Nutritional Enhancement of Biocontrol of Blue Mold on Apples

W. J. Janisiewicz, J. Usall, and B. Bors

USDA-ARS, Appalachian Fruit Research Station, 45 Wiltshire Road, Kearneysville, WV 25430.
Present address of second author: Universitat Politecnica De Catalunya, Department De Technologia D'Aliments, Avda. Rovira Roure, 177, 25506 Lleida, Spain.
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ABSTRACT

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Thirty-six carbohydrates and twenty-three nitrogenous compounds were evaluated for their effect on germination, and germ tube and radial growth of the pathogen *Penicillium expansum*, and for stimulation of growth of an antagonist *Pseudomonas syringae* (strain L-59-66). Compounds that strongly stimulated growth of the antagonist but had little or no stimulatory effect on germination and growth of the pathogen were evaluated for their effect on enhancing biocontrol of blue mold on ripe Golden Delicious apple. Seven amino acids and two carbohydrates met the required criteria. *myo*-Inositol and L-arabinose at 50 μg/ml had no effect and at concentrations ranging from 0.1 to 12.8 mg/ml actually reduced biocontrol. L-Asparagine and L-proline greatly enhanced biocontrol at concentrations of 20 and 80 mM. Enhancement effects were strongest at the highest concentrations of the antagonist (1.7 and 5.4 × 10^8 cfu/ml). Both amino acids enhanced the population of the antagonist on fruit by more than one order of magnitude. Thus, nutrients can be used to enhance antagonist population size and the resulting biocontrol of blue mold on ripe apple. Both amino acids strongly stimulated germination of *P. expansum* but had little effect on radial growth, indicating that inhibition of the mycelial phase of fungal development is the most important factor in this biocontrol system.

Additional keywords: postharvest, pome fruits, storage.

Biological control of postharvest pathogens of fruit and vegetables is a rapidly developing and relatively new area of research compared to biological control of pathogens on other plant parts including roots (15,16,25). Progress has been so rapid during the last few years that some biocontrol agents are approaching commercialization. One pilot test has been conducted on a commercial scale (21). Reliability and economics are the two major factors that will determine the feasibility of any biocontrol system. Successes in biocontrol of postharvest diseases can be attributed to highly reproducible results under controlled storage conditions. Storage practices affect biocontrol and might be manipulated to favor the antagonist. Fruit maturity also affects biocontrol because higher concentrations of the antagonist must be applied as fruit matures (W. J. Janisiewicz, *unpublished data*). However, application of the antagonist at higher concentrations makes biocontrol less economical. One alternative might be to manipulate the antagonist populations on fruit to obtain acceptable biocontrol levels. Nutrients affect populations of epiphytic microorganisms, and competition for nutrients on plant surfaces is an important aspect of the biocontrol mechanism (1–3,6–9,19). The application of a single organic compound to the surface of snap bean leaves altered the composition of the bacterial community and reduced bacterial brown spot (19). The application of organic nitrogen enhanced production of biomass and antifungal compounds by the antagonistic *Gliocladium virens* and *Trichoderma* spp., which are used to control soilborne pathogens (14). The mechanism of biocontrol of blue and gray molds on pome fruits by a saprophytic strain of *Pseudomonas syringae* (strain L-59-66) has not been explained (18), but higher populations of this antagonist on mature fruit at the wound site might enhance control of the diseases. The objective of this study was to determine which nutrients enhance the population of the antagonist *P. syringae* (L-59-66) and improve biocontrol of blue mold on ripe apples. A preliminary report has previously been presented (17).

MATERIALS AND METHODS

Antagonist. The antagonist strain L-59-66 used in this study was identified previously as a nonpathogenic strain of *P. syringae* (18). Bacterial cells were obtained from cultures grown in 250-ml Erlenmeyer flasks with 50 ml of nutrient yeast dextrose broth (NYDB) (per liter: 8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose) on a rotary shaker at 150 rpm for 24 h at 24 C. Cells were harvested by centrifugation at 7,000 rpm for 10 min, washed twice with 0.05 M phosphate buffer at pH 6.8 (11), and resuspended in aqueous solutions of 0, 5, 20, or 80 mM of L-asparagine or L-proline. The concentration of cells in the suspensions was adjusted with a PC 900 colorimeter (Brinkman Instruments, Westbury, NY) fiber optic probe.

Pathogen. Isolate MD-8 of *Penicillium expansum* Link was isolated from decayed apple and maintained on potato-dextrose agar (PDA) with periodic transfers through apple. This is the most aggressive isolate in our collection. A conidial suspension (10^4 conidia per milliliter) was prepared from 10-day-old cultures grown on PDA as previously described (18). Precautions were taken to avoid contact between the solution used for collecting conidia and PDA medium to prevent contamination of the suspension with nutrients from the medium.

