Improved Control of Postharvest Decay of Pears by the Combination of *Candida sake* (CPA-1) and Ammonium Molybdate

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Accepted for publication 1 November 2001.

ABSTRACT

Nunes, C., Usall, J., Teixidó, N., Abadías, M., and Viñas, I. 2002. Improved control of postharvest decay of pears by the combination of *Candida sake* (CPA-1) and ammonium molybdate. *Phytopathology* 92: 281-287.

The potential enhancement of *Candida sake* (CPA-1) by ammonium molybdate to control blue and gray mold caused by *Penicillium expansum* and *Botrytis cinerea*, respectively, on Blanquilla pears was investigated. In laboratory trials, improved control of blue and gray molds was obtained with the application of ammonium molybdate (1, 5, 10, and 15 mM) alone or in combination with *C. sake* at 2×10^6 or 2×10^7 CFU ml⁻¹ on Blanquilla pears stored at 20°C. In semicommercial trials at 1°C for

5 months, the efficacy of *C. sake* at 2×10^6 CFU ml⁻¹ on reducing *P. expansum* and *B. cinerea* decay was enhanced more than 88% with the addition of ammonium molybdate 5 mM in the 1999–2000 season. In two seasons, the performance *C. sake* at 2×10^6 CFU ml⁻¹ plus ammonium molybdate was similar to or greater than that of *C. sake* at 2×10^7 CFU ml⁻¹. Similar control of blue mold was obtained on pears stored under low oxygen conditions. The preharvest application of ammonium molybdate did not reduce postharvest blue mold decay. The population of *C. sake* on pear wounds significantly decreased in the presence of ammonium molybdate 1 and 5 mM at 20 and 1°C.

Additional keywords: nutritional enhancement, storage.

Synthetic fungicides are the primary means of controlling postharvest diseases, but concerns about the presence of chemical residues in food, the development of fungicide-resistant strains of postharvest pathogens, and the deregistration of some fungicides have generated interest in the development of alternative methods (9,32).

Biological control has been extensively studied in recent years and the use of yeasts and bacteria as antagonists has been reported to control many postharvest diseases of fruit (4,12,14,19,22,29). Although the use of biocontrol agents has been successful, their successful commercialization will depend on whether they are capable of effectively controlling decay of fruit from different locations and different cultivars.

Microorganisms as biocontrol agents have a relatively narrow spectrum of activity when compared with chemical fungicides (13). Greater and broader activity can be achieved by applying higher concentrations of the antagonist (8,20), but increasing the antagonist populations makes biological control less economical. Biological control of postharvest diseases is complex and involves a number of variables, such as biological and environmental factors (1,17,31). The chemical environment can be manipulated to enhance biocontrol activity of the antagonist and, in fact, several works report additives that enhance the effectiveness of microbial antagonists (3,9,11,16,33).

Preliminary assays in vitro and in vivo (18) on the combination of some nutrients with *Candida sake* strain CPA-1 in order to enhance biological control showed that the combination of the antagonist and ammonium molybdate reduced blue mold decay caused by *Penicillium expansum* on apples and pears more than other chemicals. However, the ability of ammonium molybdate to control disease development has not been fully explored.

Ammonium molybdate affects metabolic processes in several organisms (2,6,30). The basis of its biological activity was reported to be its ability to inhibit acid phosphatase, which interferes with phosphorylation and dephosphorylation (5), one of the most important processes of cell regulating (7,21).

The current study was undertaken to (i) determine the potential of ammonium molybdate to enhance biological control of *P. expansum* and *Botrytis cinerea* decay on pears with the yeast *C. sake* (CPA-1) under different storage conditions, (ii) compare the efficacy of preharvest and postharvest application of ammonium molybdate to control *P. expansum* decay of pears during cold storage, and (iii) determine the effect of ammonium molybdate on the population dynamics of *C. sake* in pear wounds.

MATERIALS AND METHODS

Yeast isolate and pathogens. The isolate used in this study, *C. sake* (Saito & Ota) van Uden and Buckley strain CPA-1 from UdL-IRTA (Spain), was isolated from the surface of an apple. This strain demonstrated antagonistic activity against *P. expansum* Link and *B. cinerea* Pers. in pome fruit (29). It is deposited in Colección Española de Cultivos Tipo, CECT-10817 (Universidad de Valencia, Campus de Burjassot, Burjassot, Valencia, Spain). Yeast cells were prepared by growing cultures in nutrient yeast dextrose broth (NYDB: nutrient broth, 8 g liter⁻¹; yeast extract, 5 g liter⁻¹; dextrose, 10 g liter⁻¹) for 30 h at $25 \pm 1^\circ\text{C}$ with shaking at 150 rpm. The medium was centrifuged (Avanti J-25; Beckman, Palo Alto, CA) at $8,315 \times g$ for 10 min and the cells were resuspended in 0.05 M phosphate buffer (pH 6.8). This was repeated twice, in order to wash the yeast cells from the nutritional compounds of the initial medium. The cells were resuspended in deionized water and the desired concentrations were obtained by adjusting the suspension cell concentration with a hemacytometer.

