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Impact of climate change environmental conditions on the resilience of different formulations of the biocontrol agent *Candida sake* CPA-1 on grapes

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Running headline: Climate change effect on *C. sake*

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Significance and impact of the study: The interaction between environmental factors that are expected to occur in response to climate change (CC) will have a significant impact on food security and availability. Little information exists on how elevated temperature, drought stress and increased CO_2 will have on the efficacy of biocontrol agents. The impact of these factors on the viability of different formulations of the biocontrol yeast *Candida sake* on the surface of grapes berries was evaluated for the first time. Such knowledge is critical for projecting the efficacy of biocontrol under climate change conditions and to identify formulations that have the necessary resilience to perform under CC conditions.

Abstract

Biocontrol agents have become components of integrated crop protection systems for controlling economically important fungal pathogens. *Candida sake* CPA-1 is a biocontrol agent of fungal pathogens of fruits, both pre- and post-harvest. While the efficacy of different formulations have been examined previously, few studies have considered the resilience of different formulations under changing climatic conditions of elevated temperature, drought stress and increased atmospheric CO_2. This study examined the effect of (a) temperature × RH × elevated CO_2 (400 vs 1000 ppm) on the temporal establishment and viability of two dry and one liquid *C. sake* CPA-1 formulations on grape berry surfaces; (b) temperature stress (25 vs 35 ºC); and (c) elevated CO_2 levels. Results indicated that temperature, RH and CO_2 concentration influenced the establishment and viability of the formulations but there was no significant difference between formulations. For the combined three-component factors,-increased temperature (35 ºC) and lower RH (40%) reduced the viable populations on grapes. The interaction with elevated CO_2 improved the establishment of viable populations of the formulations tested. Viable populations greater than Log 4 CFUs g^{-1} were recovered from the grape surfaces suggesting that these had conserved resilience for control of *Botrytis* rot in grapes.

Keywords: yeast formulations, global warming, climate change, resilience, elevated CO_2, biocontrol, *Botrytis cinerea*.

Introduction

Climate change is expected to have profound impacts on agroecosystems and thus food security (Medina et al. 2017). Maximising food production under climate change (CC) conditions will require effective crop protection systems, including biocontrol of fungal pathogens and pests. The Intergovernmental Panel on Climate Change (2014) has suggested that temperature will increase by 2-5 ºC and that more extreme fluctuations in wet and drought periods will occur, coupled with a doubling or tripling of the atmospheric CO_2 levels from 400 to 800-1200 ppm. Indeed, the EU Green paper has suggested that parts of southern Europe will be hotspots for CC impacts (European Commission 2013). The regions designated in the report are important for the production of many important agricultural and horticultural crops. It is thus important that biological control agents (BCAs) have the necessary resilience under such environmental stresses. For example, Borisade and Magan (2015) reported that entomogenous fungi used for pest control were less effective under CC scenarios than under existing environmental conditions.

The yeast *Candida sake* CPA-1 is a well-known BCA and its efficacy has been demonstrated against blue mould, grey mould, and *Rhizopus* rot on pome fruits (Viñas *et al.* 1998). CPA-1 is also effective against *B. cinerea* (Cañamás *et al.* 2011; Calvo-Garrido *et al.* 2013; Calvo-Garrido *et al.* 2014) and sour rot (Calvo-Garrido *et al.* 2013) in grapes. Laboratory-scale production of CPA-1 has been optimised (Arévalo 1998; Abadias *et al.* 2003) and both liquid (Torres *et al.* 2003; Abadias *et al.* 2003b) and solid formulations have been developed (Abadias *et al.* 2001, 2005; Cañamás *et al.* 2008; Carbó *et al.* 2017a; Carbó *et al.* 2017b). Two improved formulations of *C. sake* have been recently developed by the addition of biodegradable coatings using a fluidised-bed spray-drying system. This resulted in the production of film forming formulations that have better viability than liquid-based formulations on grapes (Carbó *et al.* 2017b).

