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Effects of Potassium Sorbate on Postharvest Brown Rot of Stone Fruit

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ABSTRACT

The effect of potassium sorbate (K-sorb), a low-toxicity chemical, to control *Monilinia* spp. was investigated. Preliminary in vitro studies found the MIC of K-sorb for conidial germination and mycelial growth was, respectively, 260 and 1,250 mg/liter. Immersion of naturally infected peach and nectarine fruit in a solution (15 g/liter) of K-sorb for 120 s reduced brown rot by over 80% in four of five trials. Although treated fruits showed a significant reduction in firmness with respect to the control, they did not reach the overripe stage and retained acceptable quality parameters. In an attempt to elucidate the mechanism of action for K-sorb, the inhibition of enzymatic activity by K-sorb was also tested. In a radial diffusion assay, the addition of K-sorb to agarose reduced polygalacturonase (PG) activity across the concentrations considered. The greatest reduction (54.3%, with respect to the control) was obtained at a sorbate concentration of 15 g/liter. PG kinetic activity of *Monilinia laxa* observed by a spectrophotometric assay peaked after 40 min in all samples tested. PG activity was significantly higher in the control than in the samples with increased K-sorb concentrations. In conclusion, based on these findings, K-sorb can be recommended as a low-toxicity antifungal compound against *Monilinia* spp. in peaches and nectarines with its mode of action probably depending in part on the inhibition of PG activity in *M. laxa.*

Brown rot is one of the main diseases in stone fruit and occurs in the field during both pre- and postharvest. Three species of *Monilinia* are recognized as causal agents of brown rot: *Monilinia laxa* (Aderhold and Ruhland), *Monilinia fructicola* (G. Winter), and *Monilinia fructigena* (Aderhold and Ruhland) *(29).* Yield losses depend on weather conditions and are especially severe if high humidity, warm temperatures, and abundant rainfall prevail prior to harvest *(5).* The plant pathogen invades the fruit through wounds and injuries caused by insects, hail, or harvesting and handling operations. The conidia can also penetrate through microlesions, stomata, and lenticels of fruit skin *(28).* In some cases, infections occurring in the field remain quiescent until the fruit reaches maturity, allowing *Monilinia* to overcome host defenses. For this reason, if weather conditions are favorable to brown rot, then important losses can occur also during the postharvest phase, frequently estimated between 5 and 10% or more *(22).* In Italy and in European countries, this plant-only pathogen is controlled by fungicide spray programs in the field, since postharvest treatment is not allowed.

Public demand to reduce the use of fungicides or to eat fungicide-free products has stimulated the search for alternative methods, safe for humans and the environment, with encouraging results. Nevertheless, the control of plant pathogens by unconventional means such as microbial antagonists, natural substances, and compounds generally recognized as safe (GRAS) requires optimization of several key features before successful use *(2, 5, 26).* GRAS is a U.S. Food and Drug Administration designation and denotes a chemical or substance (including certain pesticides) that is added to food and considered safe by experts, and for this reason is exempt from the usual Federal Food, Drug, and Cosmetic Act food additive tolerance requirements *(33).*

Several GRAS compounds such as chlorine, peracetic acid, K-sorb, sodium bicarbonate, and calcium salts offer considerable promise in postharvest technology, showing antimicrobial, antifungal, and insecticidal properties *(8, 34).* Bicarbonate salts are widely used in the food industry for leavening, pH control, taste, and texture development; they also have a broad-spectrum antimicrobial activity *(1, 3),* inhibit the growth of bacteria and yeast, and control postharvest diseases *(3).* Furthermore, the potential for bicarbonate salts to control postharvest diseases has been demonstrated on a wide range of species including sweet cherries, apples and peaches, and melons, alone or in combination with biocontrol agents *(23).*

K-sorb, the potassium salt of sorbic acid ($C_6H_7O_2K$), is a widely known antimicrobial agent in foods. Its inhibitory action is strongly influenced by the type of food and the conditions of processing and storage *(11).* Sorbates are common food preservatives used for many applications and applied after harvest control *M. laxa* on sweet cherry *(23)* and *Botrytis cinerea* on citrus *(14).*

