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Impacte ecològic i efectivitat de l'aplicació de l'antagonista *Candida sake* CPA-1 al camp per al control biològic de la podridura blava en postcollita de mançanes "Golden Delicious"

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Capítol 3

Impact of the antagonist *Candida sake* on apple surface microflora during cold and ambient (shelf life) storage

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Summary

This study examined the impact of the application of a biocontrol yeast, *Candida sake* CPA-1 (3×10^6 colony forming units (cfu) ml^{-1}) on the resident microbial populations just prior to harvest, during 7 months cold storage and subsequent ambient shelf-life in two seasons on apples untreated or treated with a preharvest pesticide programme. The changes in populations of the antagonistic yeast (*C. sake*) were also monitored. Generally, application of the antagonist in both 1995 and 1996 had little effect on the total bacterial populations which remained very low both prior to harvest and subsequently during cold storage. White yeasts were predominant on the apples during the experimental period, with lower populations of pink yeasts. When apples were removed after 7 months to ambient conditions the yeast populations increased quickly but those apples treated with *C. sake* had significantly less white yeasts than untreated controls. The dominant filamentous fungi isolated were *Cladosporium, Alternaria* and *Penicillium* spp.. This last genus, responsible for major postharvest disease was seldom isolated at preharvest but it became important during later cold storage and shelf life period. Populations of *Cladosporium* and *Penicillium* were significantly reduced by the *C. sake* treatment when removed from cold storage during the ambient shelf-life. In contrast, the *Alternaria* spp. were unaffected by the antagonist. The actual populations of *C. sake* applied decreased significantly immediately after application (24 h). However, they subsequently increased to a maximum after one month cold storage (10^3 cfu g^{-1}), and populations increased again only under ambient shelf-life conditions. The *C. sake* populations, and the resident microbial populations were unaffected by preharvest fungicide applications. This study demonstrates that preharvest application an antagonistic yeast such as *C. sake* has an impact on the resident mycoflora both during 7 months cold storage and during ambient shelf-life storage.

Key words: *Alternaria*, biocontrol, *Cladosporium*, fungicides, *Penicillium*, shelf-life

Introduction

Postharvest fruit diseases cause significant worldwide losses with blue mould caused by *Penicillium expansum* Link the most important disease of apples in Spain, followed by *Botrytis cinerea* Pers. and *Rhizopus nigricans* (Ehrenb) Lind (Palazón *et al.,* 1984). Currently, the main method used to control postharvest decay of fruits and vegetables are synthetic fungicides (Eckert and Ogawa, 1988).

The use of chemicals is becoming increasingly restricted because of the development of resistance to many fungicides by major postharvest pathogens (Bertrand and Saulie-Carter, 1978; Rosenberger and Meyer, 1979; Dekker and Georgopoulos, 1982; Spotts and Cervantes, 1986; Viñas *et al.*,1991, 1993) and concern for public safety and the environment (Norman, 1988; Wisniewski and Wilson, 1992). Because of these concerns alternative non-chemical methods of controlling postharvest diseases have been sought. Biological control using

microbial antagonists has been considered as a desirable and realistic alternative (Woodhead *et al.,* 1990).

In the past ten years particular success has been achieved by the development of microbial antagonists effective against fungal pathogens of pome and citrus fruit, some of which are now available commercially (Pusey and Wilson, 1984; Pusey *et al.*, 1988; Janisiewicz and Marchi, 1992; Janisiewicz and Bors, 1995).

Previous studies at the University of Lleida, Spain, demonstrated that a naturally occurring yeast, *Candida sake* (Saito and Ota) van Uden and Buckley (strain CPA-1), isolated from the apple surface (Usall, 1995) was demonstrated to be an effective biocontrol agent against the major postharvest pathogens of apples and pears (Usall, 1995; Viñas *et al.*, 1996).

Spurr (1994) has suggested that there is a relationship between the microbial ecology of aerial plant surfaces and natural biological control of diseases. Although considerable research has been done on phylloplane populations of leaves (Preece and Dickinson, 1971; Andrews and Kenerley, 1980, 1981; Blakeman, 1981; Pennycook and Newhook, 1981), little information exists on fruit and vegetable surfaces (Wilson and Wisniewski, 1989; Droby and Chalutz, 1992). Indeed, Spurr (1994) suggested that more knowledge and an understanding of the microbial status of fruit surfaces is a prerequisite to the development of successful microbial biocontrol systems.

Furthermore, little detailed information exists of the microflora of apples during postharvest storage. Knowledge is available of the fungi involved in postharvest diseases (Palazón *et al.* 1984), although very little is known of the postharvest ecology and interactions with the resident microflora on such apples.

The objectives of this work were to determine (a) the effect of fungicides on the natural microbial population dynamics on Golden Delicious apples at harvest, during cold storage and subsequent ambient shelf-life periods (b) the impact of application of the antagonist *C. sake* on these populations when applied alone or in combination with preharvest fungicide on the apple surfaces during these periods in two growing seasons, 1994/95 and 1995/1996, and (c) changes in the population dynamics of the antagonist *C. sake*.

Materials and Methods

Antagonist

The yeast used in this study was the strain CPA-1 of *Candida sake* obtained from UdL-IRTA, Catalonia, Spain. It was isolated from the apple surface and has previously been demonstrated to have antagonistic activity against

Penicillium expansum, Botrytis cinerea and *Rhizopus nigricans* in pome fruits (Usall, 1995; Viñas *et al.,* 1996). Stock cultures were stored at 5 °C and had been sub-cultured on nutrient yeast dextrose agar (NYDA).

Fruits and experimental apple orchards

This study was conducted during two growing seasons (1994/95 and 1995/96) in a 6-year-old apple orchard cv. Golden Delicious grown under standard cultural practices in Aitona (Lleida), Catalonia, Spain. A total of fourty-eight trees were used; twenty four were sprayed according to standard apple pest control recommendations for the area and the rest were left unsprayed. Chemical treatments were sprayed uniformly with a hand gun operating at a pressure of 10 atm. The pesticide regime used and the dates of application are shown in Table 1. The treatments used in this study were (a) untreated controls, (b) preharvest treatment with *C. sake*, (c) preharvest fungicide application, and (d) *C. sake*+preharvest fungicide application. Each treatment was replicated four times with each replicate consisting of a group of three trees to enable enough apples to be destructively sampled over the 7 month experimental treatment. The experiment was a randomised block design with guard trees to separate treatments.

Table 1. Summary of the timings and types of fungicide applications used in this study in 1994 and 1995.

CHEMICAL	Concentration (%)	Date of application	
		1994	1995
Summer oil 66% + Paration 3%	2	2 March	4 March
Sulphur 80% + Captan 50%	1 / 0.25	18 March	_
Flusilazol 40% + (Aluminates 10.5% + Borax 1.8% +Sulphur 56%)	0.12 / 1	15 April	18 April
Captan 50%	0.25	_	3 May
Flusilazol 40%	0.05	6 June	5 July

Inoculation of apple trees with the antagonist *C. sake* and sampling procedure

Two days before harvest, the antagonist *C. sake* CPA-1 (3×10^6 cfu ml^{-1}) was sprayed on relevant tree treatments. Two apples were randomly detached from the replicate groups of trees immediately after application and at 24 and 48 h to analyse *C. sake* populations on apples just prior to harvest. The fruit were harvested and put in separate boxes according to their treatment and kept in typical cold storage conditions (1 °C and 21% O_2) used commercially.

Samples to evaluate microbial and *C. sake* populations were taken at harvest, 15, 30, 60, 90, 150 and 210 days of storage. After 7 months (210 days) in cold storage, apples were taken out of the storage chambers and placed in ambient environmental conditions (25 °C) to simulate commercial (shelf-life) conditions. After a further 3 and 7 days samples were taken to evaluated microbial and *C. sake* populations.

Assessment of temporal microbial population dynamics on apples

The fruits were aseptically weighed, dissected and shaken in 200 ml sterile phosphate buffer pH 7 using a rotary shaker for 20 min at 150 rpm, and then sonicated for 10 min in an ultrasound bath. This final step was used to improve the detachment of microorganisms from the fruit surface. Serial dilutions of the washings were made with four replicates per dilution, and 0.1 ml aliquots were spread-plated onto both Potato dextrose agar (PDA)+streptomycin sulphate, and onto Plate count agar (PCA)+imazalil media for the analyses of fungi and bacteria respectively (Andrews and Kenerley, 1978). After incubation at 25 °C in the dark the bacteria, yeasts and filamentous fungi were counted. The number of colony forming units (cfu) g^{-1} of fresh weight tissue were calculated for each sample.

In all cases microscopic examination of individual colonies was carried out and sub-cultured, purified and stored at 4 °C on PDA or PCA if they were fungi or bacteria, respectively. Fungi were identified to genus level. Populations of bacteria were counted after 48-72 h and those of fungi after 7-10 days.

Assessment of temporal population dynamics of the antagonist *C. sake* on apples

The general procedure was as described in the previous section. Serial dilutions of the washings were spread-plate onto NYDA containing 0.5 g l^{-1} streptomycin sulphate to inhibit bacteria. After incubation at 25 °C in the dark for 48 h the isolated viable colonies per gram of fresh weight fruit (cfu g^{-1}) were calculated for each sample.

Treatment of results

Data of microflora and *C. sake* populations (cfu g^{-1} fresh weight) were transformed to logarithms to improve homogeneity of variances (Parbery *et al.*, 1981) and temporal populations plotted for each season. On each date statistical comparisons were made between all four treatments. For ease of presentation significant differences (P= 0.05) are mentioned in the text and the standard errors are shown for clarity on each sampling date.

Results

The frequency of isolation of bacteria, yeasts and filamentous fungi in untreated apples, in both seasons at harvest, during cold storage and ambient storage periods are shown in Figure 1. Isolation of yeasts and filamentous fungi were always greater than that of bacteria. Yeasts were in most of cases the predominant group isolated from the apple surface, especially after 2, 3 and 5 months cold storage. Frequency of isolation of bacteria was in all cases < 5%. The bacterial populations at harvest were very low (lesser than 250 cfu g^{-1}) in all treatments. These levels became even lower during cold storage conditions.

In general, the dominant filamentous fungi isolated from Golden Delicious apples during the present study were *Cladosporium* (*C. herbarum* and *C. cladosporioides*) and *Alternaria* spp. (mainly *A. alternata*). *Penicillium* spp. (mainly *P. expansum*) appeared during cold storage and their presence were significant (P= 0.05) from the second month of storage to the end of the study.

Other genera and species isolated occasionally included *Fusarium*, *Acremonium* and occasionally *Epicoccum nigrum*, *Gloeosporium*, *Aspergillus* (*A. flavus* and *A. niger*), *Trichoderma*, *Botrytis* and *Rhizopus*.

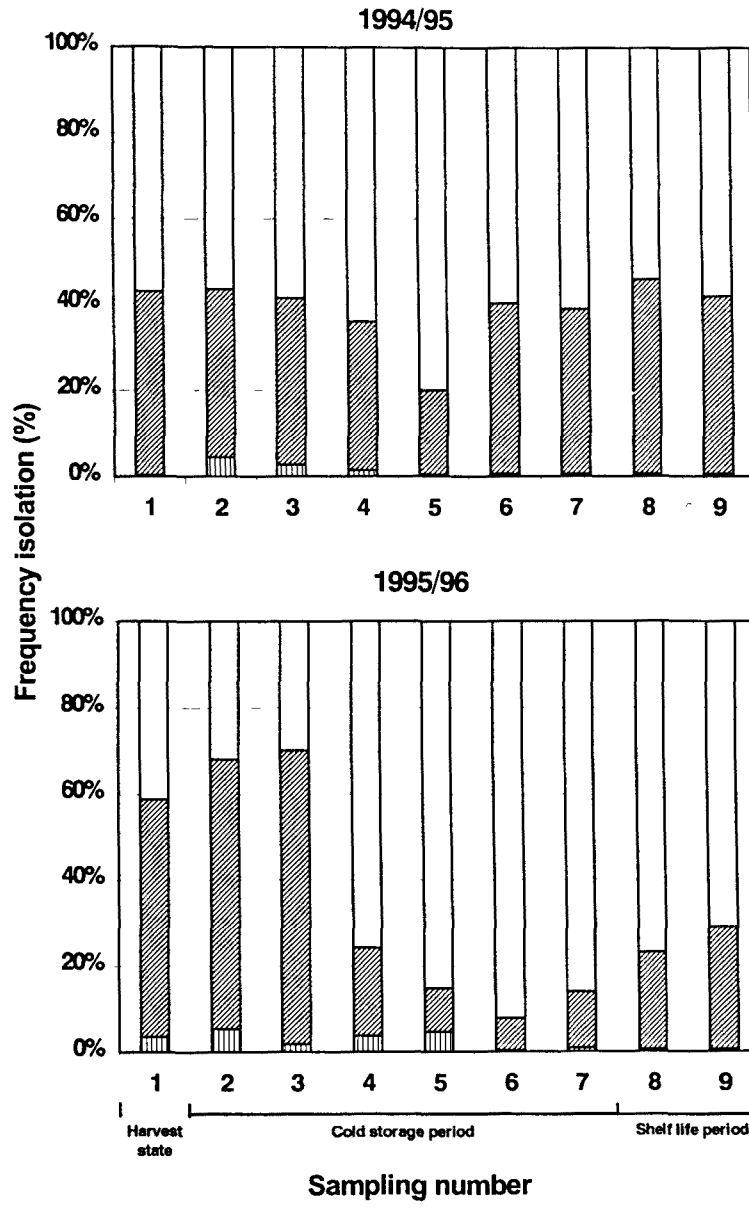

Figure 1. Frequency (%) of isolation of bacteria (▥), yeasts (□) and filamentous fungi(▨) from untreated apples cv. Golden Delicious in 1994/95 and 1995/96 seasons at harvest moment, cold storage and shelf life period.

Temporal changes in yeast populations

The total yeast populations, excluding *C. sake*, isolated from apples during cold storage and shelf-life periods in both seasons are shown in Figure 2. The most common group of yeasts isolated were white yeasts, with pink yeasts only occasionally isolated in this study. The populations of yeasts decreased significantly (P= 0.05) during the first 15 days of cold storage but recovered after one month in the lowered temperatures.

Immediately after removal from cold storage the yeast population of all treatments increased, with those treatments sprayed with the antagonist *C. sake* having significantly lower populations (P= 0.05) than those left untreated. This trend was clearer in the 1995/96 season.

Temporal changes in filamentous fungal populations

The total population of filamentous fungi, and the relative abundance of the main fungal genera isolated (*Cladosporium, Alternaria* and *Penicillium* spp.) and their temporal patterns of colonization during postharvest and shelf-life period, in both 1994/95 and 1995/96 seasons are shown in Figures 3 and 4, respectively.

Total fungal populations decreased markedly between the first and second sampling when apples were put into cold storage conditions. This pattern was observed with regard to total filamentous fungal populations, and for *Cladosporium* and *Alternaria* spp. Afterwards, at the third sampling (30 days cold storage) populations recovered and increased markedly to higher numbers than initially in the 1995/96 season, reaching the maximum populations isolated in the second year of this study.

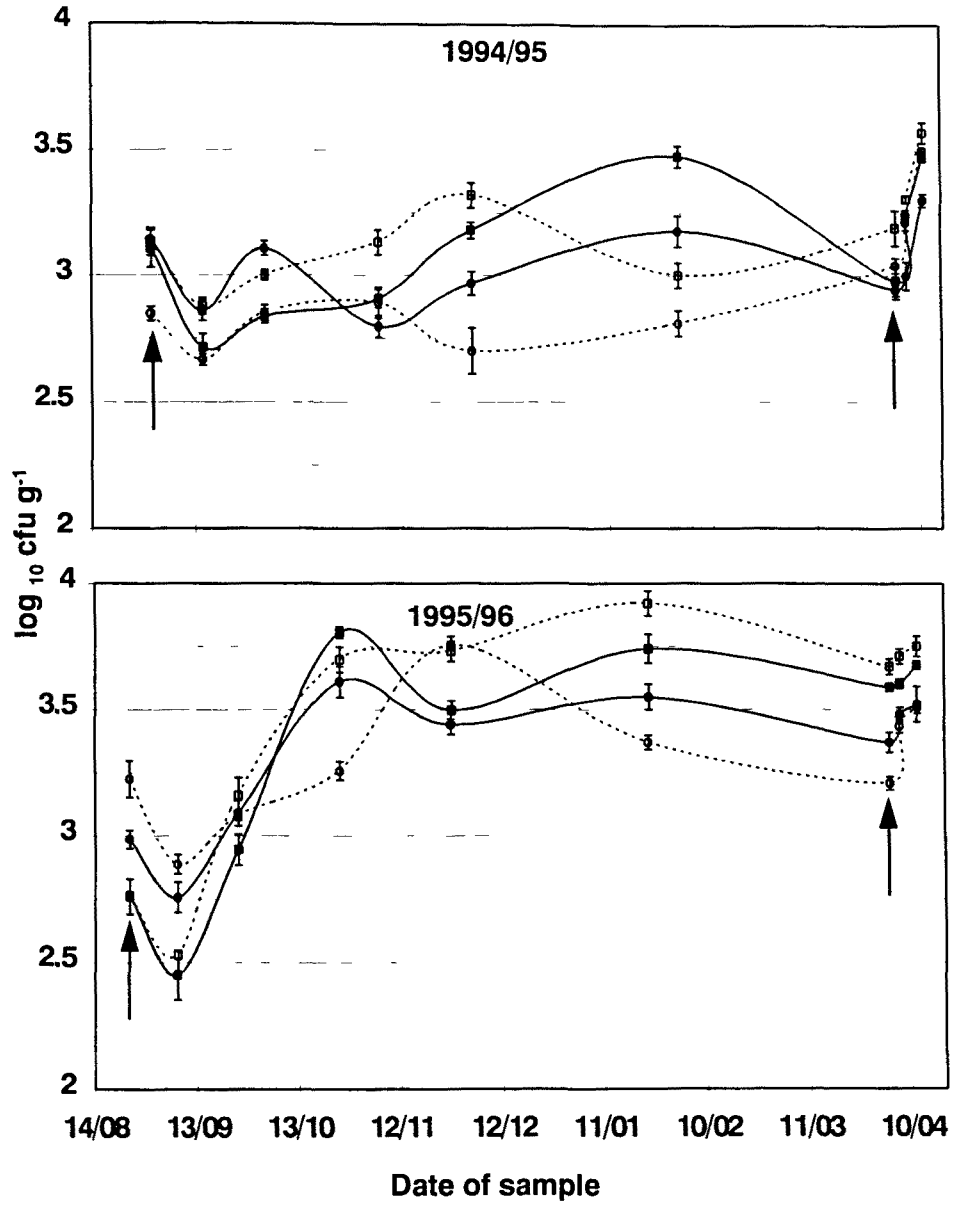

Figure 2. Populations of total yeasts(log_{10} of colony-forming units (cfu g^{-1} fresh weight) isolated from Golden Delicious apples untreated (□), treated at preharvest with *C. sake* (○), treated with preharvest fungicides (■) or treated at preharvest both with *C. sake* and fungicides (●), using the dilution plating technique, during two growing seasons (1994/95 and 1995/96). Points represent the means of four replicates on each sampling date and treatment, and vertical bars indicate standard errors of the mean. Arrows indicate the start and the end of cold storage period.

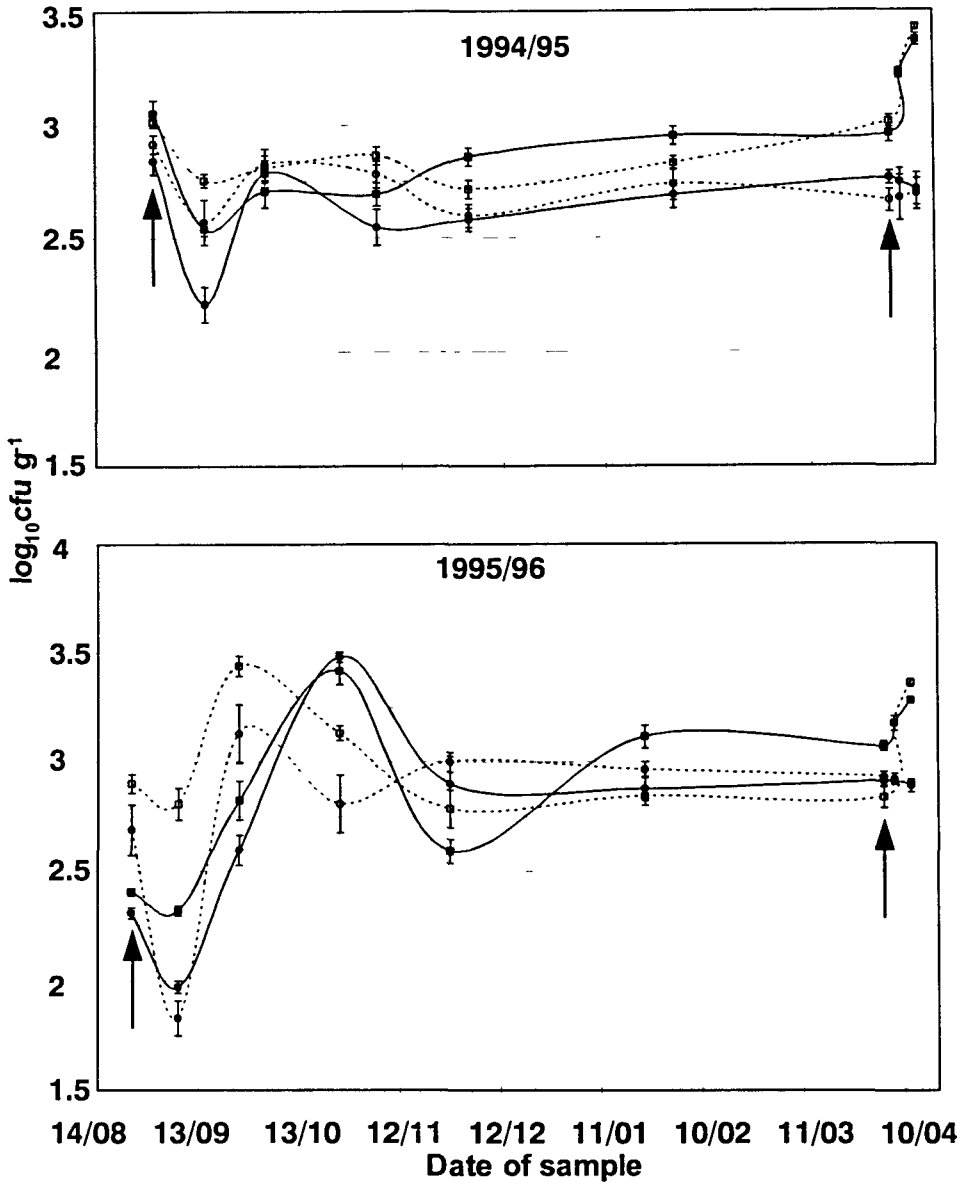

Figure 3. Populations of total yeasts(log_{10} of colony-forming units (cfu g^{-1} fresh weight) isolated from Golden Delicious apples untreated (□), treated at preharvest with *C. sake* (○), treated with preharvest fungicides (■) or treated at preharvest both with *C. sake* and fungicides (●), using the dilution plating technique, during two growing seasons (1994/95 and 1995/96). Points represent the means of four replicates on each sampling date and treatment, and vertical bars indicate standard errors of the mean. Arrows indicate the start and the end of cold storage period.

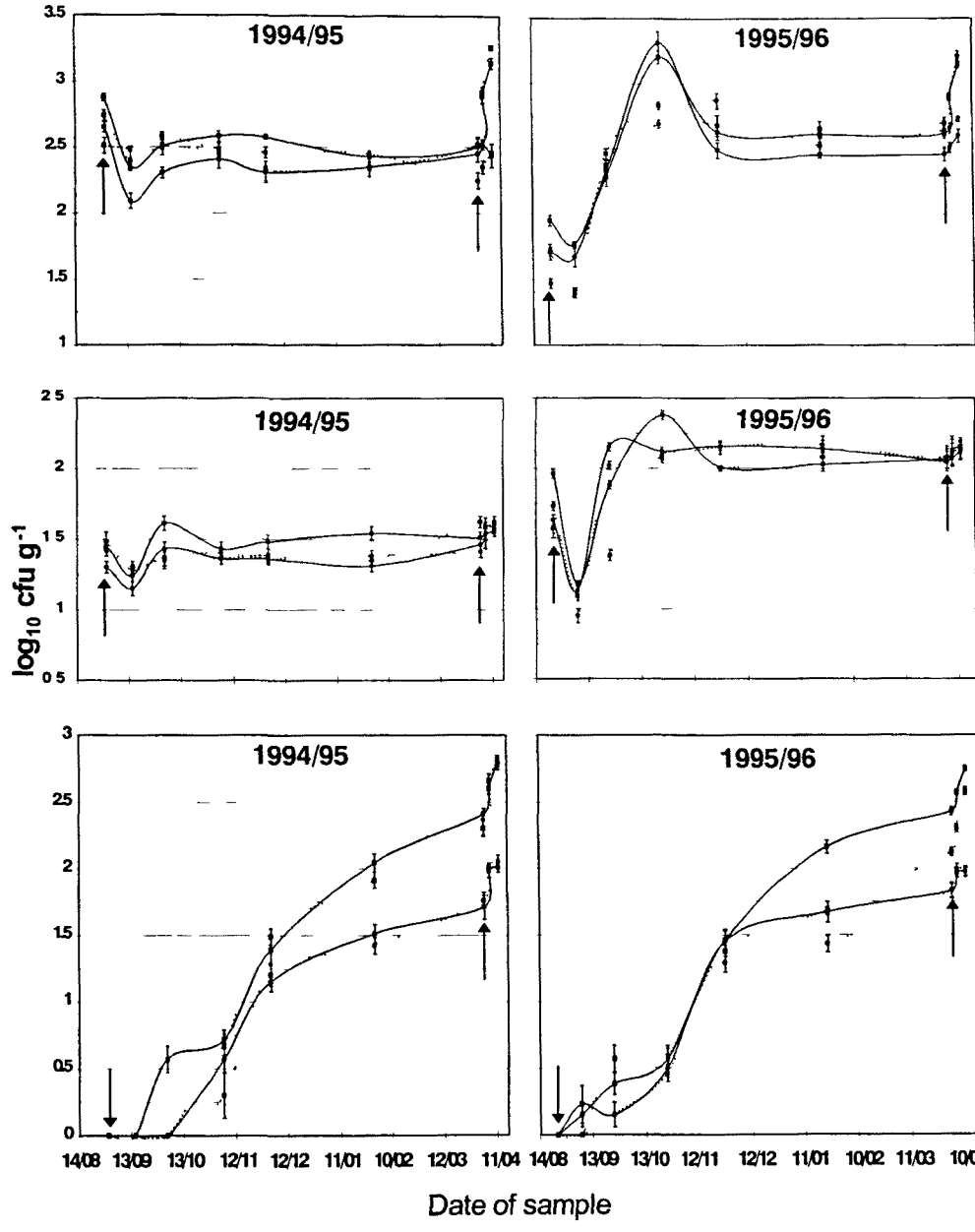

Figure 4. Populations of *Cladosporium* (a and b), *Alternaria* spp. (c and d) and *Penicillium* spp. (e and f) (log_{10} of colony-forming units (cfu g^{-1} fresh weight) isolated from Golden Delicious apples untreated (□) treated at preharvest with *C. sake* (○), treated with preharvest fungicides (■) or treated at preharvest both with *C. sake* and fungicides (●), using the dilution plating technique, during two growing seasons (1994/95 and 1995/96). Points represent the means of four replicates on each sampling date and treatment, and vertical bars indicate standard errors of the mean. Arrows indicate the start and the end of cold storage period.

