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Nutritional enhancement of biocontrol activity of *Candida sake* (CPA-1) against *Penicillium expansum* on apples and pears

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Abstract

Pome fruits are poor in nitrogenous compounds and the addition of nitrogen can improve colonisation of the fruits by antagonists. Twenty-two nitrogenous compounds were evaluated for their effect on *Candida sake* (CPA-1) growth *in vitro*. Ten compounds that induced greater growth were applied with the antagonist to wounded fruits to evaluate their effect on enhancing control of *Penicillium expansum*. Calcium chloride and 2-deoxy-D-glucose were also tested. L-serine and L-aspartic acid enhanced biocontrol by *C. sake* against *P. expansum* on apples. On apples and pears, ammonium molybdate, calcium chloride and 2-deoxy-D-glucose improved the capacity of the antagonist to control *P. expansum*. The addition of ammonium molybdate at 1 mM allowed *C. sake* to be used on apples and pears at a lower concentration without diminishing control. Similar results were observed with the addition of calcium chloride to the antagonist. 2-deoxy-D-glucose at 6 and 18 mM enhanced biocontrol on pears by over 81%, but on apples the improvement of biocontrol was observed only at 6 mM. In cold storage, the combination of ammonium molybdate and *C. sake* completely eliminated the incidence of blue mould on pears, and reduced its severity and incidence by more than 80% on apples.

Abbreviations: cfu – colony forming units; MSM – minimum salt medium; NYDB – nutrient yeast dextrose broth; OD – optical density; PDA – potato dextrose agar; RH – relative humidity.

Introduction

Postharvest fruit and vegetable diseases continue to cause losses world-wide. Fungicides are a primary means of controlling postharvest diseases (Eckert and Ogawa, 1985). The use of chemical fungicides is becoming increasingly restricted because of concerns for the environment and for human health, and it is very expensive to develop new pesticides to overcome the resistance developed by pathogens.

Biological control has advanced greatly during the last few years and microbial antagonists have been reported to control several postharvest diseases of fruits (Janisiewicz and Roitman, 1988; Janisiewicz and Marchi, 1992; Mercier and Wilson, 1994; Viñas et al., 1998; 1999; Wilson and Wisniewski, 1994).

Reliability and cost are two major factors that will determine the feasibility of any biocontrol system (Janisiewicz et al., 1992). Higher concentrations of the antagonist must be applied to achieve a more effective control (Janisiewicz, 1987; Pusey and Wilson, 1984), but increasing the antagonist populations makes biocontrol less economical. However, epiphytic antagonists are influenced by a number of biological and environmental factors (Blakeman and Fokkema, 1982; Fokkema 1984; Morris and Rouse, 1985), and storage practices. They also affect biocontrol and could be manipulated to enhance antagonist activity.

In order to be a successful competitor at the wound site, an antagonist has to be better adapted than the pathogen to various environmental and nutritional conditions, to grow rapidly at the wound site, to use nutrients effectively at low concentrations, and survive and grow at the infection site at range of temperatures, pH and osmotic conditions.

Enhancers are chemicals that serve as a food base for antagonistic microbes or are selectively toxic or fungicidal to pathogens (Spurr, 1994). Therefore, enhancers can be used to manipulate the antagonist populations on fruit and can greatly improve biocontrol levels.

Nutritional manipulation has been shown to enhance biocontrol activity of several Janisiewicz et al. (1992) reported that L-asparagine and L-proline greatly enhanced biocontrol of *Pseudomonas syringae* (strain L-59-66) against *Penicillium expansum*. McLaughlin et al. (1990) also demonstrated that calcium salts improved the efficacy of yeast biocontrol agents against *P. expansum* and *Botrytis cinerea*. Another study reported that *Candida saitoana* plus glycolchitosan enhanced the control of postharvest decay on apples and citrus (El-Ghaouth et al., 2000a).

Nutrients can affect the population of epiphytic microorganisms, and competition for nutrients on plant surfaces is an important aspect of the biocontrol mechanism (Blakeman and Brodie, 1977; Fokkema, 1984; Morris and Rouse, 1985). Furthermore, apples and pears are a rich source of carbohydrates but are poor in nitrogen. Thus nitrogen can be a growth limiting factor for the antagonist.

Previously reported work has shown that *Candida sake* (CPA-1) prevented or minimised postharvest losses on apples and pears caused by *P. expansum* and *B. cinerea* and *Rhizopus stolonifer* (Viñas et al., 1998).