Polygalacturonases (PGs) are an important factor in fungal virulence *(31),* and their reduction is associated with pathogen inhibition or, in the extreme, fungistasis. Some evidence shows that GRAS compounds can reduce the severity of diseases by limiting the activity of pectic enzyme in postharvest pathogens on rambutan *(34).*

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The aims of this study were (i) to determine the 50% effective dose (ED_{50}), 95% effective dose (ED_{95}), and MIC of K-sorb on conidia germination and mycelial growth of *M. laxa* by in vitro tests; (ii) to evaluate its efficacy on peaches and nectarines naturally infected by *Monilinia* spp.; and (iii) to investigate the inhibition of enzymatic activity of *M. laxa* PGs by K-sorb.

MATERIALS AND METHODS

Plant pathogen. An *M. laxa* strain isolated from stored peaches showing typical brown rot was grown on V8 agar (160 ml of V8 vegetable juice and 25.6 g of agar technical per 480 ml of distilled water). Petri dishes were incubated at 25°C with 12 h dark–12 h light cycles for 10 days. A conidial suspension was prepared by washing colonies in sterile water and Tween 80 (0.05%); the suspension concentrations were adjusted with a hemacytometer to 1×10^3 conidia per ml.

Fruit. Fruit (*Prunus persica* (L.) Batsch) used in this study included peaches, cv. May Crest, Maria Marta, Elegant Lady, Rich Lady, Springbelle) and a nectarine, cv. Big Top obtained from a local packinghouse. Fruits free of visible wounds and rot and homogeneous in maturity and size were stored at 0°C and used for experiments within 5 days of harvesting.

In vitro test of mycelial growth and conidial germination inhibition. In order to test the activity of K-sorb on mycelial growth and conidial germination of *M. laxa,* petri dishes containing 20 ml of malt extract agar (Oxoid, Ltd. Basingstoke, UK) plus K-sorb (Carlo Erba, Milano, Italy) were prepared. Salt concentrations ranged between 0.18 and 0.26 g/ml, and 0.078 and 1.25 g/ml for conidial germination and mycelial growth, respectively. For conidial germination, a 100-μl aliquot of the conidial suspension was spread onto petri dishes, which were incubated at 20°C in darkness. Conidial germination was examined after 3 days of incubation at 20°C, by observing with a light microscope, at least 300 conidia for each K-sorb concentration. For mycelial growth, a 6-mm-diameter plug of mycelium was taken from the periphery of an actively growing agar culture and placed at the center of a 90-mm petri dish containing malt extract agar plus K-sorb. After 7 days of incubation at 20°C without light, the diameter (millimeters) of mycelial growth was measured. Malt extract agar without K-sorb was used as a control. Each concentration was represented by five petri dishes, and the experiment was repeated at least once.

K-sorb activity on fruit naturally infected by *Monilinia* spp. Fruits, neither wounded nor inoculated, were dipped for 2 min in a K-sorb solution (15 g/ml), allowed to air dry for 60 min at room temperature and then stored at 20°C for 5 days at high humidity (80%). Fruits dipped only in water served as controls. The sample unit was represented by four replications of 25 fruits each. The incidence of infected fruits was recorded and an effectiveness index was calculated using the following formula: effectiveness index (%) = [(control infected fruits − treated infected fruits)/control infected fruits] × 100.

Analysis of fruit quality parameters. The influence of K-sorb treatment on fruit quality was evaluated on twenty noninoculated fruits for each treatment of Springbelle peaches and Big Top nectarines. The physicochemical parameters considered included firmness, soluble solid content, and titratable acidity. Firmness (in newtons) was measured after removing the skin on opposite sides of each fruit using a Chatillon digital penetrometer fitted with an 8-mm fruit tester probe. Soluble solid content (percentage) was determined using a digital refractometer (Atago Co., Tokyo, Japan) in a portion of filtrate obtained by blending each fruit. Titratable acidity (milliequivalents per 10 ml of pure juice) was determined using an automatic titrator (Crison, Barcelona, Spain) by titrating fruit juice (obtained by diluting homogenized with distilled water in a ratio 1:5 and filtering the solution in a vacuum) with a 0.1 N sodium hydroxide solution up to pH 8.1.

