meteorological conditions during the experiments (Benbow and Sugar, 1999; Lima et al., 2003, 1997; Tian et al., 2004; Zahavi et al., 2000). The different survival patterns observed in Bordeaux and Lleida field experiments are consistent with the survival pattern demonstrated in the simulated climatic conditions experiment (Fig. 7). Under simulated conditions, populations exposed to BDX conditions were approximately 0.5 Log unit higher than LDA samples, although T and RH variation between BDX and LDA regimes was reduced. Moreover, in dry Mediterranean climate regions, periods with air temperature over 35 °C are recorded in summer, and temperature is generally higher at the berry surface than the air temperature in the vineyard (Pieri and Fermaud, 2005). These facts provide harsher conditions for BCA survival because *C. sake* stops growing at 35 °C in NYDA medium (Teixidó et al., 1998c), and populations on berries also significantly decreased after 48 h at 35 °C and 60% RH in climatic chambers (data not shown). The lethal effect of limiting conditions of T and RH on *C. sake* CPA-1 was corroborated (Fig. 4) by populations rapidly decreasing at 40 or 45 °C. Thus, all our field and laboratory data indicate a lower *C. sake* survival under hot conditions, which may justify more frequent spray applications in warm-climate vineyards and would provide opportunities to reduce applications in Atlantic climate regions, such as Bordeaux vineyards.

Yeasts are considered potential BCAs due to their ability to cope with adverse environmental conditions, and there are various examples of antagonistic yeasts able to survive in a variety of pre- and/or post-harvest conditions (Benbow and Sugar, 1999; Ippolito et al., 2005; Karabulut et al., 2003; Zahavi et al., 2000). Excellent survival capacity on fruit surfaces of *C. sake* CPA-1 has been demonstrated at nearly 0 °C in apple postharvest conditions (Teixidó et al., 1998a, 1999) and, in this study, the population gradually decreased at 40 or 45 °C combined with constant 30% RH, but with measurable remaining populations after 48 or 72 h (Figs. 4 and 5). However, the combination of high temperature and high RH was extremely harmful for *C. sake* cells. This result is consistent with other studies that indicated a greater effect on yeast survival of RH than T (Lahlali et al., 2008; Teixidó et al., 1998b, 1998c). Moreover, this corroborates *in vitro* findings showing a dramatic *C. sake* population decrease in liquid medium at 40 and 45 °C (Cañamás et al., 2008). The combination of high T and high RH is not favorable for some fungal species, including BCAs (Agra et al., 2012; Cañamás et al., 2008). Similarly, fungal pathogens may be more affected under higher RH conditions at the same temperature (Teitel et al., 1989), providing opportunities for their control by means of curing treatments (Casals et al., 2010; Fallik, 2004). Low population survival under these conditions suggests reduced potential of *C. sake* for biological control in tropical regions or in association with post-harvest curing or hot water treatments.

The protective effect of the additive Fungicover on *C. sake* populations has been demonstrated in previous research (Cañamás et al., 2011); Calvo-Garrido et al., Unpublished results). However, in this study, it has been demonstrated that FC did not directly protect *C. sake* from continued exposure to high temperature and low RH. Consequently, new hypotheses should be formulated to account for the observed FC effect on *C. sake* populations, notably in the field. First, the additive FC may protect an antagonistic yeast,

such as *C. sake* CPA-1, by protecting cells from solar UV radiation, as reported for other BCA additives (Lahlali et al., 2011). Second, the microenvironment created inside the FC biofilm on berries may protect against T and RH fluctuations, notably in sub-lethal ranges, as observed for additives protecting *C. oleophila* at 75% and 98% RH (Lahlali and Jijakli, 2009).

Lastly, the survival studies conducted under different limiting conditions highlight the importance of the period immediately after the application on grape berries for *C. sake* survival. The survival pattern of BCAs during the first hours post-application has been poorly studied, although effective establishment on a natural environment is considered to be crucial for subsequent efficacy (Jijakli, 2011). In the present study, *C. sake* populations increased on the berry surface in optimal conditions for at least 48 h (Fig. 6a), whereas populations decreased less when there was an establishment period, under optimal conditions or under simulated climatic regimes, before limiting conditions were applied (Fig. 6b). Furthermore, high sensitivity of the BCA was evidenced soon after application. Indeed, a very important decrease in population level was noticeable during the first 6-hour period, and the decrease was relatively less marked after 24 h post-application for most of the conditions tested (Figs. 4, 5 and 8).