Filamentous fungi in general, and *Cladosporium* and *Alternaria* spp. in particular remained more or less stable from the 3 month sample until the end of cold storage (7 months).

Once the apples were taken out of cold storage, during the ambient shelf-life period, treatments which did not include an antagonist application had significantly (P= 0.05) greater populations of filamentous fungi than treatments sprayed with the antagonist *C. sake*. Similar patterns were observed with *Cladosporium* and *Penicillium* spp. in both seasons. However, no differences between treatments were found for *Alternaria* populations during this period.

We have paid particular attention to the behaviour of the genus *Penicillium* which is responsible for the major postharvest disease on pome fruits. Their pattern was very different from the other major genera. At harvest no *Penicillium* was isolated from apples and populations after first month storage were only 8 cfu g^{-1} fresh weight of apples. However, *Penicillium* populations increased fast and progressively during cold storage reaching about 275 cfu g^{-1} after seven months in cold storage on apples which were not treated with the antagonist. Once apples were removed and returned to ambient conditions at 25 °C, *Penicillium* population increased significantly faster and more on untreated or treated with fungicides apples when compare to those treated with the antagonist. After 7 days at ambient conditions the populations were 2-fold bigger in both untreated and preharvest fungicide treatments. However, no patterns were observed in changes in filamentous fungal populations of apple mycoflora in relation to the preharvest application of fungicides.

Temporal population dynamics of *C. sake* sprayed on apples

The population dynamics of the antagonist *C. sake* on apples (untreated and treated with preharvest fungicides) during the whole experimental period are shown in Figure 5. Populations of about 700 cfu g^{-1} and 400 cfu g^{-1} in the 1994/95 and 1995/1996 seasons, respectively, were isolated from apples after application of the antagonist. However, populations decreased significantly (approx. 28-fold) within 24 h under field conditions. Populations increased during the following twenty-four hours but did not grow to the initial levels. Populations of *C. sake* in cold storage increased progressively to reach a maximum of about 10^3 cfu g^{-1} after one month storage. Subsequently, the populations of *C. sake* decreased during the rest of the storage period. When apples were taken out of cold chamber, *C. sake* populations increased quickly during the first 3 days, reaching population levels 3- and 6-fold higher than at the end of cold storage in the 1994/95 and 1995/96 seasons respectively. After 7 days populations remained relatively stable. There

were no significant (P= 0.05) differences between *C. sake* populations isolated from apples treated with *C. sake* or with *C. sake* + fungicide treatments.

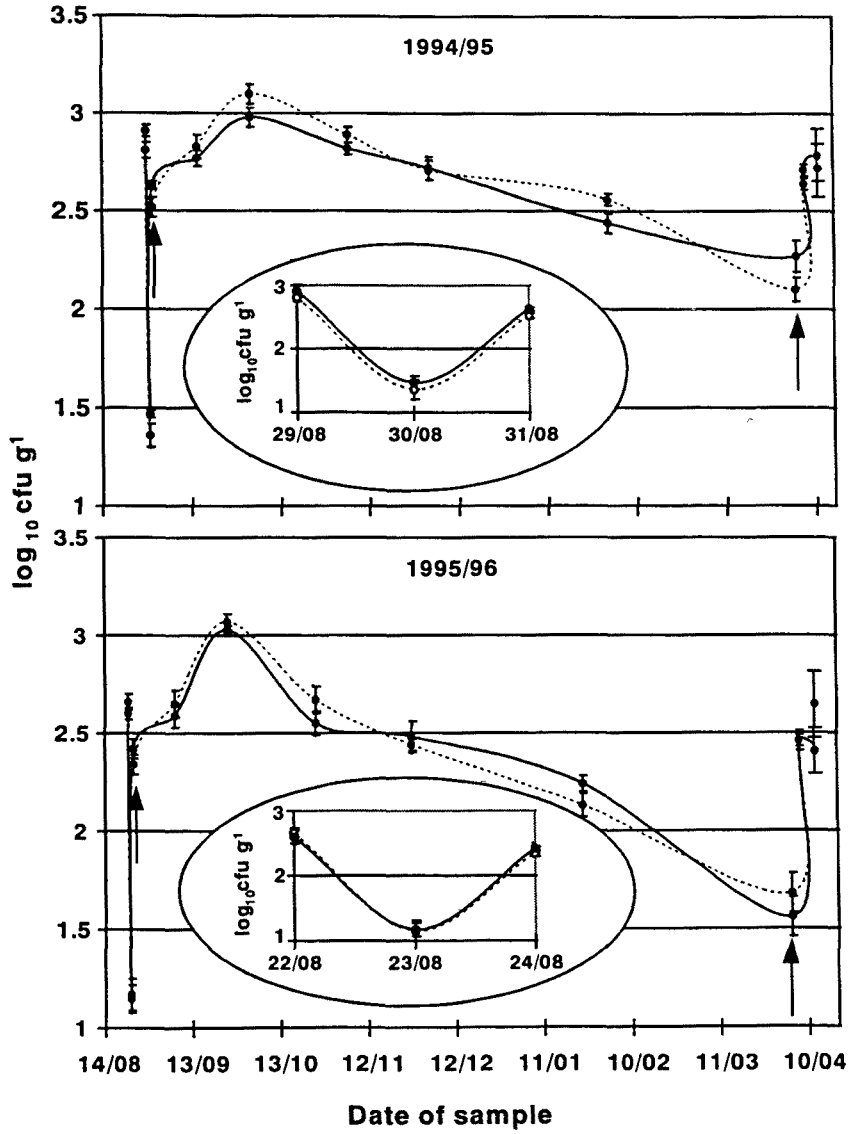

Figure 5. Populations of *C. sake* ($\log_{10}$ of colony-forming units (cfu g^{-1} fresh weight) isolated from Golden Delicious apples treated at preharvest with *C. sake* (-----), or treated at preharvest both with *C. sake* and fungicides (——), using the dilution plating technique, during two growing seasons (1994/95 and 1995/96). Points represent the means of four replicates on each sampling date and treatment, and vertical bars indicate standard errors of the mean. A magnification of *C. sake* population in the field has been represented. Arrows indicate the start and the end of cold storage period.

Discussion

Knowledge in naturally occurring microbial population on fruit surfaces has been meager and in many cases researchers have drawn primarily on studies of epiphytic populations on leaf surfaces and assumed that similar events might occur on fruits (Droby and Chalutz, 1992). However, it is important to study the microbial ecology of fruit surfaces to successfully evaluate whether a biocontrol agent can become effectively established and antagonise other microorganisms in this specialised niche. The present work was thus a detailed investigation of population dynamics of microflora associated with apples during the time periods from harvest to cold storage and subsequent shelf-life periods, and the impact of a successful yeast antagonist introduced just prior to harvest.

In this study the frequency of bacteria was in all cases less than 5% of the total microbial populations, and were seldom isolated during cold storage. In contrast, yeasts were predominant component of the apple microflora specially after two months cold storage to the end of the study. This was followed by filamentous fungi including *Cladosporium*, *Alternaria* and *Penicillium* spp.

Blakeman (1985) described bacteria as early colonizers of aerial plant surfaces such as leaves when nutrient levels on surface are low. However, as nutrients increase due to cell leakage, pollen and insect honeydew, the number of yeasts and filamentous fungi increase markedly. Furthermore, it has been known that phylloplane bacteria showed a marked decline in populations even after short periods of dry weather (Sleesman and Leben, 1976), while yeasts continued to colonize leaves under prolonged periods of high temperature and dry conditions (Fokkema *et al.*, 1979). High content of nutrients in mature fruits, high temperatures and dry weather conditions in summer at harvest could partially explain low bacteria populations isolated at the first sample. They may also be particularly sensitive to low temperature shock during cold storage.

Cladosporium, *Alternaria* and *Penicillium* were the main filamentous fungal genera isolated from apples during the study. However, studies of the mycoflora of cider apples suggested that *Aureobasidium pullulans* and *Epicoccum nigrum* were the main species present on apple surfaces in France (Bizeau *et al.*, 1990) with *Alternaria*, *Cladosporium*, *Fusarium* and *Penicillium* spp. less prevalent. The isolation technique of this experience was direct plating of epidermal samples on to the media.

Penicillium spp., particularly *P. expansum*, responsible for major postharvest disease of apples was seldom isolated in the preharvest samples in both seasons. This supports previous studies by Usall and Viñas (1989), which demonstrated the presence of *Penicillium* spp. in apple orchards just prior to harvest was

insignificant in northern Spain. However, it became very important during later cold storage and the shelf-life period.

Preharvest fungicide application alone or with the antagonist had little effect on the apple mycoflora just prior to harvest. The last fungicide application was applied in early July, with little residual effect evident two months later. Previous studies suggest that the impact of fungicides on aerial plant mycoflora often last for a maximum of between 3-6 weeks (Andrews and Kernerley, 1980).

White yeasts were always the predominant component of the mycofloral community, with a very low presence of pink yeasts. Other authors have reported a similar pattern on buds of apples (Pennycook and Newhook, 1981). During ambient storage after removal from cold storage, a consequence of the temperature rise was a rapid increase in yeast populations isolated from the apple surface. Apples not treated with the antagonist also achieved much higher populations than those sprayed with *C. sake*. This could be due to the inoculated *C. sake* cells being more competitive than resident yeast flora, and competing more effectively for nutrients on the apple surface.

It was notable that immediately after field application of *C. sake*, the populations of the antagonist were drastically decreased during the first 24 hours, probably as a consequence of high temperature and low humidity shock. Studies on preharvest biocontrol treatments using *Trichoderma harzianum* and *Ulocladium atrum* in relation to control of *Botrytis* on various crops (Elad and Kirshner, 1993; Köhl *et al.*, 1995) found, respectively, that relative humidity and leaf wetness periods had a significant effect on effective establishment. Furthermore, McKenzie *et al.*, (1991) found that unformulated pure conidial suspensions of *T. harzianum* did not survive effectively under field conditions. However, in the present work, the antagonistic yeast *C. sake* was able to survive under field conditions and increased during cold storage. This indicates good adaptation of this strain to cold temperatures. Janisiewicz (1991) has suggested that yeasts have characteristics that make them desirable candidates for biocontrol of postharvest diseases, one of those being tolerance to low temperatures.

This study has shown that it is important to examine the effect of potential biocontrol agents on resident epiphytic and pathogen populations of apples. Application of *C. sake* on apples just prior to harvest had a significant effect on filamentous fungal populations at the end of cold storage and during the ambient shelf-life period. Populations of *Cladosporium* and *Penicillium* genera increased significantly during the shelf-life period on untreated apples when compared to those treated with *C. sake*. However, no significant effects were observed on *Alternaria* populations. Previous studies (Teixidó, unpublished data) have shown

that *C. sake* had no effect against artificially inoculated *Alternaria alternata* populations on apple trees. Biocontrol agents are generally specific in their action in relation to pathogens and hosts (Janisiewicz, 1988).

This study has also demonstrated that the antagonist yeast *C. sake* was able to survive under field conditions on the surface of Golden Delicious apples and that in cold storage the antagonist populations increased significantly during the first month before progressively decreasing for the next 6 months. However, viability of the yeast was conserved as populations grew very quickly when apples were removed to ambient conditions. Furthermore, populations of *Cladosporium* and *Penicillium* were markedly reduced. Further research is needed to examine if preharvest *C. sake* treatment is able to effectively control postharvest diseases such as *P. expansum.* This study has shown that *C. sake* CPA-1 has good features to be an effective biocontrol agent in pre and postharvest conditions, and also during the subsequent ambient shelf-life period.

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Capítol 4

Efficacy of Pre and Postharvest *Candida sake* biocontrol treatments to prevent blue mold on apples during cold storage

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Enviat a: Biocontrol Science and Technology

Summary

This study compared the biocontrol efficiency of pre and postharvest applied yeast cells of *Candida sake* to apples (cv Golden Delicious) wounded before and after harvest and inoculated with *Penicillium expansum* prior to cold storage in cold storage conditions in two seasons, 1994/95 and 1995/96. The establishment of populations of *C. sake* during this period were also determined.

In both years postharvest treatment of the antagonist resulted in significant ($P< 0.05$) and effective control of *Penicillium* rot whether pre and postharvest wounds were made. The maximum control levels achieved in terms of incidence and severity were bigger than 80% diameter reduction and 50% incidence reduction However, preharvest application of the antagonistic yeast at a concentration of 3×10^6 cfu ml $^{-1}$ was less effective against *Penicillium* rot than postharvest treatment with lesion size being reduced by about 50%. No improvements in biocontrol were observed when apples were treated with the yeast antagonist both pre and postharvest.

C. sake population levels during cold storage of apples treated only preharvest with the antagonist decreased faster and before those apples receiving a postharvest application where higher populations were maintained even after 90 days in cold storage (about 7×10^3 and 1.5×10^4 cfu ml $^{-1}$ on apples treated only with *C. sake* postharvest treatment and 9×10^3 and 1.2×10^4 cfu ml $^{-1}$ on apples treated both pre and postharvest in 1994/95 and 1995/96 respectively).

Key words Biological Control, Postharvest diseases, *P. expansum*, antagonist, field treatments

Introduction

Fruits and vegetables suffer significant losses from fungal diseases after harvest (Eckert, 1975; Dennis, 1983; Snowdon, 1990, 1992). Postharvest application of synthetic fungicides has been the main method used for combating these diseases (Eckert and Ogawa, 1988). However, the development of resistance to these fungicides (Bertrand and Saulie-Carter, 1978; Dekker and Georgopoulos, 1982; Viñas *et al.*, 1991, 1993), and public demand to reduce pesticide use has provided the impetus to develop alternative and effective natural methods of controlling postharvest diseases.

Biological control has emerged in recent years as a promising solution (Janisiewicz, 1991; Fokkema *et al.*, 1993; Korsten *et al.*, 1994; Wilson and Wisniewski, 1994). Effective biological control has been reported for postharvest diseases of pome (Janisiewicz, 1987; Janisiewicz and Marchi, 1992), stone (Pusey and Wilson, 1984), citrus (Chalutz *et al.*, 1988) and various subtropical fruits (Koomen and Jeffries, 1993; Korsten *et al.*, 1993). Recently two bacteria (*Pseudomonas syringae*) and one yeast (*Candida oleophila* Montrocher) were registered by the Environmental Protection Agency in United States for control of

postharvest diseases of apple, pear and citrus fruit (Janisiewicz, 1996). Recent studies have shown that a strain of *Candida sake* (Saito and Ota) van Uden and Buckley (CPA-1) is an effective antagonist for the control of the major postharvest pathogens on pome fruits (Usall, 1995; Viñas *et al.*, 1996). While most biocontrol agents have been applied postharvest, a few studies have examined preharvest application for postharvest control of pome fruit diseases (Leibinger, *et al.*, 1997). It has been suggested that this would be advantageous as less fruit manipulation would be involved and decreases the potential for damage and injuries which may occur during any postharvest treatments. It would also reduce the time periods between harvest and cold storage required for application of treatments, and avoid additional contamination of pathogenic fungi from drenching solutions usually used during chemical treatments.

Furthermore, it has been suggested that biocontrol of postharvest diseases by yeasts is mainly due to nutrient competition (Droby and Chalutz, 1994). This implies that early preharvest establishment of the biocontrol agent could result in effective pre-emptive niche exclusion on pome fruit surfaces and improve the potential for postharvest control (Smilanick *et al.*, 1993). However, few attempts have been made to examine and compare these strategies in apple field trials.

The objectives of the present study were to evaluate and compare (a) the efficacy of the biocontrol agent *C. sake* when applied pre or postharvest to control *P. expansum* rot of apples cv. Golden Delicious during cold storage, and (b) to evaluate the population dynamics of the yeast treatments on the apple surface during three months cold storage.

Material and Methods

Fungal isolates

Antagonist: The isolate used in this study was the strain CPA-1 of *Candida sake* obtained from UdL-IRTA, Catalonia, Spain. This strain was isolated from the apple surface and has previously been demonstrated to have antagonistic activity against *Penicillium expansum, Botrytis cinerea* and *Rhizopus nigricans* (Usall, 1995; Viñas *et al.*, 1996). Antagonistic suspensions were cultured in Nutrient Yeast Dextrose Broth (NYDB) at 25 °C for 37 h.

Pathogen: Penicillium expansum was isolated from decayed apples kept in cold storage. The cultures were maintained on Potato Dextrose Agar medium (PDA). A conidial suspension (10^4 conidia ml^{-1}) was prepared from 10-day-old cultures to use in effectivity assays.

Fruits and orchards

This study was conducted during two growing seasons (1994/95 and 1995/96) in a 6 year-old apple orchard cv. Golden Delicious grown under standard cultural practices in Aitona (Lleida), Catalonia, Spain.

Temperature and Relative Humidity were measured during the field period of the study and are summarised in Table 1.

Table 1. Maximum and minimum temperature and Relative Humidity data registered over the 48 h period from preharvest *Candida sake* treatment to harvest moment.

DATA	Growing season	
	1994	1995
Maximum Temperature (°C)	26	32
Minimum Temperature (°C)	15	17
Maximum Relative Humidity (%)	80	86
Minimum Relative Humidity (%)	40	52

Field trials for pre and postharvest biocontrol of *P. expansum*

Twenty trees were selected at random to conduct this experiment. One tree (70 apples) was used as the sample unit and every treatment was repeated four times. In all cases guard trees were used to separate randomized treatments and replicates.

Five treatments were studied to compare pre and postharvest application of *C. sake* cells to control *P. expansum* disease on apples, with pre and postharvest wounds. The treatments used in this study in both seasons are detailed in Table 2 and included the following combination of factors: Preharvest inoculation with*C. sake*, with additional postharvest wounding (to simulate manipulation damage), and postharvest inoculation of the biocontrol agent *C. sake*.

Preharvest inoculation was done by spraying a 3×10^6 cfu ml^{-1} suspension of *C. sake* at 10 atm pressure on the trees, immediately after preharvest wounding.

Two days after the antagonist treatment the 70 wounded fruits of each tree were harvested from all treatments (preharvest treated, and untreated) and put in separate boxes according to their treatment.

Table 2. Treatments applied to the fruits according to the experimental design

Treatment	Sample (apples)	Preharvest wounds	Preharvest inoculation of *C. sake*	Postharvest wounds	Postharvest inoculation of *C. sake*	Postharvest inoculation of *P. expansum*
Untreated	70 × 4	+	-	-	-	+
CsPre	70 × 4	+	+	-	-	+
CsPost	70 × 4	+	-	-	+	+
CsPre+WPost	70 × 4	+	+	+	-	+
CsPre+CsPost +WPost	70 × 4	+	+	+	+	+

CsPre.- Preharvest *C. sake* treatment
CsPost.- Postharvest *C. sake* treatment
Wpost.- Postharvest Wounding

To simulate field damage each apple was wounded, 2 days before harvest, with a 1 mm diameter nail by making four equidistant wounds in the upper plane of the apple (preharvest wounding). Postharvest wounding was done by adding two wounds on the lower half of the fruits as described previously. Postharvest inoculation of the antagonistic yeast was achieved by dipping the boxes containing fruits in a 3×10^6 cfu ml^{-1} suspension of *C. sake* for 30 s, immediately after postharvest wounding.

All treatments included a final inoculation of the pathogen by dipping the boxes of fruits in a 10^4 conidia ml^{-1} suspension of *P. expansum* for 30 s.

Once dried, apples from all treatments were stored under typical cold storage conditions (1 °C and 21% O_2) used commercially. The percentage of infected wounds (incidence) and lesion diameters (severity) caused by *P. expansum* were measured two months after cold storage from all pre and postharvest treatments.

Population dynamics of *C. sake* on apple surface

Wounded apples from each of the treatments described previously which had been treated with *C. sake* were stored in the same post-harvest conditions to evaluate

population dynamics of the antagonistic yeast in relation to the different treatments. Samples were taken at day 0 (just prior to cold storage), 1, 5, 30, 60 and 90 days storage, and the *C. sake* populations on the apple surface quantified.

Isolation of cells of the biocontrol yeast was carried out as follows: apples were weighed and aseptically peeled . The peel and wound area was shaken in 200 ml sterile phosphate buffer (pH 7) on a rotatory shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasound bath. This final step was used to improve detachment of microorganisms from the apple surface. Serial dilutions of the washings were made and plated on Nutrient Yeast Dextrose Agar (NYDA) containing 0.5 g l^{-1} streptomycin sulphate as a bacteriostat. After incubation at 25 °C in the dark for 48 h the isolated viable colonies per gram of fresh weight of fruit (cfu g^{-1}) were calculated for each sample. This study was carried out with four replicates per treatment.

Data analysis

A general linear model (GLM) procedure of the Statistical Analysis System was performed on disease incidence data (percentage of infected wounds) and on severity data (lesion diameter). Statistical significance was judged at the $P< 0.01$ level. When the analysis was statistically significant the Duncan's Multiple Range Test for separation of means was performed.

Results

Efficiency of biocontrol of *Penicillium* rot by the different *C. sake* treatments

Preharvest wounds

All antagonist treatments studied significantly ($P< 0.01$) decreased development of *P. expansum* rot in both years study. Severity and incidence of the disease were significantly higher (> 80%, > 10 mm respectively) in untreated apples (Figure 1). The mean decay reduction on apples treated preharvest with *C. sake* was about 50%. No differences were observed between preharvest treatments either with pre or pre and postharvest wounding.

In both growing seasons the incidence and lesion diameter on apples treated with *C. sake* postharvest were significantly lower than apples treated preharvest only. Percentages of *Penicillium* rot control achieved with postharvest treatment alone were always greater than 80%. However, no improvement of biocontrol was observed when apples were inoculated at both pre and postharvest stages with *C. sake* in both seasons.

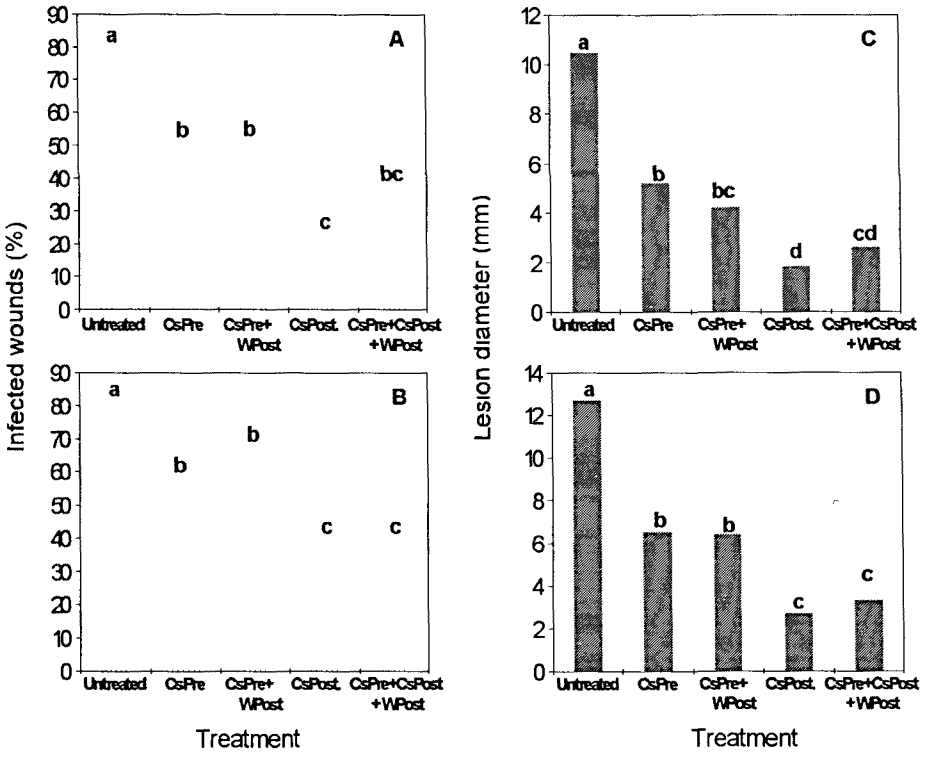

Figure 1. Effect on preharvest wounds of different *C. sake* treatments on percentage of infected wounds (□) or lesion diameters (▨), caused by *P. expansum* on Golden Delicious apples during 1994/95 (A and C) and 1995/96 (B and D) growing seasons. Fruits were stored for 60 days at 1°C. Different letters in the bars indicate ssignificant differences between means according to a Duncan's Multiple Range Test ($P<0.01$).

Postharvest wounds

After two months in cold storage conditions, inoculated postharvest wounds had in general lower incidence and smaller lesion size of blue mould than preharvest wounds.