P. expansum and *B. cinerea* were isolated from decayed apples after several months in storage and were maintained on potato dextrose agar (PDA: extract from boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g; water, 800 ml). The conidial suspensions of

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Publication no. P-2002-0115-01R

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10⁴ conidia ml⁻¹ were prepared from 10- and 14-day-old cultures of *P. expansum* and *B. cinerea*, respectively, and were determined with a hemacytometer.

Fruits and orchard. Blanquilla pears from commercial orchards in Lleida, Catalonia were used in all experiments on harvested fruit. Fruit were used following harvest or after storage at 1°C (no longer than 3 months after harvest). In the field trials, the fruit trees were from a commercial orchard in Tornabous (Lleida, Catalonia, Spain).

Chemical products. The ammonium molybdate ((NH₄)₆Mo₇O₂₄·4H₂O) used in this study was purchased from Aldrich-Chemie (D-7924 Steinheim, Germany), and cited as NH₄-Mo.

Laboratory trials. For laboratory tests where the fruit were stored at 20°C, pears were wounded (3 by 3 by 3 mm³) at the stem (top) and calyx (bottom) end. Twenty-five microliters of each treatment was applied to the wounds, followed by inoculation with 20 µl of an aqueous suspension of *P. expansum* or *B. cinerea* at 10⁴ conidia ml⁻¹. Treatments consisted of (i) distilled water; (ii) *C. sake* at 2 × 10⁶ CFU ml⁻¹; (iii) *C. sake* at 2 × 10⁷ CFU ml⁻¹; and (iv) aqueous solutions of NH₄-Mo at 1, 5, 10, or 15 mM alone or in combination with *C. sake* at 2 × 10⁶ or 2 × 10⁷ CFU ml⁻¹. Lesion diameter was measured after 7 days of incubation at 20°C and 85 ± 5% relative humidity (RH). Each treatment was repeated three times with three fruit per replication. The test was repeated three times. The most effective treatments were selected for small-scale trials to control *P. expansum* and *B. cinerea* rot under cold storage conditions.

For laboratory tests where the fruit were stored at 1°C, the fruit were treated as above, and the treatments consisted of (i) distilled

water; (ii) *C. sake* at 2 × 10⁶ CFU ml⁻¹; (iii) *C. sake* at 2 × 10⁷ CFU ml⁻¹; and (iv) NH₄-Mo at 5 mM alone or in combination with *C. sake* at 2 × 10⁶ CFU ml⁻¹. The recommended concentration of *C. sake* (CPA-1) to control blue mold is ≈10⁷ CFU ml⁻¹ (29); therefore, the concentration of *C. sake* at 2 × 10⁷ CFU ml⁻¹ was tested alone because the objective was to reduce the antagonist dose necessary to control decay. Ten fruit constituted a single replicate, and each treatment was replicated three times. The fruit were stored in a cold room at 1°C and 90 ± 5% RH for 60 days. The test was repeated twice.

Semicommercial trials under cold storage conditions. To simulate commercial damage, a wound (≈1 mm in diameter and 2 mm deep) was inflicted on each fruit at two locations (midway between the calyx and stem end) with a nail. The fruit were dipped in each treatment for 30 s; fruit were left drying under ambient conditions and then were dipped again for 30 s in a conidial suspension of *P. expansum* or *B. cinerea* (10⁴ conidia ml⁻¹). Treatments consisted of (i) distilled water; (ii) *C. sake* at 2 × 10⁶ CFU ml⁻¹; (iii) *C. sake* at 2 × 10⁷ CFU ml⁻¹; and (iv) NH₄-Mo at 5 mM alone or in combination with *C. sake* at 2 × 10⁶ CFU ml⁻¹. The fungicide imazalil (Decozil-S-7.5; Elf Atochem Agri España, Valencia, Spain) at the recommended rate for postharvest treatment (0.5%) also was tested. Twenty fruit constituted a single replicate and each treatment was repeated four times. Lesion diameters were measured 5 months after cold storage at 1°C and 21% O₂ (ambient air). This study was conducted during two storage seasons (1997–98 and 1999–2000).

Blue mold suppression on pears stored at 1°C and under low oxygen conditions (2% O₂-1% CO₂) during 5 months also was

TABLE 1. Treatments used on the control of blue mold by preharvest treatments

Treatment ^z	Preharvest treatment with NH ₄ -Mo		Postharvest		Postharvest inoculation with	
	7 days before harvest	2 days before harvest	Wounds	Treatment with NH ₄ -Mo	<i>Candida sake</i>	<i>Penicillium expansum</i>
Nontreated	–	–	+	–	–	+
NH ₄ -MoPre7d	+	–	+	–	–	+
NH ₄ -MoPre7d + CS	+	–	+	–	+	+
NH ₄ -MoPre2d	–	+	+	–	–	+
NH ₄ -MoPre7d + CS	–	+	+	–	+	+
NH ₄ -MoPost	–	–	+	+	–	+
NH ₄ -MoPost + CS	–	–	+	+	+	+
CS	–	–	+	–	+	+

^z NH₄-MoPre7d = application of ammonium molybdate ((NH₄)₆Mo₇O₂₄·4H₂O) 7 days before harvest; NH₄-MoPre2d = application of NH₄-Mo 2 days before harvest; CS = *Candida sake* treatment; NH₄-MoPost = postharvest application of NH₄-Mo.

