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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*

Significance and impact of the study: Interacting climate change environmental factors 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. This study examined the impact that such factors may have on the viability of different formulations of the biocontrol yeast *Candida sake* on the surface of grapes for the first time. Such knowledge is critical in evaluating whether biocontrol will remain effective and identify formulations which have the necessary resilience under extreme climate change environmental 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 CO_2. This study examined 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 and (b) temperature stress (25 vs 35 ºC) elevated CO_2 concentrations. This showed 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 three-way climate change environmental factors, the increased temperature (35 ºC) and lower RH (40%) reduced the viable populations on grapes. However, the interaction with elevated CO_2 improved the establishment of viable populations of the formulations tested. The viable populations recovered were higher than log 4 CFUs g^{-1} 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*.

1. 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) have suggested that there will be an increase in temperature by between 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 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). These regions are important for many important agricultural and horticultural crops. It is thus important that biological control agents (BCAs) are able to have the necessary resilience under such environmental stresses. For example, Borisade and Magan (2015) showed 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.*998). CPA-1 has also been effective against *B. cinerea* (Cañamás *et al.* 2011; Calvo-Garrido *et al.* 2013a, 2014) and sour rot (Calvo-Garrido *et al.* 2013b) in grapes. Laboratory scale production of CPA-1 has been optimised (Arévalo 1998; Abadias *et al.* 2003a) and both liquid (Abadias *et al.* 2003b; Torres *et al.*, 2003) and solid formulations have been developed (Abadias *et al.* 2001, 2005; Cañamás *et al.* 2008; Carbó *et al.* 2017a). Indeed, recently, two improved formulations of *C. sake* have been developed by the addition of biodegradable coatings using a fluidised-bed spray-drying system, which has resulted in film forming formulations on grapes which have better viability than liquid-based formulations (Carbó *et al.* 2017a,b).

There has been no attempt to examine the impact of CC environmental factors on the viability of formulations of BCAs for fungal pathogen control. Some studies have examined 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 have predominantly examined individual component factors only such as elevated CO_2 or temperature. The interaction between the three factors have been suggested to be very important in examining the effects on fungal plant pathogens and indeed pests (Medina *et al.* 2014, 2015a,b; Medina *et al.* 2017; Borisade and Magan 2015).

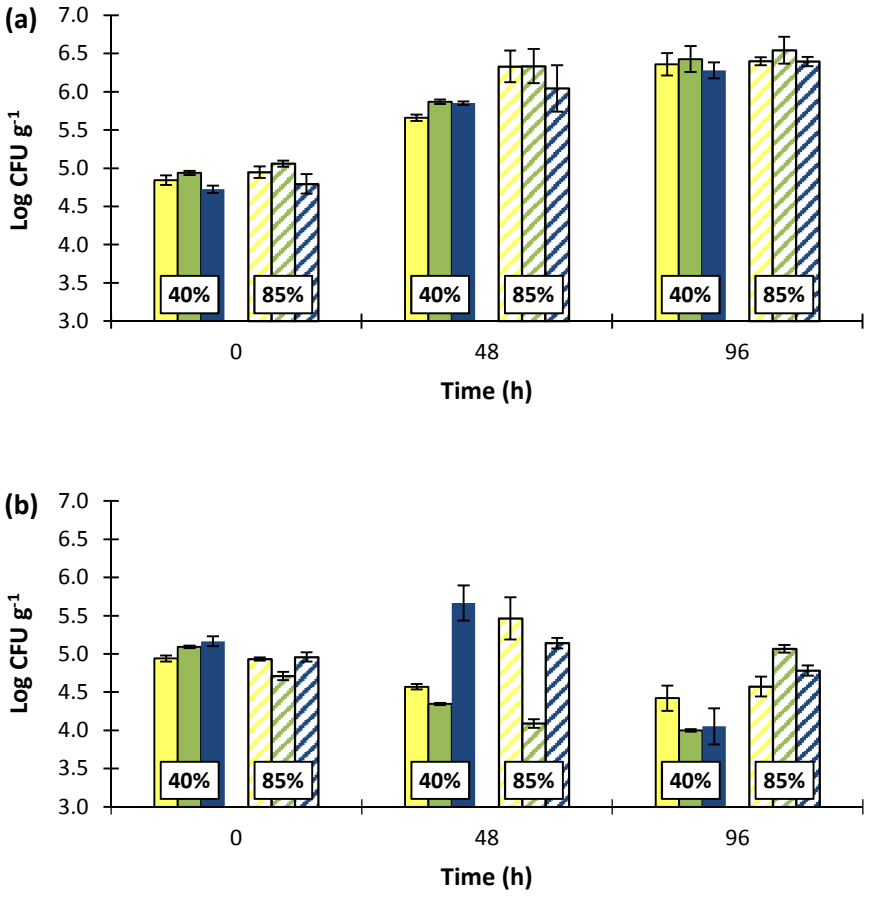
There is thus a dearth of studies on the resilience of formulations of BCAs in the context of tolerance of CC environmental factors. Recently, Carbó *et al.* (2017b) examined the population dynamics of two fluidised-bed spray-dried formulations in relation to viability on grapes. However, the potential resilience under extreme interacting environmental changes haa not previously been examined. Thus, this study examined the effect of interacting CC environmental factors (25 vs 35 ºC; 85 vs 40% RH; 400 vs 1000 ppm CO_2) on the resilience of one liquid and two dry formulations of *C. sake* CPA-1 cells in terms of population dynamics on the surface of grapes.