Treatment that included preharvest application with *C. sake* and postharvest wounds presented in both seasons bigger *P. expansum* rot development than the treatment which compile all kind of treatments (pre and postharvest wounds and antagonist inoculation) as can be seen in Figure 2. Reduction of disease severity and incidence achieved with this last treatment in postharvest wounds in relation to preharvest treatment were about 73% and 58% respectively.

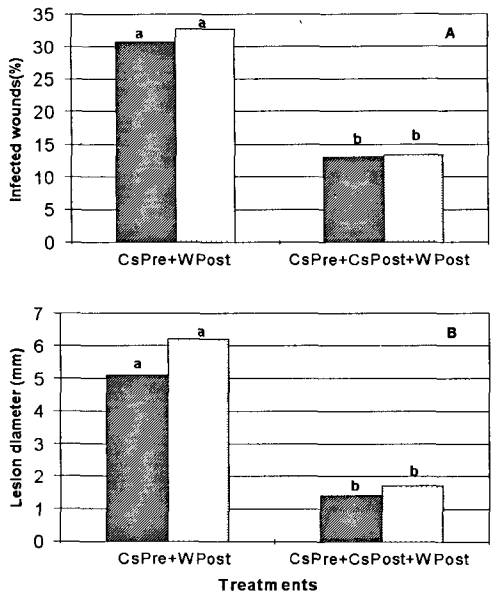

Figure 2. Comparative effect on postharvest wounds of *C. sake* preharvest treatment and both pre and postharvest on percentage of infected wounds (A) and lesion diameter (B), caused by *P. expansum* on Golden Delicious apples during 1994/95 (▨) and 1995/96 (□).

Postharvest *C. sake* treatment significativelly increased control of *P. expansum* rot in postharvest wounds.

Population dynamics of *C. sake* on wounded apples

Population dynamics of *C. sake* on wounded apple surface during the cold storage period after the different treatments assayed in this study are shown in Figure 3. Initially, 48 h after preharvest applications of *C. sake*, populations were higher in the 1994/95 (about 1750 cfu g^{-1}) than in 1995/96 (about 370 cfu g^{-1}) growing season.

The *C. sake* populations of all treatments studied decreased during the first 24 h in cold storage conditions. Subsequently, they increased progressively to reach maximum populations after about 1 month of storage. Population on apples that include postharvest antagonist application achieved higher population levels in both years than those which only received a preharvest treatment.

Where only preharvest treatments of *C. sake* were applied, total yeast populations on apples decreased faster and before than the two postharvest treatments after 60 days storage; fruits with these treatments had lower populations than fruits that had been treated postharvest with *C. sake*, especially during the 1995/96 growing

season Both postharvest treatments with *C sake* maintained higher population levels even after 90 days in cold storage (about 7×10^3 and $1\,5 \times 10^4$ cfu ml ^{1} on apples treated with *C sake* postharvest treatment only and 9×10^3 and $1\,2 \times 10^4$ cfu ml^{-1} on apples treated both pre and postharvest in 1994/95 and 1995/96 respectively)

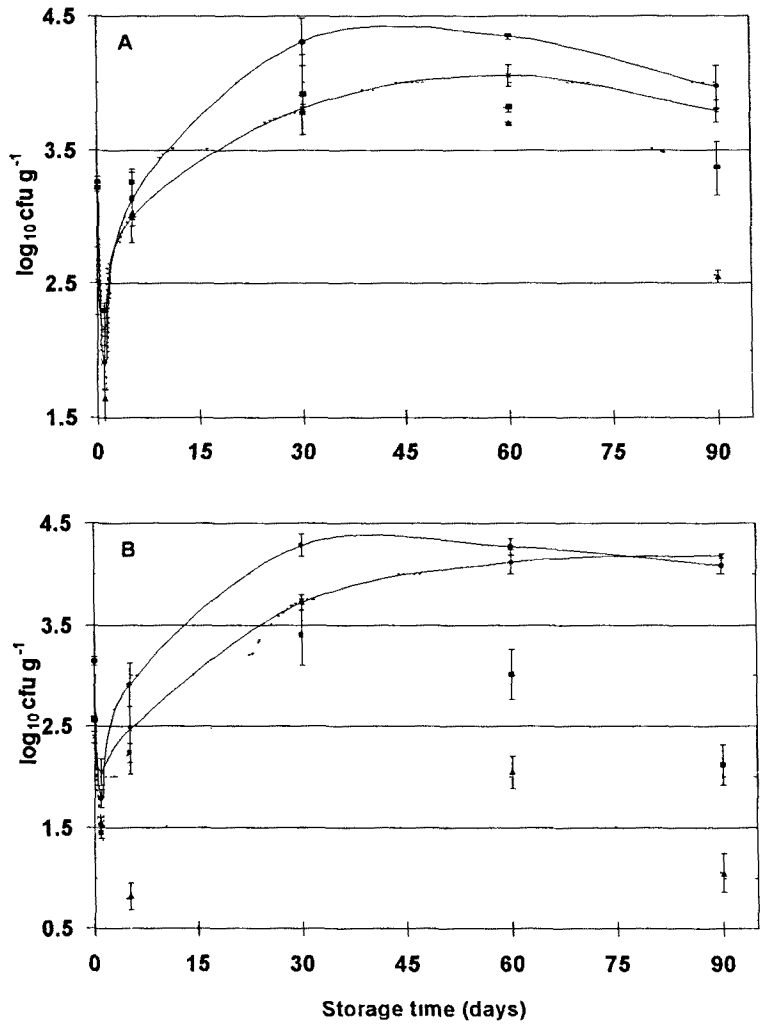

Figure 3. Population dynamics of *C. sake* on wounded apple surface during cold storage period after the different treatments assayed in this study: Preharvest treatment of *C. sake* (▲), Preharvest treatment of *C. sake* + Postharvest wounding (■), Postharvest treatment of *C. sake* (✳) and Pre and postharvest treatment of *C. sake* + Postharvest wounding (●). Growing seasons 1994/95 (A) and 1995/96 (B) Standard error bars of the means are given.

Discussion

The postharvest environment provides a special milieu for developing biological control systems because more standardized and controllable environmental conditions exist in storage packinghouses (Droby and Chalutz, 1992). In contrast, many positive biocontrol results in the laboratory were not confirmed under naturally fluctuating field conditions, because most of the microbial isolates could not become effectively established under natural conditions and develop their antagonistic effects (Wilson, *et al.*, 1991). However, some advantages could be achieved with preharvest application of biocontrol agents for postharvest control of pome fruit diseases. The aim of the present study was to compare biocontrol thus to compare pre- and post-harvest treatments to evaluate the real potential of using field inoculation to achieve good control of diseases postharvest.

Previous laboratory studies have demonstrated that *C. sake* CPA-1 is an effective agent to control the main disease on apples, *P. expansum* and other diseases (Usall, 1995). Even in very unfavourable conditions for the antagonist, by pre and postharvest wounding and artificial inoculation with *P. expansum*, the pathogen was effectively controlled by *C. sake* application on apples. However, this study consistently showed that no improvements in biocontrol were observed when *C. sake* cells were applied to apples both pre and postharvest.

The concentration of *C. sake* used in this study is lower than those recommended for other biocontrol agents with similar biocontrol potential (McLaughlin *et al.*, 1990; Wisniewski *et al.*, 1991) 10^8 or 10^7 and 10^8 cfu ml^{-1} respectively. The concentration of the antagonist needed to obtain control were low enough to be considered for commercial use.

Although preharvest treatment with *C. sake* was not as effective as postharvest treatment against *Penicillium* rot, about 50% control was achieved on preharvest woundswith the field treatment. This may partially be attributed to a lower level of yeast populations becoming established on the apple surface. Rot development was evaluated after 60 days in cold storage conditions and population levels on apples with postharvest treatments were in all cases, at this point, at least 3.5-fold bigger than *C. sake* levels present on fruits sprayed preharvest.

Preharvest wounds made on the fruit surface generally decayed before and more extensively that postharvest wounds. Metabolic changes in the fruit tissue leading to an increased ripening and subsequently, a decreasing resistance of the apple to decay, could explain this effect. Imaseki (1985) reported that a wound given to a higher plant gives rise to significant physiological as well as biochemical alteration, not only in the wounded area, but throughout the plant. Increased rate of

ethylene production after wounding and consequently during the ripening process, has been recognized in a large number of plant tissues (Abeles, 1973).

In treatments that included preharvest biocontrol treatment only, the populations of *C. sake* on apples with pre and postharvest wounds were bigger than on fruits that were only wounded before harvest. This could be due to *C. sake* colonization from postharvest wounds. Usall (1995) showed that *C. sake* had a greater growing capacity inside wounds made on Golden Delicious apples. In contrast, he found that limited development occurred on the unwounded surface of Golden Delicious apples. This may partially be due to the lower content of nutrients and greater water stress on the surface of unwounded apple skin.

In this study, preharvest *C. sake* treatment was not enough to control *P. expansum* rot on artificial postharvest wounds. Further research is needed in this aproach.

The *C. sake* populations achieved after 48 h in the field treatments were higher in the first growing-season than in the second one. This result could be related to the lower maximum temperature reached during this period (26 °C) with respect to the one reached in the season 1995/96 (32 °C). These results agree with those of Usall (1995) who demonstrated that as the temperature decreased, the maximum population level achieved by *C. sake* CPA-1 increased. This indicates good adaptation of this strain to cold storage temperatures, a necessary feature for a postharvest biocontrol agent. In the same study Usall (1995) observed that *C. sake* did not grow at temperatures higher than 34 °C.

Elad and Kirshner (1993) found that the effect of microclimatic conditions on survival of the populations of the biocontrol agent *Trichoderma harzianum* was variable and reflected the complexity of the behaviour of introduced populations in the phylloplane. Humidity and available nutrients represent the most important limitation factors (Blakeman and Fokkema, 1982). Nevertheless, Vercesi *et al.* (1990) characterized the temperature as the most important factor influencing the activity of antagonists against *B. cinerea* in vineyards. Also, Baker and Cook (1973) considered the different temperature conditions in growth chamber and field trials as the reason for the failure of biological control agents under outdoor conditions.

Further research is needed to understand the reason for the lower survival of *C. sake,* when applied preharvest, during subsequent cold storage conditions. Biological control is based on the antagonistic properties of the agent used. Apart from the agent's antagonistic activity, effective biocontrol also involves the ability of the agent to survive in the habitat where it is applied. Insufficient research effort has been directed towards selection for characteristics of biocontrol agents for

survival of environmental stresses for enhancing the activity and control potential of antagonists.

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Capítol 5

Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH estress

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Summary

The growth responses of the biocontrol agent *Candida sake* to changes in water activity (a_w), temperature and pH and their interactions, and accumulation of sugars (glucose, trehalose) and sugar alcohols (glycerol, erythritol, arabitol and mannitol) were determined *in vitro* in nutrient yeast dextrose based media. The a_w × temperature profile for growth was between 0.995 and 0.90 and 4-37 °C with the non-ionic solute glycerol, and between 0.995-0.92 and 10-30 °C with the ionic solute NaCl. Regardless of solute there was a longer lag time prior to growth as a_w was reduced and at marginal temperatures for growth. Relative growth rates were compared at different a_w levels and temperatures and found that a_w, temperature, solute and two and three-way interactions were statistically significant. By contrast, *C. sake* was tolerant of a wide range of pH levels (3-7) regardless of a_w, although growth rates were reduced at marginal temperatures and a_w. In non-stressed basal NYDB glucose and arabitol were the predominant endogenous reserves accumulated in the cells of *C. sake*. However when nutrient status was diluted (75%) and stressed by the addition of glycerol or NaCl (0.93 and 0.96 a_w) significant changes in the accumulation of sugars and sugar alcohols occurred. In glycerol-stressed media glucose and glycerol were the major compounds accumulated, with markedly lower arabitol and little trehalose or mannitol present. With NaCl-stressed media glycerol was the only sugar alcohol accumulated, with very low levels of the sugars and other sugar alcohols. This study has defined the ecological niche within which *C. sake* would be active as a biocontrol agent for the first time and suggests that endogenous reserves can be significantly modified by nutrient and a_w stress, and that such changes may be useful for improving environmental competence of such microorganisms in the environment.

Key words: Growth, water activity, temperature, pH, compatible solutes, sugars, sugar alcohols, ecophysiology, biocontrol, yeasts, *Candida sake.*

Introduction

Biological control of postharvest fungal diseases of fruits has received much attention in recent years because of concerns about the possible adverse effects of chemical pesticide residues on human health (Norman,1988; Wisniewski and Wilson, 1992). Furthermore, the development of tolerance to major fungicides by fungal pathogens (Bertrand and Saulie-Carter, 1978; Rosenberger and Meyer, 1979; Decker and Georgopoulos, 1982; Spotts and Cervantes, 1986; Viñas *et al.*, 1991, 1993) has focussed attention on alternative methods to chemical treatments. Microbial antagonists have been developed as potential alternatives to chemicals or as part of integrated crop management systems to reduce the inputs of pesticides into fruit and vegetable production. Antagonists with efficacy against fungal pathogens of both pome and citrus fruit have been reported, some of which are now being commercially developed (Pusey and Wilson, 1984; Janisiewicz, 1987,

1988; Janisiewicz and Roitman, 1988; Pusey *et al.*, 1988; Wilson and Chalutz, 1989).

The microorganisms being examined include bacteria (e.g. *Bacillus subtilis*, *Pseudomonas cepacia*), yeasts (e.g. *Debaryomyces*, *Cryptococcus*, *Pichia* and *Candida* spp.) and mycelial yeasts (*Aureobasidium pullulans*). In some cases biocontrol efficacy has not been consistent, and been markedly influenced by environmental conditions. Surprisingly, the ecological parameters in which these microorganisms might work effectively and their limits have never been determined. The most important environmental parameters are the water availability (water activity, a_w), prevailing temperatures and the pH of the fruit tissue. These three factors interact and directly influence the capability for growth and establishment on the fruit surface. It is important to identify the environmental niche in which an individual biocontrol agent can actively grow as this enables abiotic threshold criteria for efficacy to be obtained. This contrasts with work on spoilage yeasts where detailed knowledge is available on the water and temperature relations for growth, and where the physiology and mechanisms of stress tolerance of xerophilic/xerotolerant and sensitive species have been studied (Anand and Brown, 1968; Edgley and Brown, 1978; Magan and Lacey, 1986 a, b; Blomberg and Adler, 1992).

Recently, detailed studies have shown that a strain of *Candida sake* (isolate CPA-1) is antagonistic to the major postharvest pathogens on pome fruits (Usall, 1995; Viñas *et al.*, 1996). Information is available on the water relations and the role of sugar alcohols in stress tolerance of some *Candida* spp. but not those being used in biocontrol systems (Magan and Lacey, 1986a; Van Eck *et al.*, 1993). Such information on the ecological fitness of the biocontrol fungi is critical to enable the development of strains which have the competence for survival under naturally fluctuating field conditions (Deacon, 1991; Wisniewski and Wilson, 1992). Indeed, recent work with entomogenous and other filamentous biocontrol fungi has demonstrated that these are key factors influencing ecological competence and fitness and sometimes biocontrol potential in the environment (Hallsworth and Magan, 1994, 1995, 1996; Pascual *et al.*, 1996). No such knowledge is available for *C. sake* or indeed other candidate biocontrol agents being examined at the present time for controlling diseases of fruit pre or postharvest.

The objectives of this study were to (a) determine the effect of water availability, temperature, pH and their interactions on growth rates and limits for growth of *C. sake* and (b) study the effect of such interacting environmental factors on accumulation of endogenous sugars and sugar alcohols (polyols) in the yeast cells.

Materials and Methods

Yeast Isolate

The isolate used in this study was the strain CPA-1 of *Candida sake*, isolated from the apple surface and with demonstrated antagonistic activity against *Penicillium expansum, Botrytis cinerea* and *Rhizopus nigricans* in pome fruits (Usall, 1995; Viñas *et al.*, 1996).

Growth media

Basic media: The basic media were nutrient yeast dextrose agar (NYDA) and a nutrient yeast dextrose broth (NYDB). They had a pH of 7 and a water activity (a_w) of 0.995. The a_w of this and all media was determinated with a Novasina Humidat IC II (Novasina AG, Zurich, Switzerland).

Water activity × temperature profile for growth: To obtain information on the a_w × temperature profile of *C. sake*, the yeast was grown on the basic NYDA modified with an ionic (NaCl; Lang 1967) and non-ionic solute (glycerol; Dallyn, 1980) in the a_w range 0.995 to 0.85 a_w at 4-37 °C. All treatments were carried out with at least three replicates. Media of the same a_w were always sealed in plastic polyethylene bags to maintain the equilibrium relative humidity conditions and prevent water loss.

Effect of a_w × temperature on growth rates: Studies were carried with the NYDB liquid medium modified with the ionic solute NaCl and the non-ionic solute glycerol to 0.99, 0.98, 0.96 and 0.94 a_w. Temporal experiments were carried out with 50 ml of medium in 250 ml conical flasks at 10, 25 and 30 °C for a period of 196, 144 and 144 h, respectively. Growth rates of each treatment were determinated turbidimetrically by removing 1 ml subsamples and placing in cuvettes and obtaining the optical density (OD) with a spectrophotometer (CECIL CE 1020) set at 700 nm, with reference in each case to a sterile solution of the same composition and a_w as the growth medium (Anand and Brown, 1968). All experiments were carried out with three replicates.

A separate experiment was carried out to determine the effect of decreasing the concentration of nutrients in the basic NYDB medium to 1/4 strength (NYDB25), and modification with NaCl and glycerol (0.98 and 0.96 a_w) as described previously and examining the effects on both yeast growth and on the physiological accumulation of sugars (trehalose and glucose) and sugar alcohols (glycerol, erythritol, arabitol and mannitol) in the yeast cells after 24, and 48 h growth. The yeast cells were spread plate onto NYDA medium to obtain

information on the number of colony forming units (cfu) in each treatment as the OD measurements had reached a maximum value in some treatments after 24 h.

Effect of pH, water activity and temperature on growth: The a_w of the basal NYDB medium was made up using buffers (0.1 M citric acid and 0.2 M Na_2HPO_4) to pH levels of 3, 5 and 7. They were used unmodified, or after modification with NaCl and glycerol to 0.98 and 0.96 a_w. Experiments were again carried out in triplicate at 10, 25 and 30 °C as described previously over periods of 600, 336 and 360 h, respectively. Growth was measured turbidimetrically as before.

Inoculation and examination

The solid agar media were inoculated by spread plating a 0.1 ml aliquot of a 10^3 cfu ml^{-1} yeast suspension of the *C. sake* grown for 24 h in NYDB medium. Plates were examined visually and with a microscope every 24 h to determine whether colonies had grown on the various treatments. This information was used to construct a diagram of the range of a_w/temperature conditions over which *C. sake* could grow on media modified with NaCl and glycerol. The time and limits for growth were determined by examination of the treatments after 20 h, 40 h, and then daily as required.

All liquid media experiments were carried out by inoculation with a 1 ml suspension of *C. sake* yeast cells in the linear phase of growth (36 h; 10^4 cfu ml^{-1}).

Sugar and sugar alcohol extraction and analyses of yeast cells

A 30 ml subsample of each treatment was placed in a sterile plastic Universal bottle and centrifuged immediately for 15 min at 4000 rpm (MSE Cenetaur 2). The medium was decanted and the yeast cells resuspended in 5 ml of HPLC grade water, shaken vigorously and centrifuged a second time and the water decanted.

A known amount of yeast cells (10-25 mg) was mixed with 1 ml HPLC grade water in a 2 ml Eppendorf tube and sonicated for two minutes using a Soniprep 150 (Fisons), at an amplitude of 26 μm. After immersion in a boiling water bath for 5 minutes the samples were left to cool. A 0.67 ml volume of HPLC grade acetonitrile was added to each sample to get the same ratio of acetonitrile/water (40:60) as the mobile phase for HPLC analyses. The Eppendorf tubes were centrifuged for 10 min at 13000 rpm and the supernatant was filtered through 0.2 μm filters into HPLC vials sealed with plastic septa.

The sugars and sugar alcohols were analysed and quantified using a Gilson HPLC system with a RI Detector and a Hamilton HC-75 Ca^{2+} column. The mobile phase consisted of a 40:60 degassed mixture of acetonitrile:water. Standard calibration curves were constructed for each sugar and sugar alcohol using concentrations in the range 50-600 ppm. These were used to integrate the individual peaks for each sample. The results were modified to take account of the actual concentration of yeast cells extracted and the results are presented as mg g^{-1} fresh weight of yeast cells (Hallsworth and Magan, 1996). All results are the means of three replicate yeast samples per treatment.

Statistical treatment of the results

In all cases the linear regression of the increase in optical density (OD) against time (in hours) was plotted and used to obtain the relative growth rates (OD h^{-1}) under each set of treatment conditions. The growth rates were then analysed by an analysis of variance with SAS software (SAS Institute, version 6.03, Cary N.C.). Statistical significance was judged at the level $P< 0.05$. When the analysis was statistically significant the Duncan's Multiple Range Test for separation of means. This enabled the statistical significance of single, two and three-way interactions to be examined for all experiments.

Results

Water activity × temperature profiles for growth

Figure 1 shows the range of a_w and temperatures over which *C. sake* was able to grow on the NYDA medium. The a_w × temperature range for growth with the ionic solute NaCl was more limited than that with the non-ionic solute glycerol. The minimum a_w for growth in media modified with these two solutes were 0.92 and 0.90 a_w respectively at the optimum temperatures for growth (20-25 °C). The temperature profile was slightly narrower with the ionic solute especially at marginal conditions. The number of days (d) to initiation of growth demonstrated that with the ionic solute NaCl there was a longer lag time to growth than that with glycerol under optimum temperature conditions. These profiles were used in determining the steady-state a_w and temperatures for subsequent studies.

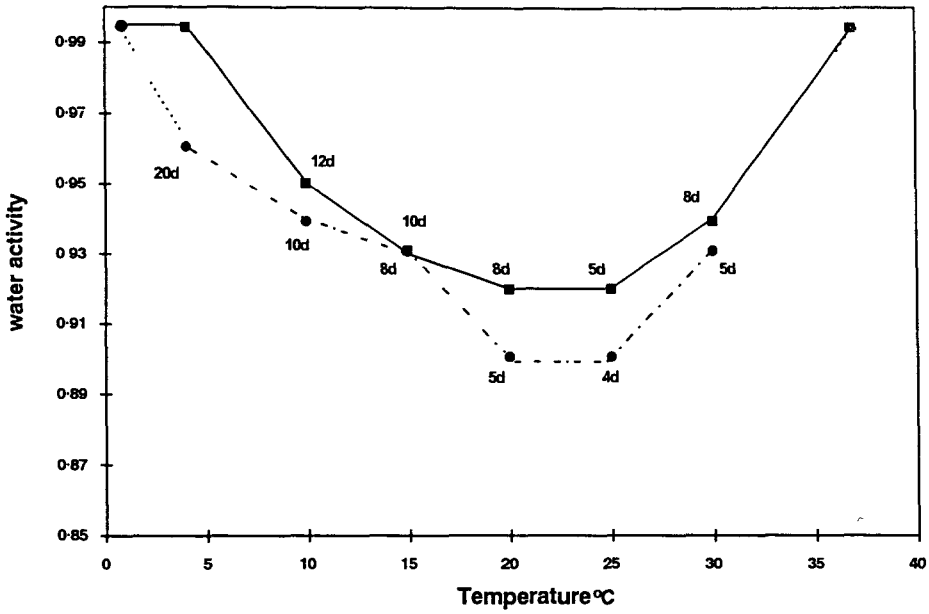

Figure 1. The minimum water activity (a_w) and temperature profile for growth of *C. sake* in a nutrient yeast dextrose medium modified with the ionic solute NaCl (■) or the non-ionic solute glycerol (●). The lag times in days (d) to growth are shown on the profile lines.

Water activity × temperature effects on temporal growth patterns of *C. sake*

The temporal increase in growth of the *C. sake* strain at different steady-state a_w levels at a single temperature when modified with glycerol or NaCl are shown in Figure 2. This clearly demonstrates that growth was very rapid at a_w levels > 0.98. Under greater water stress there was an increase in the lag times prior to growth initiation. It was also noticeable that the rates of growth were faster in glycerol than NaCl-amended media. Linear regression of this data at each temperature and for each solute was used to obtain the relative growth rates (OD h^{-1}) so that comparisons could be made.

Figure 3 shows that when a_w of the medium was reduced with NaCl or glycerol, there was a significant reduction in growth rate at all temperatures studied. There were also a marked difference in growth rates of *C. sake* dependent on solute type used. The faster growth rates (OD h^{-1}) in glycerol than NaCl-amended media reflects the temporal results obtained. In all cases, regardless of a_w or solutes *C. sake* grew better at 25 °C than 30 or 10 °C.

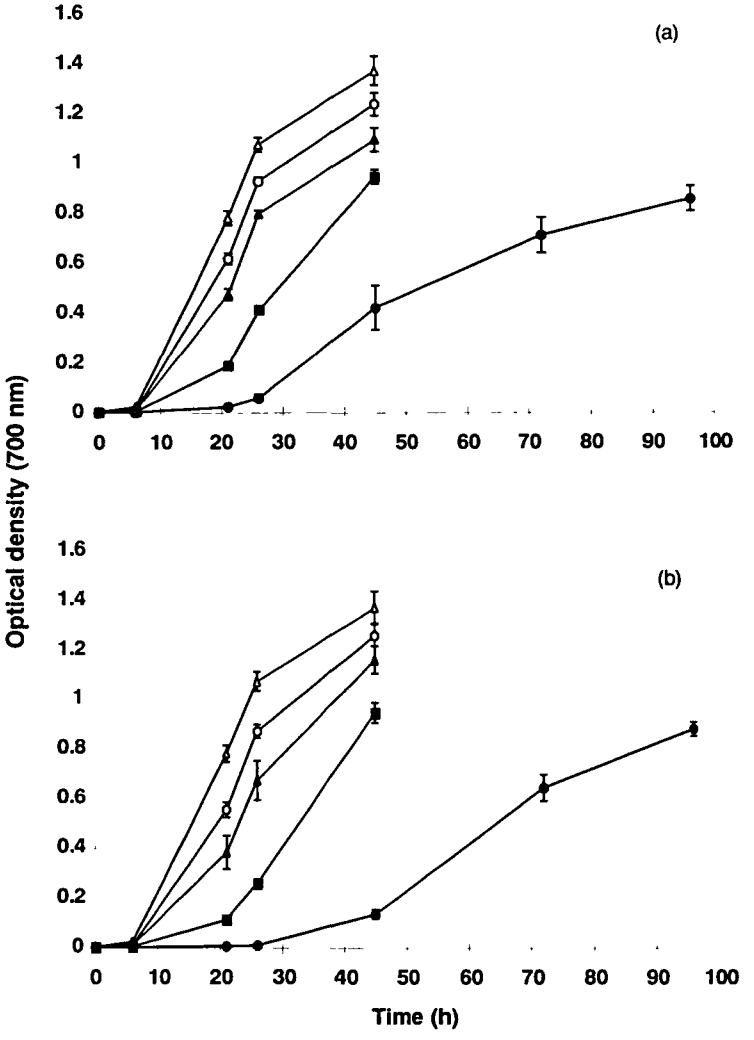

Figure 2. The temporal growth patterns of *C. sake* in relation to water activity (a_w) at 25 °C in a nutrient yeast dextrose broth measured by optical density (OD) modified (a) using the non-ionic solute glycerol and (b) the ionic solute NaCl. The bars represent the standard error of the means. Where the bars are not shown they were smaller than the symbol size. Simbols: Δ, 0.995; ○, 0.99; ▲, 0.98; ■, 0.96 and ●, 0.94.