TABLE 2. Lesion diameter (mm) and infected wounds (%) of *Penicillium expansum* and *Botrytis cinerea* decay (10⁴ conidia ml⁻¹) on Blanquilla pears protected with *Candida sake*, ammonium molybdate (NH₄-Mo), and their combination

<i>C. sake</i> (CFU ml ⁻¹)	NH ₄ -Mo (mM)	Pathogens ^z			
		<i>P. expansum</i>		<i>B. cinerea</i>	
		Lesion diameter (mm)	Infected wound (%)	Lesion diameter (mm)	Infected wound (%)
0	0	34.0 a	100 a	42.7 a	100 a
0	1	8.8 c	39 c	42.2 a	100 a
0	5	8.2 c	33 c	29.7 b	83 b
0	10	25.6 b	89 b	29.6 b	100 a
0	15	31.1 a	100 a	24.8 b	67 c
2 × 10 ⁶	0	17.2 a	72 a	24.4 ab	89 a
2 × 10 ⁶	1	1.2 b	17 b	27.8 a	78 ab
2 × 10 ⁶	5	0.0 b	0 b	15.6 b	56 c
2 × 10 ⁶	10	20.9 a	89 a	31.8 a	83 ab
2 × 10 ⁶	15	21.6 a	83 a	22.6 ab	72 b
2 × 10 ⁷	0	10.4 a	56 a	10.2 a	50 a
2 × 10 ⁷	1	1.0 bc	6 c	15.2 a	56 a
2 × 10 ⁷	5	0.0 c	0 c	0.0 b	0 b
2 × 10 ⁷	10	6.7 ab	33 ab	1.3 b	6 b
2 × 10 ⁷	15	3.2 bc	17 bc	0.0 b	0 b

^z Means of 18 wounds (three replicates, three fruit samples per treatment, and two lesions per fruit) determined after 7 days of incubation at 20°C and 85 ± 5% relative humidity. In relation to each concentration of the antagonist, different letters in the same column indicate significant difference between means using the least significant difference test.

studied in the 1997–98 season with the methodology described above. The test was repeated twice.

Suppression of blue mold during cold storage by preharvest treatments. For fruit used for preharvest treatments, 20 trees were selected at random from an orchard of Blanquilla apple trees (8 years old at a spacing of 4 by 3 m), growing at Lleida, Catalonia, Spain, with guard rows between sets of treatments. Each treatment was repeated four times and each replicate consisted of four trees (70 ripe pears). In all cases, a guard trees were used to separate randomized treatments and replicates. Eight treatments were studied to compare pre- and postharvest application of $\text{NH}_4\text{-Mo}$ in combination with postharvest application of *C. sake* to control blue mold on pears wounded at postharvest. The treatments used in this study are detailed in Table 1 and included the following combination of factors: preharvest application of $\text{NH}_4\text{-Mo}$ at 5 mM, postharvest wounding, postharvest application of *C. sake* at 10^7 CFU ml^{-1} , and postharvest application of $\text{NH}_4\text{-Mo}$ at 5 mM alone or in combination with *C. sake* at 10^7 CFU ml^{-1} .

Preharvest treatments were applied 7 and 2 days before harvest using a handgun operating at 10 atmospheres of pressure. After harvest, the fruit were placed in separate boxes according to treatment and replicate number, and each fruit was wounded as described previously. Postharvest treatments were applied by dipping the boxes containing fruit in a suspension of $\text{NH}_4\text{-Mo}$, *C. sake*, or *C. sake* plus $\text{NH}_4\text{-Mo}$ for 30 s. 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, the pears were stored at 1°C and $90 \pm 5\%$ RH in air. The number of infected wounds was determined after 4 months of storage. The test was repeated twice.

Effect of ammonium molybdate on *C. sake* (CPA-1) populations in pear wounds. On each pear, two wounds (1 mm in diameter and 2 mm deep) were made in different locations midway between the calyx and the stem end. All fruit were dipped for 30 s into the antagonist suspension containing 2×10^6 CFU ml^{-1} alone or in combination with 1 or 5 mM $\text{NH}_4\text{-Mo}$. Once dried, fruit were placed on tray packs in plastic boxes and incubated at 20°C and $85 \pm 5\%$ RH or at 1°C and $90 \pm 5\%$ RH. Yeast populations were determined at 0 (just prior to storage), 24, 48, 72, and 168 h on fruit stored at 20°C ; and at 0, 15, 30, 45, and 60 days on fruit stored at 1°C .

Four pears constituted each replicate and 20 pieces of peel surface of 166 cm^2 were removed (including the wounded areas) with a cork borer and a knife from each fruit. Peel surface pieces were shaken in 50 ml of sterile 0.05 M phosphate buffer (pH 6.5) on a rotary shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasound bath (Selecta, Abrera, Barcelona, Spain). This final step was used to improve detachment of the antagonist from the pear surface. Serial 10-fold dilutions of the washings were made with 0.05 M phosphate buffer and plated on NYDA medium (the same as NYDB plus agar at 15 g liter^{-1}). Colonies were counted after incubation at 25°C in the dark for 48 h. Population sizes were expressed as CFU cm^{-2} of pear surface. Each treatment was repeated four times and the experiment was repeated twice.