2. Results and discussion

2.1. Effect of interaction climate change environmental factors on population dynamics of *C. sake* formulations

Figure 1 shows the effect of interacting CC factors on the viability of *C. sake* CPA-1 formulations isolated from grape surfaces over a period of 96 h.

Under simulated present conditions (25 ºC, 400 ppm; and 40 and 85% RH) all the formulations became established on the grape surfaces (Figure 1a). Indeed, the recovered cells of all three formulations after 48 and 96 h showed an increase at both 40 and 85% RH. From previous ecophysiological studies of *C. sake*, 25 ºC was optimum for growth (Teixido et al., 1998). Overall, recovered formulations from grape surfaces showed that more than log 6 CFUs g^{-1} was established in the control treatment.



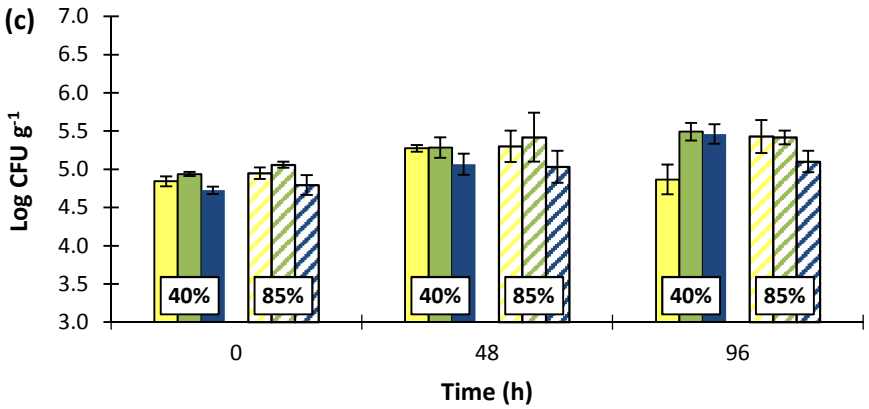


Fig. 1 Dynamics of populations of *C. sake* under the different treatment conditions: (a) current environmental conditions of 25ºC and 400 ppm of CO_2; (b) elevated temperature scenario at 35ºC and 400 ppm CO_2; and (c) interacting climate change environmental conditions of 35ºC and 1000 ppm of CO_2. 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.