Table 1 shows that in the liquid culture study there were statistically significant differences due to all single factors, and two and three-way interactions with the exception of that for a_w × solute for all test treatments.

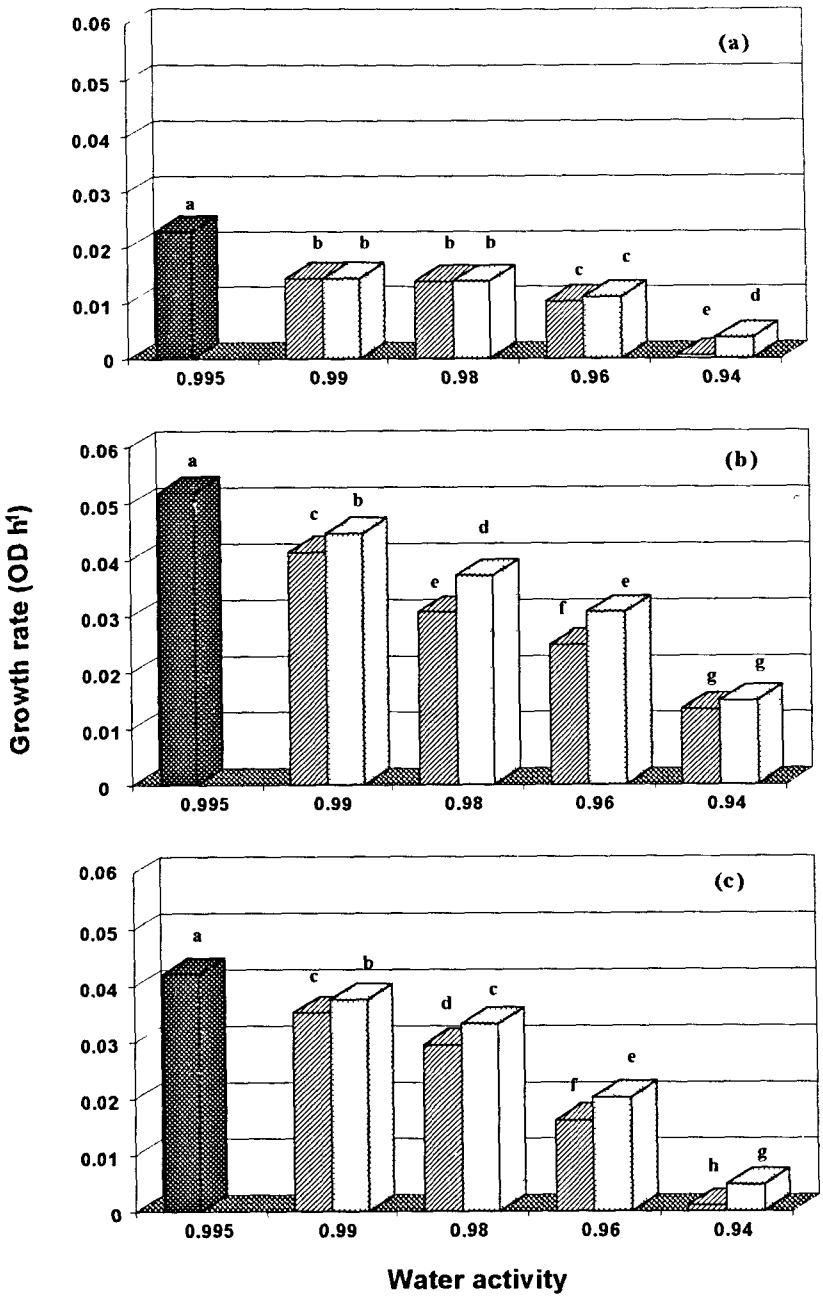

Figure 3. Comparison of the growth rate (OD h^{-1}) of *C. sake* in relation to water activity (a_w) for different temperatures (°C); 10 °C (a), 25 °C (b) and 30 °C (c), on a nutrient yeast dextrose broth (▩) and modified with the ionic solute NaCl (▨) and the non-ionic solute glycerol (□). The separation of means for each temperature was conducted according to Duncan's Multiple Range Test (P=0.05). Columns with different letters indicate significant differences between treatments.

Table 1. Analysis of variance of effect of water activity (a_w), solute (sol.) and temperature (t) two and three-way interactions on growth rate of *C. sake* in nutrient yeast dextrose broth medium.

SOURCE	DF	MS	F	*Pr*>F
t	2	0.0028372	1863.95 **	0.0001
a_w	3	0.0021164	1390.43 **	0.0001
sol	1	0.0001513	99.45 **	0.0001
t × a_w	6	0.0001547	101.65 **	0.0001
t × sol	2	0.0000182	11.95 **	0.0001
a_w × sol	3	0.0000026	1.75 NS	0.1674
t × a_w × sol	6	0.0000046	3.07 *	0.0121

Note: MS Mean square; * Significant $P< 0.05$; ** Significant $P< 0.001$; NS No significant

Effect of water activity and nutrient interactions on sugar and sugar alcohol accumulation

The total sugars (glucose+trehalose) and sugar alcohols present intracellularly in yeast cells after 24 and 48 h are shown in Table 2. Generally, the total concentrations were higher after 48 h than 24 h. The highest concentrations of sugars and sugar alcohols were present in the medium modified with glycerol after 48 h incubation. Endogenous reserves of sugars and polyols accumulated were lowest in the NaCl-modified medium, especially at 0.96 a_w.

The quantities of individual sugars and sugar alcohols accumulated in the yeast cells are shown in Figure 4. In the normal strength NYDB medium the predominant sugars/sugar alcohols were glucose (about 0.3 m g^{-1}) and arabitol (about 3.5 mg g^{-1}). Decreasing the nutrient concentration resulted in only arabitol (about 0.7 mg g^{-1}) being present in a significant amount g^{-1} yeast cells. In contrast when the weaker medium was modified with glycerol significant changes occurred in the endogenous sugar and sugar alcohol content of the yeast cells. At both 0.98 and 0.96 a_w glycerol was the predominant sugar alcohol (about 5 mg g^{-1}), with arabitol content significantly less. Glucose was present as the predominant sugar, of those quantified (about 0.8 and 0.5 mg g^{-1}), respectively. Some trehalose was detected in the glycerol treatment especially at 0.96 a_w. The use of the ionic solute NaCl at both 0.98 and 0.96 a_w significantly reduced the total and individual sugars and sugar alcohols with

only glycerol and glucose being present at the higher a_w and much reduced glycerol concentrations at the lower a_w. Many of these differences were statistically significant.

Table 2. Mean total intracellular quantities of the sugars (glucose and trehalose) and sugar alcohols (glycerol, erythritol, arabitol and mannitol) present in cells of *C. sake* grown on a nutrient yeast dextrose broth unmodified, diluted (NYDB25) and modified with glycerol and NaCl to 0.98 and 0.96 water activity. Incubated 24 and 48 h at 25 °C.

	mg g^{-1} fresh weight			
GROWTH MEDIUM (a_w)	**SUGARS**		**SUGAR ALCOHOLS**	
	24 h	**48 h**	**24 h**	**48 h**
NYDB (0.995)	0.358	0.307	1.887	3.687
NYDB25 (0.995)	0	0	1.470	0.700
NYDB25 + GLY (0.98)	0	0.968	3.400	6.437
NYDB25 + GLY (0.96)	(*)	0.811	(*)	5.865
NYDB25 + NaCl (0.98)	0	0.060	0.288	2.253
NYDB25 + NaCl (0.96)	(*)	0	(*)	0.090

(*) Endogenous reserves were not evaluated because of poor growth

Effect of water activity × pH × solute type × temperature effects on growth of *C. sake*

Figure 5 shows examples of temporal changes in growth rate (total OD) of *C. sake* at different pH levels in relation to a_w at one steady state temperature (25 °C) modified with glycerol and NaCl. There was only a small difference in the temporal rates of growth between pHs at individual steady-state a_w levels. There was very little difference in the lag times prior to growth initiation in relation to pH in contrast to the effect of a_w where marked differences were obtained. The linear regression of this data was used to determine and compare the growth rate of *C. sake* under different combinations of treatments (OD h^{-1}).

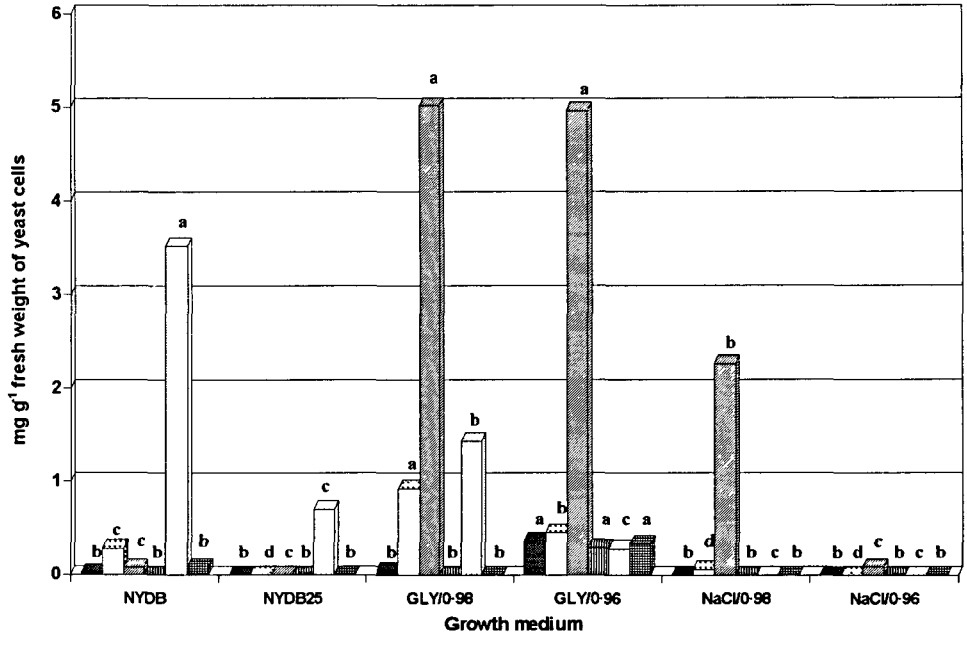

Figure 4. Effect of medium strength (100 or 25% nutrient yeast dextrose broth) and water activity (a_w) modified with NaCl or glycerol on the endogenous concentrations of sugars (trehalose ▩ and glucose □) and sugar alcohols (glycerol ▨ , erythritol ▥, arabitol □ and mannitol ▦) g^{-1} fresh weight of *C. sake* cells after 48 h incubation at 25 °C. For each individual sugar/sugar alcohol, treatments with different letters are statistically different according to Duncan's Multiple Range Test (P=0.05).

Comparisons of growth rates in relation to interactions between pH × a_w × temperature where glycerol or NaCl was used is shown in Figure 6. The best pH for growth of *C. sake* was 5 at all three temperatures (10, 25, 30 °C), regardless of a_w, although growth was slower as a_w and temperature were reduced. Statistical analyses demonstrated that there were statistically significant effects of single, two, three and four-way interactions for the factors tested (temperature, pH, a_w and solute, Table 3).

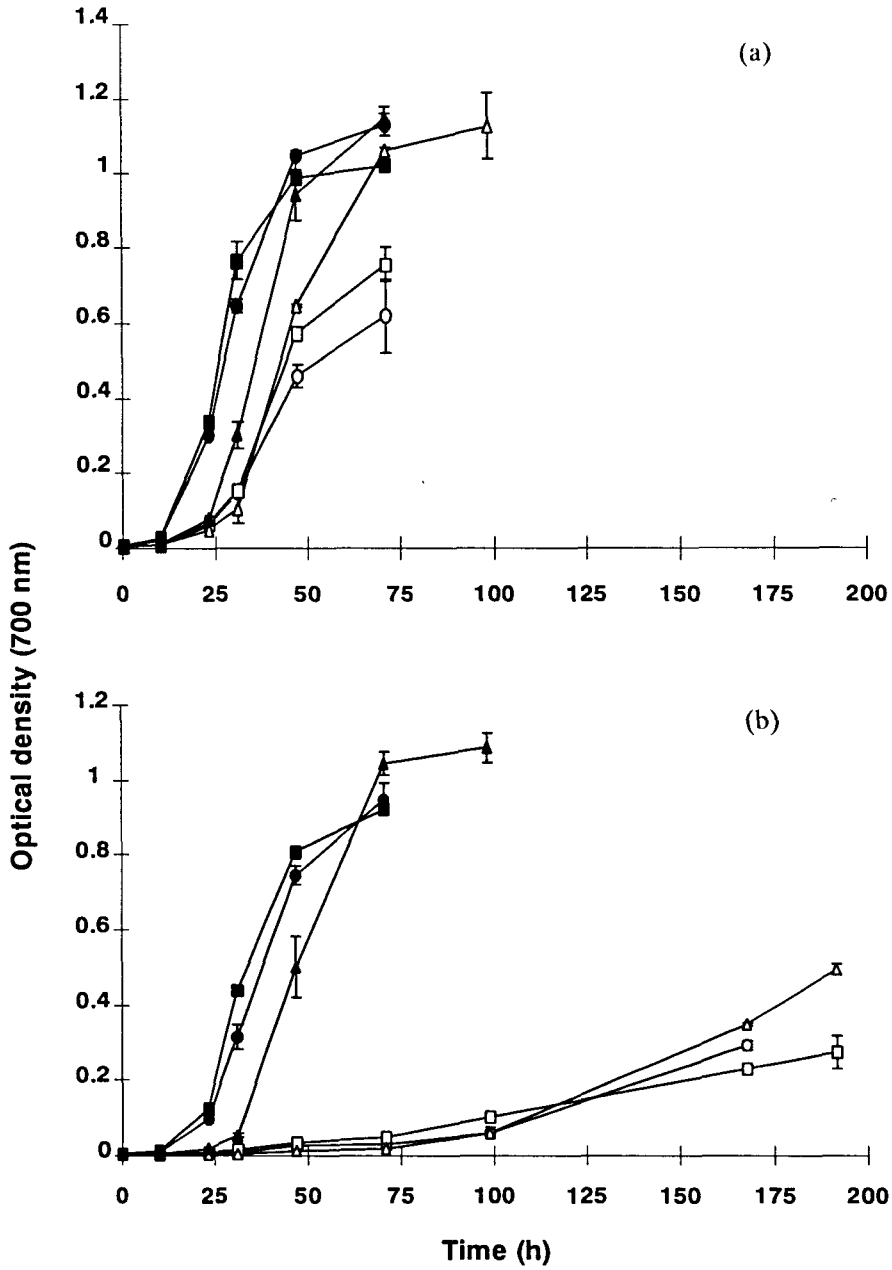

Figure 5. Temporal changes in growth of *C. sake* in relation to pH (3, 5, and 7) at 25 °C modified with NaCl and glycerol to (a) 0.98 and (b) 0.96 water activity (a_w). Standard error bars of the means are given except where they are smaller than the symbol size. Symbols; ▲, Glycerol and pH 7; ■, Glycerol and pH 5; ●, Glycerol and pH 3; Δ, NaCl and pH 7; □, NaCl and pH 5; O, NaCl and pH 3.

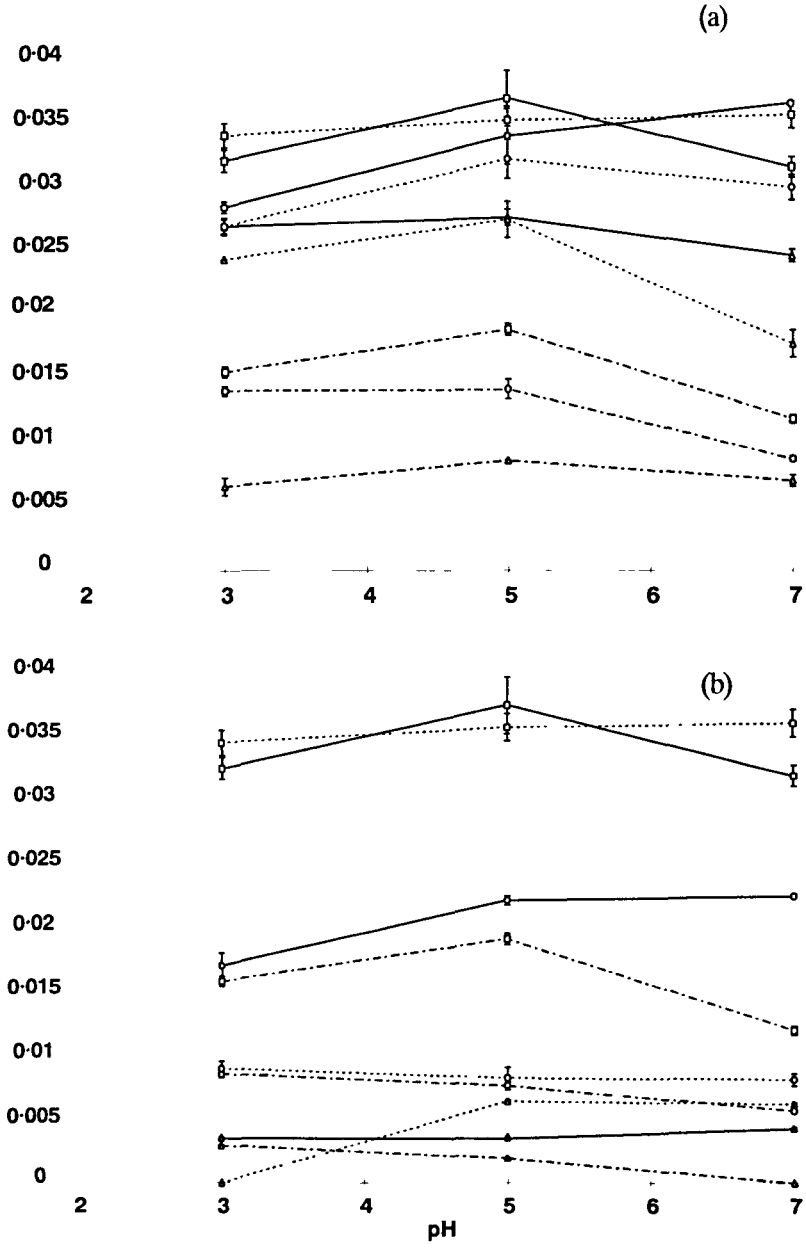

Figure 6. Comparison of the growth rate (OD h^{-1}) of *C. sake* in relation to pH (3, 5 and 7) and temperature (10, – - – - – - – , 25, ———— and 30 °C, ·····) and water activity (a_w) (□, 0.995; ○, 0.98 and Δ, 0.96) in a nutrient yeast dextrose medium modified with (a) glycerol and (b) NaCl. Standard error bars of the means are given except where they are smaller than the symbol size.

Table 3. Analysis of variance of the effect of water activity (a_w), temperature (t), pH, solute (sol) and two-, three-, and four-way interactions on growth of *C. sake* in a nutrient yeast dextrose broth medium.

SOURCE	DF	MS	F	*Pr*>F
t	2	0.0027357	3527.22 **	0.0001
pH	2	0.0000877	113.10 **	0.0001
a_w	1	0.0014837	1912.97 **	0.0001
sol	1	0.0056898	7335.97 **	0.0001
t × pH	4	0.0000232	30.03 **	0.0001
t × a_w	2	0.0001196	154.22 **	0.0001
t × sol	2	0.0005654	729.09 **	0.0001
sol × pH	2	0.0000173	22.40 **	0.0001
a_w × sol	1	0.0000500	64.49 **	0.0001
pH × a_w	2	0.0000097	12.63 **	0.0001
t × pH × a_w	4	0.0000260	33.54 **	0.0001
t × pH × sol	4	0.0000073	9.48 **	0.0001
t × a_w × sol	2	0.0000928	119.71 **	0.0001
pH × a_w × sol	2	0.0000174	22.54 **	0.0001
t × pH × a_w × sol	4	0.0000254	1.37 **	0.0001

Note: MS Mean square; ** Significant $P< 0.001$; NS No significant

Discussion

This study is the first detailed investigation of the water, temperature and pH relations of growth, and on the physiological accumulation of endogenous sugars and sugar alcohols of *C. sake*. This has shown clearly that the environmental niche within which this biocontrol agent will effectively grow is limited by an a_w of about 0.90-0.92 a_w, a temperature range of 4-30 °C with a relatively wide pH tolerance even at lowered a_w and temperature. *C. sake* has however previously been demonstrated to grow slowly at 1 °C (Usall, 1995), a temperature condition not tested in the present study. Anand and Brown (1968) suggested that

osmotolerant species generally had slower growth rates than non-osmotolerant species of yeasts, with the former having broad a_w optima for growth, and the latter having very sharp narrower optima. The lower a_w minima with glycerol than with NaCl for *C. sake* is similar to that observed previously. For example, *C. guilliermondii* was shown to have optima of 0.995 a_w and a similar a_w minima range with the same ionic and non-ionic solutes (Magan and Lacey, 1986b). Detailed studies on a_w minima for a range of other *Candida* spp. demonstrated lower tolerances in the presence of glucose than NaCl, respectively, for *C. cacaoi* (0.83/0.84), *C. magnoliae* (0.82/0.88), *C. tropicalis* (0.88/0.89; Van Eck *et al.,* 1993). Thus *C. sake* is less tolerant of low a_w than some other *Candida* spp..

The tolerance of lowered pH was quite striking and showed that this biocontrol agent can grow effectively in acidic environments characteristic of damaged fruits, particularly apples. Although growth rates were optimum at pH 5 regardless of temperature and a_w, there was no significant difference. This contrasts with information on *C. guilliermondii* which had significantly longer generation times at pH 4 than at the optimum pH 6 at both 0.995 and 0.95 a_w (Magan and Lacey, 1986a). Unfortunately, there are no other comparable studies on effects of a_w × temperature × pH interactions on growth of other *Candida* spp.

Under unstressed a_w conditions (0.995) in a relatively rich NYDB medium the predominant endogenous compatible solutes of *C. sake* cells were arabitol, glucose and smaller amounts of mannitol and glycerol. However, in a weaker NYDB medium arabitol was the only dominant polyol accumulated in the yeast cells. Reduction of the a_w with ionic or non-ionic solutes NaCl and glycerol, respectively, to either 0.98 or 0.96 a_w, significantly altered the sugar and sugar alcohol content. In glycerol-amended media glycerol concentrations significantly increased, with a marked reduction in arabitol and a smaller increase in glucose. Small amounts of trehalose were accumulated at both a_w levels. By contrast in NaCl-modified media glycerol was the only sugar alcohol accumulated together with small concentrations of glucose. This suggests that *C. sake* is more sensitive to ionic solutes. This is supported by the very low total level of sugars and sugar alcohols accumulated in the yeast cells over 48 h growth. Limited comparisons can be made with work on other *Candida* spp. where accumulation of glycerol, arabitol and mannitol were examined in *C. cacaoi* and *C. magnoliae* using glucose and NaCl (Van Eck *et al.,* 1993). In both cases intracellular glycerol was demonstrated to increase as a_w was decreased from 0.998 to 0.92 with some increase in arabitol in the former and mannitol in the latter species. Glucose or trehalose accumulation or changes in carbon:nitrogen limitation as well as a_w were not compared in their study. However, other studies with *Hansenula anomala* and filamentous fungi has demonstrated that carbon source can have a bearing on the accumulation of sugar

alcohols other than glycerol (Van Eck *et al.*, 1989; Hallsworth and Magan, 1995). Interestingly, the concentration ratios of external to internal concentrations were found to change markedly as the level of stress was increased. However, previous studies have not quantified trehalose accumulation under different a_w levels except for those involving the industrial baker's yeasts *Saccharomyces cerevisiae (*Van Dijck *et al.*, 1995*)*. In *S. cerevisiae* trehalose levels greater than 10% dry weight is considered critical for stress resistance to freezing and freeze drying. Thus the accumulation of trehalose in cells of *C. sake* could be important in attempts to produce cells which have greater ecological competence provided biocontrol activity can be conserved.

In summary, this study has shown that from an ecological point of view *C. sake* has a very wide tolerance of a_w, temperature and also pH which should enable the species to actively grow over a wide range of environmental conditions. This knowledge should contribute to the development of methods for improving environmental stress tolerance by manipulation of growth conditions to physiologically channel specific endogenous compounds which may enable better environmental stress tolerance and competence.