Statistical treatment. Data of separate experiments were pooled when statistical analysis determined the absence of significant interactions between treatments and experiments. The effect of combining $\text{NH}_4\text{-Mo}$ with *C. sake* at room temperature was analyzed by two-way factorial analysis. Data of antagonist populations (CFU cm^{-2}) were transformed to logarithms to improve the homogeneity of variances. The incidence and severity of decay and yeast population dynamics were analyzed by an analysis of variance with SAS Software (version 6.08; SAS Institute, Cary, NC). Statistical significance was applied at the level $P < 0.05$. When the analysis was statistically significant, the least significant difference test was applied to separate means.

RESULTS

Laboratory trials. From all $\text{NH}_4\text{-Mo}$ tested concentrations, the application of 5 mM alone was the only one that significantly reduced the severity and incidence of both blue ($P = 0.0001$) and gray ($P = 0.0012$ and $P = 0.0001$) molds (Table 2). When combined with *C. sake* at 2×10^6 or 2×10^7 CFU ml^{-1} , $\text{NH}_4\text{-Mo}$ at 1 or 5 mM markedly enhanced the efficacy of this biocontrol agent against blue mold decay. In addition, complete control of blue mold was achieved with the addition of 5 mM $\text{NH}_4\text{-Mo}$ to *C. sake* at 2×10^6 or 2×10^7 CFU ml^{-1} . In gray mold assays, the application of $\text{NH}_4\text{-Mo}$ alone at 5 or 15 mM significantly reduced the severity ($P = 0.0012$) and incidence ($P = 0.0001$) of the decay. The combination of *C. sake* at 2×10^6 CFU ml^{-1} and $\text{NH}_4\text{-Mo}$ at 5 mM enhanced the biocontrol agent activity on reducing incidence and severity of *B. cinerea*. Also, complete control was achieved with *C. sake* at 2×10^7 CFU ml^{-1} plus $\text{NH}_4\text{-Mo}$ at 5 or 15 mM. The best and more consistent results were obtained with the addition of $\text{NH}_4\text{-Mo}$ at 5 mM to *C. sake*; therefore, this concentration was selected for further experiments.

In laboratory trials under cold storage, all treatments significantly reduced the incidence ($P = 0.0001$) and severity ($P = 0.0001$) of blue and gray molds on pears stored at 1°C for 60 days (Fig. 1). The combined treatment (*C. sake* at 2×10^6 CFU ml^{-1} plus $\text{NH}_4\text{-Mo}$ at 5 mM) significantly enhanced the efficacy of this biocontrol agent against both pathogens. Complete control of blue mold was obtained in pears inoculated with the combination (Fig. 1A), and this performance was superior to the application of *C. sake* at 2×10^6 or 2×10^7 CFU ml^{-1} alone. The application of $\text{NH}_4\text{-Mo}$ alone reduced severity and incidence of blue mold by 91 and 86%, respectively, compared with the control treatment. In the case of gray mold, the combined treatment enhanced the capacity of *C. sake* by 83% to reduce infected wounds (Fig. 1B). Similar control was obtained with the combination and *C. sake* at a higher concentration (2×10^7 CFU ml^{-1}).

Fig. 1. Incidence (■) and severity (▲) of **A**, blue mold and **B**, gray mold on wounded Blanquilla pears treated with *Candida sake* at 2×10^6 and 2×10^7 CFU ml^{-1} , $\text{NH}_4\text{-Mo}$ at 5 mM, and the combination of *C. sake* at 2×10^6 CFU ml^{-1} and $\text{NH}_4\text{-Mo}$ at 5 mM, followed by inoculation with the pathogens (10^4 conidia ml^{-1}) after 60 days of incubation at 1°C and $90 \pm 5\%$ relative humidity. Within incidence, columns with the same letter are not significantly different according to the least significant difference test.

Semicommercial trials under cold storage conditions. The effectiveness of the combined treatment was observed in semi-commercial tests and also was compared with the postharvest fungicide imazalil (Figs. 2 and 3). The combined treatment significantly reduced incidence and severity of blue and gray molds under cold storage conditions in air (Fig. 2), as well as in reducing incidence and severity of blue mold during 5 months of storage under cold conditions in a low oxygen atmosphere (Fig. 3). In ambient air, all treatments significantly reduced blue mold in both seasons ($P = 0.0001$) (Fig. 2A). The efficacy of *C. sake* in reducing blue mold severity and incidence was enhanced by 92 and 88%, respectively, with the addition of $\text{NH}_4\text{-Mo}$, in the 1999–2000 season. In both seasons, the efficacy of the combination was greater than *C. sake* at 2×10^7 CFU ml^{-1} alone. Moreover decay among pears stored in the 1999–2000 season were not significantly different than that after application of *C. sake* plus $\text{NH}_4\text{-Mo}$ or imazalil. On pears treated with $\text{NH}_4\text{-Mo}$ at 5 mM alone, the reduction of blue mold severity was greater than 91% in both seasons, and incidence was reduced by 73 and 87% in the 1997–98 and 1999–2000 seasons, respectively.