No attempts have been made to examine the impact of the environmental factors predicted to occur during CC on the viability of formulations of BCAs for fungal pathogen control. Some studies have examined the effects of CC factors on BCAs for pest control (Johns *et al.* 2003; Diaz *et al.* 2012; Wang *et al.* 2014; Reeves *et al.* 2015). However, these studies have predominantly examined individual factors, such as elevated CO_2 or temperature. The interaction between three environmental factors (temperature, drought, and elevated CO_2) have been suggested to be critical in examining the effects of CC on fungal plant pathogens and insect pests (Medina *et al.* 2014; Borisade and Magan 2015; Medina *et al.* 2015a; Medina *et al.* 2015b).

There is thus a dearth of studies on the resilience of BCA formulations to CC environmental parameters. Recently, Carbó *et al.* (2017b) examined the population dynamics of two fluidised-bed spray-dried formulations on grapes. However, the potential resilience of different BCA formulations under extreme interacting environmental parameters has not been previously examined. Therefore, the present study examined the effect of the interaction between different environmental factors (25 vs 35 ºC; 85 vs 40% RH; and 400 vs 1000 ppm CO_2) on the resilience of one liquid and two dry formulations of *C. sake* CPA-1 by examining the population dynamics of *C. sake* on the surface of grapes.

Results and discussion

Combined effect of multiple climate change environmental factors on the population dynamics of *C. sake* formulations

Figure 1 shows the combined effect of interacting CC factors (temperature, RH. And CO_2) on the viability of *C. sake* CPA-1 formulations isolated from grape surfaces over a 96 h time period after application.

Under simulated conditions of 25 ºC, 400 ppm, and either 40 or 85% RH) all the formulations allowed *C. sake* to became readily established on the surface of grape berries (Figure 1a). All three formulations showed an increase in the number of viable cells after 48 and 96 h at both 40 and 85% RH. Previous ecophysiological studies of *C. sake* have indicated that 25 ºC is optimum for growth

(Teixidó *et al.* 1998). Overall, more than Log 6 CFUs g^{-1} of *C. sake* was established under conditions that represented the control treatment.

Figure 1b shows the impact of increasing the temperature to 35 ºC and maintaining the CO_2 concentrations at 400 ppm at both 40 and 85% RH. The elevated temperature (35ºC) generally reduced the number of recovered cells for all of the applied formulations, indicating that *C. sake* had a lower resilience at this temperature regardless of the formulation. There was a significant difference in cell survival between the different formulations after 48 h. The conditions under which all formulations had the least resilience was after 96 h at 35 ºC and 40% RH when the number of viable cells had been reduced by Log 0.52- Log1.12 from the number of cells originally applied. After 96 h at 85% RH, the number of viable cells was between Log 4.5-5 CFUs g^{-1}. At 40% RH this was between Log 4-4.5 CFUs g^{-1} in all the formulations. It has been previously shown that *C. sake* cells cannot survive at 37 ºC but is able to grow at 30 ºC, although not as well as at 20-25 ºC (Teixidó *et al.* 1998). Calvo-Garrido *et al.* (2014a) previously applied *C. sake* cells plus Fungicover® (a commercial coating) to grape berries and observed a decline of Log 5.6 units at extreme temperatures of 40 ºC and 100% RH after 72 h and a reduction of Log 2.7 units at 40 ºC and 30% RH after 48 h.

The resilience of the three formulated cell treatments was improved at 35ºC when the cells were exposed to elevated CO_2 (1000 ppm) at both RH levels with a similar trend for all the formulations (Figure 1c). Approximately Log 5 CFUs g^{-1} of viable cells were recovered from the surface of grape berries for all the formulations of *C. sake* CPA-1, with the exception of the liquid formulation at 40% RH. The population of viable cells in all the formulations increased after 96 h. This suggests that exposure to combined conditions of elevated temperature, RH, and increased CO_2 resulted in better resilience of the formulated cells on the surface of grape berries than under elevated temperature and RH stress alone. Thus, the colonisations and survival of CPA-1 formulated yeast cells at 35 ºC differed depending on the concentration of CO_2, with better resilience at 1000 ppm of CO_2 than at 400 ppm.