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Capítol 6

Improving ecological fitness and environmental stress tolerance of the biocontrol yeast *Candida sake* (strain CPA-1) by manipulation of intracellular sugar alcohol and sugar content

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Enviat a: Mycological Research

Summary

Candida sake was cultured on nutrient yeast dextrose broth (NYDB) medium, which was diluted and/or modified by the addition of either glycerol, glucose to 0.96 or trehalose to 0.97 water activity (a_w) to modify endogenous sugar alcohol and sugar content. Sugar alcohols (glycerol, erythritol, arabitol and mannitol) and sugars (trehalose and glucose) were extracted from the yeast cells and quantified using HPLC. Total polyol and sugar content varied significantly between treatments with the maximum in unmodified NYDB being about 6 and 1 mg g^{-1} fresh weight yeast cells. This was significantly increased in NYDB+glucose media to 8 mg g^{-1} and 3.5 mg g^{-1}, respectively. The major intracellular polyols/sugars in cells grown on unmodified NYDB were arabitol, trehalose and glucose with small amounts of glycerol and erythritol. This was changed by reducing a_w of the growth medium, particularly with glucose or glycerol. The major polyols in *C. sake* cells grown on glucose-modified media were arabitol and the low molecular weight glycerol, with smaller amounts of glucose. In glycerol-amended full strength normal and diluted media, glycerol was the major intracellular polyol with lower amounts of the other polyols and sugars. The viability of the yeast cells with various endogenous reserves were compared in two experiments, on water-stressed medium (0.935 a_w), and on both unstressed (0.995 a_w) and stressed media (0.95 a_w). These experiments demonstrated that endogenously modified yeast cells were significantly more tolerant of water stress than those from unmodified richer media. Improvements in water stress tolerance varied with age of cells (24 or 48 h), with the best treatments being obtained from yeast cells grown for 48 h in NYDB media modified with glucose, glycerol and trehalose. This suggests that tolerance to environmental stress may be improved by increasing intracellular reserves of the low molecular weight polyol glycerol, the high molecular weight polyol arabitol, and the sugars trehalose and glucose. Small amounts of erythritol and mannitol were also present in the best treatments. Such modification of endogenous reserves were also shown to preserve or improve the biocontrol potential of the yeast against *Penicillium expansum* rot of apples. Thus physiological manipulation of intracellular polyol and sugar content may provide a means for developing biocontrol agents with improved ecological fitness in field environments.

Key words: ecophysiology, sugar alcohols, sugars, water stress tolerance, improved viability, ecological fitness, biocontrol potential, *Candida sake*.

Introduction

Biological control using microbial antagonists have attracted much interest as an alternative to chemical methods of controlling pre and postharvest plant pathogens and pests of agricultural and horticultural crops (Janisiewicz, 1988, 1990; Wilson and Chalutz, 1989; Wilson and Wisniewski, 1989). There has been much research effort into optimizing spore yields and improvement of formulations of inocula but surprisingly little on improving physiological quality of inocula. Indeed, biocontrol in the field has often been severely limited by a narrow range of relative

humidity and temperature conditions over which successful establishment and effective pest or disease control can occur (Doberski, 1981; Heale, 1988; Hallsworth and Magan, 1994 a, b).

In recent years particular success has been achieved by the development of microbial antagonists effective against fungal pathogens of pome and citrus fruit, some of which are being commercially developed (Pusey and Wilson, 1984; Pusey *et al.*, 1988; Janisiewicz and Marchi, 1992; Janisiewicz and Bors, 1995). However, application pre-harvest in the field to such crops have not been successful because of the environmental sensitivity of biocontrol strains (Wilson *et al.*, 1991).

Recently, detailed studies have shown that a strain of *Candida sake* (Saito and Ota) van Uden and Buckley (CPA-1) is an effective antagonist to the major fungal pathogens of apples and pears (Usall, 1995; Viñas *et al.*, 1996). It is particularly effective at high humidity (> 98% R.H.). However, at reduced water availability, particularly in the field, establishment of the antagonist and biocontrol is more difficult to achieve. Thus we are interested in mechanisms which can improve the environmental competence of this biocontrol agent to allow effective establishment and survival under fluctuating field conditions and thus improve its biocontrol potential. Physiological methods for improving tolerance to such environmental stresses are considered to be fundamental to enable the development of effective and consistent microbial biocontrol agents (Deacon, 1991).

Low and high molecular polyhydroxy alcohols (polyols) such as glycerol, erythritol, and arabitol and mannitol, respectively, are often accumulated in fungal cells under low water availability (water activity, a_w) (Beever and Laracy, 1986; Hocking, 1986; Ellis *et al,.* 1991; Kelly and Budd, 1991; Van Eck *et al,.* 1993). Intracellular accumulation of these polyols reduces cytoplasmic a_w but does not disrupt enzyme structure and function, thus allowing metabolic activity to continue during periods of water stress (Brown, 1978). Recent ecophysiological studies on entomogenous biocontrol fungi have demonstrated that it is possible to physiologically manipulate growth conditions, carbon sources and carbon:nitrogen (C:N) ratios to channel specific low molecular weight polyols such as glycerol and erythritol into mycelium and propagules of filamentous fungi which resulted in improved and more rapid germination under water stress conditions (Hallsworth and Magan, 1994a, 1995, 1996). Conidia modified in this way may be more pathogenic to target pests at low relative humidity than those containing traces of these low molecular weight polyols (Hallsworth and Magan, 1994b, c). Initial studies with physiologically manipulated inocula of the biocontrol agent *Epicoccum nigrum* Link containing high concentrations of glycerol and erythritol have also been found to give better control of brown rot (*Monilinia laxa*) of peaches than unmodified inocula (Pascual *et al.*, 1996).

The disaccharide trehalose protects membrane and protein structure during dehydration and upon rehydration (Crowe *et al.*, 1984; Carpenter and Crowe, 1988; Colaco *et al.*, 1992; Leslie *et al.*, 1994). It has been suggested that trehalose enhances desiccation tolerance of conidia of the biocontrol fungi *Trichoderma harzianum* Rifai (Harman *et al.*, 1991), and *Aspergillus japonicus* Saito (Gornova *et al.*, 1992). Trehalose concentrations of greater than 10% have been found to be critical for stress resistance to freezing and freeze-drying of the industrial yeast *Saccharomyces cerevisiae* Hansen (Van Dijck *et al.*, 1995). However, Hallsworth and Magan (1995) showed that elevated trehalose concentrations in conidia of the filamentous fungi *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Paecilomyces farinosus* Bainier did not improve stress tolerance at lowered water availability, although it did prolong shelf-life.

This study was carried out to determine (a) the effect of modifying nutrient substrates and their concentration, and a_w on the intracellular accumulation of polyols (glycerol, erythritol, arabitol and mannitol) and sugars (glucose and trehalose) in cells of *C. sake*; (b) to evaluate the relationship between physiologically modified *C. sake* inocula and improved stress tolerance competence; and (c) the conservation of biocontrol efficacy of such modified inocula against the pathogen *Penicillium expansum* Link.

Materials and Methods

Organisms and media

The isolate used in this study was *Candida sake* (strain CPA-1) from UdL-IRTA. Stock cultures were stored at 5 °C and had been sub-cultured on nutrient yeast dextrose agar (NYDA). A nutrient yeast dextrose broth (NYDB; nutrient broth, 8 g l^{-1}; yeast extract, 5 g l^{-1}; dextrose 10 g l^{-1}; 0.995 a_w) was used as the basal medium in this study. This medium was modified by the addition of glucose (398 g l^{-1}), glycerol (184 g l^{-1}) to obtain 0.96 a_w and trehalose (95 g l^{-1}) to obtain 0.97 a_w. The basic medium was also diluted by 1:4 (NYDB25, 25% strength) and modified as above to 0.96 and 0.97 a_w respectively. The a_w of media was determinate with a Novasina Humidat IC II (Novasina AG, Zurich, Switzerland).

In all experiments 100 ml of media in 250 ml Erlenmeyer flasks of each treatment were inoculated with a known concentration of *C. sake* (10^4 cfu ml^{-1}) and were cultured with agitation on a rotary shaker (150 rpm) at 25 °C. After 24 and 48 h of incubation three replicates of each treatment were destructively sampled to obtain

yeast cells for quantifying polyol and sugar concentrations, and for comparison of a_w stress tolerance of treatments. All experiments were carried out in triplicate and repeated.

Evaluation of viability and growth of physiologically characterized *C. sake* in unstressed and stressed media

Two separate experiments were carried out to determine the environmental competence of modified *C. sake* cells to grow under various a_w stress conditions. In the first experiment *C. sake* cells grown in different media were removed after 24 and 48 h and spread plate on to the surface of 9 cm Petri plates containing NYDA modified with Polyethylene Glycol (PEG 200/300; 1.25 M and 1.0 M respectively) to 0.935 a_w. Plates of the same a_w were sealed in polyethylene bags to prevent water loss and incubated at 25 °C. The colonies were counted after 48 h.

The second experiment was conducted to compare the viability and growth of *C. sake* obtained from both unstressed (0.995 a_w) and stressed (0.95 a_w) NYDA-based media. A known concentration of the antagonist was inoculated in 250 ml Erlenmeyer flasks containing: NYDB, NYDB50 (diluted by 50%) and NYDB25 (diluted by 75%) and supplemented with glycerol or glucose only to 0.96 a_w as described previously. All treatments were carried out in triplicate and were incubated with agitation (150 rpm) at 25 °C. After 24 and 48 h samples were spread-plate on unstressed (0.995 a_w) and stressed (0.95 a_w ;1.25 M PEG 200/0.5 M PEG 300) NYDA media and incubated at 25 °C. The number of cfu were determined after incubation for 24 and 48 h. All treatments were carried out in triplicate.

Extraction and detection of polyols and sugars

Suspensions of yeast cells of *C. sake* were placed in sterile 30 ml Universal bottles and centrifuged immediately for 15 min at 4000 rpm in a MSE Cenetaur 2. The yeast cells were resuspended in AnalaR water and centrifuged again to remove any residual liquid medium.

A known amount of fresh weight of *C. sake* cells (10-25 mg) was mixed with 1 ml AnalaR water in a 2 ml Eppendorf tube and sonicated for 2 min using a Soniprep 150 (Fisons), at an amplitude of 26 μm. After immersion in a boiling water bath for 5 min the samples were left to cool and 0.67 ml acetonitrile was added to each sample to obtain the same ratio of acetonitrile:water as the mobile phase (40:60).The tubes were centrifuged for 10 min at 13000 rpm and the supernatant was filtered through 0.2 μm filters and finally injected in the HPLC for quantification of endogenous polyols and sugars.

Solutes were analyzed and quantified by high performance liquid chromatography (HPLC) with a Gilson RI Detector using a Hamilton HC-75 Ca^{2+} column, specifically for sugars/polyol separation. The mobile phase used was acetonitrile:water (40:60). A total of six solutes were analyzed: the polyols glycerol, erythritol, arabitol and mannitol and the sugars trehalose and glucose. In all cases three replicates of all treatments were analyzed. The peak areas were integrated and compared with calibration curves constructed with standards of 50-600 ppm of each solute. Polyols, trehalose and glucose content were calculated as mg g^{-1} fresh weight of *C. sake* cells (Hallsworth and Magan, 1995).

Antagonistic activity of characterized inocula of *C. sake* treatments against *P. expansum* in apples

The antagonist suspensions from the different treatments above were grown in 250 ml flasks cointaining 100 ml of media with various a_w as described above and harvested after 24 and 48 h. The cells were centrifuged at 7000 rpm for 10 min and cells were resuspended in 50 ml of water. The concentrations of the suspensions were adjusted to 7.5×10^5 and 1.6×10^6 cfu ml^{-1} according to a standard curve obtained spectrophotometrically by measuring transmittance at 420 nm (Usall, 1995).

The apple cultivar Golden Delicious used in this experiment was obtained from commercial orchards in Lleida, Catalonia, Spain which was grown under standard cultural practices. Surface sterilized Golden Delicious apples were wounded, at the stem (top) and calyx (bottom) end. The wounds were $3 \times 3 \times 3$ mm. A 25 µl suspension of appropriate concentration of the antagonists from each treatment was applied to each wound and followed by inoculation with 20 µl of an aqueous suspension of *P. expansum* (1×10^4 cfu ml^{-1}). Three apples constituted a single replicate and each treatment was replicated three times.

Treated apples were incubated at 25 °C and 75% relative humidity for 7 days after which the diameter of decay were measured. *P. expansum* strain was isolated from decayed apples after several months in storage and it was cultured on potato dextrose agar (PDA).

Statistical treatment of the results

All results were analyzed by an analysis of variance with SAS software (SAS Institute, version 6.03, Cary N.C.). Statistical significance was judjed at the level $P< 0.05$. When the analysis was statistically significant Duncan's Multiple Range Test was used for separation of the means.

Pearson correlation coefficients between endogenous reserves and biocontrol effectivity or viability in a_w stressed media were calculated respectively.

Results

Nutrients and water availability in relation to polyol, trehalose and glucose content of *C. sake* cells

The accumulation of polyols and sugars in *C. sake* cells in the NYDB medium, either diluted or modified with glucose, glycerol to 0.96 a_w or trehalose to 0.97 a_w after 24 and 48 h are shown in Figure 1 A and B respectively. In normal unstressed NYDB medium, after 24 h, arabitol (< 1 mg g^{-1} fresh weight) and trehalose (< 0.4 mg g^{-1}) were the major compounds accumulated while that of arabitol increased to > 5 mg g^{-1} after 48 h, with glucose concentration (0.5 mg g^{-1}) also increased. Dilution of the medium concentration alone affected the accumulation of endogenous reserves, arabitol (1.5 mg g^{-1}) and erythritol were the major polyols and glucose the main sugar at 48 h. Either modifying the a_w only, or dilution medium+reduced a_w modification resulted in significant changes in the accumulation patterns of polyols and sugars. For example, glycerol content of cells increased significantly, especially in the glycerol-modified media (5-7 mg g^{-1}fresh weight) with a significant reduction in arabitol content (1.25 mg g^{-1}), and some glucose and trehalose. However, no data are presented for the NYDB25+glycerol treatment after 24 h because of poor growth. By contrast, in the normal strength NYDB100+glucose treatment arabitol was still the major polyol (> 6 mg g^{-1}) after 48 h, but glycerol, trehalose and glucose increased significantly in relation to unmodified medium. Dilution of the medium and modification of thea_w resulted in most accumulation of glycerol, arabitol and trehalose. In normal strength medium and trehalose treatments, arabitol (4.5-6 mg g^{-1}) and trehalose were the major compounds accumulated by the yeast cells. In most cases in the weaker medium (NYDB25) trehalose content decreased after 48 h of incubation when compared to that present after 24 h. Erythritol and mannitol contents were always low (< 0.9 mg g^{-1} fresh weight) in cells of *C. sake*. Mannitol concentrations generally increased in low a_w and diluted media.

There were significant differences in total sugars in NYDB100 and NYDB25 with various amendements depending on time of incubation and treatment (Figure 2). Glucose and trehalose-modified media had the highest intracellular concentration of sugars present (up to 3.5 mg g^{-1} fresh weight), with the glycerol and unmodified treatments the least.

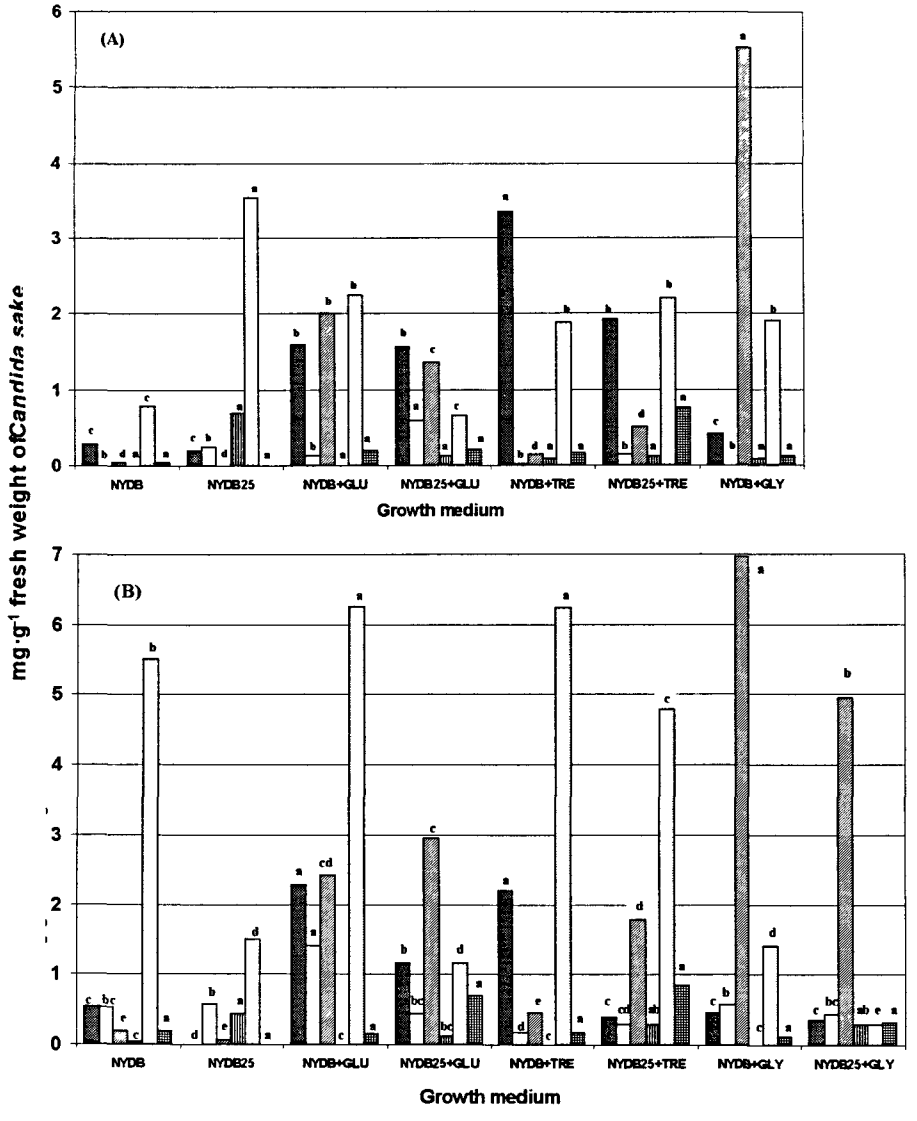

Figure 1. Accumulation of intracellular sugars trehalose (▩), glucose (□), and the polyols glycerol (▨), erythritol (▥), arabitol (▦) and mannitol (▦) in *Candida sake* grown on nutrient yeast dextrose based media (NYDB), either diluted to 25% strength NYDB25), or modified with glucose (+GLU) and glycerol (+GLY), and trehalose (+TRE) to achieve 0.96 and 0.97 water activity (a_w) respectively. The treatments were incubated for 24 (A) and 48 (B) hours at 25 °C. Results are means of three replicates per treatment. The separation of means are based on Duncan's Multiple Range Test for each endogenous reserve. Columns with different letters indicate significant differences ($P< 0.05$).

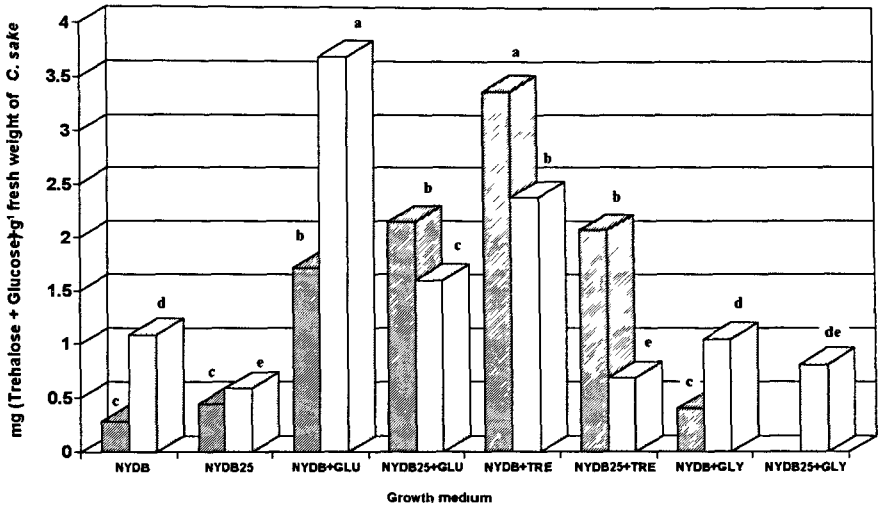

Figure 2. Comparison of total intracellular sugars (glucose and trehalose) present in cells of *C. sake* grown on either NYDB, or modified with glucose, glycerol or trehalose. The separation of means was conducted according to Duncan's Multiple Range Test ($P< 0.05$) and are shown for the time of incubation; 24 ( ) and 48 ( ) hours. Columns with different letters indicate significant differences between treatments.

Comparison of the total amounts of polyols accumulated in the different treatments (Figure 3) shows clearly that there are significant increases in accumulation in the yeast cells in some treatments after both 24 and 48 h incubation. Generally, higher amounts were synthesized after 48 than 24 h regardless of treatments. The highest concentrations (7-9 mg g^{-1} fresh weight) were present in the richest medium (NYDB100) modified either with glucose or glycerol to 0.96 a_w and in NYDB25+TRE treatment after 48 h.

Stress tolerance of *C. sake* cells in low water availability media

In the first experiment, the cells from each treatment described previously were plated after both 24 and 48 h on to 0.935 a_w medium modified with PEG200/300 to examine tolerance of low a_w. There were significant differences between the viability of the cells of the *C. sake* treatments after both 24 and 48 h (Figure 4 A and B). When data was superimposed on the percentages of polyols in each treatment for comparison, it was evident that larger populations were recovered on the 0.935 a_w medium from the treatments NYDB25+glucose, NYDB25+trehalose and NYDB25+glycerol at 48 h.

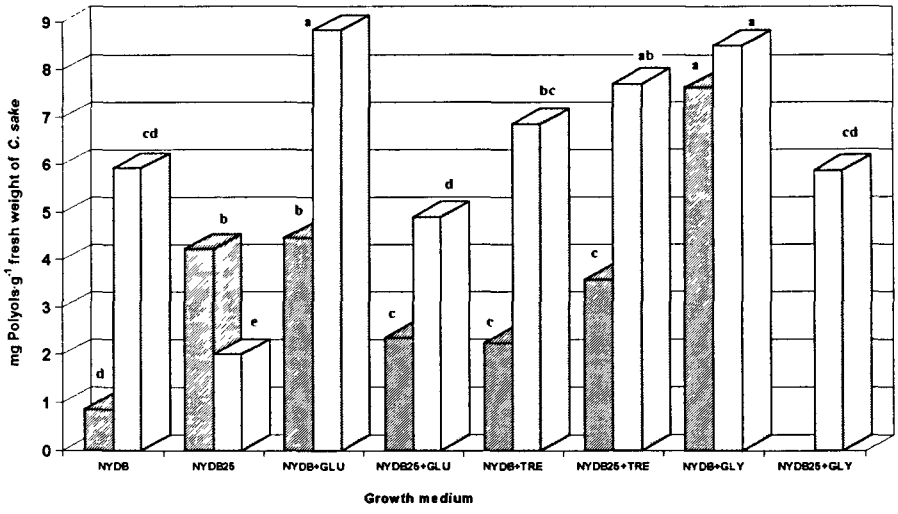

Figure 3. Comparison of total intracellular polyols present in cells of *C. sake* grown on either NYDB, or that modified with glucose, glycerol or trehalose. The separation of means for the time of incubation of 24 (▨) and 48 (▩) hours was conducted according to Duncan's Multiple Range Test ($P< 0.05$).

There was a statistically significant correlation between viability in stressed media and arabitol (Pearson coefficient= -0.51, $P< 0.05$) and with mannitol (Pearson coefficient= 0.84, $P< 0.01$) respectively. No correlation with other polyols or sugars was found.

In a second experiment the study was expanded to evaluate the capability of a wider range of treatments for viability on both normal unstressed (0.995 a_w) and on stressed 0.95 a_w media. In this experiment the medium was diluted to 50 or 25% of the normal strenght and again modified with glucose or glycerol. *C. sake* cells from NYDB50+glucose, NYDB25+glucose and NYDB25+glycerol treatments grew better on the 0.95 a_w medium after 48 h incubation (Figure 5). Interestingly, significant improvements in viability were also observed on unstressed media.

Biocontrol and antagonistic activity of modified *C. sake* treatments against *P. expansum* on apples

All *C. sake* treatments (NYDB, diluted and modified a_w) strongly inhibited development of *P. expansum* rot (Table 1). Percentage rot reduction was in all cases greater than 60%. However, cells grown on 50% and 25% diluted NYDB medium amended with glucose to 0.96 a_w with modified endogenous reserves gave a significative increased control in the range 80-96%.