The objectives of the present work were to determine (i) which nitrogenous compounds improved *C. sake* (CPA-1) growth *in vitro* and (ii) if the nitrogenous compounds that increased *C. sake* growth enhanced its efficacy to control *P. expansum* decay on apples and pears. Calcium chloride and 2-deoxy-D-glucose that have been reported to induce significant effects in other systems were also tested.

Material and methods

The yeast isolate used in this study was the strain CPA-1 of *C. sake* isolated from the apple surface (Viñas et al., 1998). Yeast cells were grown in nutrient yeast dextrose broth (NYDB) (nutrient broth 8 g l^{-1}, yeast extract 5 g l^{-1}, dextrose 10 g l^{-1}) for 24–48 h at 25 ± 1 °C with shaking at 150 rpm. The medium was centrifuged (Avanti J-25, Beckman, Palo Alto, California) at 8315 × *g* for 10 min and the cells were resuspended in 50 ml of 0.05 M phosphate buffer. This step was repeated twice in order to wash the yeast cells from the nutritional compounds of the initial medium. After a second washing, the cells were resuspended in 50 ml of de-ionised water. The desired concentrations were obtained by adjusting the suspension with a haemacytometer.

P. expansum was isolated from decayed apples after several months in storage. This isolate was the most aggressive one in our collection and produced the largest lesions on inoculated apples. The fungi were maintained on potato dextrose agar (PDA) with periodic transfers through apple. The concentration of the conidial suspensions was determined with a haemacytometer. The conidial suspensions were prepared from 10-day-old cultures of *P. expansum*.

'Golden Delicious' apples and 'Blanquilla' pears were obtained from commercial orchards in Lleida, Catalonia. The fruits were used just after harvest or after storage at 1 °C for no longer than 4 months.

The nitrogenous compounds, calcium chloride and 2-deoxy-D-glucose used in this study were purchased from Aldrich-Chemie (D-7924 Steinheim).

Effect of nitrogenous compounds on antagonist growth in vitro

Minimum salt medium (MSM) for *C. sake* (CPA-1) with one source of carbon (glucose) and one source of nitrogen (ammonium sulphate) (Usall, 1995) was used in tests to determine the utilisation of nitrogenous compounds. This medium contained per litre: 5 g ammonium sulphate, 10 g glucose, 850 mg KH_2PO_4, 150 mg K_2HPO_4, 500 mg $MgSO_4 \cdot 7H_2O$, 100 mg NaCl, 67 mg $CaCl_2 \cdot 2H_2O$, traces of elements (500 µg H_3BO_3, 40 µg $CuSO_4 \cdot 5H_2O$, 200 µg $FeCl_3 \cdot 6H_2O$, 303 µg $MnSO_4 \cdot H_2O$, 200 µg $Na_2MoO_4 \cdot 2H_2O$, and 400 µg $ZnSO_4 \cdot 7H_2O$), 20 µl biotin, 400 µg thiamine and 400 µg pyridoxine. The glucose and salts were autoclaved, the other solutions were sterilised by filtration.

Tests for utilisation of nitrogenous compounds were conducted on Seru-well microplates (BibbySterlin Ltd, Stone, Staffs, UK). The MSM was prepared without the nitrogenous source. Nitrogen compounds were tested

at 20 mM and each compound was concentrated 2× to compensate the dilution when adding the antagonist suspension. The suspensions of the antagonist were prepared at a concentration of 6.6×10^6 cfu ml^{-1}. One hundred microlitres of the antagonist suspension and 100 µl of the tested nutrient solution were added to each microplate well. The control was the antagonist suspension in MSM without nitrogen source. There were three replicates per treatment. The plates were incubated at 28 ± 1 °C for 48 h. The optical density (OD) of the wells was determined with a PLUS MK II Titertek Multiskan (Labsystem) reader at 405 nm. Higher levels of OD were considered to indicate a higher yield of cells. The experiment was repeated twice.

Nutritional enhancement of biocontrol of blue mould

Room temperature trials

Ten nitrogenous compounds that best stimulated growth of *C. sake* (CPA-1) *in vitro* were selected to determine their effect on biocontrol of blue mould on fruit. Calcium chloride (McLaughlin et al., 1990) and 2-deoxy-D-glucose (Janisiewicz, 1994) were also tested.

