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Integration of Biocontrol Agents and Food-Grade Additives for Enhancing Protection of Stored Apples from *Penicillium expansum*

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ABSTRACT

Forty-nine compounds currently used as additives in foods were tested in combination with three biocontrol agents, the yeasts *Rhodotorula glutinis*, *Cryptococcus laurentii*, and the yeastlike fungus *Aureobasidium pullulans*, to increase their antagonistic activity against *Penicillium expansum*, the causal agent of blue mold on apples. Twelve additives dramatically improved the antagonistic activity of one or more of the tested biocontrol agents. In a two-way factorial experiment with these selected additives the percentage of *P. expansum* rots on apples was significantly influenced by the antagonist and the additive as well as by their interaction. The combination of the biocontrol agents and some additives resulted in a significantly higher activity with respect to the single treatments applied separately, producing additive or synergistic effects. Some of the selected additives combined with a low yeast concentration (10^6 cells per ml) had comparable or higher efficacy than the biocontrol agents applied alone at a 100-fold higher concentration (10^8 cells per ml). Some organic and inorganic calcium salts, natural gums, and some antioxidants displayed the best results. In general, the effect of each additive was specific to the biocontrol isolate used in the experiments. Possible mechanisms involved in the activity of these beneficial additives and their potential application in effective formulations of postharvest biofungicides are discussed.

Blue mold due to *Penicillium expansum* Link is the most important postharvest rot of pome fruits *(35).* The use of fungicides immediately before or after harvest to prevent rots is being increasingly limited by legislation because of risks for consumers' health *(2),* environmental pollution, and the onset of resistant pathogen strains *(1, 38).* An additional health risk for consumers of pome fruits and derived food is the accumulation of the mycotoxin Patulin, which is putatively carcinogenic and causes immunodepression *(19).*

Several biocontrol agents (BCAs), particularly yeasts and yeastlike fungi, have been extensively studied as alternative means to control postharvest fungal diseases *(17, 22).* Few strains of these antagonists, however, have been developed as biofungicides for commercial applications because of inconsistent activity when applied alone under large-scale conditions against natural infections by pathogens *(13, 30)* and because of the high cost of development and biomass production. For these reasons, research is being carried out to improve the activity of antagonists and to use lesser amounts of their biomass by optimizing the formulation and application of these BCAs. Nevertheless, research aimed at developing suitable formulations has been relatively limited *(22).* Attempts to improve biocontrol activity involve preharvest application of BCAs (to also control latent infections) *(20, 32)* and the combination of BCAs with physical treatments or chemical adjuvants *(14, 23).* Regarding adjuvants, the enhancement of biocontrol yeast activity was observed by combining the antagonists with low dosages of fungicides *(7, 13, 30)* or natural compounds *(14).* Moreover, the presence of specific additives seems to be an essential prerequisite for the commercial success of recently developed yeast-based biofungicides, such as the biofungicide ''Shemer'' registered in Israel and based on the yeast *Metschnikowia fructicola (24, 26),* which provides high protection against a wide range of pathogens on different crops only in the presence of a natural additive included in its formulation *(12).*

Among the potential adjuvants, some food-grade additives improved BCA activity *(15, 23).* Because food-grade additives are already authorized for applications in food systems and also have a well-known toxicological profile *(18, 33),* they appear particularly promising for application on fruits and vegetables. However, the use of these compounds in biofungicide formulations is to be preceded by a specific evaluation of their compatibility with BCAs and of their interaction with host and pathogen to improve and stabilize the biocontrol activity at a level comparable with that of synthetic fungicides.

The main objective of this work was to select and evaluate the effect of food-grade additives on three biocontrol yeasts to enhance their activity against *P. expansum* on apples.