Effect of temperature on *C. sake* population dynamics on grape berries (after 96h)

Overall, an analysis of the relative effect of 25 vs 35 ºC indicated that no significant differences were observed in the number of viable *C. sake* cells recovered from grapes between the three different formulations (Table 1). This suggests that the drying temperatures used to make the solid formulations did not influence the resilience of the rehydrated cells on grapes. This also indicates that the fluidised-bed spray-dried formulations are more user-friendly to utilise than the liquid formulation, mainly because of the easier downstream handling of the biological product.

The interaction between temperature and RH (25 or 35 ºC; 40 or 85% RH) had a significant impact on the number of viable cells recovered from the surface of grape berries. The three-way interaction (temperature × RH × formulation) was not significant (see Table 1). The number of viable cells recovered from the grape berries was significantly better at 25 ºC than 35 ºC, regardless of treatment, and also higher at 85% RH than 40% RH. However, the formulations provided a measure of resilience to the *C. sake* cells exposed to the CC conditions examined. Previously, Calvo-Garrido *et al.* (2014) found that formulations of *C. sake* CPA-1 allowed the yeast to become established and survived under relatively dry Mediterranean climatic conditions when the maximal daily temperature reached 31 ºC, and the average minimum daily RH value was 39%.

In the present study, the highest number of *C. sake* cells recovered from grapes was achieved from the 25 ºC treatment. Previously, Teixidó *et al.* (1998) in ecological studies demonstrated that 20-25 ºC was optimum for the growth of unformulated cells of *C. sake*. With regard to the effect of RH on viability of cells from the formulations, the dry formulations gave better results than the liquid one at 85% RH. However, no differences were observed between the formulations at 40% RH (see Table 1).

Effect of CO_2 concentration on *C. sake* population dynamics on grapes berries (after 96h)

Little difference was observed between the formulations in the viability of *C. sake* cells in the different CO_2 treatments (400 vs 1000 ppm; Table 2). Due to the significant impact of the high temperature (35^{o}C) on cell viability of CPA-1 formulations, the effect of CO_2 at this temperature was

also examined. Results indicated that the drying process did not influence the resilience of the rehydrated *C. sake* CPA-1 cells on the surface of grape berries.

Regardless of the formulation, the viability of CPA-1 was significantly better at 1000 ppm CO_2 than at 400 ppm of CO_2 treatment; and also higher at 85% RH than at 40% RH. No significant differences were observed in the interaction between formulation and RH (Candifruit, Potato starch or Maltodextrin; 40 or 85% RH), or in the interaction between formulation and CO_2 (Candifruit, Potato starch or Maltodextrin; 400 or 1000 ppm CO_2). However, the interaction between RH and CO_2 (40 or 85%; 400 or 1000 ppm CO_2) resulted in a significant decrease in the populations of cells recovered from the surface of grape berries. Regardless of the RH, the resilience of *C. sake* in the different formulations was better under elevated CO_2 conditions. Also, the three-way interaction among formulation × RH × CO_2 was significant (see Table 2).

Previously, other fungal BCAs such as *Puccinia aprupta* var. *partheniicola* was shown to perform more effectively under elevated CO_2 levels than under existing atmospheric levels (Shabbir *et al.* 2014). However, this may vary with BCAs, as CC factors were shown to have a negative impact on the efficacy of some entomopathogenic fungi for pest control (Borisade and Magan 2015).