In order to determine the lowest effective dose, several concentrations of each nitrogenous compound (1–80 mM) were tested, except for L-arginine concentration which was tested at 5–20 mM (Usall, 1995). This wide range of concentrations was tested in order (i) to find the minimal concentration required for enhancing biocontrol and (ii) to identify concentrations determining toxic effects. Calcium chloride was tested between 10 and 100 mM and 2-deoxy-D-glucose at 6 and 18 mM. The antagonist was suspended in an aqueous solution of each tested compound. For apples, the tested yeast concentrations were 2×10^5 and 2×10^6 cfu ml^{-1}, and for pears 6.6×10^6 cfu ml^{-1}. Higher concentrations were used on pears because *C. sake* (CPA-1) is usually less effective against *P. expansum* than on apples. The fruits were wounded by removing blocks of 3 × 3 × 3 mm at the stem (top) and calyx (bottom) end. Twenty-five microlitres of an aqueous suspension of each compound-antagonist mixture was applied to the wounds and followed by inoculation with 20 µl of an aqueous suspension of *P. expansum* at 10^4 conidia ml^{-1}. The control treatments were distilled water, each concentration of the antagonist (2×10^5 and 2×10^6 cfu ml^{-1} on apples and 6.6×10^6 cfu ml^{-1} on pears) and each nutrient compound at the tested concentrations. All the above experimental treatments were compared to *C. sake* applied at the concentration (2×10^7 cfu ml^{-1}) usually recommended to control blue mould (Usall et al., 2000; 2001) and allow the comparison with the lower concentrations, since one of the objectives was to reduce the antagonist dose necessary to control decay. However the statistical analysis with this treatment was not included in the figures. The lesion diameter was measured after 7 days of incubation at 20 ± 1 °C and 85 ± 5% relative humidity (RH). Each treatment was repeated three times with three fruits per replication. The test was repeated twice. The treatment showing the best inhibition of the pathogen was selected for small-scale trials to control *P. expansum* rot under cold storage conditions.

Cold storage trials

The fruits were treated as above. The pathogen was *P. expansum* at 10^4 conidia ml^{-1}. The *C. sake* concentrations tested were 2×10^5 and 2×10^6 cfu ml^{-1} on apples and 2×10^6 cfu ml^{-1} on pears. Ten fruits constituted a single replicate, and each treatment was replicated three times. The fruits were stored in a cold room at 1 °C and 90 ± 5% RH for 60 days. The test was repeated twice.

Statistical analysis

The incidence and severity of decay were analysed by an analysis of variance with SAS Software (SAS Institute, version 6.08, Cary N.C.). Effects of treatments were considered to be statistically significant when $P < 0.05$. When the analysis of variance was statistically significant, Duncan's Multiple Range Test was used for separation of means. Data from the two replicate experiments were pooled when statistical analysis determined that variances were homogeneous.

Results

Effect of nitrogenous compounds on antagonist growth in vitro

Of 22 nitrogenous compounds tested, 17 were utilised by the antagonist at 20 mM concentration (Table 1). In general, at the higher concentration, utilisation by the antagonist increased significantly, with the exception of L-arginine, for which utilisation declined from OD = 1.6463 at 5 mM to OD = 1.2717 at 20 mM

Table 1. Effect of nitrogenous compounds at 20 mM on growth of *Candida sake* (CPA-1)

Compound	Optical density	Standard error
Ammonium sulphate	1.6760 a	0.0191
L-Alanine	1.4807 ab	0.0338
Ammonium Molybdate	1.4293 bc	0.0472
L-Aspartic acid	1.4083 bc	0.0180
L-Glutamic acid	1.3740 bcd	0.0399
L-Serine	1.2740 bcd	0.0096
L-Arginine	1.2717 bcd	0.1739
L-Glutamine	1.2103 cde	0.3430
Glycin	1.1190 def	0.0017
L-Isoleucine	1.0007 efg	0.0107
L-Valine	0.9653 efg	0.0064
L-Leucine	0.9470 fg	0.0177
L-Phenylalanine	0.9430 fg	0.0032
Nicotinic acid	0.9343 fg	0.0432
L-Methionine	0.9257 fg	0.0084
L-Lysine	0.8463 g	0.0189
L-Threonine	0.8343 g	0.0098
L-Cysteine	0.5690 h	0.0142
Hydroxyproline	0.5090 h	0.0143
L-Histidine	0.4773 h	0.0035
Thiamine hydrochloride	0.4267 h	0.0087
Pyridoxine	0.3937 h	0.0087
Control	0.3763 h	0.0162

Means of growth of *C. sake* at 20 mM of nitrogenous compounds (3 microplate wells per compound) measured as optical density after 48 h incubation at 28 ± 1 °C. Means followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

(data not shown). The most stimulatory compounds were ammonium sulphate and L-alanine at 20 mM.