K-sorb inhibition of *M. laxa* enzymatic activity: fungus growth. A modified liquid Pratt's medium *(32)* was prepared that contained (grams per liter): 13.6 KH_2PO_4, 4.0 NH_4NO_3, 1.25 $MgSO_4 \cdot 7H_2O$, 0.02 $FeSO_4 \cdot 7H_2O$, 0.001 $CuSO_4 \cdot 5H_2O$, 0.002 $ZnSO_4 \cdot 7H_2O$, 0.0013 $(NH_4)_2MoO_4$, 0.016 $MnSO_4 \cdot H_2O$, 0.028 H_3BO_3, and 1% yeast extract. After adjusting to pH 4.5 with HCl, the medium was dispensed in flasks, autoclaved for 15 min at 105 Pa, inoculated with 1 ml of a spore suspension of *M. laxa* (10^3 conidia per ml), and then stored at 20°C. Flasks containing only Pratt's medium were used as a control. Each concentration was represented by three flasks (replications), and the experiment was repeated once. Before inoculation, different aliquots of K-sorb were added to flasks to obtain six concentrations ranging from 31.25 to 1,000 mg/liter. After 7 days the suspension was filtered using a Millipore glass filter (0.45 μm; MF-Millipore membrane filters, Millipore, Inc., Bedford, Mass.), and *M. laxa* growth was evaluated as dry mycelium, weighing the filter after completely drying at 70°C for 1 day. In order to extract the *M. laxa* enzymatic complex, the filtration liquid was centrifuged at 13,000 × *g* for 30 min. The pellet was diluted with 1 ml of buffer (0.05 M NaCl in 1 liter of 0.1 M sodium acetate pH 5), centrifuged (13,000 × *g* for 30 min), and supernatants were subsequently collected, filtered, dialyzed with Spectra/Por membrane tubing (6,000 to 8,000 molecular weight cutoff; Spectrum Laboratories, Inc., Rancho Dominguez, Calif.) overnight at 4°C against 0.1 M sodium acetate (pH 5), and finally concentrated by polyethylene glycol compound (Sigma, St. Louis, Mo.) at 4°C for about 1 h. Enzyme extract was stored at 4°C until use.

K-sorb inhibition of *M. laxa* enzymatic activity: cup-plate assay. Based on previous work showing that *Aspergillus niger* (pectinase, Sigma) has endo-PG (EC 3.2.1.15) activity, the endo-PG inhibition at different K-sorb concentrations was determined by an agarose diffusion assay (evaluating a square-millimeter halo area), in accordance with Tamura et al. *(35),* with some modifications. In the control, an agarose sheet was prepared by pouring 100 ml of microwave oven–melted 1 g of agarose, 0.001 g of polygalacturonic acid (Fluka, Milano, Italy), and 0.365 g of EDTA (Sigma) in 0.1 M sodium acetate buffer (pH 5.0) into a Plexiglas frame (23 by 23 cm). In the same way, inhibition activity by different concentrations of K-sorb against pectinase from *A. niger* was analyzed, dissolving 0.375, 0.75, and 1.5 g of K-sorb in 100 ml of agarose solution. When the agar solidified, 4-mm-diameter wells were cut in the agar sheet, using a cork borer. Each K-sorb concentration was represented by three samples of 15 μl of total volume, loaded into wells. Pectinase from *A. niger* represented the standard. The plate was incubated overnight at 37°C and dyed with ruthenium red (Fluka, Buchs, Switzerland), staining PG substrate for PGs. After 1 h, the plate was rinsed with distilled water several times over 1 h to remove excess (i.e., unbound) dye. PG activity was then assessed by measuring the area around wells that had been cleared by ruthenium red.

K-sorb inhibition of *M. laxa* enzymatic activity: spectrophotometer assay. PG kinetic activity of *M. laxa* was detected using a spectrophotometer (Agilent Technologies, Santa Clara, Calif.), in accordance with Wang et al. *(37),* with some modifications.

