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

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Abstract

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 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. *Penicillium* spp. which is responsible for major postharvest diseases 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 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 of 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.

Introduction

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

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

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 an effective biocontrol agent against 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 population on 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 for 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, (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. 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 experiments were arranged in a randomised block design with guard trees to separate treatments. Preharvest chemical applications were sprayed uniformly with a hand gun operating at a pressure of 10 atm. The fungicide regime used and the dates of application are shown in Table 1.

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

The antagonist *C. sake* CPA-1 (3×10^6 cfu ml^{-1}) was sprayed two days before harvest. Two apples were

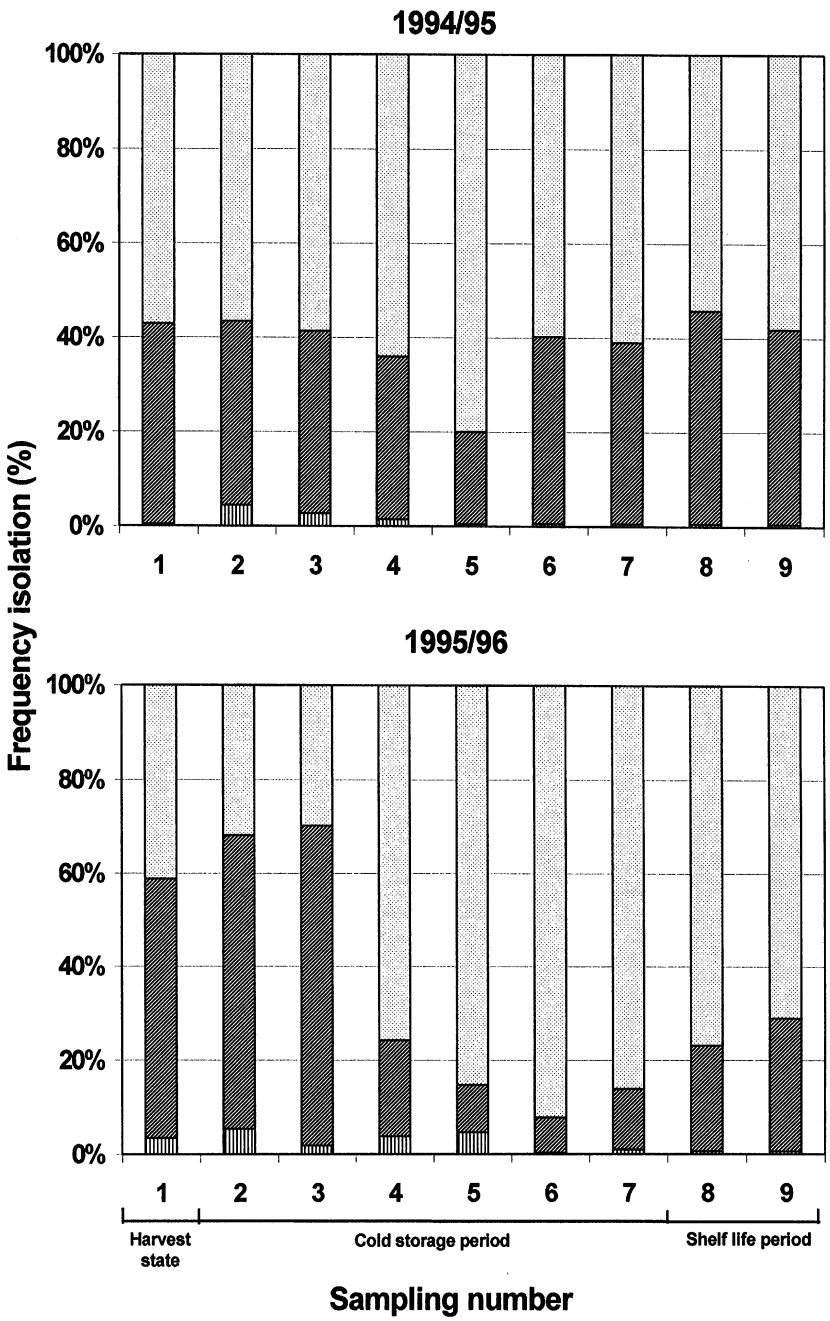

Figure 1. Frequency (%) of isolation of bacteria (▥), yeasts (□) and filamentous fungi (▨) from untreated apples cv Golden Delicious in 1994/95 and 1995/96 seasons at harvest moment, cold storage and shelf life period.

randomly detached from the replicate groups of trees immediately after application, at 24 and 48 h to determine *C. sake* populations on apples surface. The fruits were harvested and put in separate boxes according to their treatment and kept at 1 °C and 21% O_2 a typical cold storage conditions 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 environment (25 °C) to simulate shelf-life conditions. Samples were taken to evaluated microbial and *C. sake* populations after 3 and 7 days.