Estimated capacity of *C. sake* to control *Botrytis* rot under climate change scenarios

Previously, it was shown that populations of at least Log 3 to 5 CFUs g^{-1} of *C. sake* cells had to be recovered from grape surfaces after the BCA application for effective control of *Botrytis* bunch rot on grapes (Calvo-Garrido *et al.* 2013a). In the present study, the three formulations tested would result in the establishment of Log 4.87 to 5.49 CFUs g^{-1} under CC conditions. This is a range at which *Botrytis* rot would be expected to be effectively controlled (Cañamás *et al.* 2011); Calvo-Garrido *et al.* 2014).

Indeed, even in the high temperature scenario (35^{o}C), which represented the most stressed condition, the recovered population levels after 96 h were almost Log 4 CFUs g^{-1}. Therefore, the three formulations could be effective against *B. cinerea* on grapes under any tested climate scenario.

Thus, the resilience and viability of the yeast cells is maintained above the necessary threshold on the surface of grape berries to effectively control of *Botrytis*.

In summary, the present study demonstrated that the formulations of *C. sake* CPA-1 provided a sufficient level of resilience to the BCA under the CC conditions that allowed to yeast cells to retain a level of viability and population size within the range necessary for the control of *Botrytis* rot on grapes. Additionally, elevated levels of CO_2 boosted cell viability in the different formulations, even at the elevated temperature of 35ºC, regardless of RH. The fluidised-bed spray-drying process used to produce dry formulations of CPA-1 did not significantly affect the resilience and viability of *C. sake* cells on the surface of grape berries. It may be prudent to examine the relative resilience provided by ~~of~~ different formulations of BCAs to ensure that control levels achieved under existing environmental conditions can be maintained under future CC scenarios. In addition, the ecophysiology and pathogenicity of the pathogen may also change under CC scenarios and this may affect the relative efficacy of formulations of BCAs in the future (Váry *et al.* 2015).

Material and methods

Biocontrol agent and formulations

The yeast strain CPA-1 of *Candida sake* used in this study was obtained from University of Lleida-IRTA, Catalonia, Spain, and it was deposited at the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot. *C. sake* stock cultures were stored at 4 ºC on nutrient yeast dextrose agar 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}).

All assays were carried on with three different formulations of the BCA: (i) a liquid formulation registered in Spain under de name Candifruit™; (ii) a dry formulation based on potato starch; and (iii) a dry formulation based on maltodextrin. Both dry formulations were dried using a fluidised-bed spray-drying system by the addition of biodegradable coatings to enhance the survival under environmental stress conditions. The formulation process was done using the protocol described by Carbó *et al.* (2017b).

The number of CFUs ml^{-1} was determined by plating 100 µl of serial dilutions on NYDA and incubating at 25 ºC for 48 h. The viability of formulations was also checked by serial dilutions to calculate the required amount of product to achieve the final concentration of 2.5×10^7 CFUs ml^{-1}. The applied concentration of each treatment was also checked by serial dilutions on NYDA plates.

Inoculation and incubation conditions

The study was conducted using white seedless grapes washed with tap water to remove possible residues. Afterwards, grape bunches were left to dry in a flow bench and then cut into three-berry clusters leaving the pedicel attached. Three clusters formed one replicate and each treatment consisted of three replicates.

For each treatment, the required amount of formulation was dissolved in 200 ml of water to obtain a concentration of 2.5×10^7 CFUs ml^{-1}. To inhibit bacterial growth, 500 mg l^{-1} of ampicillin was added to each treatment. Each formulation was placed into a glass beaker and clusters immersed three times into the treatment using sterile forceps, then the clusters were hung on glass rods and allowed to dry at room temperature. When the grape surfaces were dry, each replicate was placed into a glass container and they were all placed in a plastic box and incubated in each climate environmental condition.

Environmental chamber conditions

Treated grapes were exposed to three different climatic scenarios: (i) the current conditions of 25 ºC and 400 ppm CO_2; (ii) elevated temperature of 35 ºC and existing CO_2 conditions of 400 ppm and (iii) interacting future climate change scenario of 35 ºC and 1000 ppm CO_2. In addition, two relative humidity (RH) conditions were tested for each scenario: (i) 40% and (ii) 85% RH.