TABLE 1. *Effect of treatments with K-sorb on conidial germination and mycelial growth of* M. laxa[a]

Effect	MIC (mg/liter)	ED_{50} (mg/liter)	ED_{95} (mg/liter)
Mycelial growth	1,250 ± 3.3	160 ± 6.9	500 ± 5.55
Conidial germination	260 ± 1.6	210 ± 2.91	260 ± 1.16

[a] Fungicidal activity on mycelial growth and conidia germination was evaluated 7 and 3 days, respectively, after inoculation. Values are means of five replicates ± standard errors.

The method was based on the hydrolytic release of reducing groups from polygalacturonic acid. The reaction mixture contained 3 ml of substrate 0.2% (wt/vol) polygalacturonic acid, 3 ml of buffer (0.1 M sodium acetate, pH 5), and 50 μl of a solution containing concentrated enzyme from K-sorb concentrations. K-sorb concentrations ranged from 0 (control) to 1,000 mg/liter. The reaction mixture was incubated in a water bath at 30°C for 0, 20, 40, and 60 min. The reaction was stopped by adding 0.2 ml of reaction mixture to 1 ml of sodium borate (0.1 M, pH 9.6) and 0.3 ml of 1% 2-cyanoacetamide (Sigma), and immersion in a boiling water bath for 10 min. Blank enzyme samples and blank substrate samples were determined in the same way without addition of substrate or without enzyme, respectively.

The standard reaction mixture contained polygalacturonic acid solution (0.2%) as reagent and was heated in a boiling water bath for 10 min. Absorbance of the samples, blanks, and standards was measured in triplicate at 276 nm.

K-sorb inhibition of *M. laxa* enzymatic activity: protein assay. Protein was determined by the method of Bradford *(7)* using a protein assay dye reagent concentrate (Bio-Rad, Hercules, Calif.) and bovine serum albumin (Sigma) as a standard, by recording the absorbance at 595 nm.

Statistical analysis. ED_{50} and ED_{95} were calculated using probit analysis applied to the percentages of germination and mycelial growth inhibition resulting from in vitro experiments. Regression lines between the logarithm of the K-sorb concentrations and the effectiveness indices transformed in probit were calculated *(13).* All data were processed with analysis of variance using SYSTAT 5.3 *(38).* Separation of means was performed according to the least significant difference (LSD) test at $P \leq 0.05$. All experiments were carried out in a completely randomized design.

RESULTS

In vitro test of mycelial growth and conidial germination inhibition. The regression lines between the logarithm of the compound concentrations and the effectiveness indices transformed in probit, used to calculate ED_{50} and ED_{95}, were highly significant. The correlation coefficients were 0.93 for mycelial growth and 0.90 for conidial germination (data not shown). The MIC of K-sorb for *M. laxa* mycelial growth (Table 1) was 1,250 mg/liter. At this concentration, K-sorb was fungicidal because no mycelial growth was observed after 14 days at 20°C (data not reported). Furthermore, ED_{50} and ED_{95} were 160 and 500 mg/liter, respectively. The K-sorb MIC for conidial germination was 260 mg/liter. No conidia germinated after 8 days at 20°C. The ED_{50} of 210 mg/liter was higher than the ED_{50} for mycelial growth and the ED_{95} of 249 mg/liter was about half the ED_{95} of mycelial growth. However, the colonies produced by conidial germination were noted to have a smaller diameter than the control, probably referable to a fungistatic action of K-sorb.

TABLE 2. *Effect of K-sorb treatment (15 g/liter) on* Monilinia *rot in naturally infected fruits after 4 days at 20°C*[a]

Cultivar	Control (% of infected fruit)	K-sorb (% of infected fruit)	EI (%)[b]
May Crest (peach)	45 A		
		6.2 B	92
Elegant Lady (peach)	81.2 A	8.3 B	89.8
Springbelle (peach)	48.3 A	5 B	89.6
Big Top (nectarine)	42.5 A	5 B	88.2

[a] Fruits were treated by dipping for 2 min. Values for each treatment correspond to the mean of 20 fruits per five replicates. Within each cultivar, the values followed by the same letters are not significantly different according to the least significant difference test ($P \leq 0.05$).

[b] EI, effectiveness index (%) = [(control infected fruits − treated infected fruits)/control infected fruits] × 100.