The first 10 nitrogenous compounds from the top of the list were selected for studies to enhance the efficacy of *C. sake* (CPA-1) on the control of blue mould on fruits.

Nutritional enhancement of biocontrol of blue mould

Room temperature trials

Of the 10 nitrogenous compounds tested, only ammonium molybdate enhanced biocontrol on pears, and L-serine, L-aspartic acid and ammonium molybdate on apples. On fruits protected with *C. sake*, the addition of nitrogenous compounds had the greatest effect on enhancing biocontrol at the lower concentrations tested (data not shown). Calcium chloride and 2-deoxy-D-glucose significantly enhanced biocontrol on apples and pears.

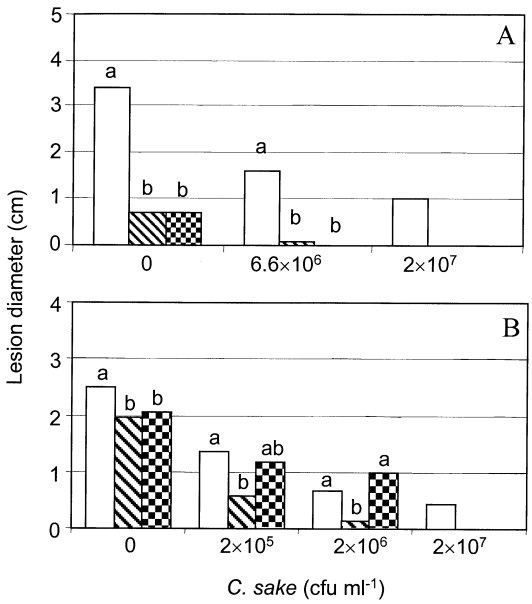

Figure 1. Effect of ammonium molybdate and *C. sake* (CPA-1) on suppressing *P. expansum* on (A) pears and on (B) apples. The antagonist was suspended in ammonium molybdate. The fruits were wounded (3 × 3 × 3 mm), inoculated with the antagonist and ammonium molybdate suspension, and then inoculated again with a suspension of the pathogen (10^4 conidia ml^{-1}) and incubated for 7 days at 20 °C. Ammonium molybdate concentrations were (□) 0 mM, (▨) 1 mM and (▩) 5 mM. Bars represent means lesion diameter for three replicate samples. For a given concentration of the antagonist, bars associated with the same letter represent means that are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

On pears treated with ammonium molybdate (Figure 1A), a significant reduction (80%) in severity of blue mould decay was observed at 1 and 5 mM. Ammonium molybdate at 1 and 5 mM significantly enhanced biocontrol by *C. sake* (CPA-1) at 6.6 × 10^6 cfu ml^{-1} and control of blue mould decay was significantly greater than that observed with control treatment of *C. sake* (2 × 10^7 cfu ml^1).

On apples, the application of ammonium molybdate alone at 1 and 5 mM significantly reduced the severity of *P. expansum* decay (Figure 1B). The combination of *C. sake* at 2 × 10^6 cfu ml^{-1} and ammonium molybdate at 1 mM provided a significant reduction in lesion size (95%), and, compared with fruits treated only with the antagonist, the enhancement of biocontrol was 81%. At 2 × 10^5 cfu ml^{-1} of *C. sake* the addition of ammonium molybdate at 1 mM significantly reduced lesion size (77%). No differences in severity were observed