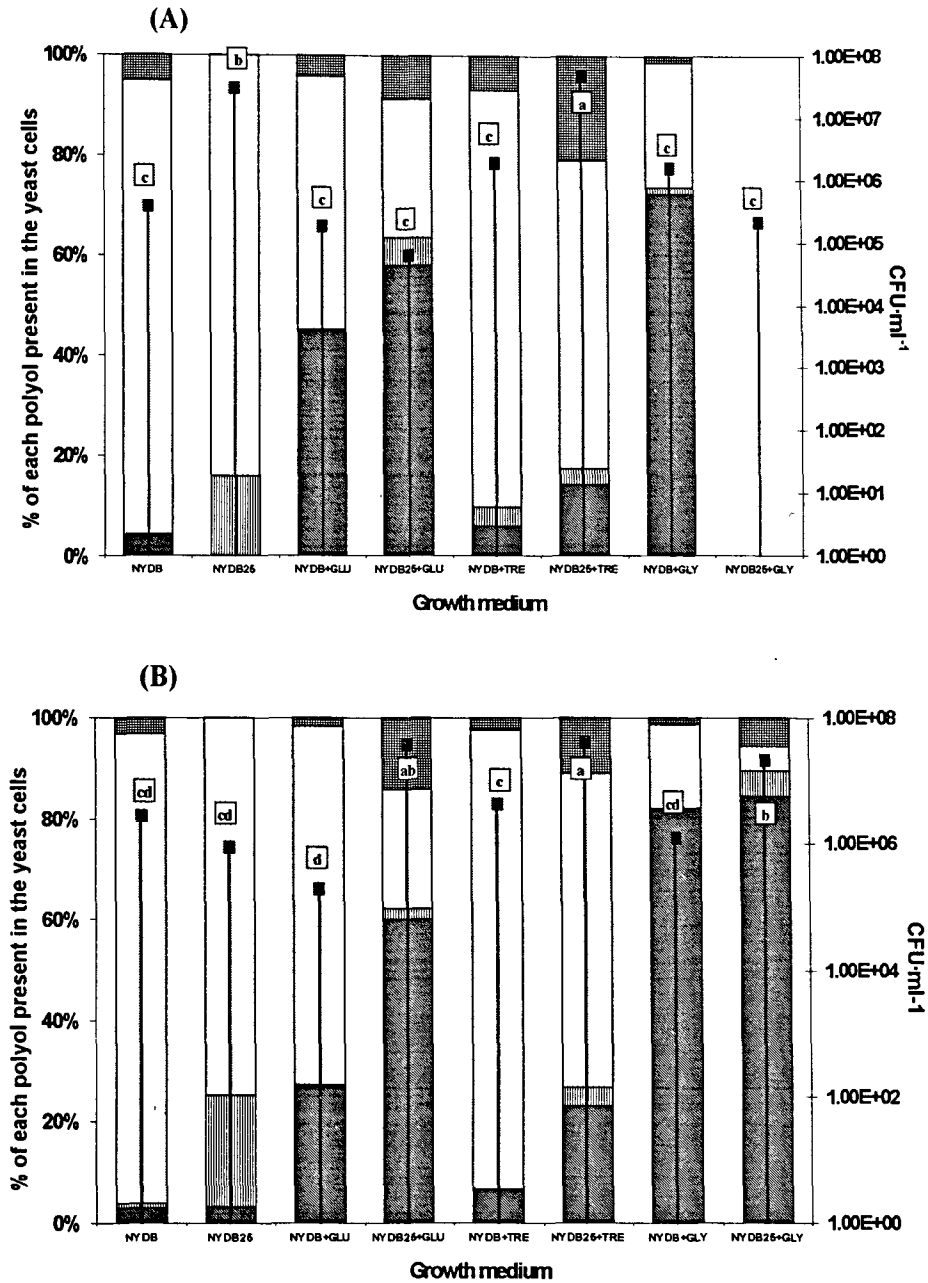

Figure 4. Viability of *C. sake* cells from different treatments at 0.935 a_w on NYDA modified with PEG (colony forming units (cfu) ml^{-1}) superimposed on relative percentage of individual polyols present in the yeast cells after 24 (A) and 48 (B) hours. Points with the same letter are not significantly different according to Duncan's Multiple Range Test ($P< 0.05$). Symbols: glycerol (▧), erythritol (▥), arabitol (▩), mannitol (▦) and *C. sake* growth (■).

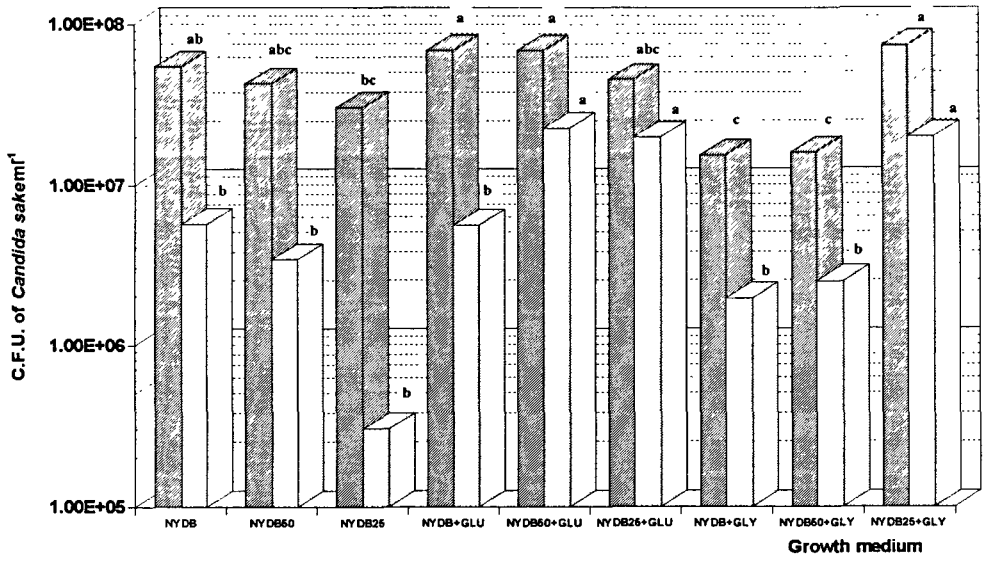

Figure. 5. Viability of *C. sake* cells of each treatment in unstressed (0.995 a_w, (▨)) and stressed (0.95 a_w, (▩))media. The means for each medium are separated according to Duncan's Multiple Range Test ($P< 0.05$).

Table 1. Biocontrol efficacy of *C. sake* (% rot reduction) grown in different modified and unmodified media against *P. expansum* rot of apples. Fruits were wounded, inoculated with the antagonist and challenged with 1×10^4 cfu ml^{-1} of *P. expansum,* and incubated for 7 days at 25 °C. The separation of means for % of rot reduction was conducted according to Duncan's Multiple Range Test ($P< 0.05$). Treatments in each column with different letters are significantly different.

TREATMENTS	*C. sake* concentration	
	7.50×10^5 cfu ml^{-1}	1.60×10^6 cfu ml^{-1}
NYDB	80.2 d	76.4 e
NYDB50	81.8 cd	84.8 c
NYDB25	72.8 f	82.0 cd
NYDB+GLU	82.9 cd	91.9 b
NYDB50+GLU	92.2 b	96.7 a
NYDB25+GLU	82.9 cd	95.8 a
NYDB+GLY	68.8 g	64.0 h
NYDB50+GLY	72.7 f	69.5 g
NYDB25+GLY	90.5 b	80.9 d

Glycerol treatments had better biocontrol efficacy at modified strenght than unmodified one, being medium diluted to 25% which presented the best results in relation to other glycerol treatments

Statistically significant correlations were found with trehalose (Pearson coefficient= 0.58, $P< 0.05$) and with total sugars (Pearson coefficient= 0.52, $P< 0.05$). For other polyols and sugars there was no correlation.

Discussion

This study has shown that manipulation of the growth of the biocontrol yeast *C. sake* by changing either nutrient concentration alone or by modifying both nutrient status and water stress significantly affected intracellular accumulation of both individual and total polyols and glucose and trehalose. Increasing the carbon concentration not only changes the C:N ratio but also reduces a_w markedly (Hallsworth and Magan, 1994b). The significant increase in the total polyols and sugars demonstrated that under certain nutrient/water stress conditions greater amounts of these endogenous reserves are accumulated than in rich media commonly used for their cultivation. However, when considering the function of polyols in intracellular osmotic adjustment, the roles of individual polyols becomes important as they are differentially effective as compatible solutes. High molecular weight polyols (e.g. mannitol) cause slight inhibition of enzyme activity compared to low molecular polyols (e.g. glycerol) at equivalent concentrations (Chirife *et al.*, 1984). Thus it was interesting to discover that yeast cells with a particular mixture of polyols/sugars are more tolerant of lowered a_w than others. For example, yeast cells with a mixture of predominantly glycerol, the higher m.weight polyol arabitol, and trehalose and glucose, and with very much smaller concentrations of mannitol and erythritol were significantly more tolerant of low water availability.

Besides reducing a_w, excess amounts of exogenously supplied carbohydrates can lead to N-limitation which can result in increased polyol concentration, particularly in mycelial fungi. In rich media with *Candida albicans* (Robin) Berkhout (Pfyffer and Rast, 1988), and in low a_w media with *C. cacaoi* Buckley and van Uden and *C. mangniolia* (Lodder and Kreger-van Rij) Meyer and Yarrow (Van Eck *et al.*, 1993), it was demonstrated that high m.weight arabitol and low m.weight glycerol were the major polyols accumulated intracellularly. In the latter study a_w was modified with glucose and NaCl only. However, Van Eck *et al.* (1989) did demonstrate that intracellular and extracellular concentration ratios of the low m.weight glycerol/erythritol were markedly changed as the level of water stress was increased. Work with *Dendryphiella salina* (Sutherland) Pugh and Nicot and *Neurospora crassa* Shear and Dodge suggested that the total soluble carbohydrates

in fungi in relation to water stress is constant (Ellis *et al.*, 1991; Jennings, 1995). With *N. crassa* grown in glucose media modified with NaCl, although the same total concentration of glycerol and mannitol were produced the ratio was 9:1. However, with fructose and the same water stress the ratio was about 2:1. In the present study different patterns were observed with significant (P= 0.05) increases in both total, and some individual polyols, especially where media were modified with glucose or glycerol to 0.96 a_w or in combination with medium dilution.

We have paid attention specifically to intracellular accumulation of polyols and sugars because of the interest in finding ways for conservation of quality and effective viability of the biocontrol agent. Previously, no attempt has been made to evaluate the effect that such manipulation of their physiology for improving tolerance of water stress and the potential for improving ecological competence in the environment. This is the first detailed study to demonstrate that physiologically modified and characterized yeast cells with known concentrations of polyols and sugars can improve viability of the cells under low water stress conditions. Previously, sclerotia with modified polyols had faster germination than unmodified sclerotia, but was only tested in normal unstressed medium (Al Hamdani and Cooke, 1987) while significantly improved germination was demonstrated for conidia of some entomogenous fungi at reduced a_w (< 0.90) when they contained elevated concentrations of glycerol and erythritol (Hallsworth and Magan, 1995). It is important to note that the accumulations of polyols and sugars observed in our study in relation to combined manipulation of nutrient status and a_w are significantly greater than that recently found in propagules of entomogenous biocontrol fungi in relation to pH and temperature (Hallsworth and Magan, 1996). Culture age also can have an impact on accumulation of endogenous reserves as in some treatments after 48 h there was a decrease relative to that present after 24 h. Thus time of incubation can also have a profound effect on the final quality and viability of the biocontrol agent and this may differ for species. In yeasts, depending on the solute used polyols are often actively released from yeast cells depending on the amount of water stress and the culture age (Blomberg and Adler, 1992; Jennings, 1995). In propagules of filamentous fungi polyols may be slowly metabolized and ultimately used in respiration or converted to higher molecular weight compounds such as glycogen. They can also move out of the conidia by transport to the mycelium or by diffusion into the conidiophore wall (Kiyosawa, 1991).

More attention has been paid to physiologically increasing the concentrations of sugars, particularly trehalose, in the industrial baker's yeast *Saccharomyces cerevisiae* (Van Dijck *et al.*, 1995). By manipulating growth conditions this yeast increased intracellular concentrations of trehalose (on a dry weight basis) to more

than 10%, which is the threshold above which stress resistance to freezing and freeze drying is optimum. Trehalose can be rapidly metabolized and be effective and efficient protectant, enhancing the resistance of cellular components against adverse conditions such as extreme temperatures, dehydration, or osmotic stress (Van Laere, 1989; Miekle *et al.*, 1991; Piper, 1993).

The demonstration that biocontrol potential can also be conserved is of particular significance. This suggests that significant ecophysiological changes in the modified yeast cells does not affect biocontrol potential, and indeed may improve control. This indicates that modified stress-tolerant inocula of such yeasts may survive and become more effectively established on the surfaces of fruit in naturally fluctuating environmental conditions and that this could give the biocontrol agent a competitive advantage enabling more effective preemptive exclusion of pathogens such as *P. expansum* from such niches. This could provide a method for improving consistency and efficacy of such biocontrol agents in the field. The findings reported here demonstrate that ecophysiological manipulation of such yeasts has the potential for significantly improving the quality of such biocontrol agents by improving stress tolerance, and perhaps field performance and biocontrol.

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Capítol 7

Preharvest application of *Candida sake* grown in media with different water activity for control of blue mold of apples in storage

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Summary

Unmodified and low water activity (a_w) tolerant cells of *Candida sake* CPA-1 applied before harvest were compared for ability to control blue mold of apples (Golden Delicious) caused by *Penicillium expansum* under commercial storage conditions. The population dynamics of strain CPA-1 on apples was studied in the orchard and during storage following application of 3×10^6 cfu ml^{-1} of each treatment two days prior to harvest. In the field population sizes of unmodified treatment remained relatively unchanged, while the low a_w-modified CPA-1 cells increased. During cold storage the populations in all treatments increased from 10^3 cfu g^{-1} to 10^5 cfu g^{-1} after 30 days, and then declined to about 2.5×10^4 cfu g^{-1} apple. Laboratory studies showed that the low a_w-tolerant cells provided significantly better disease control when compared with the unmodified treatment, with reductions in infected wounds and lesion diameter being bigger than 75% and 90% respectively, when compared with untreated controls. When *C. sake* treated apples were evaluated after 4 month cold storage both unmodified and low a_w-tolerant cells were equally effective against *P. expansum* (> 50% reduction in infected wounds). Our data demonstrate that application of a_w-tolerant strains of antagonistic microorganisms may be a promising method for improving field establishment while conserving biocontrol efficacy for postharvest diseases of apples.

Key words: Compatible solutes, ecophysiology, growth, *Penicillium expansum*, population dynamics, postharvest diseases, sugars, sugar alcohols, water activity.

Introduction

The development of resistance in fungal pathogens to fungicides (Bertrand and Saulie-Carter, 1978; Rosenberg and Meyer, 1979; Dekker and Georgopoulos, 1982; Spotts and Cervantes, 1986; Viñas *et al.*, 1991, 1993) and the growing public concern over health and environmental hazards from the high levels of pesticide inputs into fruit orchards (Norman, 1988; Wisniewski and Wilson, 1992) have resulted in a significant interest in the development of alternative non-chemical methods of control. Biological control using microbial antagonists has emerged as one of the most promising alternatives, either alone or as part of an integrated control strategy to reduce pesticide inputs. Recently, many antagonists with efficacy against fungal pathogens of fruits have been reported (Pusey and Wilson, 1984; Janisiewicz and Roitman, 1988; Pusey *et al.*, 1988; Wilson and Chalutz, 1989; Smilanick and Denis-Arrue, 1992; Janisiewicz and Bors, 1995). Biocontrol of postharvest pathogens has been very successful as indicated by a number of commercial products on the market including Aspire (*Candida oleophila* strain 182, Ecogen Inc., Langhome, PA) and Bio-save 10 and 11 (*Pseudomonas syringae*

strains ESC30 and ESC 11, Ecoscience Corp., Worcester, MA) (Koch, 1996). Detailed studies in Spain have shown that the strain CPA-1 of *Candida sake* (Saito and Ota) van Uden and Buckley is an effective antagonist of the major postharvest pathogens of pome fruits, including *Penicillium expansum* Link and *Botrytis cinerea* Pers.:Fr (Usall, 1995; Viñas *et al.*, 1996).

Some studies have been carried out to apply biocontrol agents in the field to control foliar diseases (Elad and Kirshner, 1992, 1993). Infection of fruit by postharvest pathogens often occurs in the field prior to harvest (Roberts, 1994; Biggs, 1995) and it would be advantageous to apply antagonists before harvest. Recently Leibinger *et al.* (1997) have examined this approach by using mixtures of yeasts and bacteria for control of apple postharvest diseases and obtained control of *P. expansum* and *B. cinerea* similar to that of a fungicide. Application before harvest are also of the interest, because European regulations of integrated pest management do not allow postharvest treatments of apples. A report in Postharvest News and Information (Rendall-Dunn, 1991) indicated that European Parliament has voted a favour of a total ban on postharvest treatments of fruits and vegatables with chemicals as soon as this practice becomes feasible. However, for this approach to be successful inocula applied in the field need to have key characteristics including tolerance of environmental stresses particularly of temperature and water activity (a_w), low nutrient conditions, and UV light for effective establishment and disease control (Deacon, 1991). However, few studies have tried to improve the competence, survival and activity of such biocontrol agents in the field for improving subsequent disease control (Windels and Lindow, 1985; Elad, 1990).

Recent physiological studies with cells of *C. sake* CPA-1 have shown that it is possible to modify the growth conditions such that specific endogenous compounds, particularly of sugar alcohols and trehalose accumulate in the yeast cells resulting in improved viability over a wider relative humidity range and with conserved biocontrol efficacy (Teixidó *et al.*, 1997). Accumulation of low (glycerol, erythritol) and high (arabitol, mannitol) molecular weight sugar alcohols occur in many fungi grown under conditions of environmental stress (Beever and Laracy, 1986; Hocking, 1986; Ellis *et al.*, 1991; Kelly and Budd, 1991; Van Eck, 1993). Intracellular accumulation of these polyols reduces cytoplasmatic a_w and enables enzymes to remain active during periods of water stress (Brown, 1978). Conidia of entomopathogenic biocontrol fungi with elevated concentrations of polyols (erythritol and glycerol) tolerated lower water activities and were more pathogenic than unmodified conidia (Hallsworth and Magan, 1994, 1995). Physiologically manipulated inocula of the biocontrol agent *Epicoccum nigrum* Link containing high concentrations of glycerol and erythritol provided better field control of *Monilinia laxa* (Aderh. and Ruhl.) Honey of peaches than unmodified inocula (Pascual *et al.*,

1996). Some studies have also suggested that the disaccharide trehalose may also be important as it enhances desiccation tolerance and increases viability and germination of fungi at reduced water activity (Panek, 1963; Van Laere, 1989; Harman *et al*, 1991).

The objectives of this study were to (a) compare the establishment and temporal population dynamics of unmodified and two physiologically modified low a_w-tolerant inocula of *C. sake* CPA-1 on apple surfaces in the orchard and subsequently during postharvest cold storage, (b) compare the efficacy of unmodified and the low a_w-tolerant treatments of the yeast applied in the field to Golden Delicious apples for control of blue mold caused by *P. expansum* after postharvest cold storage.

Materials and Methods

Biocontrol strains and pathogen

The biocontrol strain CPA-1 of *Candida sake* obtained from UdL-IRTA centre, Catalonia, Spain was used in this study. This strain was originally isolated from the apple surface cv. Golden Delicious, and demonstrated previously to have antagonistic activity against *Penicillium expansum, Botrytis cinerea* and *Rhizopus nigricans* Ehrenb. on pome fruits (Usall, 1995; Viñas *et al.*, 1996). Stock cultures were stored at 5 °C and were sub-cultured on nutrient yeast dextrose agar (NYDA). The pathogen *P. expansum* was isolated from decayed apples after several months in storage and maintained on potato dextrose agar (PDA, 200 ml extract from boiled potatoes, 20 g dextrose, 20 g agar and 800 ml water) with periodic transfers through apple. This is the most virulent isolate in the University of Lleida-IRTA collection and was used in all experiments. A conidial suspension was prepared by decanting 10 ml sterile water over the surface of ten day-old cultures grown on PDA and agitating with a sterile glass spreader. The cells were counted in a haemocytometer and diluted to 10^4 cfu ml^{-1} to use in disease control assays.

Fruits and orchards

Apples cv. Golden Delicious, were used in all experiments. Fruit trees, and fruits (freshly harvested, 1996) were from a commercial orchard in Aitona (Lleida), Catalonia, Spain, and were grown under standard cultural practices.

Growth media

An unmodified nutrient yeast dextrose broth (NYDB, 0.995 a_w) was used as the basal medium in this study. Low a_w-tolerant modified inocula were cultured on NYDB amended with (a) glucose (398 g l^{-1} to modify the medium to 0.96 a_w and

diluted by 50% prior to a_w modification (NYDB50+GLU) and (b) with glycerol (184 g l^{-1}) to 0.96 a_w and diluted by 75% prior to a_w modification (NYDB25+GLY) for 48 h at 25 °C on a rotary shaker at 150 rpm. The a_w of all media was determined with a Novasina Humidat IC II (Novasina AG, Zurich, Switzerland).

In previous studies inocula of *C. sake* grown on these media contained elevated amounts of glycerol and trehalose and were found to be the most viable cells in in vitro assays at different a_w levels (Teixidó, Viñas, Usall and Magan, *unpublished data*).

Isolation of *C. sake* from apple surfaces

Treated apples were weighed and aseptically peeled. The peels and wounded areas were shaken in 200 ml sterile phosphate buffer (pH 7) on a rotatory shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasound bath. This final step was used to improve detachment of microorganisms from the apple surface. Serial dilutions of the washings were made and plated on NYDA containing 0.5 g l^{-1} streptomycin sulphate to inhibit bacteria. Colonies were counted after incubation at 25 °C in the dark for 48 h. Population sizes were expressed as cfu per gram fresh weight fruit.

Preliminary comparison of modified and unmodified *C. sake* for growth and survival on the apple surfaces

Growth and survival of unmodified cells of *C. sake* grown in NYDB and two modified treatments grown in NYDB50+GLU and NYDB25+GLY were evaluated at concentrations of 5×10^5, 10^6 and 10^7 cfu ml^{-1} on apples in the orchard. The treatments were sprayed with a hand gun operating at a pressure of 10 atmospheres, onto attached apple fruits with four wounds equidistant from each other in the equatorial plane of the apple each made with a 1 mm diameter nail to a depth of 1 mm. In this study, two apples from one tree constituted a replicate and there were four replicate trees per treatment. In all cases guard trees were used to separate the randomized treatments. Samples were harvested immediately after treatment (0 h) and 48 h later. The experiment was carried out twice in 1996 on different trees. Apple surfaces were washed as described before and washings serially diluted and plated onto NYDA medium to determine populations of *C. sake* isolated from the apple surfaces.

A $3 \times 2 \times 3$ factorial analysis of variance (ANOVA) with SAS software (SAS Institute, version 6.03, Cary N.C., USA) was carried out to analyse survival and growth on apple surfaces with different *C. sake* treatments. This enabled the statistical significance of single, two and three-way interactions to be examined.

Statistical significance was judged at the level $P = 0.0001$. Least significant difference Test (LSD) was used for means separation.

Population dynamics of *C. sake* on the apple surface in the orchard and during postharvest cold storage

Antagonist suspensions of the three treatments were prepared in beakers at a concentration of 3×10^6 cfu ml^{-1} as described previously. Each treatment consisted of four replicate trees, with a total of 20 apples per replicate being artificially wounded as detailed above.

Wounded apples were dipped into the antagonist suspension for 30 s just after wounding. Apples were harvested when dry and then were taken to the laboratory to isolate *C. sake* populations from the apple surface as described above.

Each treatment was replicated four times with a single apple constituting a single replicate, and the experiment was carried out twice. Trees were sampled at 0, 24 and 48 h after application of each antagonist treatment. After 48 h, the rest of the treated apples were harvested and kept in cold storage (1 °C and 21% O_2). Population sizes of *C. sake* cells on apple surfaces were determined after 1, 7, 15, 30, 60 and 120 days in cold storage.

Data of *C. sake* populations (cfu g^{-1} fresh weight) were transformed to logarithms to improve homogeneity of variances (Parbery *et al.*, 1981), and an analysis of variance (ANOVA) for each sample date was conducted with SAS software. Statistical significance was judged at the $P = 0.001$ level and the Dunnet procedure used for means separation between modified and unmodified cells.

Antagonistic effect of *C. sake* sprayed on apples in the field for controlling *P. expansum* during postharvest storage

(a) ***In situ experiment***: Antagonist suspensions (unmodified, two a_w-tolerant treatments) were prepared at concentrations of 10^7 cfu ml^{-1}. Each treatment consisted of four replicates (70 apples per tree) arranged in a random block design. Each replicate consisted of three trees. In all cases a guard row of trees was used to separate the randomized treatments.

Apples were wounded as described previously. Two days before harvest each treatment was applied at 10^7 cfu ml^{-1} of the antagonist treatments using a hand gun operated at 10 atmospheres pressure.

After two days in the orchard, apples were harvested and placed in storage boxes according to replicate and treatment. Before cold storage apples were sprayed for 30 s with a suspension of *P. expansum* at a concentration of 1×10^4 conidia ml^{-1}.

Once dried, apples were stored at 1 °C and 21% O_2 which is typical of commercial cold storage. There were thus a total of 70 fruits per replicate, and four replicates per treatment. The number of infected wounds and the lesion diameters (mm) were measured 2 and 4 months after cold storage.

An analysis of variance (ANOVA) from SAS was performed on disease incidence and on severity data (P= 0.0001). Least significant difference Test (LSD) was used for means separation.

(b) ***Laboratory storage test***: Apples from the same orchard were used in the laboratory for short-term disease control assays. Two concentrations of *C. sake* cells were used in this study, 7.5×10^5 cfu ml^{-1} and 1.6×10^6 cfu ml^{-1}. Surface sterilized apples were wounded, at the stem (top) and calyx (bottom) with each wound 3 × 3 mm and 3 mm deep. 25 µl of appropriate aqueous suspension of each antagonist treatment (unmodified and low a_w-tolerant treatments) was applied to each wound and then 20 µl of an aqueous suspension of *P. expansum* (1×10^4 conidia ml^{-1}) was added. Each treatment was replicated four times with three apples per replicate and the experiment was repeated twice. Treated apples were incubated at 25 °C and 75% relative humidity for 7 days after which the percentage of infected wounds (incidence) and lesion diameters (severity) caused by *P. expansum* were measured.

A 3 × 3 factorial analysis of variance (ANOVA) with SAS software, was carried out to analyse disease incidence and severity data. This enabled the statistical significance of single and two-way interactions to be examined. Statistical significance was judged at the level P= 0.0001. Least significant difference Test (LSD) was used for means separation.

Results

Preliminary comparison of *C. sake* treatments for growth and survival on the apple surface

Statistical analysis of population at 0 and 48 h after *C. sake* treatment on apples showed that single treatments; treatment (treat), inoculum concentration (conc) and sample time (t) and two-way interaction of treat × t were statistically significant. However, there was no significant interaction between treat × conc. Thus the results for the different concentrations were pooled and the result of the analyses are shown in Figure 1.