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 for fungi and bacteria, respectively. Fungi were identified to genus level. Populations of bacteria were counted after 48–72 h and 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-plated 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.

Data analysis

Populations of microflora and *C. sake* (cfu g^{-1} fresh weight) were log transformed to improve homogeneity of variances (Parbery et al., 1981) and plotted for each season. On each date statistical comparisons (ANOVA analysis of variance) were made between treatments in every sampling date. 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 data.

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. The frequency of isolation of yeasts and filamentous fungi was always greater than that of bacteria. Yeasts were in most cases the predominant group isolated from the apple surface, especially after 60, 90 and 150 days cold storage. Frequency of isolation of bacteria was in all cases < 5%. The bacterial populations at harvest were very low (less 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*.

Temporal changes in yeast populations

The most common group of yeasts isolated during cold storage and shelf-life periods were white yeasts, with pink yeasts only occasionally isolated in this study (Figure 2). The populations of yeasts decreased significantly ($P = 0.05$) during the first 15 days of cold storage but recovered after one month later.

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

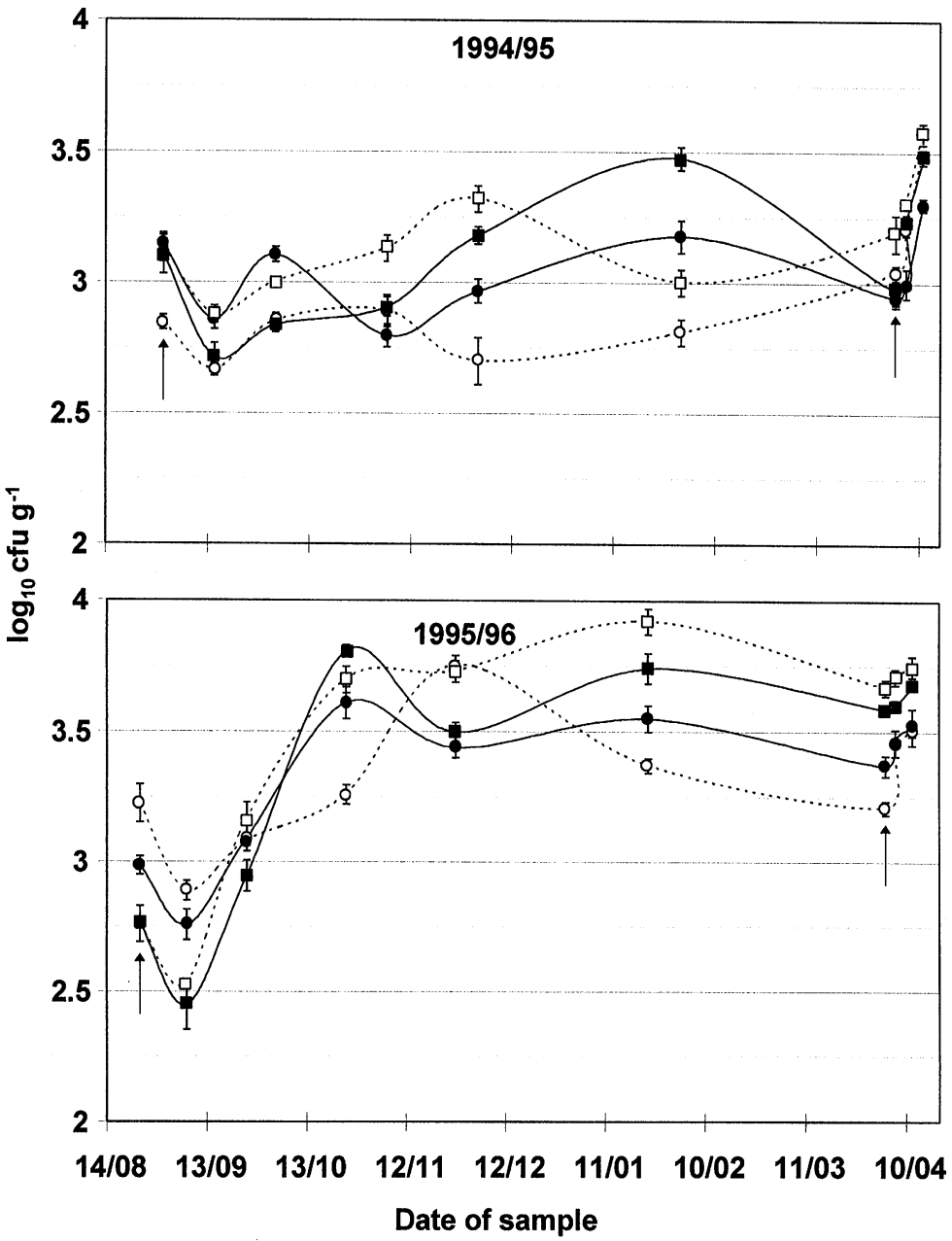

Figure 2. Populations of total yeasts ($\log_{10}$ of colony-forming units (cfu) g^{-1} fresh weight) isolated from Golden Delicious apples untreated (□), treated at preharvest with *C. sake* (○), treated with preharvest fungicides (■) or treated at preharvest both with *C. sake* and fungicides (●), 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.