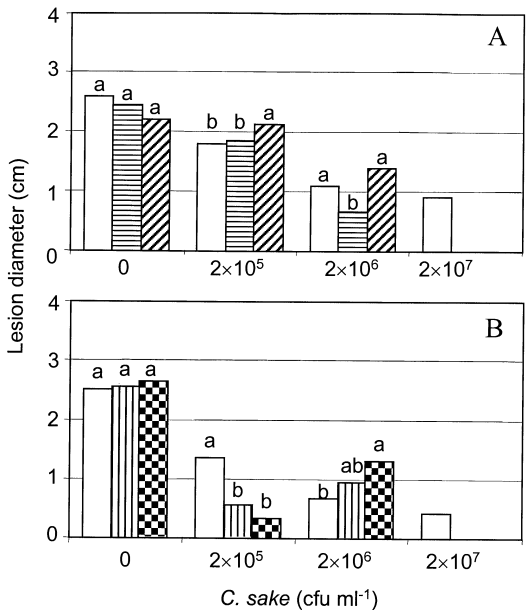

Figure 2. Effect of *C. sake* (CPA-1) and (A) L-serine and (B) L-aspartic acid on suppressing *P. expansum* on apples. The antagonist was suspended in L-serine or in L-aspartic acid. The apples were wounded (3 × 3 × 3 mm), inoculated with the antagonist and the compound suspension, and then inoculated again with a suspension of the pathogen (10^4 conidia ml^{-1}) and incubated for 7 days at 20 °C. L-serine concentrations were (□) 0 mM, (▤) 5 mM and (▨) 20 mM and L-aspartic acid concentrations were (□) 0 mM, (▥) 1 mM and (▩) 5 mM. Bars represent means lesion diameter for three replicate samples. For a given concentration of the antagonist, bars associated with the same letter represent means that are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

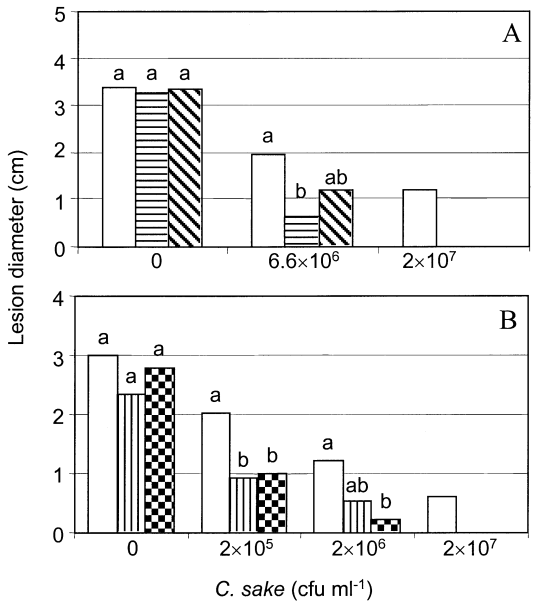

Figure 3. Effect of calcium chloride and *C. sake* (CPA-1) on suppressing *P. expansum* on (A) pears and on (B) apples. The antagonist was suspended in calcium chloride. The fruits were wounded (3 × 3 × 3 mm), inoculated with the antagonist and calcium chloride suspension, and then inoculated again with a suspension of the pathogen (10^4 conidia ml^{-1}) and incubated for 7 days at 20 °C. Calcium chloride concentrations were on pears (□) 0 mM, (▤) 50 mM and (▧) 100 mM, and on apples (□) 0 mM, (▥) 10 mM and (▩) 20 mM. Bars represent means lesion diameter for three replicate samples. For a given concentration of the antagonist, bars associated with the same letter represent means that are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

between *C. sake* amended with ammonium molybdate at 1 mM and the control treatment by *C. sake* (2×10^7 cfu ml^{-1}). Increasing the concentration of ammonium molybdate did not enhance biocontrol of *C. sake*.

L-serine (Figure 2A) and L-aspartic acid (Figure 2B) alone had no effect on blue mould on apples. The combination of *C. sake* at 2×10^6 cfu ml^{-1} and L-serine at 5 mM enhanced biocontrol by 40%. L-aspartic acid at 1 and 5 mM enhanced biocontrol by 59% and 75% respectively at the lowest concentration of the antagonist. In contrast, at *C. sake* 2×10^6 cfu ml^{-1}, the addition of L-aspartic acid reduced the effectiveness of the antagonist.

Calcium chloride did not affect the severity of blue mould on pears (Figure 3A) and apples (Figure 3B), but significantly enhanced biocontrol of *C. sake*. The minimum concentration needed to enhance biocontrol was 50 mM on pears and 10 mM on apples. On pears, no differences were observed between the control treatment of *C. sake* (2×10^7 cfu ml^{-1}) and *C. sake* (6.6×10^6 cfu ml^{-1}) amended with calcium chloride at 50 mM. On fruits protected with *C. sake* and calcium chloride at 50 mM, biocontrol was enhanced by 67% (Figure 3A). Addition of 10 and 20 mM of calcium chloride to *C. sake* at 2×10^5 cfu ml^{-1} on apples enhanced biocontrol by more than 50%. When the *C. sake* concentration was 2×10^6 cfu ml^{-1}, the strongest effect was observed at the 20 mM concentration of calcium chloride with an enhancement of biocontrol by 81% (Figure 3B).