K-sorb activity on fruit naturally infected by *Monilinia* spp. and its influence on fruit quality. Fruit treated with K-sorb at 15 g $liter^{-1}$ showed a significant reduction in *Monilinia* infections after 5 days at 20°C (Table 2). The effectiveness index of K-sorb ranged between 92.5% in Maria Marta and 35.3% in May Crest peaches. No visible damage was observed on the fruit skin after K-sorb treatment.

Fruit quality was influenced by treatment with K-sorb (15 g/liter), firmness was significantly reduced (−25%), and soluble titratable acidity (−7%) (Table 3). In treated fruit, the soluble solid showed a different behavior in relation to the cultivar: it increased in peaches and decreased in nectarines.

Inhibition of *M. laxa* enzymatic activity by K-sorb treatment. As shown in Figure 1, fungal dry weight was significantly reduced, with respect to the control, by K-sorb at all tested concentrations. After 7 days of incubation at 20°C, in the control (without K-sorb), the mycelium dry weight was 5.5 mg, while in the solution of K-sorb at 31.25

TABLE 3. *Effect of K-sorb treatment (15 g/liter) on quality parameters of a peach and nectarine*[a]

Cultivar	Firmness (N)	Acidity (meq/10 g pulp)	Total solid soluble (°Brix)
Springbelle (peach)			
Control	8.5 A	1.31 A	11 A
K-sorb	6.4 B	1.22 B	11.2 B
Big Top (nectarine)			
Control	10.4 A	0.8 A	13.4 A
K-sorb	7.6 B	0.7 B	11.5 B

[a] Values for each treatment correspond to the mean of 20 fruits per five replicates. Within each cultivar, the values followed by the same letters are not significantly different according to the least significant difference test ($P \leq 0.05$).

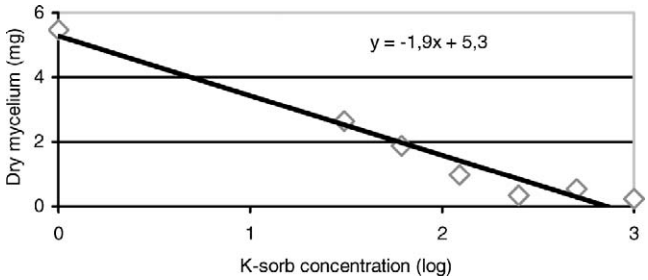

FIGURE 1. *Effect of K-sorb on* M. laxa *dry weight. After 7 days of incubation at 20°C, mycelium was filtered, and pathogen growth was determined by filter weight (milligrams) after completely drying at 70°C for 1 day. Values for each concentration correspond to the means of three flasks (replicates). Correlation coefficient* (R) = −0.98 ($P < 0.05$).

mg/liter it was only 2.63 mg (−52.2%). A greater reduction in pathogen growth was obtained at higher K-sorb concentrations.

In the cup-plate assay, the enzymatic activity of commercial PGs from *A. niger* showed considerable activity on the pectinase medium, producing a halo of 891 mm^2 (Fig. 2). The addition of K-sorb to agarose reduced PG activity across the tested concentrations. The greatest reduction (54.3%) was obtained with the 15 g/liter concentration (halo of 407 mm^2). Lower concentrations of K-sorb (3.75 and 7.5 g/liter) reduced PG activity by 28.8 and 42.1%, respectively. As expected, there was no activity in the negative controls (buffer and water, alone; data not shown). PG kinetic activity (Fig. 3) of *M. laxa* peaked after 40 min in all samples tested. PG activity was significantly higher in the control than in fruit treated with K-sorb. The 0.5 g/liter K-sorb solution reduced PG activity by 60% with respect to the control.

DISCUSSION

In this study, data showed the activity of K-sorb against *Monilinia* spp. Sorbic acid and its potassium salt have long been used as food additives in order to prevent microbial growth. Recently, since the interest for alternative chemicals increased, K-sorb has been tested, compared with other food additives, on fresh fruits in order to control postharvest decay. Preliminary investigations in in vitro trials against *Geotrichum candidum (16), Penicillium expansum (36), B. cinerea (39),* and *Aspergillus flavus (20)* showed a wide range of MICs, between 400 and 5,000 μg/ml, depending on the fungus. In this study, the MIC of *M. laxa* for mycelial growth fell in the same range (1,250 μg/ml), although the MIC or conidial germination was almost fivefold less (260 μg/ml).