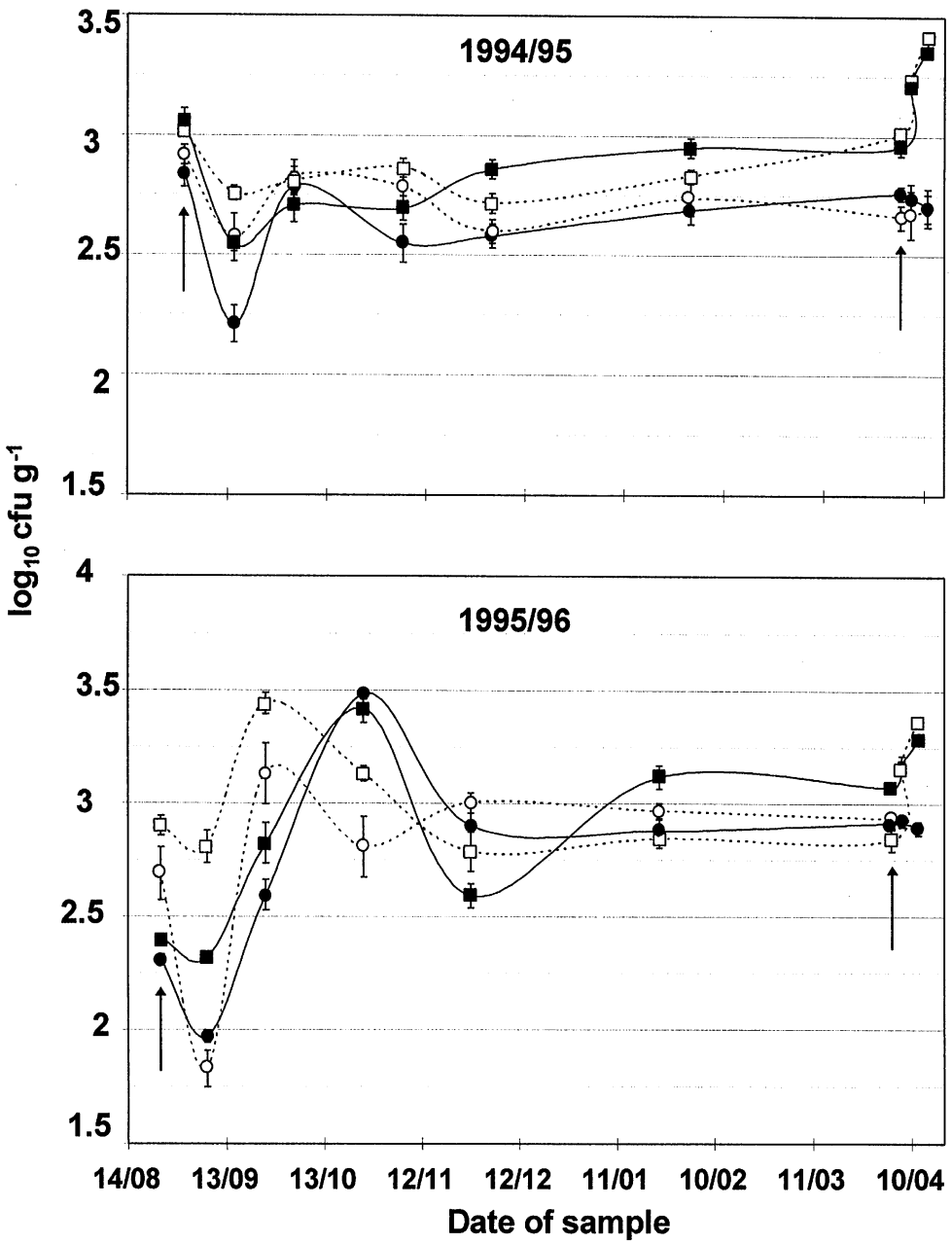

Figure 3. Populations of total filamentous fungi ($\log_{10}$ of colony-forming units (cfu) g^{-1} fresh weight) isolated from Golden Delicious apples untreated (□) treated at preharvest with *C. sake* (○), treated with preharvest fungicides (■) or treated at preharvest both with *C. sake* and fungicides (●), 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.