MATERIALS AND METHODS

BCAs. *Cryptococcus laurentii* strain LS28 and *Aureobasidium pullulans* strain LS30 (both isolated from apples), and *Rhodotorula glutinis* (LS11) (isolated from olives), which had previ-

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TABLE 1. *Type, concentration and source of additives used in this study*

Additive	Concentration (%, wt/vol)	Source
Soybean lecithin	0.1	Carlo Erba Reagents, Milan, Italy
Ascorbic acid	0.3	
Calcium acetate, calcium lactate, calcium propionate, propionic acid, sodium phosphate dibasic	0.5	
Acetic acid, ammonium bicarbonate, Ammonium phosphate dibasic, calcium chloride, calcium citrate, citric acid, glucono-δ-lactone, lactic acid, magnesium sulfate·$7H_2O$), potassium carbonate, potassium-sodium carbonate, potassium-sodium tartrate, sodium bicarbonate, sodium silicate	1	
Calcium ascorbate	0.03	Sigma, St. Louis, Mo.
Alginic acid, arabic gum, benzoic acid, calcium alginate, guar gum, karaya gum, locust bean gum, methyl cellulose, pectin from citrus fruits, postassium aspartate, tragacanth gum, xanthan gum	0.1	
Corn starch, potassium bicarbonate, potassium phosphate dibasic, potato starch, sorbic acid	0.5	
Ammonium sulfate, calcium gluconate, calcium silicate, D-sorbitol, manganese sulfate, potassium acetate, potassium phosphate monobasic, potassium sorbate, sodium acetate, sodium alginate	1	

ously been characterized for their antagonistic activity *(28–30),* for some mechanisms of actions *(3–5),* and on a molecular basis *(10, 30),* were used in this study. Growth of the BCAs and production of cell suspensions for biocontrol assays were performed as reported elsewhere *(29).*

Pathogen. A highly virulent isolate of *P. expansum* isolated from decayed apples was used in all the experiments. Conidial suspensions for fruit inoculation were obtained as follows. The pathogen was grown on potato dextrose agar (PDA) under fluorescent light for 5 to 7 days at 21°C. Five milliliters of sterile distilled water containing 0.05% Tween 20 was poured in a petri dish, conidia were scraped from the agar by a sterile loop, and the suspension was filtered through four layers of cheesecloth. Concentration was adjusted to 2×10^4 conidia ml^{-1}.

Food-grade additives. Forty-nine compounds, alone or combined with the BCAs, were screened. In all the experiments, the concentration used for each additive was chosen from the range of values that may be legally added to foods. Additives, concentrations, and source are listed in Table 1. These compounds had previously been tested in vitro for their compatibility with the antagonists and antifungal activity against *P. expansum* and *Botrytis cinerea (31).*

Fruits. Apples cv. Golden were purchased from a local distributor and kept in a cold room at 3°C, with 95 to 98% relative humidity before using. Fruits were removed from cold storage 24 h before performing each experiment. During storage (from 20 to 90 days), average values of fruit firmness and soluble solid concentration were 3.7 kg and 12.2% (wt/vol), respectively, with a low variability throughout the period.

Activity of BCAs and adjuvants against *P. expansum* on apples. Apples were superficially disinfected by immersion for 1 min in a sodium hypochlorite solution (2% active chlorine), rinsed twice with sterile distilled water, and dried at room temperature. Four wounds (3 mm wide by 3 mm deep) on each fruit were produced around the blossom end. In all the assays, the following treatments were placed in each wound: 30 μl of distilled water containing (i) BCA alone (at a concentration of 10^6 cells ml^{-1}), (ii) additive alone, or (iii) BCA (at a concentration of 10^6 cells ml^{-1}) plus additive. In all the experiments, the control was represented by fruits in which wounds were treated with 30 μl of sterile distilled water. Additives and their concentration tested are reported in Table 1.

A preliminary screening was performed on 49 additives (Table 1). Twelve of these compounds, selected on the basis of their effectiveness, were used in the subsequent experiments. Finally, in experiments with three of the best performing additives selected for each BCA (Fig. 3), the different treatments were also compared with the BCAs applied alone at the higher concentration of 10^8 cells ml^{-1}.

In all experiments, after 2 h at room temperature from application of each treatment, all wounds were each inoculated with 15 μl of spore suspensions of *P. expansum.* Fruits were incubated in the dark at 20°C for 5 days with 95 to 98% relative humidity. Each treatment included three replications and each replication consisted of three fruits in preliminary experiments or six fruits in the subsequent assays. The number of wounds showing rot symptoms was periodically assessed and the assays were stopped when control fruits reached 90 to 100% of infected wounds.