In *B. cinerea* assays in cold storage in ambient air, the incidence and severity of gray mold in the 1997–98 season was very low after all treatments (Fig. 2B). In this season, no significant reduction in gray mold was observed after the application of $\text{NH}_4\text{-Mo}$ at 5 mM or imazalil. Significantly similar but modest control was achieved with *C. sake* at both tested concentrations and the combined treatment. In the 1999–2000 season, all treatments significantly reduced gray mold ($P = 0.0001$), and similar control was achieved with the application of $\text{NH}_4\text{-Mo}$ alone or in combination with *C. sake* and imazalil (Fig. 2B). The combined treatment reduced the percentage of infected wounds from 50 to 6% and the mean lesion diameter from 58 to 4 mm. The combined treatment enhanced 86% the efficacy of the *C. sake* on reducing incidence, and the reduction was superior to that observed with the application of *C. sake* at 2×10^7 CFU ml^{-1} .

Under controlled atmosphere, all treatments significantly reduced blue mold ($P = 0.0001$) (Fig. 3). The combined treatment reduced blue mold by 86% and was not significantly different from the application of $\text{NH}_4\text{-Mo}$ alone or from the imazalil. The reduction in disease incidence achieved with the combination was 55% higher than that observed with *C. sake* at 2×10^6 or 2×10^7 CFU ml^{-1} .

Suppression of blue mold during cold storage by preharvest applications. Preharvest application of $\text{NH}_4\text{-Mo}$ treatments alone did not reduce blue mold incidence (Fig. 4). On untreated pears and those treated with a preharvest application of $\text{NH}_4\text{-Mo}$, the incidence of blue mold was significantly higher (89%) than on pears treated with $\text{NH}_4\text{-Mo}$ followed by postharvest *C. sake* application (44%). No differences in incidence of blue mold were observed among preharvest applications of $\text{NH}_4\text{-Mo}$ followed by postharvest applications of *C. sake*, postharvest applications of $\text{NH}_4\text{-Mo}$, or postharvest applications of *C. sake*. The percentage of infected wounds in the postharvest treatment of *C. sake* plus $\text{NH}_4\text{-Mo}$ was significantly less than all treatments and was 90% less than the nontreated control.

Effect of ammonium molybdate on *C. sake* (CPA-1) populations in pear wounds. The growth rate of *C. sake* was reduced by $\text{NH}_4\text{-Mo}$ in pears stored at 20°C (Fig. 5A) and 1°C (Fig. 5B). At room temperature during the first 24 h, *C. sake* populations increased 13-fold; whereas, in the presence of $\text{NH}_4\text{-Mo}$, the population increased only sevenfold (Fig. 5A). After this period, *C. sake* populations in presence of $\text{NH}_4\text{-Mo}$ at 5 mM decreased. At the end of the experiment, the population was only fourfold higher than the initial population.

The effect of $\text{NH}_4\text{-Mo}$ on the growth of *C. sake* was greater on pears stored at 1°C than at 20°C. At this temperature, *C. sake* alone during the first 15 days increased 20-fold and, at the end of the experiment, the population was 300-fold higher than the initial population (Fig. 5B). Meanwhile, in the presence of $\text{NH}_4\text{-Mo}$ during the first 15 days, population of *C. sake* increased 10-fold

Fig. 2. Severity (▲) and incidence of **A**, blue mold and **B**, gray mold during the 1997–98 season (■) and the 1999–2000 (□) season on wounded Blanquilla pears treated with *Candida sake* at 2×10^6 and 2×10^7 CFU ml^{-1} , $\text{NH}_4\text{-Mo}$ at 5 mM, *C. sake* at 2×10^6 CFU ml^{-1} combined with $\text{NH}_4\text{-Mo}$ at 5 mM, and imazalil (0.5%), followed by inoculation with *Penicillium expansum* (10^4 conidia ml^{-1}) after 5 months of incubation at 1°C in air. Within each season, letters are applied to incidence, and columns with the same letter are not significantly different according to the least significant difference test.

and remained stable until the end of the experiment both at 1 and 5 mM concentrations.

Under both storage conditions, increasing the concentration of $\text{NH}_4\text{-Mo}$ from 1 to 5 mM in general did not affect the growth of *C. sake*.