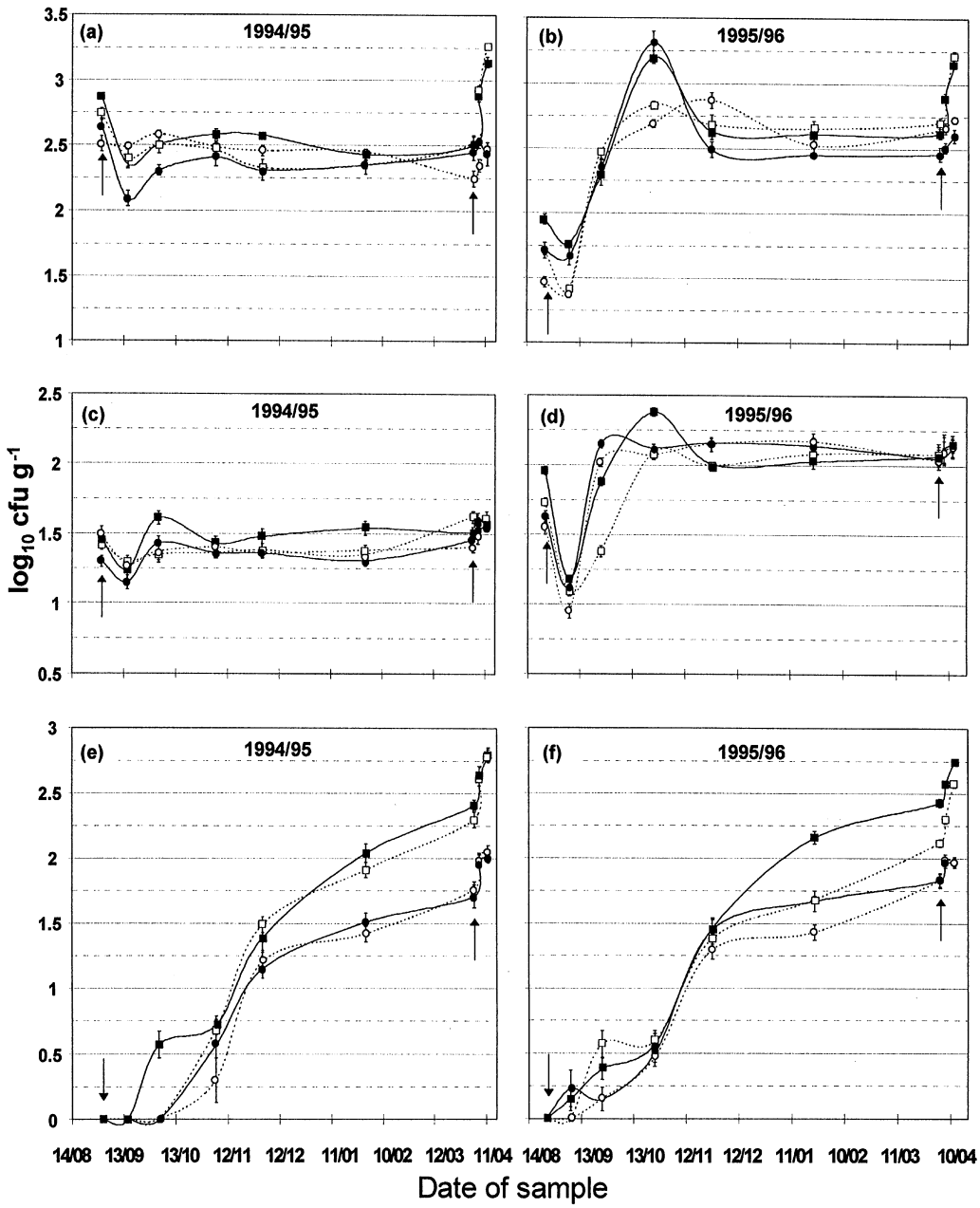

Figure 4. Populations of *Cladosporium* (a and b), *Alternaria* spp. (c and d) and *Penicillium* spp. (e and f) ($\log_{10}$ of colony-forming units (cfu) g^{-1} fresh weight) isolated from Golden Delicious apples untreated (○) treated at preharvest with *C. sake* (□), treated with preharvest fungicides (■) 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.