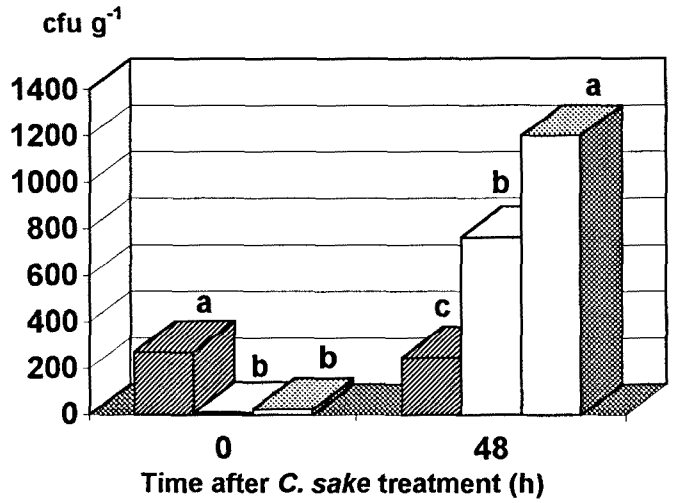

Figure 1. Yeast populations on apple surface at 0 h and 48 h after the application of unmodified cells of *C. sake* (NYDB ▨) and two water activity (a_w)-tolerant inocula grown on NYDB50+GLU (□) and NYDB25+GLY (□) media. The separation of means according to Least Significative Differences Test (LSD) are shown for every time of sampling. Columns with different letters indicate differences between treatments. Maximum temperature reached during the field assay was 37 °C.

Immediately after application (0 h), populations of unmodified *C. sake* were higher than the two low a_w-stress tolerant inocula. However, after 48 h under field conditions, the population sizes of the two a_w-tolerant inocula were significantly greater than the unmodified treatment (P= 0.0001). Populations of *C. sake* cells from glucose-amended media were isolated in largest numbers, followed by those from glycerol-amended treatment. Populations of unmodified cells did not increase over this period.

Population dynamics of *C. sake* on apple surfaces in the orchard and during postharvest storage

The temporal changes in the populations of *C. sake* cells of each treatment in the field on Golden Delicious apples are shown in Figure 2A. Initially, the population size of the unmodified inoculum was significantly (P= 0.001) greater on apples in the orchard when compared with the two a_w-tolerant treatments. However, after 24 h under field conditions, population sizes of the unmodified treatment decreased markedly while that of the two low a_w-tolerant treatments increased. After 48 h the numbers of modified a_w-tolerant *C. sake* cells isolated from the apple surface were significantly (P= 0.001) greater than those of the unmodified treatment.

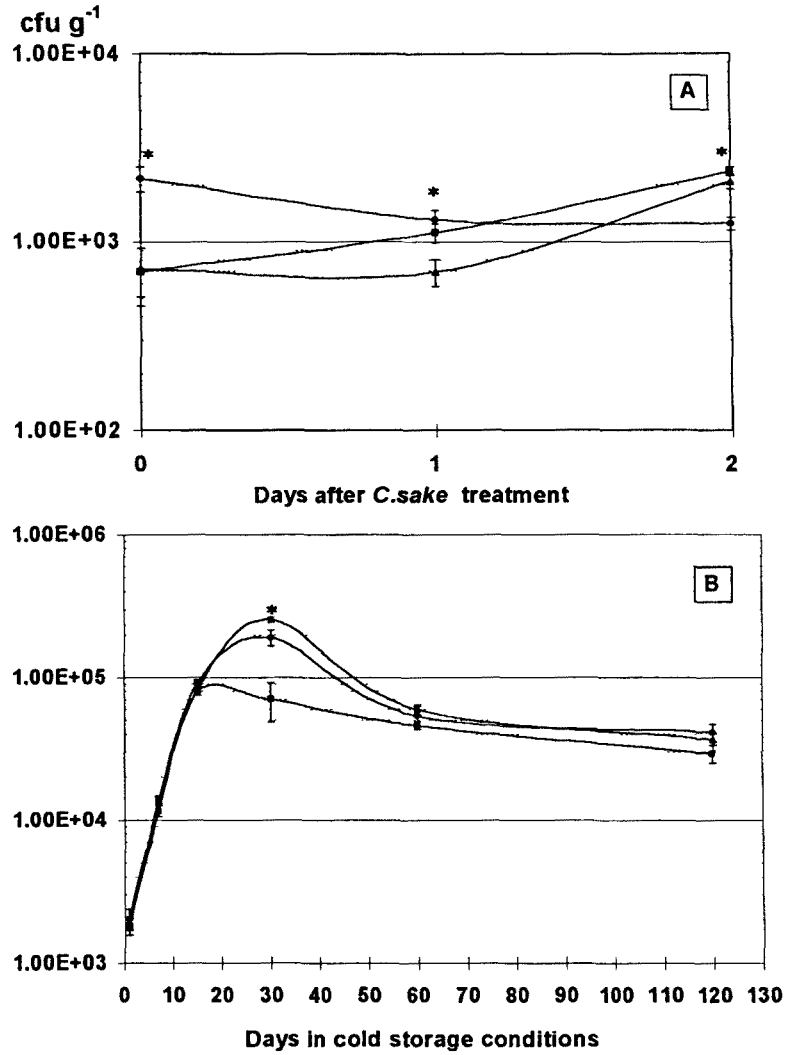

Figure 2. Population dynamics of *C. sake* CPA-1 grown in different media on Golden Delicious apple surface during two days in the field (A) and during the cold storage period (B). Antagonist suspensions were inoculated on wounded apples in the field two days prior to harvesting. Fruit samples were removed at various times to isolate antagonists from the surface. Symbols: ●, NYDB; ■, NYDB50+GLU; ▲, NYDB25+GLY. Statistical differences between unmodified and both modified inocula with the Dunnet procedure are shown with asterisks. Maximum temperature reached during the field assay was 34 °C.

However, in cold storage the population sizes of all three treatments were similar for about one month (Figure 2B). Subsequently, the glucose-modified a_w-tolerant treatment had lower populations of *C. sake* on apples than either the unmodified

cells or the glycerol-modified a_w-tolerant treatment, which had the highest *C. sake* populations. At the end of the four month period in cold storage all treatments had similar populations.

Antagonistic activity of *C. sake* sprayed on apples in the field against *P. expansum* rot during postharvest storage

Results of the field tests showed that there were statistically significant differences (P= 0.0001) between control apples (treated with *P. expansum* only) and apples sprayed with the three different *C. sake* treatments (Figure. 3). This was demonstrated both as control of lesion diameter (severity) and the percentage of infected wounds (incidence). However, no differences were observed between different *C. sake* treatments.

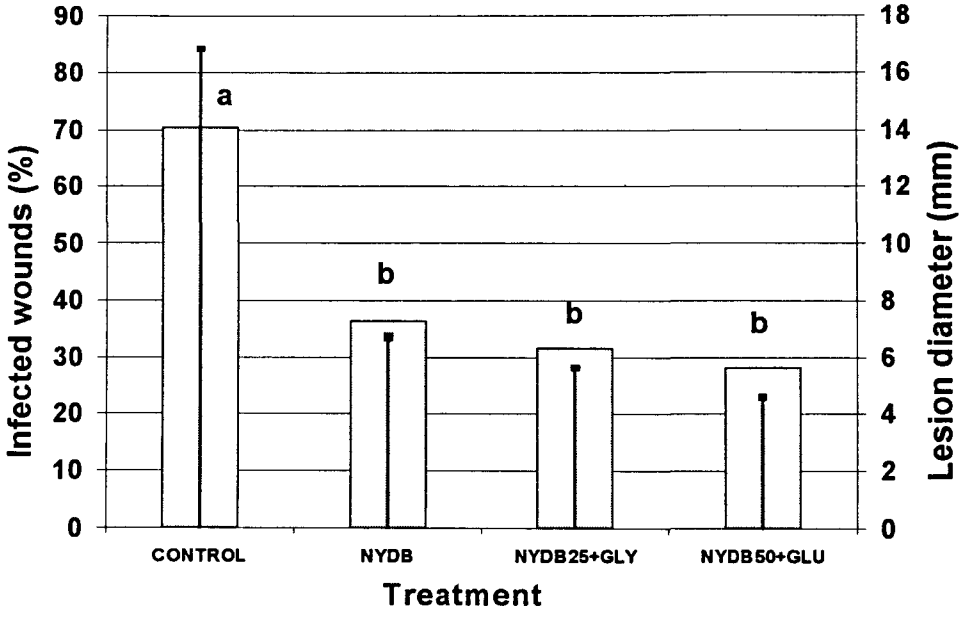

Figure 3. Efficacy of *C. sake* CPA-1 treatmens grown in different media on the development of *P. expansum* decay in Golden Delicious apples. Fruits were wounded in the field and antagonist suspensions (10^7 cfu ml^{-1}) sprayed on the trees two days prior to harvest. After harvesting, fruits were sprayed with an aqueous suspension of *P. expansum* at 10^4 conidia ml^{-1} and kept in cold storage for 4 months. The separation of means for percentage of infected wounds (⬚) and lesion diameter (■) according to Lest significant Difference Test (LSD) (P< 0.0001) are shown.

In the laboratory experiments all *C. sake* treatments significantly inhibited development of blue mold (Figure 4). The absence of statistical interaction between treatments and concentrations allowed analyses of both concentrations of each treatment together.

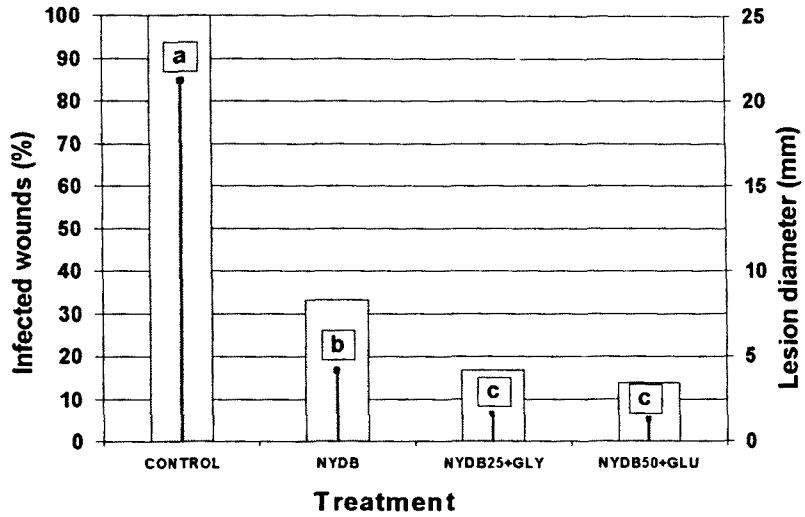

Figure 4. Biocontrol efficacy of *C. sake* CPA-1 grown in an unmodified and two different modified treatments against *P. expansum* rot on Golden Delicious apples. Fruits were wounded, inoculated with the three antagonist treatments and with 10^4 conidia ml^{-1} of *P. expansum*, and incubated for 7 days at 25 °C. The separation of means according to Least Significative Differences Test (LSD) are shown to infected wounds (☐) and lesion diameters (■). Columns with different letters indicate differences between treatments.

Severity and incidence of the disease were significantly lower in apples inoculated with low a_w-tolerant glucose and glycerol cells of *C. sake*. Control of lesion diameter were bigger than 90% and percentage infected wounds was bigger than 75% with both concentrations of the a_w-tolerant treatments. Interestingly, at the lower antagonist concentration of the unmodified treatment the control achieved was lower (72% and 49% respectively).

Discussion

This study is the first detailed investigation of the comparison of unmodified and physiologically modified a_w-tolerant inocula applied in the field to try and enhance establishment of antagonists on the apple surface for postharvest disease control. We have demonstrated that it is possible to apply biocontrol antagonists in the field prior to harvest to enable colonization to occur during storage for postharvest control of wound pathogens. Both unmodified and low a_w-tolerant treatment populations survived when sprayed in the field and were subsequently able to increase their populations over time (up to 30 days, postharvest) and thus effectively

control blue mold, the most important postharvest disease of apples in Spain. The initial survival of the populations of the all three inocula in the preliminary experiment were lower than in the subsequent full field trial although the final populations achieved prior to harvest were similar. Previously, Teixidó *et al.* (1997) demonstrated that it was possible to significantly alter the endogenous sugar alcohols and sugars present in the cells of *C. sake*. Those treatments grown under water stress with either glucose or glycerol contained a mixture of the sugar alcohols glycerol, arabitol, and the disaccharide trehalose, which appeared to be important in improving viability of the yeast cells and tolerance to lowered a_w stress. The polyols are known to be important compatible solutes in enabling cell functioning under environmental stress, particularly lowered a_w, and the trehalose is critical for preventing desiccation and has a role as a cryoprotectant (Van Laere, 1989). Previously, filamentous fungal biocontrol agents of pests containing significantly elevated concentrations of the low molecular weight polyols, glycerol and erythritol, have been shown to significantly improve germination and biocontrol efficacy of insect larvae (Hallsworth and Magan, 1994, 1995).

This study has also shown that the two a_w-tolerant inocula controlled *P. expansum* rot significantly better that the unmodified inoculum, specially at lower concentrations in laboratory studies. The potential for using lower threshold concentrations for achieving control could be very important from a economic point of view and also in relation to conserving viability and inoculum quality during formulation.

The only other study on preharvest biocontrol treatments for postharvest control of apple diseases is that by Leibinger *et al.* (1997) involving the use of mixtures of strains of *Aureobasidium pullulans* (de Bary) G. Arnoud and *Rhodotorula glutinis* (Fresen) F.C. Harrison, and of *Bacillus subtilis* and *A. pullulans*. By using three preharvest applications they were able to increase populations on the apple surface prior to harvest. However, during cold storage these rapidly decreased. At a treatment concentration of a mixture of 10^6 (*A. pullulans*) and 10^8 cells ml^{-1} (bacteria) they were able to get similar control of *P. expansum* and *B. cinerea* to that with a fungicide. Other studies have concentrated on foliar disease control with preharvest application of *Trichoderma harzianum* (Rifai) and *Ulocladium atrum* for *B. cinerea* on various crops (Elad and Kirshner, 1993; Köhl *et al.*, 1995a, b). They found, respectively, that relative humidity and leaf wetness periods had a significant effect on effective establishment, although the use of low a_w-tolerant strains were not investigated in these studies. Indeed, McKenzie *et al.* (1991) found that unformulated pure conidial suspensions of *T. harzianum* did not survive effectively under field conditions. However, Smilanick *et al.* (1993) has suggested

that this approach may enable early colonization by biocontrol agents and give protection against latent infections.

In the present study it is noteworthy that immediately after application (up to 24 h) in the field the populations of the unmodified yeast decreased rapidly. However, although the a_w-tolerant treatments developed more slowly, over the crucial 2-3 days prior to harvest their populations increased markedly, particularly that of the glycerol-modified treatment.

In these experiments it was also observed that unmodified yeast cells initially adhered better to the apple surface than the two low a_w-tolerant treatments. There is little detailed knowledge of the characteristics of the matrix produced by yeasts such as *C. sake*. However, previous studies with matrices of spores of other fungi suggest that they may have a number of important ecological properties, including protection against temperature extremes, desiccation and short wave radiation (Louis and Cooke, 1983, 1985). It is possible that the energy requirements for the production of high concentrations of endogenous reserves such as polyols and trehalose (Teixidó *et al.*, 1997) could result in a modification of the concentration or characteristics of the matrix. More knowledge may be needed to identify these subtle changes during the production of modified inocula under water stress conditions.

We believe that there may be a number of advantages to preharvest application of biocontrol agents for postharvest control of pome fruit diseases provided they are ecologically competent or the inoculum quality can be conserved. This would result in less fruit manipulation and decrease the potential for damage and injuries which may occur during postharvest treatment. It would decrease the time periods between harvest and cold storage required for application of treatments and also avoid additional contamination by pathogenic fungi from drenching solutions usually used during chemical treatments.

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Capítol 8

Discussió general

DISCUSSIÓ GENERAL

1. Microflora pròpia de la mançana "Golden Delicious" tant a camp com a cambra

L'objectiu principal d'aquest treball era el poder aplicar l'antagonista *Candida sake* CPA-1 a camp per a poder controlar el fong responsable de la principal malaltia de fruita de llavor en postcollita, *Penicillium expansum.*

Era lògic que abans d'aplicar el nostre agent de biocontrol en les mançanes intentessim averiguar quina era la microflora pròpia d'aquestes, amb què es trobaria el nostre antagonista. De fet, alguns autors com ara Spurr (1994), ja havien suggerit que existia una relació entre la microflora de les superfícies aèries de les plantes i el control biològic natural de les malalties. I que el coneixement sobre l'estat microbià de la superfície dels fruits era un prerequisit per aconseguir sistemes de biocontrol efectius.

Així doncs, durant les dues primeres campanyes vam intentar assolir el coneixement de la microflora de la mançana tant a camp al llarg del seu desenvolupament com a cambra. D'aquesta manera s'intentava conèixer el medi on hauria d'establir-se i desenvolupar-se l'antagonista per a dur a terme la seva acció més endavant en postcollita.

Els borrons foren l'estat fenològic on les poblacions bacterianes van ser més elevades, això coincidia amb l'esmentat per Blakeman (1985) que localitzava les bactèries en els estadis de desenvolupament amb nivells de nutrients més baixos, com ara els borrons o el moment d'emergència de les fulles. Després, a mesura que avança l'estació i augmenten les poblacions de llevats, comença una competència pels aminoàcids i les poblacions de bactèries disminueixen. A més a més el pH menys àcid del borró també pot explicar en part el predomini de les bactèries sobre els fongs (Mossel *et al.*, 1991).

Així mateix, les poblacions de bactèries ja no van tornar a assolir els nivells de l'etapa de borró, ben al contrari, van tenir un petit pic en l'estadi de quallat del fruit per anar disminuint després progressivament fins el moment de collita, moment que localitzat a les darreries de l'estiu i després de períodes de calor i dessecació elevada, les poblacions de bactèries foren realment minces. És conegut que les bactèries sofreixen fortes decaigudes quan són sotmeses a períodes d'altes temperatures i condicions seques (Fokkema *et al.*, 1979). Més baixes foren encara les poblacions bacterianes durant el procés de cambra frigorífica, i és que aquest tipus de microorganismes són especialment sensibles a les temperatures baixes.

Les principals floridures aïllades dels diferents estadis de desenvolupament a camp van ser *Cladosporium* spp. i *Alternaria* spp., i en menor quantitat *Epicoccum* spp., *Fusarium* spp. i *Acremonium* spp. entre d'altres. Prèviament Pennycook i Newhook (1981) havien trobat que el 60-80 % de colònies aïllades de borrons i fulles de mançanera eren de *Cladosporium* spp. En canvi, estudis en micoflora de mançanes per a sidra suggerien *Aureobasidium pullulans* i *Epicoccum nigrum* com les principals espècies presents en la superfície de mançanes a França (Bizeau *et al.*, 1990).

Durant tot l'estudi tant de camp com de cambra, els llevats blancs van ser sempre més numerosos que els rosats. Hislop i Cox (1969), Warren (1976) i Pennycook i Newhook (1981) van trobar el mateix comportament en borrons de diferents espècies d'arbres de fulla caduca.

És important destacar l'efecte negatiu dels fungicides sobre les poblacions fúngiques en general, sobretot durant les diferents fases de creixement del fruit. Així les aplicacions fungicides disminueixen les poblacions de floridures en general, de *Cladosporium, Alternaria* i llevats blancs per un màxim de 15-30 dies després del tractament. En canvi les poblacions bacterianes solien presentar-se més numeroses en les mançanes tractades amb fungicides que amb les no tractades, segurament per un fenomen de manca de competència amb els fongs. Andrews i Kenerley (1978) en un estudi sobre l'impacte dels pesticides sobre fulles de mançanera cv. McIntosh, van veure que tant bactèries, llevats com floridures eren significativament reduides pel programa de pesticides utilitzat. De totes formes *A. pullulans*, un colonitzador dominant de les fulles de mançanera a Wisconsin USA, no va ésser afectat pels tractaments pesticides.

En canvi, els efectes dels fungicides sobre els constituents de la microflora de fulles fou estudiat per Blakeman (1985) i suggeria que el creixement de fongs sapròfits era inhibit per un ampli espectre de fungicides, però que aquests tenien poc efecte en les bactèries.

En l'estadi de borró ja es va trobar *Alternaria* spp. a l'interior, com a infecció endofítica. Si tenim en compte que aquest gènere és el causant de la podridura del cor en mançanes sembla important el tenir en compte aquest resultat. I més quan els estudis previs suggerien que la infecció per *Alternaria* en el cor de la mançana es donava 3 o 6 setmanes abans de la collita (Taylor, 1955; Raina *et al.*, 1971) o poc després de la floració (Marshall i Walkley, 1951; Ellis i Barrat, 1983; Combrick *et al.*, 1985). El nostre estudi prova que *Alternaria* spp. ja està present en l'estadi de borró, en forma latent dins el teixit de la mançana, protegit dels fungides i esperant condicions adequades per causar la podridura.

És important destacar també que els principals fongs causants de podridures en postcollita de mançanes, com ara *P. expansum* o *B. cinerea* rarament van ser aïllats de les mançanes durant el seu desenvolupament. Això confirma els estudis duts a terme per Usall i Viñas (1989) que van trobar que la presència de *Penicillium* spp. en camps de mançanes de Lleida estudiats just abans de la collita era insigficant. No obstant la presència del gènere *Penicillium* esdevingué important a partir del segon mes d'emmagatzematge i va augmentar progressivament fins al final del període en fred i durant el període de simulació de la comercialització o també comunament anomenat "shelf life".

Al igual del què passava en precollita les floridures dominants en cambra van ser *Cladosporium* spp. i *Alternaria* spp. I cap al final de la conservació *Penicillium* spp. tal i com ja s'ha esmentat.

Els fungicides aplicats en precollita no van tenir cap efecte apreciable sobre els diferents tipus de microorganismes una vegada collida la fruita. El darrer tractament havia estat dut a terme a començaments de juliol i estudis previs sobre el tema suggerien que l'impacte dels fungicides en la micoflora de la superfície aèria de les plantes sovint restava com a màxim durant 3-6 setmanes (Andrews i Kenerley, 1980).

2. Efecte de l'antagonista *C. sake* CPA-1 sobre la microflora pròpia de la mançana

L'aplicació de *C. sake* en precollita va tenir poc efecte sobre les poblacions bacterianes en postcollita, de fet aquestes van ser molt minces (inferiors al 5% del total de la població microbiana) durant tot el període de frigoconservació.

L'efecte de *C. sake* sobre la micoflora va notar-se principalment durant els darrers mostratges de frigoconservació (després de 5 i 7 mesos en cambra) i durant el període de "shelf life". Així per exemple, durant aquesta darrera etapa de simulació de la comercialització, com a conseqüència de la temperatura adequada per al creixement dels microorganismes, hi va haver un ràpid increment de la població de llevats sobre la superfície de les mançanes. Malgrat tot, les fruites no tractades amb antagonista van assolir nivells molt més elevats que les que havien estat tractades amb *C. sake*. Això podria ser degut a què *C. sake* resulta ser més competitiva que els llevats propis de la fruita i competeix més efectivament pels nutrients de la superfície de la mançana, evitant en part el creixement dels llevats propis d'aquesta.

Un efecte similar va poder observar-se amb les floridures pertanyents als gèneres *Cladosporium* i *Penicillium*, tant durant el darrer mostratge com en el període de "shelf life". Això resulta de particular importància si tenim en compte que *P. expansum* és el fong responsable de la principal malaltia de postcollita de mançanes, i que *C. sake* ha estat seleccionada com agent de biocontrol efectiu per a controlar aquest patogen.

En canvi, no va apreciar-se cap efecte significatiu de l'antagonista sobre les poblacions d'*Alternaria* spp. De fet estudis previs (Nogueras, 1996) van mostrar que *C. sake* no tenia cap efecte en les poblacions d'*Alternaria alternata* inoculades artificialment en mançanes a camp. Els agents de biocontrol són generalment específics en la seva acció en relació tant al patogen com a l'hoste (Janisiewicz, 1988).

El fet que l'antagonista mostri també el seu efecte durant el període de simulació de la comercialització és força important de cara a la seva utilització, ja que ens asseguraria una protecció del producte fins el moment en què aquest arribés al consumidor.

3. Evolució de *C. sake* CPA-1 sobre mançanes "Golden Delicious"

Veure com s'estableixen les poblacions de l'antagonista sobre el substracte que han de protegir és força interessant, i en el nostre cas que l'aplicació vol fer-se directament a camp encara ho és més, donat que les condicions de camp no solen ser les òptimes per als microorganismes; altes temperatures i condicions de dessecació.

És important destacar que durant les primeres 24 h a camp després del tractament les poblacions de *C. sake* van disminuir dràsticament, probablement com a conseqüència del "shock" produït per les altes temperatures i la baixa humitat relativa. De fet, estudis duts a terme per a l'aplicació en precollita de *Trichoderma harzianum* i *Ulocladium atrum* per al control de *Botrytis* a camp en diferents conreus (Elad i Kirshner, 1993; Köhl *et al.*, 1995a, b) van mostrar respectivament que la humitat relativa i la humitat de la fulla tenien un efecte significatiu en l'establiment efectiu de les poblacions a camp. A més a més, McKenzie *et al.* (1991) van trobar que suspensions de conidis de *T. harzianum* sense formular no sobreviuen efectivament sota condicions de camp. Malgrat tot, en el present treball es va veure que el llevat *C. sake* era capaç de sobreviure a camp i augmentar la seva població un cop en condicions de cambra frigorífica. Tot plegat indica una bona adaptació de la soca antagonista a les condicions de fred pròpies de postcollita. Janisiewicz (1991) suggeria que els llevats reunien les característiques

apropiades per ser desitjables candidats per al biocontrol de malalties en postcollita, i una d'aquestes característiques era la tolerància a les baixes temperatures. El llevat *C. sake* una vegada en fred augmenta progressivament la seva població assolint el màxim després d'un mes en cambra, després disminueix paulatinament per a tornar a augmentar de forma destacada durant el període de simulació de la comercialització.

A més a més també va comprovar-se en aquest estudi que les poblacions de *C. sake* no es veuen afectades en absolut pels tractaments en precollita aplicats de forma habitual a la zona.

4. Eficàcia de l'aplicació de *C. sake* CPA-1 en precollita sobre el control en postcollita de *P. expansum* inoculat artificialment

El proper pas en aquest treball fou intentar veure l'eficàcia de l'aplicació de *C. sake* en precollita en el control de la podridura causada per *P. expansum* que havíem inoculat just abans d'emmagatzemar la fruita en cambra frigorífica, en mançanes foradades artificialment per tal d'afavorir encara més la podridura.