DISCUSSION

Detailed studies have shown that *C. sake* CPA-1 is an effective antagonist of the major postharvest pathogens of pome fruit (25, 27, 29) and has demonstrated a high degree of efficacy in controlling postharvest blue mold on apples in pilot tests (28). However, the capacity of *C. sake* to control postharvest diseases is greater on apples than on pears (26). In the present study, we demonstrated that $\text{NH}_4\text{-Mo}$ has great potential for controlling postharvest diseases on pears and could be used to enhance the antagonistic ability of *C. sake* (CPA-1) against the most important postharvest pathogens on pears. The efficacy trials at room temperature with *C. sake* plus $\text{NH}_4\text{-Mo}$ generally showed higher blue and gray mold control than applications of *C. sake* alone. Based

on these results, $\text{NH}_4\text{-Mo}$ at 5 mM was chosen to combine with *C. sake* at 2×10^6 CFU ml^{-1} to determine the efficacy of $\text{NH}_4\text{-Mo}$ on enhancing biocontrol activity of *C. sake*. Under cold storage during 60 days, the combined treatment usually offered better control of decay on pears artificially wounded than did *C. sake* at 2×10^7 CFU ml^{-1} alone or $\text{NH}_4\text{-Mo}$ at 5 mM alone. In simulating commercial conditions under cold storage in air or a low oxygen atmosphere for 5 months, the consistency of laboratory trial results was maintained. The effectiveness of the combined treatment was usually comparable to that observed with the fungicide imazalil and in general provided a higher reduction in decay than the treatment of *C. sake* at 2×10^6 or 2×10^7 CFU ml^{-1} alone. The application of $\text{NH}_4\text{-Mo}$ alone also provided great control, and was usually comparable to *C. sake* at 2×10^7 CFU ml^{-1} .

Enhancement of biocontrol activity has been reported with several additives. Janisiewicz et al. (13) reported the enhancement of *Pseudomonas syringae* biocontrol activity of blue mold on apples by the addition of nitrogenous compounds at room temperature. Wisniewski et al. (33) achieved the enhancement of *C. oleophila* at 10^6 CFU ml^{-1} by the addition of calcium salts. However, in that study, the concentration of calcium chloride needed to improve the efficacy of *C. oleophila* to control decay on apples was very high (90 and 180 mM). El-Ghaouth et al. (3) also reported that the combination of *C. saitoana* with 0.2% glycochitosan on semicommercial trials on apples stored at 18°C during 28 days enhanced the biocontrol activity of the antagonist. In that study, the concentration of the biocontrol agent was 10^8 CFU ml^{-1} ; they did not compare the effectiveness of the combination at a lower concentration of the antagonist.

Reliability and cost are two major factors that will determine the feasibility of any biocontrol system (13); thus, the concentration of the compounds needed to enhanced biocontrol activity as well as the concentration of the antagonist are very important. The biocontrol agent must be effective at concentrations reasonable for commercial development (8). From a practical point of view, the high limit of CFU ml^{-1} for many yeasts seems to be $\approx 5 \times 10^7$ CFU ml^{-1} (10) and the recommended concentration of *C. sake* conformed to this prerequisite by being generally effective at 10^7 CFU ml^{-1} (28). The results from the present study showed that the addition of $\text{NH}_4\text{-Mo}$ to *C. sake* allowed the antagonist concentration to be reduced at least to 2×10^6 CFU ml^{-1} to confer a level disease control equivalent to a commercial recommended fungicide with the subsequent economic benefits, and facilitate industrial production of *C. sake* by reducing the total volumes that need to be produced.

Fig. 3. Incidence (■) and severity (▲) of blue mold on wounded Blanquilla pears treated with *Candida sake* at 2×10^6 or 2×10^7 CFU ml^{-1} , $\text{NH}_4\text{-Mo}$ at 5 mM, *C. sake* at 2×10^6 CFU ml^{-1} combined with $\text{NH}_4\text{-Mo}$ at 5 mM, and imazalil (0.5%), followed by inoculation with *Penicillium expansum* (10^4 conidia ml^{-1}), after 5 months of incubation at 1°C with low oxygen atmosphere (2% O_2 –1% CO_2) during the 1997–98 season. Within incidence, columns with the same letter are not significantly different according to the least significant difference test.

Fig. 4. Comparative effect of preharvest and postharvest applications of $\text{NH}_4\text{-Mo}$ on percentage of infected wounds caused by *Penicillium expansum* on Blanquilla pears. Fruit were stored for 4 months at 1°C in air. Columns with the same letter are not significantly different according to the least significant difference test.

We did not find references reporting $\text{NH}_4\text{-Mo}$ as inhibitor of fungi growth apart from one study carried out on 1969 by Singh and Khanna (23). They observed that in vitro $\text{NH}_4\text{-Mo}$ inhibited mycelial growth of *Alternaria tenuis* but only at a concentration of or superior to 20 mM. When we tested $\text{NH}_4\text{-Mo}$ in vitro, we observed an inhibition of the spore germination of *P. expansum* and *B. cinerea* (C. Nunes, J. Usall, N. Teixidó, X. Ochoa de Eribe, and I. Viñas, unpublished data).

The ability of *C. sake* to colonize wounded fruit was affected in the presence of $\text{NH}_4\text{-Mo}$; however, the decrease in population did not seem to influence the control of decay. The observed increase in the *C. sake* population in the presence of $\text{NH}_4\text{-Mo}$ during the first 15 days at 1°C could possibly restrict the nutrient availability to the pathogens, and made it possible to exploit both the antagonistic activity of *C. sake* and antifungal properties of $\text{NH}_4\text{-Mo}$. Some studies reported that one of the reasons for the biocontrol enhancement by additives is their effect on increasing antagonist population (9,13,33). In this study, the effectiveness of the combination of *C. sake* with $\text{NH}_4\text{-Mo}$ may be due to the biological activity of *C. sake* and the antifungal property of $\text{NH}_4\text{-Mo}$. Ammonium molybdate is essential for growth of fungi (15); however, it seems that concentrations beyond the tolerance level of fungus disturbed its metabolic processes, resulting in its inhibition.