Assays with selected compounds were performed twice and data of repeated experiments were pooled and processed by statistical analysis.

Data analysis. The percentages of *P. expansum*–infected wounds assessed from all experiments were converted into Bliss angular values (arcsine $\sqrt{\%}$) before analysis.

Data of experiments with three BCAs and 12 selected substances were submitted to factorial analysis of variance using the PROC GLM of SPSS (release 12.0 for Windows, SPSS Inc., Chicago, Ill.). The average values of the main factors (antagonist and additive) were separated by Duncan's multiple range test, while the means of the interaction (antagonist × additive) were compared by the least significant difference (LSD) test.

To ascertain the advantage of mixtures (BCA and adjuvant)

TABLE 2. *Synergy factor (SF) values of the combined activity of twelve adjuvants with the biocontrol agents (BCAs)* R. glutinis *(LS11),* C. laurentii *(LS28), and* A. pullulans *(LS30) against* P. expansum *on wounded apples*[a]

Additive	SF values for each BCA[b]		
	LS11	LS28	LS30
None (water)	1.0 bc	1.0 C	1.0 c
Alginic acid	0.4 d	1.5 A	1.1 bc
Calcium acetate	0.1 d	1.4 AB	1.4 abc
Calcium ascorbate	0.5 d	1.5 A	1.3 abc
Calcium chloride	1.4 a	1.6 A	1.2 abc
Calcium citrate	0.9 c	1.6 A	1.8 a
Calcium propionate	0.1 d	1.5 A	1.7 a
Calcium silicate	1.0 bc	0.9 C	1.2 bc
Corn starch	1.2 abc	1.5 A	1.4 abc
Guar gum	1.3 ab	0.9 C	1.5 abc
Locust bean gum	1.4 a	1.0 BC	1.7 ab
Potassium phosphate dibasic	0.4 d	1.5 A	1.4 abc
Xanthan gum	1.4 a	0.8 C	1.2 bc

[a] SF values: 1, the interaction is additive; <1, the interaction is antagonistic; >1, the interaction is synergistic.

[b] BCAs were applied at 10^6 cells per ml. Values represent the SF of the combined activity (BCA + adjuvant) according to the Abbott's formula. In each column, values marked by the same letters are not statistically different at $P = 0.05$ (lowercase letters) or at $P = 0.01$ (capital letters), according to Duncan's test.

with respect to treatments applied separately, the type of activity (additive, synergistic, or antagonistic) was evaluated. For this purpose values of infected wounds were transformed into percentage of control efficacy (CE) as follows: $CE = [(C - T)/C] \times 100$, where C was the number of infected wounds in the control (water plus pathogen) and T was the number of infected wounds in the examined treatment (antagonist alone or antagonist plus additive). Values ranged between 0 (no CE, corresponding to 100% infected wounds) and 100 (maximum CE, corresponding to lack of evident infection). The synergy factor (SF) was calculated according to the Abbott's formula *(27),* as follows: $SF = E_{obs.}/E_{exp.}$ where $E_{obs.}$ and $E_{exp.}$ are observed and expected CE of the mixture (antagonist plus adjuvant), respectively. $E_{exp.}$ was calculated as follows: $a + b - a \times b/100$, where a = CE of the factor a (antagonist) applied alone; b = CE of the factor b (adjuvant) applied alone. If SF = 1, the interaction between antagonist and adjuvant is additive; if SF < 1, the interaction between antagonist and adjuvant is antagonistic; if SF > 1, the interaction between antagonist and adjuvant is synergistic.

SF values of the different combinations of BCAs and additives were submitted to one-way analysis of variance (SPSS 12.0) and the average values were compared by using Duncan's multiple-range test.

RESULTS

In preliminary experiments, 12 of 49 additives tested against *P. expansum* on apples (Table 1) significantly improved the activity of the biocontrol yeasts *R. glutinis* (LS11) and *C. laurentii* (LS28) and of the yeastlike fungus *A. pullulans* (LS30) (data not shown).