Aquest assaig es va dur a terme tant amb forats fets a camp, simulant els danys de precollita que puguin tenir les fruites, com amb forats fets en postcollita, simulant els danys de la manipulació de la fruita durant la collita i passos previs a l'emmagatzematge.

A més a més, es tractava d'un estudi comparatiu entre el tractament en precollita i el de postcollita, i fins i tot la combinació d'ambdós.

El tractament de postcollita amb l'antagonista va controlar significativament tant les ferides de pre com les de postcollita, amb reduccions del diàmetre de podridura superiors al 80 %. El tractament de precollita va ser menys efectiu que el de postcollita, malgrat tot van assolir-se reduccions del diàmetre de podridura del 50%. Si tenim en compte que les condicions testades van ser totalment desfavorables per al microorganisme (fruites foradades artificialment, concentració elevada de patogen directament aplicada abans de l'entrada en cambra,...), aquest resultat tot i no ser del tot satisfactori, dóna força esperances de cara a un futur.

La possible explicació a aquesta diferència d'efectivitat podria tenir el seu origen i explicació en les diferències observades entre l'evolució de les poblacions sobre les mançanes dels diferents tractaments. Així, es va observar que la corba de creixement de l'antagonista en mançanes dels tractaments aplicats només en precollita disminuia més ràpid i abans, que en les mançanes tractades en postcollita, en les quals es continuaven mantenint nivells de població elevats tot i

després de 90 dies en cambra frigorífica. Clarament es necessita més investigació per a poder esbrinar les raons d'aquesta menor supervivència durant les condicions de frigoconservació de l'antagonista quan és aplicat a camp.

Potser el fet que les poblacions disminueixin tant en el camp podria tenir alguna relació amb aquesta menor supervivència més endavant. S'hauria d'aconseguir doncs, una major resistència a les condicions adverses amb què el microorganisme ha d'enfrontar-se en precollita.

No van observar-se millores de control quan es tractava en ambdós moments; pre i postcollita.

Un aspecte interessant observat durant el desenvolupament d'aquest assaig és el fet que les ferides fetes en els fruits en precollita es podreixen abans i assoleixen diàmetres de podridura més grans que les realitzades en postcollita. Aquest fet podria tenir relació amb els canvis metabòlics que tenen lloc en el teixit de la mançana i que comporten un augment en la maduració del fruit i com a conseqüència una disminució en la resistència dels teixits de la mançana a l'atac per part dels patògens. Imaseki (1985) afirmava que una ferida en algun òrgan d'una planta superior donava lloc a certes alteracions tant fisiològiques com bioquímiques, no només en l'àrea danyada sinó en la totalitat de la planta. Així mateix, està àmpliament reconegut en un gran nombre de teixits vegetals que quan se'ls hi practica una ferida hi ha un augment en el nivell de la producció d'etilè i per tant un avançament en la maduració del teixit (Abeles, 1973).

Després d'aquesta experiència durant dues campanyes consecutives, es va veure que el microorganisme assajat realment presentava unes característiques adequades per a poder ésser aplicat com agent de biocontrol en precollita per controlar malalties de postcollita. No obstant encara hi havia una sèrie d'interrogants per respondre i certes limitacions a millorar en un futur, com ara la tolerància de l'agent de biocontrol a les condicions estressants de temperatura i d'humitat amb què s'enfrontava quan l'aplicàvem a camp. Malgrat tot, abans de plantejar cap tipus de millora era necessari conèixer bé els límits de temperatura, activitat aigua i pH (com aspectes importants en aquest cas) als quals el microorganisme pot desenvolupar-se, establir-se i per tant exercir un biocontrol eficaç. Aquest doncs va ser el següent pas.

5. Caracterització de l'agent de biocontrol *C. sake* CPA-1 enfront a condicions d'estrès hídric, temperatura i pH

El perfil d'a_w × temperatura per al creixement de *C. sake* CPA-1 va estar situat entre 0.995-0.90 i 4-30 °C quan s'utilitzava glicerol per ajustar l' a_w, i 0.995-0.92 i

10-30 °C amb el solut iònic NaCl. Com es pot veure *C. sake* tolera a_w més baixes en presència de soluts no-iònics com ara el glicerol que de soluts iònics com ara el NaCl. Resultats similars es van poder observar en estudis duts a terme per Van Eck *et al.* (1993) amb altres espècies del gènere *Candida*, que també van mostrar una major tolerància a a_w més baixes en presència de glucosa que de NaCl. Així respectivament les a_w mínimes de creixement amb *C. cacaoi* van ser 0.83/0.84, per a *C. magnoliae* 0.82/0.88 i per a *C. tropicalis* 0.88/0.89. Com pot apreciar-se totes tres espècies són més tolerants a baixes a_w que *C. sake*.

L'interval de temperatures de creixement per a *C. sake* van ser en aquest estudi entre 4 i 30 °C. Cal tenir però en compte, que *C. sake* creix perfectament a les temperatures típiques de frigoconservació de mançanes, que estan situades a 1 °C aproximadament (Usall, 1995). En canvi, no creix a temperatures superiors a 36 °C, tal i com va demostrar en els seus estudis Usall (1995), deduint així que no podia créixer a la temperatura corporal de l'ésser humà (37 °C).

C. sake va demostrar ésser tolerant a un ampli interval de pH, 3-7, independentment de l'a_w i la temperatura, encara què les velocitats de creixement eren inferiors per a temperatures i a_w extremes. Aquest resultat contrasta amb els estudis duts a terme amb *C. guilliermondii*, que creixia més lentament a pH 4 que amb el seu òptim (pH 6) tant a 0.995 com a 0.95 d'a_w (Magan i Lacey, 1986).

Cal destacar la bona tolerància que presenta l'antagonista a pH baixos i que mostra que podrà créixer i exercir efectivament el seu biocontrol en medis àcids com ara les fruites i en especial la mançana que presenta un pH de 3-4. Aquesta característica resulta força positiva de cara a la utilització de *C. sake* en fruita.

6. Caracterització de les reserves endògenes (sucres i polihidroxialcohols) acumulades pel llevat *C. sake* i efecte de la temperatura i de l'a_w sobre aquestes

Una vegada caracteritzat l'agent de biocontrol enfront a la temperatura i l'a_w, ja estàvem en disposició de poder intentar millorar-lo enfront a condicions d'estrès, utilitzant l'enfoc de què quan fem créixer un microorganisme en condicions d'estrès acumula una sèrie de substàncies, ja siguin poliols (Brown, 1978) o sucres (Van Laere,1989; Miekle *et al.*, 1991; Piper, 1993), que el fan més resistent a les condicions adverses esmentades. Aquest enfoc ha estat utilitzat en alguns agents de biocontrol per tal de fer-los més resistents a les condicions extremes de camp (Hallsworth i Magan, 1994b, 1994c; Pascual *et al.*, 1996).

Els principals poliols/sucres acumulats en cèl.lules de *C. sake* crescudes en medi no modificat (NYDB) a_w=0.995, i per tant no restrictiu, foren arabitol, trealosa i

glucosa amb petites quantitats de glicerol i eritritol. A més a més, el contingut total màxim de poliols i sucres assolit fou de 6 i 1 mg g^{-1} pes fresc de cèl.lules de llevat respectivament. Això va variar significativament en el moment en què el microorganisme es va fer créixer en medis amb a_w baixa (0.96), aconseguint un contingut total de poliols i sucres en el cas d'utilitzar glucosa per ajustar l'a_w, de 8 i 3.5 mg g^{-1} respectivament.

Els principals soluts compatibles acumulats en cèl.lules crescudes en medi modificat amb glucosa foren arabitol, glicerol, trealosa i glucosa. Mentre que quan el solut utilitzat fou el glicerol, també va ser aquest mateix glicerol el principal poliol intracel.lular acumulat amb petites quantitats d'altres poliols i sucres.

En estudis previs en medi modificat amb *Candida albicans* (Pfyffer i Rast, 1988) i en medis amb baixa a_w amb *C. cacaoi* (Van Eck *et al.*, 1993), es va demostrar que l'arabitol i el glicerol eren els principals poliols acumulats intracel.lularment.

Cal esmentar també que en estudis duts a terme amb llevats i floridures s'havia observat que en condicions d'estrès els soluts compatibles sintetitzats principalment eren el glicerol i l'arabitol com a resposta a baixes a_w (Griffin, 1981). En canvi la sacarosa, la glucosa i el sorbitol eren acumulats quan es trobaven presents en el medi (Corry, 1987).

Amb el present estudi quedava demostrat que manipulant el creixement de l'agent de biocontrol *C. sake* canviant la concentració de nutrients del medi, l'a_w del mateix o ambdós factors, es podia afectar significativament l'acumulació intracel.lular de poliols, glucosa i trealosa. Calia veure ara, si realment les cèl.lules crescudes en medis restrictius i que havien acumulat unes reserves endògenes diferents a les que acumularia en medi normal, presentaven una major tolerància a condicions d'estrès hídric gràcies a la síntesi d'aquets soluts compatibles esmentats. Això és el que va intentar-se veure en la següent experiència del present treball.

7. Millora del microorganisme *C. sake* CPA-1 enfront a condicions d'estrès hídric.

En aquesta experiència es va comparar la viabilitat de les cèl.lules del llevat antagonista modificades amb les no modificades, en medis amb a_w normal i altres amb a_w baixa, i va comprovar-se que les cèl.lules del llevat crescudes en medis modificats i per tant amb reserves endògenes diferents dels crescuts en medi no-modificat, eren significativament més tolerants a l'estrès hídric que les cèl.lules no modificades. Estudis previs a aquest havien demostrat que la germinació de conidis de fongs entomopatògens era millorada significativament sota condicions

d'estrès hídric (a_w<0.90) en conidis que contenien elevades concentracions de glicerol i d'eritritol (Hallsworth i Magan, 1995). Cal remarcar que les quantitats de sucres i poliols acumulats per l'antagonista *C. sake* en el nostre estudi en medis modificats (per nutrients o a_w) van ser superiors a les trobades per Hallsworth i Magan (1996) en propàguls de fongs entomopatògens utilitzats en el control biològic de plagues.

En llevats, depenent del solut utilitzat els poliols sovint són activament alliberats de les cèl.lules en relació al nivell d'estrès hídric i l'edat del cultiu (Blomberg i Adler, 1992; Jennings, 1995).

En propàguls de fongs filamentosos els poliols poden ésser lentament metabolitzats i finalment utilitzats en la respiració o transformats en components de pes molecular més alt com ara el glicogen. Els poliols també poden sortir dels conidis per transport cap el miceli o per difusió a l'interior de la paret del conidiòfor (Kiyosawa, 1991).

Els tractaments que van mostrar una major tolerància a l'estrès hídric van ser les cèl.lules de llevat crescudes en el medi diluit al 50% i al 75% respectivament i amb a_w=0.96 ajustada mitjançant glucosa (NYDB50+GLU i NYDB25+GLU) i finalment les cèl.lules crescudes en el medi diluit al 75% i ajustat a 0.96 d'a_w mitjançant glicerol (NYDB25+GLY). Aquests doncs van ser els tractaments elegits per continuar treballant més endavant i per testar-los a la pràctica en les condicions de camp.

Cal esmentar que també s'aconseguien resultats força bons quan s'utilitzava el solut trealosa per ajustar l'a_w, de cara a la pràctica, però resulta econòmicament inviable pensar en un medi ajustat amb grans quantitats de trealosa per a fer créixer un agent de biocontrol. Fou per aquest motiu que vàrem quedar-nos amb aquesta informació només a nivell de laboratori i no vàrem continuar amb ella en els tractaments de camp.

Un altre aspecte important era que no solament havia d'aconseguir-se un medi en el qual les cèl.lules crescudes de l'antagonista fossin més tolerants a l'estrès hídric, sinó que també era important que a part d'aquesta major qualitat d'inòcul també s'aconseguís una quantitat d'inòcul econòmicament rendible. Els tres tractaments esmentats abans, a part de créixer millor en medis amb baixa a_w, aconseguien amb el mateix temps d'incubació creixements significativament iguals que en el medi NYDB sense modificar. És a dir, aconseguíem amb ells la mateixa massa microbiana. Cal dir que això és força interessant de cara a una possible comercialització de l'antagonista, a fi i efecte de no incrementar el cost de producció del mateix.

De totes formes tot l'esmentat no serviria de res si les cèl.lules de llevat modificades enfront a l'estrès hídric haguéssin perdut o disminuit la seva eficàcia de cara a controlar els principals fongs causants de podridura en postcollita, que era la finalitat per a la què havien estat aïllats i seleccionats. Així doncs, la propera tasca fou esbrinar si realment el fet d'haver sofert un canvi en les seves reserves endògenes havia afectat d'alguna manera la seva eficàcia com agent de biocontrol.

8. Comprovació del manteniment de l'activitat antagònica de *C. sake* CPA-1 per a controlar la podridura blava en mançanes.

Van dur-se a terme varis assaigs en laboratori per tal de comprovar el manteniment de l'efectivitat de les cèl.lules de *C. sake* millorades enfront l'estrès sobre *P. expansum* inoculat en mançanes "Golden Delicious". En tots els casos va observar-se que l'eficàcia com agent de biocontrol es mantenia, i fins i tot en alguns casos era millor que la soca sense modificacions, especialment a baixes concentracions. Aquest fet realment era força important, ja que feia suposar que els inòculs tolerants a la dessecació podrien sobreviure millor i esdevenir més efectius que l'inòcul normal en condicions d'aplicació directa en el camp. Tanmateix, la possibilitat de ser utilitzat a concentracions més baixes per aconseguir nivells de control adequats és força important des d'un punt de vista econòmic.

Mancava tan sols veure si realment aquests avantatges es manifestaven a la pràctica aplicant les cèl.lules millorades directament en els arbres a camp com qualsevol tractament normal de precollita, i aquest era el darrer esglaó del present treball.

9. Estudi de l'eficàcia de l'aplicació en precollita de les soques adaptades a l'estrès en el control de *P. expansum* en poscollita.

L'únic estudi apart del present que havia intentat utilitzar tractaments amb agents de biocontrol en precollita per al control de malalties en poscollita és el dut a terme per Leibinger *et al.* (1997), en el qual s'avaluava l'eficàcia de les barreges dels antagonistes: *Aureobasidium pullulans; Rhodotorula glutinis* i *Bacillus subtilis* en el control de diferents patògens causants de podridura en postcollita de mançana "Golden Delicious". En aquest estudi es va veure que amb vàries aplicacions de barreges dels antagonistes a camp s'obtenia un control de *P. expansum* i *B. cinerea* similar al dels productes fungicides.

Estudis previs amb fongs entomapatògens utilitzats en el control biològic de plagues havien demostrat que els conidis modificats de la mateixa forma utilitzada

en el present treball, podien ser més patogènics per a controlar plagues en condicions d'humitat relativa baixa que aquells que no havien estat modificats (Hallsworth i Magan, 1994a, 1994b, 1994c). Així mateix, també s'han obtingut resultats esperançadors amb inòculs modificats de l'antagonista *Epicoccum nigricans* en el control de la podridura causada per *Monilinia laxa* en préssecs a camp (Pascual *et al.*, 1996).

Quan van aplicar-se els tractaments (tant els modificats com el normal) a camp per a controlar la podridura de *P. expansum,* inoculada de forma artificial en mançanes prèviament foradades, es va veure que després de quatre mesos en cambra frigorífica, tots els tractaments d'antagonista disminuien de forma significativa el percentatge de forats infectats pel patogen (aproximadament en un 50%). Malgrat tot, no van observar-se millores amb els tractaments fets amb els inòculs modificats respecte als fets amb el no modificats. De totes formes es va veure clarament que immediatament després de l'aplicació en camp les cèl.lules no-modificades s'adherien a la superfície de les mançanes millor que les modificades, d'aquesta forma l'inòcul inicial per mançana era força superior en els tractaments no modificats. Malgrat tot, al cap de poques hores de permanència sota condicions de camp les poblacions de *C. sake* modificades creixien significativament mentre que les poblacions de *C. sake* normal disminuien o restaven al mateix nivell inicial. Aquest resultat sembla doncs corroborar el fet que realment les cèl.lules modificades s'adapten millor a les condicions de camp i són més resistents a les temperatures elevades i a la humitat relativa baixa, pròpies de l'època de la collita de mançana en la nostra zona.

El fet que les poblacions inicials en les mançanes tractades amb l'inòcul millorat enfront a l'estrès fossin força més baixes que les tractades amb el no modificat, podria ser el motiu pel qual no va aconseguir-se una millora en l'efectivitat. Al cap i a la fi, la quantitat de *C. sake* per mançana al moment d'entrada a cambra era aproximadament la mateixa per a tots els tractaments.

És possible que la menor adherència de l'inòcul adaptat a l'estrès fos deguda a algun canvi en la matriu extracel.lular dels llevats. L'augment de la síntesi de reserves endògenes es fa probablement en detriment de la síntesi d'algun altre component com podrien ser per exemple, els polisacàrids responsables de l'adherència en els llevats. Existeix poquíssima informació sobre les característiques de la matriu produida per llevats com ara *C. sake.* De totes maneres, estudis previs amb matrius d'espores d'altres fongs suggereixen que aquestes són les responsables d'un nombre important de propietats ecològiques, com ara són la protecció enfront a temperatures extremes, la dessecació, les radiacions d'ona curta i l'adhesivitat entre d'altres (Louis i Cooke, 1983, 1985). És possible que els requeriments d'energia per a la producció elevada de reserves

endògenes com ara poliols i trealosa pugui donar lloc a una modificació de la concentració o de les característiques de la matriu. És clarament conegut que l'energia és limitada i si és emprada en una direcció, no podrà ésser utilitzada per una altra finalitat.

Probablement si fos possible millorar l'adherència de les cèl.lules adaptades a condicions d'estrès, les poblacions a l'entrada de cambra serien també superiors i podria ser que l'efectivitat en el control de la podridura blava pugués augmentar-se. De fet la majòria de productes químics aplicats actualment, porten en la seva formulació compostos totalment inerts que afavoreixen un millor recobriment de la superfície tractada i una millor adhesió en general. Caldria doncs tenir en compte aquest aspecte alhora de desenvolupar la futura formulació de l'antagonista.

Tot això de moment són hipòtesis i es fa necessària molta més investigació sobre aquest tema. Malgrat tot, en aquest estudi s'ha demostrat que existeix un potencial important per utilitzar medis ecofisiològics en la millora del comportament ambiental dels agents de biocontrol aplicats a camp, i tot plegat és una nova via per continuar millorant el desenvolupament d'agents de biocontrol eficients que puguin ser utilitzats en tot tipus de condicions, incloses les de camp; variables i totalment incontrolables.

CONCLUSIONS

1. **Les floridures predominants en la microflora de la mançana "Golden Delicious" al llarg del seu desenvolupament són *Cladosporium* spp. i *Alternaria* spp., i en molta menor quantitat els gèneres *Epicoccum*, *Fusarium* i *Acremonium*.**

2. ***P. expansum*, el principal fong causant de podridura en postcollita de fruita de llavor, rarament es troba a la superfície de les mançanes "Golden Delicious" durant el seu desenvolupament. No obstant, la seva presència esdevè important al final del període de frigoconservació i durant el de simulació de la comercialització ("shelf life").**

3. **Tant durant el desenvolupament com en la frigoconservació de mançanes "Golden Delicious" els llevats blancs es troben en major quantitat formant part de la microflora que no pas els rosats.**

4. **La població bacteriana en mançana "Golden Delicious" és màxima durant l'estadi de borró, disminueix després durant el període de creixement del fruit, essent minça finalment durant la collita i la posterior conservació en fred.**

5. **Les aplicacions de fungicides en precollita disminueixen les poblacions de floridures en general, *Cladosporium* spp., *Alternaria* spp. i llevats blancs, per un període màxim de 15-30 dies després del tractament. Així mateix, no s'observa cap efecte apreciable d'aquests sobre la microflora a partir de la recol.lecció de la fruita.**

6. **La temperatura d'1 ºC utilitzada en frigoconservació de mançanes provoca una davallada general en les poblacions naturals associades a la mançana "Golden Delicious".**

7. **El fong *Alternaria* spp., causant de la podridura del cor en mançanes, es troba a l'interior dels borrons i en la resta d'estadis de desenvolupament com a infecció endofítica.**

8. **L'aplicació de *C. sake* CPA-1 dos dies abans de la collita en mançanes "Golden Delicious", disminueix significativament la població natural de *Cladosporium* spp. i *Penicillium* spp. al final del període de conservació i durant el de simulació de la comercialització. Així mateix, un efecte similar s'observa en les poblacions de llevats durant el "shelf life". En canvi, no produeix cap efecte sobre les poblacions d'*Alternaria* spp.**

9. **La població del llevat *C. sake* CPA-1 dos dies abans de collita, disminueix durant les primeres 24 h a camp, però augmenta un cop en cambra frigorífica assolint el seu màxim poblacional als 30 dies per anar disminuint després progressivament fins al final de la conservació i augmentar de forma destacada un cop a temperatura ambient durant el període de "shelf life".**

10. **El tractament en postcollita mitjançant bany de l'antagonista *C. sake* CPA-1 controla efectivament (amb reduccions de la podridura superiors al 80%) la podridura causada per *P. expansum* inoculat artificialment, tant en ferides de pre com de postcollita en mançanes "Golden Delicious".**

11. **El tractament en precollita mitjançant pulverització de l'antagonista *C. sake* CPA-1 a la concentració 3×10^6 ufc ml^{-1} dos dies abans de collita, és menys efectiu en el control de *P. expansum* que el tractament en postcollita. Malgrat tot presenta reduccions de la podridura del 50% en ferides fetes en precollita en mançanes "Golden Delicious" inoculades pel patogen mitjançant bany i després de dos mesos en cambra frigorífica.**

12. **La població de *C. sake* CPA-1 sobre mançanes "Golden Delicious" amb danys artificials disminueix abans i més ràpidament després d'assolir el màxim poblacional durant la conservació en fred, quan s'aplica mitjançant pulverització en precollita (dos dies abans de collita) que quan es fa mitjançant bany en postcollita.**

13. **Les condicions ambientals de creixement de l'antagonista *C. sake* CPA-1 estan limitades per una a_w entre 0.90 i 0.92, depenent si és en presència de NaCl o glicerol respectivament. En canvi, tolera un ampli rang de pH (entre 3 i 7) independentment de l'a_w i la temperatura.**

14. **Els principals poliols i sucres acumulats per les cèl.lules de *C. sake* CPA-1 crescudes en medi NYDB (no modificat) són l'arabitol, la trealosa i la glucosa amd petites quantitats de glicerol i eritritol.**

15. El creixement de *C. sake* CPA-1 en medi amb a_w reduida (0.96) provoca una modificació de les reserves endògenes acumulades i una millora de la viabilitat a baixes a_w en assaigs *in vitro* quan es compara amb cèl.lules crescudes en medi NYDB no modificat.

16. Els principals soluts compatibles acumulats en les cèl.lules de *C. sake* CPA-1 crescudes en medi modificat amb glucosa són arabitol, glicerol, trealosa i glucosa. Mentre que el glicerol és el principal solut compatible acumulat quan s'ajusta el medi amb glicerol.

17. Les cèl.lules de *C. sake* CPA-1 modificades mantenen la seva efectivitat de biocontrol enfront la podridura causada per *P. expansum* en mançanes "Golden Delicious" en assaigs de laboratori i en molts casos fins i tot la milloren.

18. L'aplicació a camp de les cèl.lules d'antagonista modificades enfront a condicions d'estrès presenten la mateixa efectivitat que les no modificades en el control de *P. expansum* en postcollita de mançanes. Aquest inòcul sobreviviu millor a les condicions de camp però presenta una menor adherència a la mançana que l'inòcul no modificat.

19. *C. sake* reuneix les condicions adequades i es presenta com un bon candidat a ser utilitzat com agent antagònic de postcollita en aplicacions en precollita.

PERSPECTIVES DE FUTUR

Un cop arribats a aquest punt de la tesi no podem dir que això sigui el final, més aviat podria dir-se que el tema just acaba d'encetar-se, i que aquest treball només ha estat la porta que s'obre cap un ampli ventall d'idees i experiències futures. Abans de posar punt i final a aquest trajecte que m'ha portat a escriure aquesta tesi, voldria esmentar breument algunes d'aquestes possibilitats a endegar a partir d'ara.

En primer lloc caldrà tenir en compte el tema de l'adherència, veure quins compostos en el llevat en són els responsables i què passa amb ells quan el fem créixer en condicions de baixa a_w. Tanmateix, dur a terme diferents experiències amb mullants i adhesius inerts, utilitzats normalment en formulació, per tal de veure si és possible millorar l'adherència a la mançana de les cèl.lules de microorganisme millorades enfront l'estrès. Un cop trobada la formulació oportuna, hauria d'estudiar-se la seva aplicació a la pràctica en condicions de camp, per tal de veure si realment s'aconsegueixen millores en el control del *P. expansum*.

Un altre tema pendent i també relacionat amb la producció i la formulació de l'agent de biocontrol és l'aplicació d'aquest enfoc per millorar microorganismes enfront l'estrès, amb medis de creixement més econòmics i que puguin resultar rendibles de cara a una posterior comercialització. Es tractaria de medis provinents de subproductes de la indústria alimentària com ara són les melasses, els concentrats de mançana de l'elaboració de sucs, el bagàs de cerveseria, entre d'altres.

L'enfoc de la millora de microorganismes enfront l'estrès podria aplicar-se a altres agents de biocontrol amb què es conta actualment al nostre laboratori. I també fer-lo extensiu a altres cultius, la problemàtica de podridura dels quals és específicament de camp, com ara les maduixes i la vinya amb el patogen *B. cinerea* com exemples.

Finalment esmentar només, que seria interessant un major aprofondiment en l'estudi dels microorganismes endofítics. El fet d'haver trobat presència del gènere *Alternaria* ja en l'estadi de borró de les mançanes és força important. Seria bò intentar veure quin és el moment d'infecció d'aquests fongs endofítics per tal de veure la millor forma de controlar-los.

Breument, aquestes serien algunes de les primeres idees que se'n desprenen d'aquest treball. El control biològic de malalties però, és un tema nou i amb grans posibilitats per a continuar investigant i avançant.

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