K-sorb treatments were effective on artificially infected fruits such as table grapes inoculated with *B. cinerea (15),* lemons inoculated with *G. candidum (16),* and citrus fruits inoculated with *Penicillium digitatum* and *Penicillium italicum (30).* In our trials, we obtained good control (over 80% in four of five trials) on peaches and nectarines naturally infected with *Monilinia* spp. Brown rot infections occur over the season, remaining latent and developing during the postharvest phase, right up to the time the fruit reaches the consumer's home. They are extremely dangerous for at least three reasons: first, their control with preharvest fungicide applications is difficult; second, in Europe postharvest chemical treatments are not allowed; and third, the retail value of harvested fruit is high. In Italy, in a scheduled program, the early cultivars of peaches and nectarines (Springbelle, Rich Lady, and Big Top) are subjected to only one fungicide treatment, at bloom, but it frequently fails to control postharvest rot, in particular when weather conditions favorable to the plant pathogen occur. In our trials, untreated fruits showed a disease incidence of over 40% up to 81.2%; K-sorb treatment, however, showed good control (89.8%) of brown rot even in the worst case.

All of our fruit reached the eating quality standard ($N < 13$) after 4 days at 20°C *(27).* Although K-sorb treated fruit showed a significant reduction in firmness with respect to control, they did not reach the overripe stage ($N = 5$) *(25).*

The mode of action of sorbic acid has been investigated for a long time, and many hypotheses have been discussed *(9, 12, 17).* One of these suggested that the activity of sorbates against fungi and yeast relies on a decreasing pH value in the intercellular part, after ionization of acid molecules *(19),* but this hypothesis is in contrast with the study of Bracey et al. *(6)* on *Saccharomyces cerevisiae.* The authors found that growth inhibition of this yeast correlated with activation of an energy-dependent mechanism, which compensated for alterations of intercellular pH homeostasis,

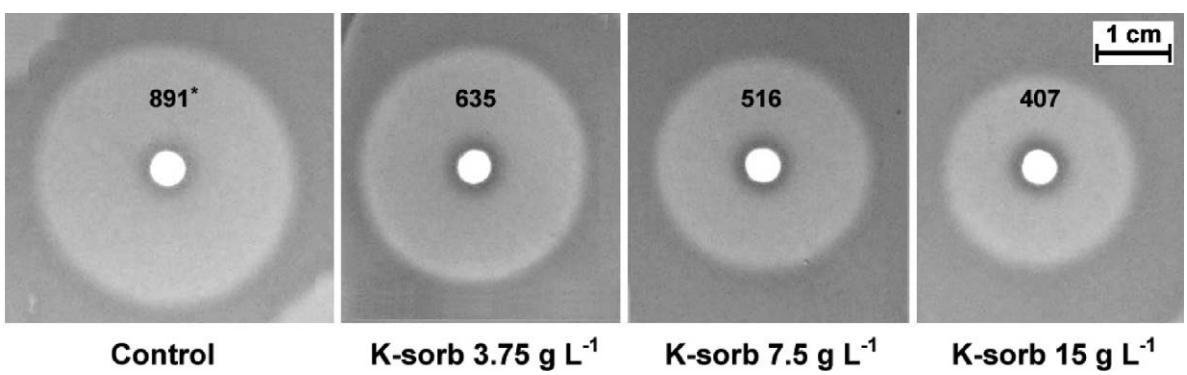

FIGURE 2. *Radial diffusion assay of different K-sorb concentrations against* A. niger *from commercial pectinase. *, Area halo (square millimeters) is the mean of three repetitions.* R = −0.98 ($P < 0.05$).