The selected compounds (listed in Table 2) were tested further against *P. expansum* on apples in combination with the BCAs. In a factorial experiment, the percentage of infected wounds was significantly influenced ($P < 0.001$) by antagonist (factor A) and additive (factor B) and by the interaction between the main factors (antagonist × additive) (Table 3). Regarding the BCA factor, all three isolates used significantly reduced the percentage of infected wounds with respect to untreated control (Fig. 1a). The most effective isolates were LS28 and LS30, which reduced infected wounds by 78.3 and 73.2%, respectively, whereas isolate LS11 reduced the infection by 50.0%. The activity of the additive factor is reported in Fig. 1b; seven additives, among 12 compounds tested, significantly reduced *P. expansum* rot with respect to the control without additive. These additives were calcium silicate (−65.4%, reduction of infected wounds), locust bean gum (−50.6%), potassium phosphate dibasic (−34.7%), calcium citrate (−35.7%), corn starch (−36.7%), calcium chloride (−27.5%), and calcium ascorbate (−23.8%).

The average activities of each BCA-additive combination (factorial interaction A × B) are reported in Fig. 2. At the concentration used, the additives without the BCAs were poorly effective, except for calcium silicate, which showed a significant reduction of infected wounds (−60.7%). The reduction of rots produced by antagonists LS11, LS28, and LS30 applied alone were −52.3, −58.9, and −54.9%, respectively. The addition of adjuvants to cell suspensions of the BCAs, in general, enhanced their antagonistic activity, but the influence of each additive was different, depending on the biocontrol isolate.

Some of the better additives, i.e., the ones reducing more appreciably the percentage of infected wounds when applied in combination with BCAs compared with each BCA applied alone, were locust bean gum (−100%), calcium silicate (−88.4%), corn starch (−81.5%), calcium chloride (−77.4%), and xanthan gum (−76.8%), for isolate

TABLE 3. *Analysis of variance for the influence of three biocontrol agents (factor A) and twelve additives (factor B) on percentage of apple wounds infected by the postharvest pathogen* Penicilium expansum

Source of variability	Degrees of freedom	Mean square	*F* value	Significance (*P*)
Biocontrol agents (A)[a]	3	19,583.90	285.40	<0.001
Additives (B)[a]	12	766.66	11.17	<0.001
Interaction A × B	36	767.23	11.18	<0.001
Error	104	68.62		
Total	156			

[a] Untreated apple wounds (H_2O) are also included as a control.

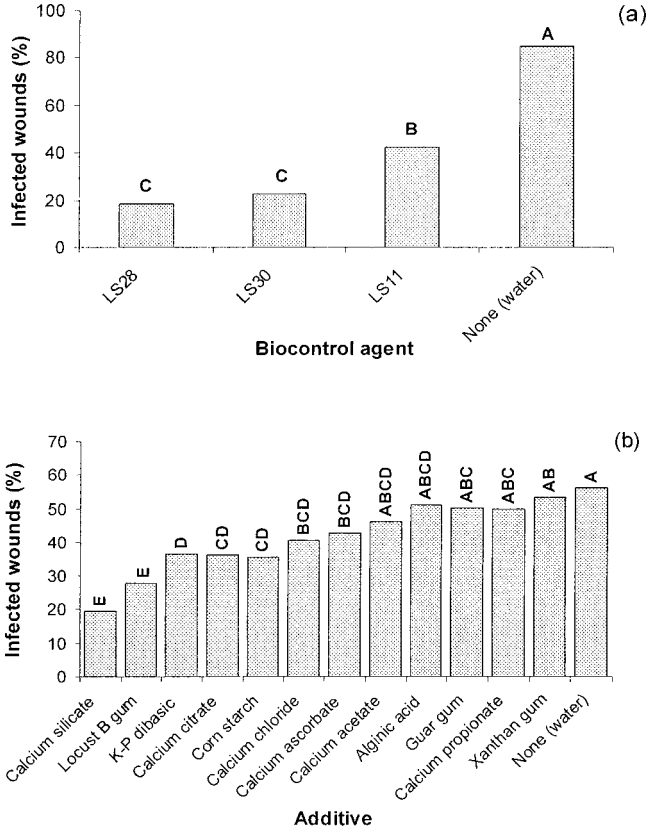

FIGURE 1. *Main effects of the factorial influence of the three biocontrol agents* R. glutinis *(LS11),* C. laurentii *(LS28), and* A. pullulans *(LS30) (a) and twelve additives (b) on the percentage of apple wounds infected by the postharvest pathogen* P. expansum*. Values marked by common letters are not statistically different at* P *= 0.01 (Duncan's test).*