When CO_2 concentrations of atmospheric air (400 ppm) were tested, the RH was controlled by introducing 2×500 ml beakers of glycerol/water solution with the same water activity (a_w) as the treatment condition to maintain the equilibrium relative humidity during incubation.

An incubator flushed with the required CO_2 concentration (1000 ppm) was used to simulate the possible climate change scenario. In this situation, the air moisture was controlled by inserting a container with 2 l glycerol/water solution with the same a_w as the treatment condition.

Evaluation of C. sake populations growth on grapes surface

Populations on grape berry surface were recovered after 0, 48 and 72 h. At the recovering time, the three berries of each cluster were separated cutting the pedicels with sterile scissors. The nine berries of each replicate were weighed and then placed into a sterile plastic bag containing 50 ml of sterile distilled water amended with Tween 80 (one drop per litre). Then, the bags were homogenised in a Stomacher 400 (Seward Ltd, Worthing, West Sussex, U.K.) for 10 min. Torres *et al.* (2012) recommended the use of the Stomacher as a consistent and rapid method for recovering the BCA populations from the fruit surface. Serial dilutions were then prepared as described previously to determine the CFUs ml^{-1}, with the results presented as CFUs g^{-1}. All tests were carried out with three replicates and repeated.

Statistical analyses

The results of CFUs g^{-1} data were transformed to logarithmic values prior to analyses to improve the homogeneity of variances. Data were analysed by multiple-factor ANOVA using JMP8 software (SAS Institute Inc., NC, U.S.A.). When the analysis was statistically significant (P<0.05), Student's test was used for means separation.

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Conflict of interest

The authors of this work declare that there is not conflict of interest.

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Tables

Table 1 Analysis of variance of effect of formulations, temperature, RH (relative humidity) and two- and three-way interactions on growth of *C. sake* over grapes. Significant sources were itemised and different letters indicate significant differences (P<0.05) according to Student's test.

Source	DF	SS	F Ratio	Prob > F
Formulation	2	0.119262	1.1055 NS	0.3480
Temperature	1	31.464600	583.3040 **	<.0001
25 ºC [A]				
35 ºC [B]				
Formulation × Temperature	2	0.003171	0.0294 NS	0.9711
RH	1	1.236696	22.9264 **	<.0001
85% [A]				
40% [B]				
Formulation × RH	2	0.399328	3.7015 *	0.0404
Potato starch, 85% RH [A]				
Maltodextrin, 85% RH [AB]				
Candifruit, 85% RH [BC]				
Candifruit, 40% RH [BCD]				
Potato starch, 40% RH [CD]				
Maltodextrin, 40% RH [D]				
Temperature × RH	1	0.712771	13.2136 *	0.0014
25 ºC, 85% RH [A]				
25 ºC, 40% RH [A]				
35 ºC, 85% RH [B]				
35 ºC, 40% RH [C]				
Formulation × Temperature × RH	2	0.289837	2.6866 NS	0.0894

Note: SS, sum of square; * significant P<0.05; ** significant P<0.001; NS, not significant

Table 2 Analysis of variance of effect of formulations, CO_2, RH (relative humidity) and two- and three-way interactions on growth of *C. sake* over grapes. Significant sources and two-way interactions were itemised and different letters indicate significant differences (P<0.05) according to Student's test.