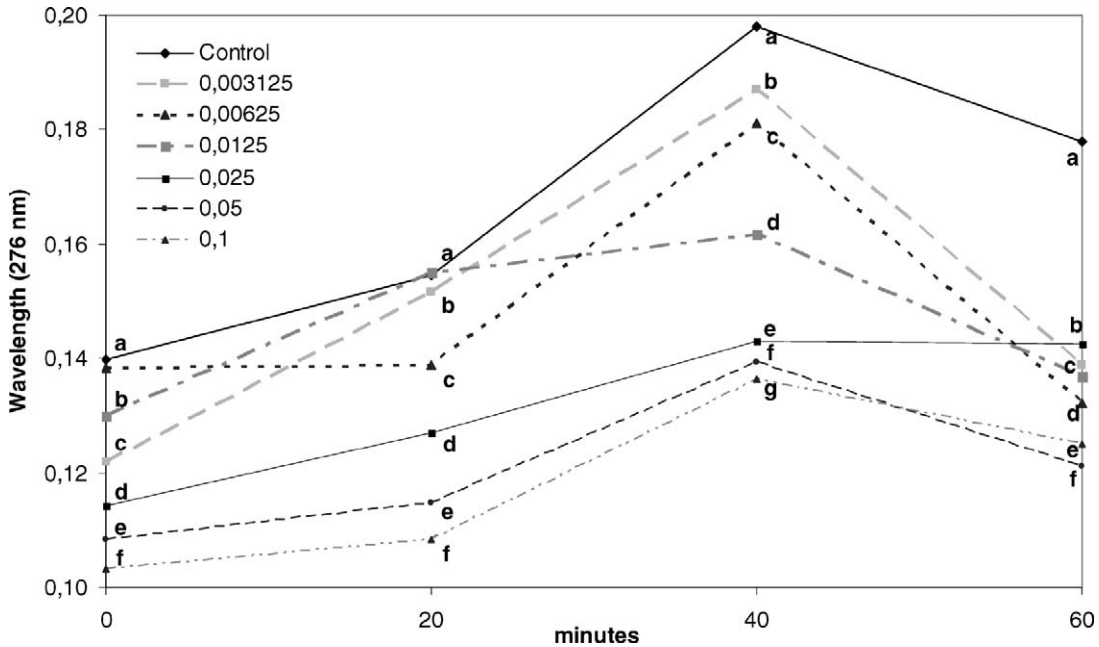

FIGURE 3. *Effect of K-sorb concentrations against PG activity of* M. laxa. *Within each period, values followed by the same letters are not significantly different according to the least significant difference test ($P \leq 0.05$).*

reducing the available energy for normal growth. Furthermore, sorbate and benzoate are compounds with lipophilic features and can affect the permeability of microbial cell membranes by inhibition of proton gradients across the cell and mitochondrial membranes, which followed is by their disorganization *(15).* Another mechanism involved in the inhibition of microbial growth by sorbic acid is the effect on enzymes. Fumarase, aspartase, succinic dehydrogenase, ficin, and alcohol dehydrogenase are only a few of the enzymes inhibited by sorbic acid *(25, 40).* In in vitro trials, Lyon and McGill *(21)* found that the mode of action of benzoic acid, another weak organic acid–like sorbic acid, is the inhibition of PGs and polygalacturonic acid lyase from *Erwinia carotovora.* These results are partially in agreement with our data on the *M. laxa* PG activity reduction induced by K-sorb. Nevertheless, no reduction in polygalacturonic acid lyase was observed (data not reported). The effect of different K-sorb concentrations on fungus dry weight accumulation in a liquid Pratt's medium correlated with PG activity; the same results were obtained with calcium salts on *M. fructicola (4).* PGs are an important factor of virulence *(31),* and the reduction of their activity can delay the infection process and produce a fungistatic effect (data not reported).

Based on the results obtained in our study and because of some K-sorb features, K-sorb can be recommended as an antifungal compound for peaches and nectarines. It is in fact the most readily soluble of the sorbates and suitable for the postharvest handling practices that use water to float fruit out of field bins and to remove field heat from fruit by hydrocooling. Sorbic acid and its derivates are commonly used to control mould growth and extend the shelf life of several foods (cheeses, vegetables, sauces, and meats) and also to inhibit growth and mycotoxin production by the mycotoxigenic molds *A. flavus, A. parasiticus (10), P. expansum,* and *P. patulum (18).*

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