The preharvest application of $\text{NH}_4\text{-Mo}$ did not affect blue mold decay development; therefore, the reduction observed with treatments of preharvest application of $\text{NH}_4\text{-Mo}$ (at 7 and 2 days be-

fore harvest) followed by postharvest application of *C. sake* seems to be due to the effect of the antagonist. The preharvest application of $\text{NH}_4\text{-Mo}$ was made on unwounded pears and the fact that this treatment did not reduce blue mold decay is probably because a residue of $\text{NH}_4\text{-Mo}$ must remain on the wound to inhibit the infection. Smilanick et al. (24) found that the capacity of sodium carbonate or bicarbonate to control green mold on citrus was significantly reduced when the fruit were rinsed at high pressure. They concluded that this reduction occurred because the high pressure removes the residues of the compounds necessary to achieve control of green mold. Further research is needed in this area.

Toxicology data was determined by the Centre d'Investigació i Desenvolupament Aplicat (Barcelona, Catalonia, Spain), calculating the rat oral median lethal dose (LD_{50}). This work showed that the LD_{50} of ammonium molybdate is 1,714.3 mg kg^{-1} of live weight of Sprague Dawley rat and, at this concentration, no mortality or alterations in tested animals were observed (data not shown).

In conclusion, our data indicated that manipulating the chemical environment by adding ammonium molybdate results in an improvement of *C. sake* antagonistic activity and provides a significant reduction in the amount of the yeast biomass required to achieve postharvest disease control of pears, with the consequent reduction of costs. The level of control conferred by dipping fruit in the combined treatment was superior or equivalent to imazalil, so we believe that *C. sake* could be applied with $\text{NH}_4\text{-Mo}$ as an additive by the traditional application method in packing-houses of dipping or drenching and facilitate their practical implementation.

ACKNOWLEDGMENTS

This work was supported by Subprograma Ciência e Tecnologia do 2º Quadro Comunitário de Apoio from Portugal, by CICYT Comisión Interministerial de Ciencia y Tecnología grant (ALI96-0567) from Spain, and by the Council of Lleida (La Paeria). We thank X. Ochoa de Eribe for his collaboration on field trials.

LITERATURE CITED

- Blakeman, J. P., and Fokkema, N. J. 1982. Potential for biological control of plant diseases on phylloplane. Annu. Rev. Phytopathol. 20:167-192.
- Bodart, J. F., Béchard, D., Bertout, M., Rousseau, A., Gannon, J., Vilain, J. P., and Flament, S. 1999. Inhibition of protein tyrosine phosphatases blocks calcium-induced activation of metaphase II-arrested oocytes of *Xenopus laevis*. FEBS Lett. 457:175-178.
- El-Ghouth, A., Smilanick, J. L., Brown, G. E., Ippolito, A., Wisniewski, M., and Wilson, C. L. 2000. Application of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. Plant Dis. 84:243-248.
- Filonow, A. B., Vishniac, H. S., Anderson, J. A., and Janisiewicz, W. J. 1996. Biological control of *Botrytis cinerea* in apple by yeasts from various habitats and their putative mechanisms of antagonism. Biol. Control 7:212-220.
- Glew, R. H., Saha, A. K., Das, S., and Remaley, A. T. 1988. Biochemistry of the *Leishmania* species. Microbiol. Rev. 52:412-432.
- Grangeasse, C., Doublet, P., Vincent, C., Vaganay, E., Riberty, M., Duclos, B., and Cozzzone, A. J. 1998. Functional characterization of the low-molecular-mass phosphotyrosine-protein phosphatase of *Actinobacter johnsonii*. J. Mol. Biol. 278:339-347.
- Hunter, T. 1995. Protein kinases and phosphatases: The Yin and Yang of protein phosphorylation and signaling. Cell 80:225-236.
- Janisiewicz, W. J. 1987. Postharvest biological control of blue-mould on apples. Phytopathology 77:481-485.
- Janisiewicz, W. J. 1994. Enhancement of biological control of blue mold with the nutrient analog 2-deoxy-D-glucose on apples and pears. Appl. Environ. Microbiol. 60:2671-2676.
- Janisiewicz, W. J. 1997. Biological control of postharvest diseases of temperate fruits. Pages 171-198 in: Plant-Microbe Interactions and Biological Control. G. J. Boland and L. D. Kuykendall, eds. Marcel Dekker, New York.

Fig. 5. Population dynamics of *Candida sake* on wounded pears treated with 20 μl of: *C. sake* at 2×10^6 CFU ml^{-1} (●), or the combination of *C. sake* at 2×10^6 CFU ml^{-1} with $\text{NH}_4\text{-Mo}$ at 1 mM (▲) or 5 mM (■), and incubated at **A**, 20°C and 85 ± 5% relative humidity (RH) and **B**, 1°C and 90 ± 5% RH. Points represent the means of four replications and vertical bars indicate standard errors of the means. Within times, populations with the same letter are not significantly different according to the least significant difference test.