Source	DF	SS	F Ratio	Prob > F
Formulation	2	0.2024381	1.5088 NS	0.2432
RH	1	1.0437026	15.5572 *	0.0007
85% [A]				
40% [B]				
Formulation × RH	2	0.1516857	1.1305 NS	0.3409
CO_2	1	5.3613231	79.9149 **	<.0001
1000 ppm of CO_2 [A]				
400 ppm of CO_2 [B]				
Formulation × CO_2	2	0.0973929	0.7259 NS	0.4951
RH × CO_2	1	0.8041026	11.9858 *	0.0022
85% RH, 1000 ppm of CO_2 [A]				
40% RH, 1000 ppm of CO_2 [A]				
85% RH, 400 ppm of CO_2 [B]				
40% RH, 400 ppm of CO_2 [C]				
Formulation × RH × CO_2	2	1.1582690	8.6325 *	0.0017

Note: SS, sum of square; * significant P<0.05; ** significant P<0.001; NS, not significant

Figure caption

Figure 1 Dynamics of populations of *C. sake* under the different treatment conditions: (a) current environmental conditions of 25 ºC and 400 ppm of CO_2; (b) elevated temperature scenario at 35 ºC and 400 ppm CO_2; and (c) interacting climate change environmental conditions of 35 ºC and 1000 ppm of CO_2. Key to treatments: Candifruit (■); potato starch formulation (■), and maltodextrin formulation (■) are represented as histograms for the 40% RH (solid colours) and 85% RH (striped bars) conditions. Mean values of three replicates are represented and vertical bars indicated standard error of the means.

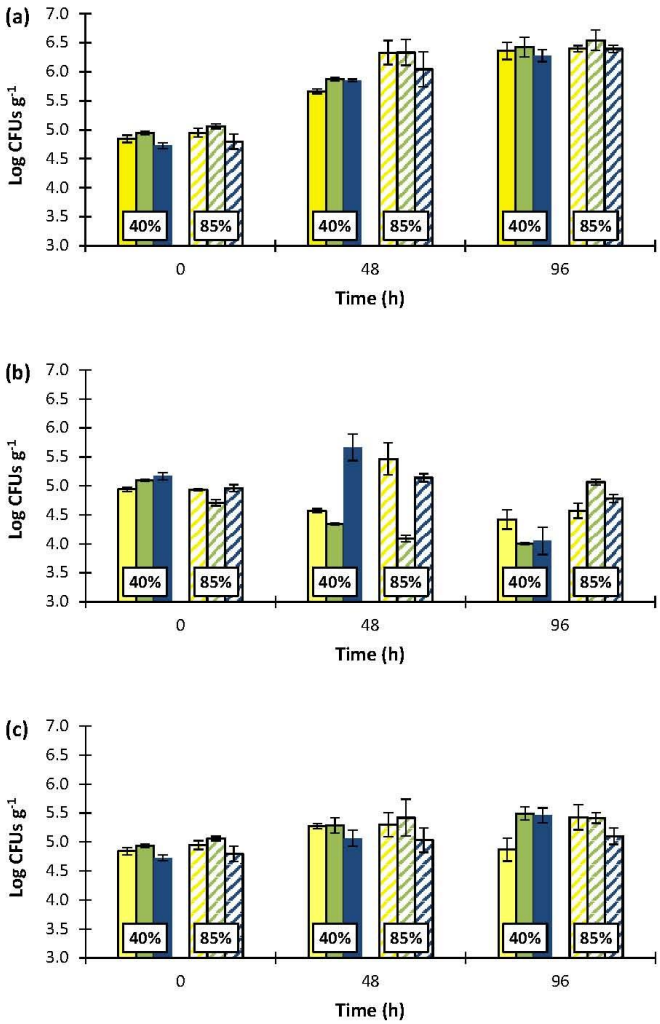

Fig. 1 Dynamics of populations of *C. sake* under the different treatment conditions: (a) current environmental conditions of 25 ºC and 400 ppm of CO_2; (b) elevated temperature scenario at 35 ºC and 400 ppm CO_2; and (c) interacting climate change environmental conditions of 35 ºC and 1000 ppm of CO_2. Key to treatments: Candifruit (■); potato starch formulation (■), and maltodextrin formulation (■) are represented as histograms for the 40% RH (solid colours) and 85% RH (striped bars) conditions. Mean values of three replicates are represented and vertical bars indicated standard error of the means.