The sugar analog 2-deoxy-D-glucose did not affect blue mould decay on pears (Figure 4A), and it reduced the severity of blue mould only at 6 mM on apples (Figure 4B). The combination of *C. sake* at

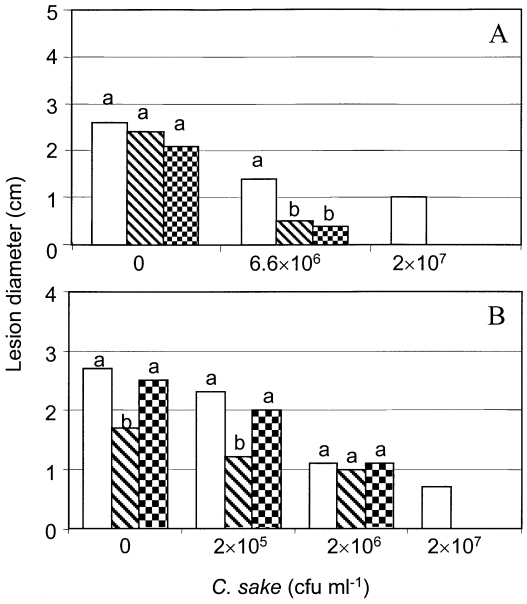

Figure 4. Effect of 2-deoxy-D-glucose and *C. sake* (CPA-1) on suppressing *P. expansum* on (A) pears and on (B) apples. The antagonist was suspended in 2-deoxy-D-glucose. The fruits were wounded (3 × 3 × 3 mm), inoculated with the antagonist and 2-deoxy-D-glucose suspension, and then inoculated again with a suspension of the pathogen (10^4 conidia ml^{-1}) and incubated for 7 days at 20 °C. 2-deoxy-D-glucose concentrations were (□) 0 mM, (▨) 6 mM and (▩) 18 mM. Bars represent means lesion diameter for three replicate samples. For a given concentration of the antagonist, bars associated with the same letter represent means that are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

6.6×10^6 cfu ml^{-1} and 2-deoxy-D-glucose at 6 and 18 mM enhanced biocontrol on pears by 64% and 72% respectively. Control of blue mould decay with *C. sake* plus 2-deoxy-D-glucose at both tested concentrations was significantly greater than that observed with the control treatment of *C. sake* (2×10^7 cfu ml^1) (Figure 4A). On apples, only the combination of *C. sake* at 2×10^5 cfu ml^1 and 2-deoxy-D-glucose at 6 mM enhanced biocontrol (47%) (Figure 4B).

Cold storage trials

Ammonium molybdate was selected for studies to enhance *C. sake* on the biocontrol of blue mould on apples and pears stored at 1 °C for 60 days. The tested ammonium molybdate concentrations were 1 mM and 5 mM, on apples and pears, respectively.

The combination of *C. sake* at 2×10^6 cfu ml^{-1} and ammonium molybdate at 5 mM completely eliminated the incidence of blue mould on pears (Figure 5A,B). Ammonium molybdate alone at 5 mM reduced decay severity by 94% (Figure 5A) and decay incidence by 92% (Figure 5B) on pears stored at 1 °C.

Ammonium molybdate at 1 mM significantly reduced the severity of blue mould on apples (Figure 5C). The combination of *C. sake* at 2×10^5 cfu ml^{-1} and 2×10^6 cfu ml^{-1} with ammonium molybdate at 1 mM reduced severity by 80% and 86% respectively (Figure 5C), and incidence by 58% and 63% respectively (Figure 5D). The addition of ammonium molybdate enhanced biocontrol of both tested concentrations of *C. sake*.

Discussion

Our study demonstrates that nutritional amendments increase biocontrol activity of *C. sake* (CPA-1). Similar results have been reported with other antagonists on apple and pear (Janisiewicz et al., 1992; Janisiewicz, 1994).

Improved biocontrol with nutritional amendments can result from stimulation of antagonist growth and inhibition of the pathogen. Janisiewicz (1988) indicated that yeasts can colonise the fruit surface for long periods under dry conditions, produce extracellular polysaccharides that enhance their survivability and restrict both colonisation sites and the flow nutrients to fungal propagules, use available nutrients rapidly, and proliferate well at wound site. Competition for nutrients and/or site is an important mechanism of biocontrol of plant pathogens. Competition for limiting nutritional sources has been reported between yeasts and phytopathogenic fungi (Dick et al., 1992; Janisiewicz et al., 2000).