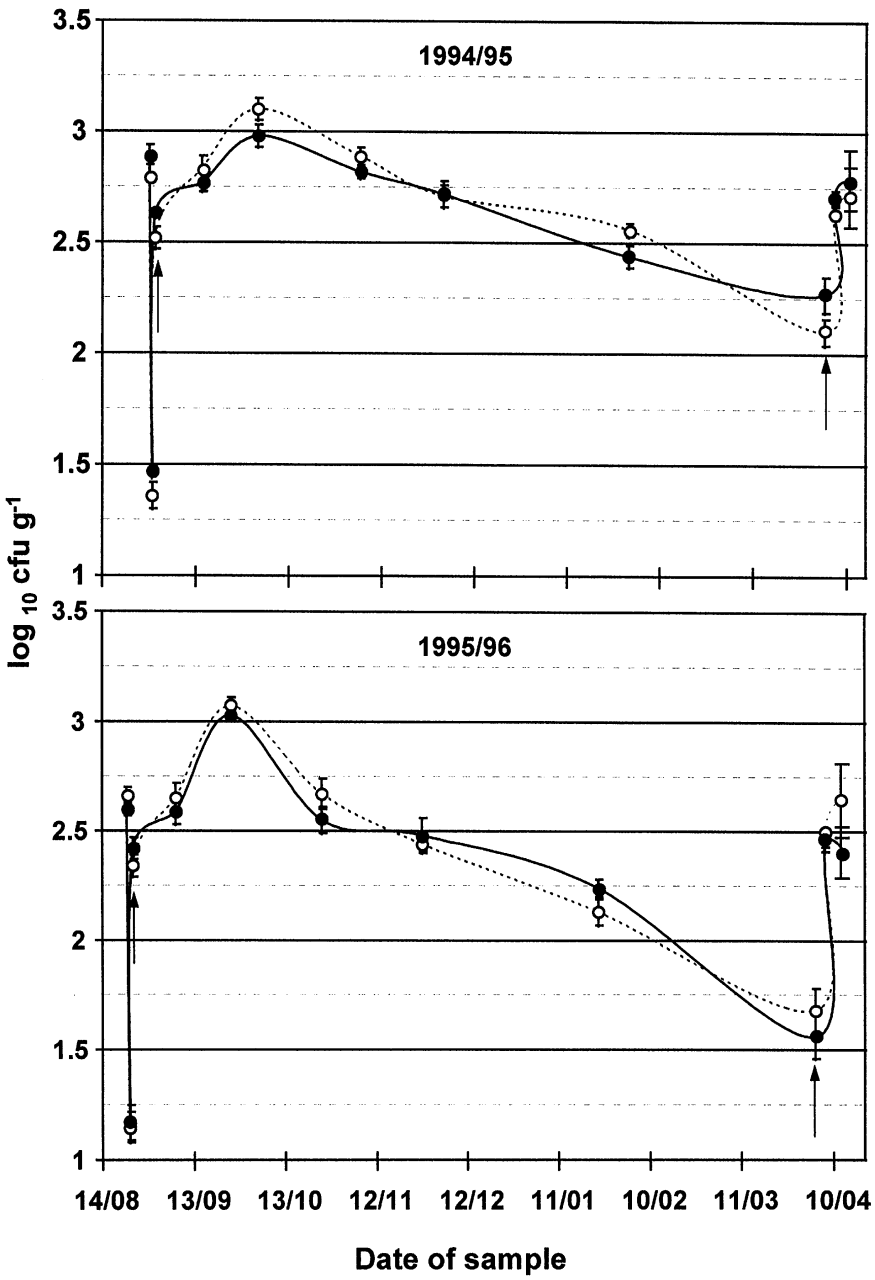

Figure 5. Populations of *C. sake* ($\log_{10}$ of colony-forming units (cfu) g^{-1} fresh weight) isolated from Golden Delicious apples treated at preharvest with *C. sake* (○), or both *C. sake* and fungicides (●), during two growing seasons (1994/95 and 1995/96). Points represent the means of four 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.

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.

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

After cold storage, during the ambient shelf-life period, populations of filamentous fungi were significantly (P = 0.05) greater on fruits without *C. sake* application. 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.

Behaviour pattern of the genus *Penicillium* 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 rapidly 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. *Penicillium* populations increased significantly faster at 25 °C and more on untreated or treated with fungicides apples than on those treated with the antagonist. After 7 days at ambient conditions the populations were 2-fold larger in both untreated and preharvest fungicide treated fruits. However, filamentous fungal populations of apple mycoflora did not change 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 7×10^2 cfu g^{-1} and 4×10^2 cfu g^{-1} in the 1994/95 and 1995/1996 seasons, respectively, were isolated from apples after application of the antagonist. Populations decreased significantly (approx. 28-fold) within 24 h under field conditions followed by increase during the next twenty-four hours but below 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 the 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 *C. sake* + fungicide.

Discussion

Knowledge in naturally occurring microbial population on fruit surfaces has been limited 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 bacteria were seldom isolated during cold storage. In contrast, yeasts were the predominant component of the apple microflora specially after two months cold storage until 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 but the isolation technique of this last experience was direct plating of epidermal samples on to the media.