These findings highlight the importance of choosing the right moment to perform field applications. To provide more favorable conditions for *C. sake* establishment during the first 6 h post-application, evening applications may be desirable to avoid periods of high temperatures. Similarly, if very hot and dry field conditions are forecast, spraying 48 h before would also minimize the population decrease.

In conclusion, the present study under laboratory and field conditions corroborates the significant efficacy of treatments associating *C. sake* and Fungicover to control different virulent strains of *B. cinerea* as well as wild vineyard populations under conducive conditions for BBR in Bordeaux vineyards. In the field, monitoring the *C. sake* population and the newly evidenced FC effect on *L. botrana* populations provided interesting information to better account for the field efficacy of treatments. Lastly, for the first time, survival studies have quantified the dynamics of *C. sake* populations under limiting and simulated climatic conditions, which led us to devise possible strategies to maximize the efficacy of field applications.

Acknowledgments

The authors are grateful to the University of Lleida for a Ph.D. grant to Carlos Calvo Garrido (CCG) and for the organic production research project, to the European Union for their financial support through the INTERREG REDBIO project and to the Catalan government for the travel grant to CCG. They are also grateful to the INRA UMR-Save for welcoming CCG in 2012 to conduct all of the experiments presented in this paper by using the INRA research facilities in Bordeaux and to the Conseil Interprofessionnel du Vin de Bordeaux (CIVB) for its financial support of the project. They are indebted to UMR SAVE-Bordeaux staff: J.M. Liminana and J.M. Brustis for their technical support associated with climatic chambers and vineyard plot, respectively. Also to J. Jolivet, O. Bonnard, S. Capdevielle, J. Poidatz and L. Delbac for their valuable help with laboratory assays. The authors thank Dr. Stephen Hoyte for his valuable review of the manuscript.