In previous studies, Viñas et al. (1998) suggested that the ability of *C. sake* (CPA-1) to rapidly colonise wounded fruits may indicate biocontrol by nutrient competition and/or site exclusion.

Pome fruits are poor in nitrogenous compounds, so we hypothesized that addition of nitrogen could improve the colonisation of the antagonist. For this reason, first we tested *in vitro* 22 nitrogenous compounds to determine their effect on *C. sake* growth, and then the compounds that induced greater growth were tested in combination with the antagonist to evaluate their effect on enhancing control of *P. expansum* on apples and pears. All the nitrogenous compounds tested in our study were utilised by the antagonist, and 17 of them greatly stimulated antagonist growth *in vitro*

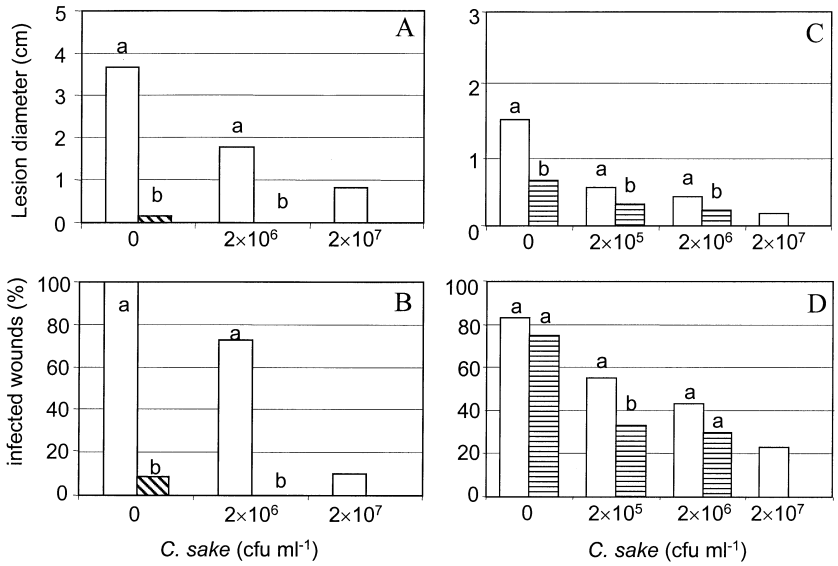

Figure 5. Effect of ammonium molybdate and *C. sake* (CPA-1) on reducing severity (A) and incidence (B) of *P. expansum* on pears and on reducing severity (C) and incidence (D) of *P. expansum* on apples. The antagonist was suspended in ammonium molybdate. The fruits were wounded ($3 \times 3 \times 3$ mm), inoculated with the antagonist and ammonium molybdate suspension, and then inoculated again with a suspension of the pathogen (10^4 conidia ml^{-1}) and incubated for 60 days at 1 °C. Ammonium molybdate concentrations were (□) 0 mM, (▨) 5 mM on pears and (□) 0 mM and (▤) 1 mM on apples. Bars represent means for three replicate samples. For a given concentration of the antagonist, bars associated with the same letter represent means that are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

at the concentration of 20 mM. Of the 10 nitrogenous compounds selected for testing in fruits, three of them enhanced biocontrol on apples and one on pears. In contrast to *in vitro* assays, most of the compounds tested at the highest concentrations on fruits increased lesion size. The high concentration of these compounds probably prevents nitrogen from being a limiting factor to the pathogen or might be toxic for the antagonist.

In this work, all the combinations of *C. sake* with nutrients compounds were compared to *C. sake* applied at the concentration (2×10^7 cfu ml^{-1}) usually recommended to control blue mould (Usall et al., 2000; 2001) to determine the possible reduction in the concentration of our antagonist without diminishing control, however the statistical analysis of this treatment was not included in the figures. L-serine and L-aspartic acid enhanced biocontrol by *C. sake* on apples and ammonium molybdate enhanced biocontrol on both fruits. The combination of L-serine at 5 mM and *C. sake* at 2×10^6 cfu ml^{-1} resulted in a similar efficacy as *C. sake* at 2×10^7 cfu ml^{-1}. In the presence of L-serine at 5 mM, it would then possible to apply ten times less *C. sake*. The addition of L-aspartic acid at 1 and 5 mM greatly enhanced biocontrol when the antagonist concentration was 2×10^5 cfu ml^{-1}, and allowed the use of 100 times less *C. sake* with a similar efficacy against *P. expansum*. However, when the concentration of the antagonist was 2×10^6 cfu ml^{-1}, the addition of L-aspartic acid at 5 mM resulted in unexpected increased of lesion diameter.