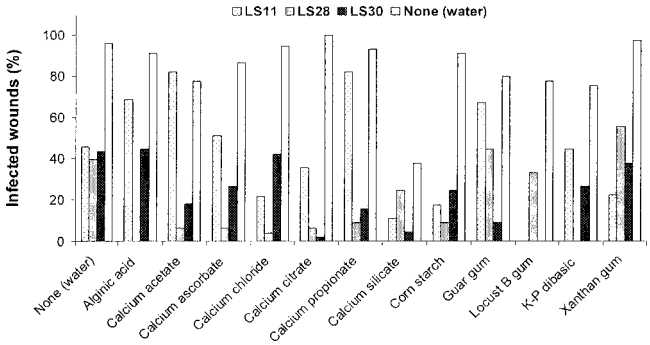

FIGURE 2. *Two-way interaction between the biocontrol agents* R. glutinis *(LS11),* C. laurentii *(LS28), and* A. pullulans *(LS30) and twelve additives and its effect on* Penicillium *rot of wounded apples. Means were separated by the least significant difference (LSD). At* P *= 0.05 and 0.01, LSD values were 13.41 and 17.75, respectively.*

LS11; alginic acid (−100%), potassium phosphate dibasic (−100%), calcium chloride (−95.9%), calcium ascorbate (−93.0%), and alcium citrate (−93.0%), for isolate LS28; and locust bean gum (−100%), calcium citrate (−97.7%), calcium silicate (−95.4%), guar gum (−90.7%) and calcium propionate (−83.8%), for isolate LS30.

To ascertain the type of interaction (antagonistic, additive, or synergistic), data regarding the selected additive-BCA combinations were further analyzed. Combination of additives with one or more BCA showed a synergistic activity, whereas some combinations produced additive or, in few cases, antagonistic activity (Table 2). The most effective mixtures, i.e., those with higher and significant values of synergistic factor (SF), were isolate LS11 plus calcium chloride (SF = 1.4), locust bean gum (SF = 1.4), or xanthan gum (SF = 1.4); isolate LS28 plus calcium chloride (SF = 1.6), calcium citrate (SF = 1.6), alginic acid (SF = 1.5), calcium ascorbate (SF = 1.5), calcium propionate (SF = 1.5), corn starch (SF = 1.5) or potassium phosphate dibasic (SF = 1.5); and isolate LS30 plus calcium citrate (SF = 1.8), locust bean gum (SF = 1.7), or calcium propionate (SF = 1.7).

Some BCA (10^6 cells per ml)-additive combinations with higher SF values were tested again on wounded apples to compare them with the two treatments applied separately and with the antagonist applied alone at a higher concentration (10^8 cells per ml) (Fig. 3). Regarding isolates LS11 and LS28, all additives confirmed the increase in BCA activity with respect to the BCAs applied alone at 10^6 cells per ml; moreover, the activity of yeast-additive combinations was comparable, or even higher (i.e., LS11–calcium chloride) than that of the yeasts applied alone at 10^8 cells per ml. Calcium citrate and calcium propionate significantly improved the activity of isolate LS30 applied at 10^6 cells per ml, although these mixtures were less effective than the yeastlike fungus applied alone at 10^8 cells per ml. The activity of locust bean gum combined with isolate LS30 was not statistically different from the activity displayed by the same BCA applied alone at 10^6 cells per ml.