References

Agra, O., David, D.R., Borenshtein, M., Shulchany, R., Elad, Y., 2012. Climate effect on pathogen – biocontrol agents interaction in the tomato – powdery mildew (*Oidium neolycopersici*) pathosystem. IOBC/WPRS Bulletin 78, 233–237.
Andrews, J.H., 1992. Biological control in the phyllosphere. Annu. Rev. Phytopathol. 30, 603–635.
Benbow, J.M., Sugar, D., 1999. Fruit surface colonization and biological control of postharvest diseases of pear by preharvest yeast applications. Plant Dis. 83, 839–844.
Calvo-Garrido, C., Elmer, P.A.G., Viñas, I., Usall, J., Bartra, E., Teixidó, N., 2013. Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. Plant. Pathol. 62, 510–519.
Cañamás, T.P., Viñas, I., Usall, J., Magan, N., Solsona, C., Teixidó, N., 2008. Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast *Candida sake* CPA-1 and viability after spray-drying. J. Appl. Microbiol. 104, 767–775.
Cañamás, T.P., Viñas, I., Torres, R., Usall, J., Solsona, C., Teixidó, N., 2011. Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. Biol. Control 56, 150–158.
Casals, C., Teixido, N., Vinas, I., Llaurado, S., Usall, J., 2010. Control of *Monilinia* spp. on stone fruit by curing treatments Part I. The effect of temperature, exposure time and relative humidity on curing efficacy. Postharvest Biol. Technol. 56, 19–25.
Deytieux-Belleau, C., Geny, L., Roudet, J., Mayet, V., Doneche, B., Fermaud, M., 2009. Grape berry skin features related to ontogenic resistance to *Botrytis cinerea*. Eur. J. Plant Pathol. 125, 551–563.
Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: is it time for a new paradigm? Postharvest Biol. Technol. 52, 137–145.
Elmer, P.A.G., Michailides, T.J., 2004. Epidemiology of *Botrytis cinerea* in orchard and vine crops. In: Elad, Y., Williamson, B., Tudzynski, P., Delen, N. (Eds.), Botrytis: Biology, Pathology and Control. Kuwer academic publishers, Dordrecht, The Netherlands, pp. 243–272.
Elmer, P.A.G., Reglinski, T., 2006. Biosuppression of *Botrytis cinerea* in grapes. Plant. Pathol. 55, 155–177.
Fallik, E., 2004. Prestorage hot water treatments (immersion, rinsing and brushing). Postharvest Biol. Technol. 32, 125–134.
Fermaud, M., Lemenn, R., 1992. Transmission of *Botrytis cinerea* to grapes by grape berry moth larvae. Phytopathology 82, 1393–1398.
Fermaud, M., Gaunt, R.E., 1995. *Thrips obscuratus* as a potential vector of *Botrytis cinerea* in kiwifruit. Mycol. Res. 99, 267–273.
Fermaud, M., 1998. Cultivar susceptibility of grape berry clusters to larvae of *Lobesia botrana* (Lepidoptera: Tortricidae). J. Econ. Entomol. 91, 974–980.
Filonow, A.B., 1998. Role of competition for sugars by yeasts in the biocontrol of gray mold of apple. Biocontrol Sci. Tech. 8, 243–256.
Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E., Dinoor, A., 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology 92, 976–985.
Ippolito, A., Nigro, F., 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. Crop Prot. 19, 715–723.
Ippolito, A., Schena, L., Pentimone, I., Nigro, F., 2005. Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. Postharvest Biol. Technol. 36, 245–252.
Jijakli, M.H., 2011. *Pichia anomala* in biocontrol for apples: 20 years of fundamental research and practical applications. Antonie Van Leeuwenhoek Int. J. General Mol. Microbiol. 99, 93–105.
Karabulut, O.A., Smilanick, J.L., Gabler, F.M., Mansour, M., Droby, S., 2003. Near-harvest applications of *Metschnikowia fructicola*, ethanol, and sodium bicarbonate to control postharvest diseases of grape in central California. Plant Dis. 87, 1384–1389.
Kinay, P., Yildiz, M., 2008. The shelf life and effectiveness of granular formulations of *Metschnikowia pulcherrima* and *Pichia guilliermondii* yeast isolates that control postharvest decay of citrus fruit. Biol. Control 45, 433–440.
Ky, I., Lorrain, B., Jourdes, M., Pasquier, G., Fermaud, M., Geny, L., Rey, P., Doneche, B., Teissedre, P.L., 2012. Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. Aust. J. Grape Wine Res. 18, 215–226.
Lahlali, R., Massart, S., Serrhini, M.N., Jijakli, M.H., 2008. A Box-Behnken design for predicting the combined effects of relative humidity and temperature on antagonistic yeast population density at the surface of apples. Int. J. Food Microbiol. 122, 100–108.
Lahlali, R., Jijakli, M.H., 2009. Enhancement of the biocontrol agent *Candida oleophila* (strain O) survival and control efficiency under extreme conditions of water activity and relative humidity. Biol. Control 51, 403–408.
Lahlali, R., Massart, S., De Clercq, D., Serrhini, M.N., Creemers, P., Jijakli, M.H., 2009. Assessment of *Pichia anomala* (strain K) efficacy against blue mould of apples when applied pre- or post-harvest under laboratory conditions and in orchard trials. Eur. J. Plant Pathol. 123, 37–45.
Lahlali, R., Brostaux, Y., Jijakli, M.H., 2011. Control of apple blue mold by the antagonistic yeast *Pichia anomala* strain K: screening of UV protectants for preharvest application. Plant Dis. 95, 311–316.
Lima, G., Ippolito, A., Nigro, F., Salerno, M., 1997. Biological control of grey mould of stored table grapes by pre-harvest applications of *Aureobasidium pullulans* and *Candida oleophila*. Difesa delle Piante 20, 21–28.
Lima, G., Curtis, F.d., Spina, A.M., Cicco, V.d., 2002. Survival and activity of biocontrol yeasts against powdery mildew of cucurbits in the field. Bull. OILB/SROP 25, 187–190.
Lima, G., De Curtis, F., Castoria, R., De Cicco, V., 2003. Integrated control of apple postharvest pathogens and survival of biocontrol yeasts in semi-commercial conditions. Eur. J. Plant Pathol. 109, 341–349.

Magan, N., 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents. In: Butt, T.M., Jackson, C., Magan, N. (Eds.), Fungi as Biocontrol Agents: Progress, Problems and Potential. CABI Publishing, pp. 239–251.

Manso, T., Nunes, C., 2011. *Metschnikowia andauensis* as a new biocontrol agent of fruit postharvest diseases. Postharvest Biol. Technol. 61, 64–71.

Martinez, F., Blancard, D., Lecomte, P., Levis, C., Dubos, B., Fermaud, M., 2003. Phenotypic differences between vacuma and transposa subpopulations of *Botrytis cinerea*. Eur. J. Plant Pathol. 109, 479–488.