Of all the nitrogenous compounds tested on fruit, only ammonium molybdate enhanced biocontrol of blue mould on apples and pears. For this reason it was selected for testing on fruits under storage conditions, where a similar efficacy against blue mould as on room temperature assays was observed. Under cold storage, equal control was observed with *C. sake* 2×10^7 cfu ml^{-1}. The combination of *C. sake* at a lower concentration with ammonium molybdate, allowed the concentration of *C. sake* to be reduced 10-fold when applied in combination with ammonium molybdate at 5 mM on pears and could be reduced 100-fold in combination with ammonium molybdate at 1 mM on apples. Application of ammonium molybdate alone greatly reduced the incidence and severity of *P. expansum*. This effect was particularly strong on pears, where no difference was observed between fruits inoculated or not with the antagonist when treated with ammonium molybdate. This suggests that besides the enhancement

of biocontrol efficacy of *C. sake*, ammonium molybdate may reduce fungal infection through direct inhibition of spore germination. However, further research needs to be conducted to clarify this point.

The addition of calcium chloride to the antagonist greatly enhanced biocontrol on apples but less on pears. On apples, the best result was observed with the addition of $CaCl_2$ at 20 mM. Increasing $CaCl_2$ concentration up to 100 mM did not enhance biocontrol more than with 20 mM. Wisniewski et al. (1995) reported enhanced biocontrol of *Candida oleophila* by adding $CaCl_2$ on apples, but in their study the improvement of biocontrol was achieved with the addition of $CaCl_2$ at more than 90 mM. Other authors using the yeast *Pichia guilliermondii* also observed improvement of the biocontrol agent using $CaCl_2$ (Chalutz et al., 1992). McLaughlin et al. (1990) found that the ability of $CaCl_2$ to improve biocontrol was dependent on the yeast strain and the concentration of $CaCl_2$ used.

Glucose reduced the inhibitory effect of $CaCl_2$ on spore germination of *P. expansum* and *B. cinerea in vitro* (Wisniewski et al., 1995), suggesting that the effect of $CaCl_2$ on more mature fruit may decline. This could explain why the concentration of $CaCl_2$ necessary to enhance biocontrol on pears was higher than on apples, because in our experiments the pears were at a more mature stage than the apples. The mode of action of $CaCl_2$ at these concentrations could thus based on improved colonisation of the antagonist at the wound site and/or on inhibition of pathogen growth.

We suggest that calcium could be used as part of the formulation of this biocontrol agent in order to reduce costs by using lower concentrations of the antagonist and to improve the efficacy of the control.

Our results demonstrate that combining *C. sake* with 2-deoxy-D-glucose in pears and apples greatly reduced the population of yeast required to give effective control. Similar results were reported on enhanced biocontrol of *C. saitona* (El-Ghaouth et al., 2000b) and of *Pseudomonas syringae* (L-59-66) (Janisiewicz, 1994). In contrast, in our study, the strongest effect was observed on apples, and an increased concentration of the compound resulted in an increased control of decay.

The sugar analog 2-deoxy-D-glucose was also effective in controlling blue mould decay on apples (Janisiewicz, 1994; El-Ghaouth et al., 1995). This carbohydrate is known to interfere with the growth of several filamentous fungi, including major postharvest pathogens (El-Ghaouth et al., 1995; 1997).

The present study indicates that of all the compounds tested, ammonium molybdate is the best candidate for enhancing biocontrol of *C. sake* (CPA-1). Even in the absence of *C. sake*, ammonium molybdate reduced the severity and incidence of blue mould on apples and pears under storage conditions. The enhanced biocontrol activity appears to result from direct inhibition of the pathogen by this nitrogenous compound and may be from an increase in the *C. sake* population.

Our research showed that manipulating the chemical environment by adding ammonium molybdate results in improved antagonistic activity and provides a significant reduction in the amount of the yeast biomass required to achieve postharvest disease control of pome fruits. This may be useful in commercial development of the biocontrol agent, as this would facilitate industrial production of *C. sake* by reducing the total volumes that need to be produced. More research is necessary on the mode of action of ammonium molybdate and its effects on fruit, on biocontrol agents and on pathogens.

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