DISCUSSION

The aim of this study was to identify adjuvants that would enhance the activity of three selected biocontrol agents against *P. expansum* on apples. This objective was successfully achieved, because different compounds dramatically improved the biocontrol activity of the tested antagonists. Factorial analysis showed that *Penicillium* rot on apples was highly influenced by both BCA and additive. Among the three BCAs examined, isolates LS28 and LS30 showed higher antagonistic activity than LS11, thus confirming our previous reports *(3, 28, 29).*

The interaction between BCA and additive was also highly significant, and the activity of each BCA was improved by different degrees by the same additive. This result is in agreement with Wisniewski et al. *(39)* who found that the addition of 90 or 180 mM, corresponding approximately to 1 or 2% (wt/vol), $CaCl_2$ as additive improved the activity of a biocontrol yeast strain, while it had no effect on another strain. However, in our study some additives had a positive effect to more than one biocontrol isolate. Some additives, in fact, improved the activity of two different biocontrol isolates (i.e., calcium chloride for LS11 and LS28, calcium citrate and calcium propionate for LS28 and LS30, locust bean gum for LS11 and LS30) and, therefore, are of particular interest.

At the tested concentrations (from 0.03 to 1%, wt/vol), the selected additives applied without the BCAs did not

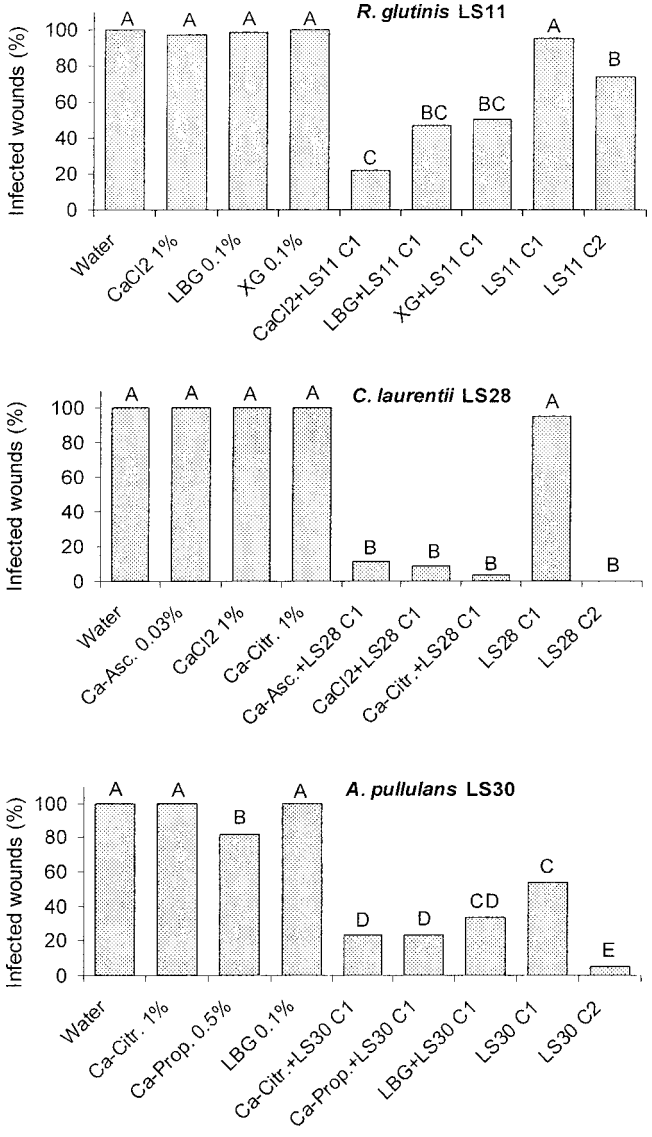

FIGURE 3. *Biocontrol activity of* R. glutinis *(LS11),* C. laurentii *(LS28), and* A. pullulans *(LS30), against* P. expansum *on apples, used alone or combined with some selected additives ($CaCl_2$, calcium chloride; LBG, locust bean gum; XG, xanthan gum; Ca-Asc., calcium ascorbate; Ca-Citr., calcium citrate; Ca-Prop., calcium propionate). Biocontrol agents alone were applied at a low concentration (C1, 10^6 cells ml^{-1}) and at a high concentration (C2, 10^8 cells ml^{-1}). Values marked by common letters are not statistically different for* $P = 0.01$ *(Duncan's test).*