Figure 1b shows the impact of increasing the temperature to 35 ºC when exposed to existing CO_2 concentrations of 400 ppm at both RH conditions. In the high temperature stress, the recovered populations of each of the formulations generally decreased indicating a lower resilience at this temperature. There was a significant difference between survival of formulations after 48 h. The conditions under which all formulations had the least resilience was after 96 hrs at 35ºC and 40% RH (log 0.52-Log1.12). After 96 h at 85% RH the formulations had recovered to 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 were 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.
At 35 ºC, the resilience of the three formulated cell treatments was better when exposed to elevated CO_2 at both RH levels with a similar trend (Figure 1c). The formulations of *C. sake* CPA-1 were recovered from the grape surfaces with viable numbers of at least around log 5 CFUs g^{-1} and, with the exception of the liquid formulation at 40% RH, all the formulations had increased their populations after 96 h. This suggests that exposure to interacting conditions of elevated temperature × RH and increased CO_2 together resulted in better resilience of formulated cells on grape surfaces than under elevated temperature × RH stress alone. Thus, the CPA-1 formulated yeast cells colonisation at 35 ºC was different depending on the CO_2 concentration with better resilience at 1000 ppm of CO_2 than at 400 ppm.

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

Overall, when examining the relative effect of 25 vs 35 ºC there were no significant differences in the populations of *C. sake* cells recovered from the grapes for the three

different formulations tested (Table 1). This suggests that the initial drying temperatures used to make the dry formulations did not influence the resilience of the rehydrated cells on the grapes. Moreover, this also suggests that the fluidised-bed spray-dried formulations were more user-friendly than the liquid formulation, mainly because of the easier downstream handling of the biological product.

However, the interaction between temperature and RH (25 or 35; 40 or 85% RH) had a significant impact on the populations of cells recovered from the grape surfaces. The three-way interaction was not significant (see Table 1). The viable populations recovered from the grapes, regardless of treatment, was significantly better at 25 ºC than 35 ºC; and also higher in 85% RH than 40% RH. However, the formulations were resilient in the CC conditions examined. Previously, Calvo-Garrido *et al.* (2014) found that formulations of *C. sake* CPA-1 were able 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%.

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

In the present study, the *C. sake* populations were recovered from grapes in the highest numbers from the 25 ºC treatment. Previously, Teixido *et al.* (1998) in ecological studies

showed that 20-25 ºC was optimum for 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 formulations at 40% RH (see Table 1).

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

There was little difference in the viability of the recovered *C. sake* cells from the formulations in the different CO2 treatments (400 vs 1000 ppm; Table 2). However, because of the significant impact of the high temperature (35oC) on CPA-1 formulations, the effect of CO_2 at this temperature was also examined. This showed that the drying process did not influence the resilience of the rehydrated CPA-1 formulations on the grape surfaces.

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

Despite this, regardless of the formulation, the viability of CPA-1 was significantly better in the elevated 1000 ppm CO_2 than 400 ppm of CO_2 treatment; and also higher at 85% RH than under water stress conditions. 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) had a significant impact on the populations of cells recovered from the grape surfaces. Regardless of the RH, the resilience of the formulations of *C. sake* 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 increased CO_2 concentrations than existing levels (Shabbir *et al.*, 2014). However, this may vary with BCAs, as CC factors were shonw to

have a negative impact on the efficacy of some entomopathogenic fungi for pest control (Borisade and Magan, 2015).

2.4. 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, under CC scenarios the three formulations tested would result in the establishment of Log 4.87 to 5.49 CFUs g^{-1}. This would be in the range over which *Botrytis* rot would 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 populations 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, provided conservation of the resilience of the yeast cells is maintained above the threshold necessary on the berry surface control of *Botrytis* rot would be achieved.

In summary, it has been demonstrated that the formulations of *C. sake* CPA-1 were resilient under the CC conditions examined and the viability of populations would be within the range necessary for *Botrytis* rot control on grapes. Indeed, under elevated CO_2 concentration, more viable cells were isolated from the formulations, even at the elevated temperature, regardless of RH. In addition, the fluidised bed drying process to produce dry formulations of CPA-1 did not significantly affect the resilience of *C. sake* cells on grape surfaces. It may be prudent to examine the relative resilience of different formulations of BCAs to ensure that control levels achieved under existing conditions will be as effective 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 (Vary *et al.* 2015).

3. Material and methods

3.1. *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.

3.2. *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.

3.3. *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.

3.4. *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.

3.5. *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
There are no conflicts of interest.

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