Martinez, F., Dubos, B., Fermaud, M., 2005. The role of saprotrophy and virulence in the population dynamics of *Botrytis cinerea* in vineyards. Phytopathology 95, 692–700.

Martinez, F., Corio-Costet, M.F., Levis, C., Coarer, M., Fermaud, M., 2008. New PCR primers applied to characterize distribution of *Botrytis cinerea* populations in French vineyards. Vitis 47, 217–226.

Meng, X., Tian, S., 2009. Effects of preharvest application of antagonistic yeast combined with chitosan on decay and quality of harvested table grape fruit. J. Sci. Food Agric. 89, 1838–1842.

Nally, M.C., Pesce, V.M., Maturano, Y.P., Munoz, C.J., Combina, M., Toro, M.E., Castellanos de Figueroa, L.I., Vazquez, F., 2012. Biocontrol of Botrytis cinerea in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. Postharvest Biol. Technol. 64, 40–48.

Nicot, P.C., 2011. Classical and augmentative biological control against diseases and pests: critical status analysis and review of factors influencing their success. IOBC/WPRS.

Parry, F., Elmer, P., Wood, P., Agnew, R., Saunders, J., Wurms, K., Hoyte, S., Chee, A., Chee, A.A., 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 2: developing a new biologically-based product for late-season botrytis control: the BCA-L1 story. Wine Viticulture J. 26, 73–78.

Pieri, P., Fermaud, M., 2005. Effects of defoliation on temperature and wetness of grapevine berries. Proc. Seventh Int. Symposium Grapevine Physiol. Biotechnol., 109–116.

Romanazzi, G., Lichter, A., Gabler, F.M., Smilanick, J.L., 2012. Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. Postharvest Biol. Tec. 63, 141–147.

Teitel, D.C., Aharoni, Y., Barkaigolan, R., 1989. The use of heat-treatments to extend the shelf-life of Galia melons. J. Horticult. Sci. 64, 367–372.

Teixidó, N., Usall, J., Gutierrez, O., Viñas, I., 1998a. Effect of the antagonist *Candida sake* on apple surface microflora during cold and ambient (shelf life) storage. Eur. J. Plant Pathol. 104, 387–398.

Teixidó, N., Viñas, I., Usall, J., Magan, N., 1998b. Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. Phytopatology 88, 960–964.

Teixidó, N., Viñas, I., Usall, J., Sanchis, V., Magan, N., 1998c. Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH stress. J. Appl. Microbiol. 84, 192–200.

Teixidó, N., Usall, J., Viñas, I., 1999. Efficacy of preharvest and postharvest *Candida sake* biocontrol treatments to prevent blue mould on apples during cold storage. Int. J. Food Microbiol. 50, 203–210.

Teixidó, N., Usall, J., Palou, L., Asensio, A., Nunes, C., Viñas, I., 2001. Improving control of green and blue molds of oranges by combining *Pantoea agglomerans* (CPA-2) and sodium bicarbonate. Eur. J. Plant Pathol. 107, 685–694.

Teixidó, N., Usall, J., Nunes, C., Torres, R., Abadias, M., Viñas, I., 2010. Preharvest strategies to control postharvest diseases in fruits. In: Prusky, D., Gullino, M.L. (Eds.), Postharvest Pathology. Springer, pp. 89–106.

Thiery, D., Gabel, B., Farkas, P., Jarry, M., 1995. Egg dispersion in codling moth: influence of egg extract and of its fatty acid constituents. J. Chem. Ecol. 21, 2015–2026.

Tian, S.P., Qin, G.Z., Xu, Y., 2004. Survival of antagonistic yeasts under field conditions and their biocontrol ability against postharvest diseases of sweet cherry. Postharvest Biol. Technol. 33, 327–331.

Valdés-Gómez, H., Fermaud, M., Roudet, J., Calonnec, A., Gary, C., 2008. Grey mould incidence is reduced on grapevines with lower vegetative and reproductive growth. Crop Prot. 27, 1174–1186.

Viñas, I., Usall, J., Teixidó, N., Sanchis, V., 1998. Biological control of major postharvest pathogens on apple with *Candida sake*. Int. J. Food Microbiol. 40, 9–16.

Zahavi, T., Cohen, L., Weiss, B., Schena, L., Daus, A., Kaplunov, T., Zutkhi, J., Ben-Arie, R., Droby, S., 2000. Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. Postharvest Biol. Technol. 20, 115–124.