11. Janisiewicz, W. J., Conway, W. S., Glen, D. M., and Sams, C. E. 1998. Integrating biological control and calcium treatment for controlling post-harvest decay of apples. *HortScience* 33:105-109.
12. Janisiewicz, W. J., and Marchi, A. 1992. Control of storage rots on various pear cultivars with a saprophytic strain of *Pseudomonas syringae*. *Plant Dis.* 76:555-560.
13. Janisiewicz, W. J., Usall, J., and Bors, B. 1992. Nutritional enhancement of biological control of blue mold on apples. *Phytopathology* 82:1364-1370.
14. Madrigal, C., Pascual, S., and Melgarejo, P. 1994. Biological control of peach twig blight (*Monilinia laxa*) with *Epicoccum nigrum*. *Plant Pathol.* 43:554-561.
15. Mathews, C. K., and Van Holle, K. E. 1996. *Biochemistry*. 2nd ed. Benjamin/Cummings, Menlo Park, CA.
16. McLaughlin, R. J., Wisniewski, M. E., Wilson, C. L., and Chalutz, E. 1990. Effect of inoculum concentration and salt solutions on biological control of postharvest diseases of apple with *Candida* sp. *Phytopathology* 80:456-461.
17. Morris, C. E., and Rouse, D. I. 1985. Role of nutrients in regulating epiphytic bacterial population. Pages 63-82 in: *Biological Control on the Phylloplane*. C. E. Windels and E. Lindow, eds. The American Phytopathological Society, St. Paul, MN.
18. Nunes, C., Usall, J., Teixidó, N., Miró, M., and Viñas, I. 2001. Nutritional enhancement of biocontrol activity of *Candida sake* (CPA-1) against *Penicillium expansum* on apples and pears. *Eur. J. Plant Pathol.* 107:543-551.
19. Nunes, C., Usall, J., Teixidó, N., and Viñas, I. 2001. Biological control of postharvest pear diseases using a bacterium *Pantoea agglomerans* CPA-2. *Int. J. Food Microbiol.* 70:53-61.
20. Pusey, P. L., and Wilson, C. L. 1984. Postharvest biological control stone fruit brown rot by *Bacillus subtilis*. *Plant Dis.* 68:753-756.
21. Remaley, A. T., Das, S., and Campbell, P. I. 1985. Characterization of *Leishmania donovani* acid phosphatases. *J. Biol. Chem.* 260:880-886.
22. Roberts, R. G. 1990. Postharvest biological control of gray mold of apple by *Cryptococcus laurentii*. *Phytopathology* 80:526-530.
23. Singh, R. S., and Khanna, R. N. 1969. Effect of certain inorganic chemicals on growth and spore germination of *Alternaria tenuis* Auct., the fungus causing core rot of mandarin oranges in India. *Mycopathol. Mycol. Appl.* 37:89-96.
24. Smilanick, J. L., Margosan, D. A., Mlikota, F., Usall, J., and Michael, I. F. 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. *Plant Dis.* 83:139-145.
25. Teixidó, N., Viñas, I., Usall, J., and Magan, N. 1998. Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. *Phytopathology* 88:960-964.
26. Usall, J. 1995. Control biològic de *Penicillium expansum* en postcollita de fruita de llavor. Ph.D. thesis. Universitat de Lleida, Lleida, Spain.
27. Usall, J., Teixidó, N., Fons, E., and Viñas, I. 2000. Biological control of blue mold on apple by a strain of *Candida sake* under several controlled atmosphere conditions. *Int. J. Food Microbiol.* 58:83-92.
28. Usall, J., Teixidó, N., Torres, R., Ochoa de Eribe, X., and Viñas, I. 2001. Pilots tests of *Candida sake* (CPA-1) applications to control postharvest blue mold on apple fruit. *Postharvest Biol. Biotechnol.* 21:147-156.
29. Viñas, I., Usall, J., Teixidó, N., and Sanchis, V. 1998. Biological control of major postharvest pathogens on apple with *Candida sake*. *Int. J. Food Microbiol.* 40:9-16.
30. Wang, G., Morré, D. J., and Shewfelt, R. L. 1995. Isolation of plasma membrane from *Capsicum annum* fruit tissue: Prevention of acid phosphatase contamination. *Postharvest Biol. Biotechnol.* 6:81-90.
31. Wilson, C. L., and Wisniewski, M. E. 1989. Biological control of post-harvest diseases of fruits and vegetables: An emerging technology. *Annu. Rev. Phytopathol.* 27:425-441.
32. Wilson, C. L., Wisniewski, M. E., Droby, S., and Chalutz, E. 1993. A selection strategy for microbial antagonists to control postharvest diseases of fruits and vegetables. *Sci. Hortic. (Amsterdam)* 53:183-189.
33. Wisniewski, M. E., Droby, S., Chalutz, E., and Eilam, Y. 1995. Effects of Ca^{2+} and Mg^{2+} on *Botrytis cinerea* and *Penicillium expansum* in vitro and on the biological control activity of *Candida oleophila*. *Plant Pathol.* 44:1016-1024.