reduce *P. expansum* infections, with the exception of calcium silicate. Similarly, the BCAs applied alone at lower concentrations provided an unsatisfactory control of *P. expansum.* Conversely, combinations of additives and low concentrations of BCAs were, in most cases, highly effective and, in addition, displayed a synergistic effect. These findings may suggest that the mechanism of biocontrol activity enhancement involves multifactor interactions among additive, biocontrol agent, host, and pathogen. Some of the most effective additives are calcium salts such as acetate, ascorbate, chloride, citrate, gluconate, lactate, propionate, and silicate. Among these compounds, only calcium chloride *(34, 39)* and propionate *(15)* have already been reported as enhancers of biocontrol activity. Other effective compounds that significantly enhanced biocontrol of *P. expansum* were alginic acid, corn starch, dibasic potassium phosphate, and different natural gums (guar, locust bean, and xanthan gum). Alginic acid, corn starch, and gums are natural polymers widely used in the food industry as stabilizers, thickeners, gelling agents, or emulsifiers, and many prepared and semiprepared foods could not be commercialized without using these compounds *(25).* Some of them, particularly alginic acid and its salts, were previously reported as suitable carriers for biocontrol bacteria and fungi *(11).* In various studies, xanthan gum was found to improve survival rate and BCA activity when applied in the field for the control of postharvest rots of strawberries and table grapes *(20).*

Different hypotheses can be made about the possible mechanisms by which the selected additives improve the activity of the examined BCA. Regarding calcium-based compounds, it is well known that Ca^{2+} ions are well tolerated by some biocontrol yeasts and act both on the host, by increasing tissue resistance *(9),* and on fungal pathogens such as *P. expansum,* by inhibiting its production of pectinolytic enzymes *(8, 39).* Calcium chloride is one of the most investigated salts and its enhancement of biocontrol activity was attributed to the Ca^{2+} ion, whereas the Cl^- anion does not seem to be involved *(39).* However, for some calcium-based additives examined in this investigation, a positive influence by the associated respective anions, e.g., a direct inhibition of the pathogen, cannot be ruled out. In fact, our previous in vitro assays showed that some acids (i.e., acetic, ascorbic, lactic, citric, and propionic), which in fruit wounds are mainly present as anions after application of respective Ca salts, drastically inhibited the growth of *P. expansum,* but they caused only a moderate or no inhibition of yeast growth *(31).* Accordingly, some of these acids such as acetic or ascorbic acid were also found to reduce infections of fungal pathogens on different fruits *(16, 37).* The positive influence of natural gums and other selected polymers could be due to their protection exerted on BCA cells. Xanthan gum, for example, has the capacity for moisture retention and, therefore, could reduce desiccation of antagonist cells and enhance survival of biocontrol yeasts when applied on fruit surfaces in the field *(21, 25).* With respect to alginates, these compounds appear to be able to protect antagonist cells from desiccation by enveloping them in their complex structure in which these microbes remain viable and physiologically active *(36).* Furthermore, high-molecular-weight polymers, such as xanthan gum, could induce resistance in pretreated host plants *(6).*

In the case of some tested additives, such as ascorbic acid, calcium ascorbate, and soybean lecithin, which are commonly used in foods as antioxidants, another mechanism could be suggested. Reactive oxygen species and free radicals are generated/induced at the wound site as a consequence of wounding or by necrotroph fungal pathogens *(3, 16).* It is conceivable that the activity of antioxidant additives could help BCA to cope with oxidative stress and

more efficiently colonize apple wounds, thus outcompeting the fungal pathogen.

In conclusion, this study has attempted to address the demand for a safer and environmentally friendly means for controlling postharvest diseases. We identified a range of candidate adjuvants displaying synergistic effects on BCAs and we demonstrated that an appropriate additive can provide a satisfactorily high level of fruit protection even using a moderately effective BCA, as for example, isolate LS11. The most effective compounds applied with low concentrations of BCA cells could, in turn, reduce the cost of fermentation processes for biomass production, thus encouraging the development of new and highly effective biofungicides.

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