Penicillium spp., particularly *P. expansum*, 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 and had little residual effect 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 Kenerley, 1980).

In our study 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 of buds of apples (Pennicook and Newhook, 1981). During ambient storage, after removal from cold storage yeast populations had a rapid increase. Apples not treated with the antagonist also had much higher populations of yeasts, than those sprayed with *C. sake*. This could result from greater competence of *C. sake* than resident yeast flora.

It was notable that immediately after field application of *C. sake*, the populations of the antagonist drastically decreased during the first 24 h, probably as a result of high temperature and low humidity after application. Relative humidity and leaf wetness periods had a significant effect on effective establishment of *Trichoderma harzianum* and *Ulocladium atrum* in relation to control of *Botrytis* on various crops (Elad and Kirshner, 1993; Köhl et al., 1995). 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. *C. sake* reduced populations of *Cladosporium* and *Penicillium* genera during the shelf-life period. However, no significant effects were observed on *Alternaria* populations. Previous studies (N. Teixidó, unpubl.) have shown that *C. sake* had no effect against artificially inoculated *Alternaria alternata* populations on apple trees. Biocontrol agents are relatively specific in their action in relation to pathogens and hosts (Janisiewicz, 1988).

This study has demonstrated that the antagonist yeast *C. sake* can survive under field conditions on the surface of Golden Delicious apples and increase in cold storage for the 6 months, the duration of the experiment. The viability of the yeast on fruit in cold storage 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 *Penicillium expansum*. This study has shown that

C. sake CPA-1 has features important for an effective biocontrol agent in pre and postharvest conditions, and also during the subsequent ambient shelf-life period.

Acknowledgements

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