Fruits. Golden Delicious apples were harvested from a commercial orchard in Kearneysville, WV, maintained under standard cultural practices, and stored in regular storage at 1 ± 1 C. At harvest the firmness of the fruit was 75.6 N as determined by an Effegi penetrometer (Effegi, 4801 Alfonsine, Italy). At the beginning of the biocontrol and antagonist recovery tests, fruit firmness was 44.5 N, starch-iodine was mainly at stage six (20), and soluble solids were 12.4%. For the second biocontrol experiment and for inhibition of biocontrol with streptomycin, fruit were purchased from a local packinghouse. At the beginning of the experiment fruit firmness was 55.6 N, while starch-iodine was at stage five, and soluble solids were 11.7%.

Chemicals. The carbohydrates, amino acids, and other nitrogen compounds used in this study were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI.

Effect of nutrients on the pathogen. Tests for conidia germination, germ tube, and mycelial growth were conducted in petri

plates (30 mm diameter) with 1.2% Phytagel (Sigma Chemical Company, St. Louis, MO) medium containing the desired nutrient concentrations. Aqueous solutions of the nutrients were filter sterilized and added to the cool medium before pouring. The final concentration of carbohydrates in the medium was 50 μg/ml, and of amino acids and other nitrogen compounds were 1 mM. Phytagel medium containing dextrose or L-alanine were used as positive controls for carbohydrates and nitrogenous compounds, respectively. Phytagel alone was used as a negative control. For germination and germ tube growth tests, 100 μl of a conidial suspension (10^6 conidia per milliliter) was spread over the surface of the medium. Plates were incubated at 20 C for 24 h, after which a drop of lactophenol-cotton-blue (5) was placed in the center of the plate and covered with a slide coverslip. Conidia germination and germ tube length were determined microscopically. Conidia were considered germinated when germ tubes exceeded the conidium diameter. Two plates were used for each compound, and 50 conidia were observed per plate. For colony growth measurements, plaques 3 mm in diameter and approximately 1 mm thick were removed from the margin of a fungal colony growing on PDA and placed in center of each plate. Plates were incubated for 168 h at 20 C, and the diameter of the colony was measured. To determine radial growth, the diameter of the plaque was subtracted from the measured colony diameter. The positive and negative controls were the same as in the germination tests.

Effect of nutrients on antagonist growth. The test for utilization of amino acids and other nitrogenous compounds and carbohydrates was conducted in regular and in Biolog MT microtiter plates (Biolog Inc., Hayward, CA) containing tetrazolium violet as an indicator of oxidation of a substrate by bacteria. A minimum salt medium with no carbon or nitrogen source was used for each test. This medium contained per liter: 3.49 g of $Na_2HPO_4 \cdot 2H_2O$, 2.77 g of KH_2PO_4, 20 ml of trace elements (Hutner's vitamin-free mineral base [12], 0.01 mg of biotin; 1.0 mg of nicotinic acid; and 0.5 mg of thiamine hydrochloride). The medium was adjusted to pH 6.8 before autoclaving. Carbohydrate utilization was determined in minimum salts basal medium supplemented with NH_4CL (1 g/L) as the nitrogen source and various stock solutions of carbohydrates. To test for utilization of amino acid and other nitrogenous compounds, the minimum salt-based medium was supplemented with the carbon source, glucose (1 g/L), and various stock solutions of amino acids and other nitrous compounds. All solutions were filter sterilized. Concentration of the tested compounds in the media was 2× the desired final concentration to compensate for the dilution when adding the antagonist suspension. Compounds containing nitrogen were tested at 1, 5, and 20 mM concentrations, and carbohydrates were tested at 0.1, 0.5, and 1.0 mg/ml. The above concentration ranges were selected because they supported good growth of the antagonist with the most stimulatory compounds and allowed detection of the toxic effect of some compounds at the higher concentrations. The antagonist suspension was prepared as previously described, except that after two washings in phosphate buffer, cells were suspended in a solution of minimum salt basic medium containing NH_4Cl or glucose for testing carbohydrates and nitrogenous compounds, respectively. Concentration of the antagonist was adjusted to 5.4×10^8 cfu/ml with the PC 900 colorimeter. Seventy-five microliters of the antagonist suspension and 75 μl of the tested nutrient solution were added to each well of microtiter plates. Controls consisted of the antagonist suspension mixed with appropriate minimal salts basic medium (containing either NH_4Cl or glucose). Plates were incubated at 24 C for 24 h, after which absorption of the wells was determined with the SLT (SLT-Labinstruments Ges.m.b.H. Salzburg, Austria) plate reader at 590 nm. Higher levels of absorption indicated greater utilization of a compound. Each well consisted of a single replicate, and there were six replicates per treatment. The experiment was repeated using regular microtiter plates, and the ranking of the compounds was similar. Results from 20 mM and 0.5 mg/ml concentrations for the nitrogenous and carbohydrate compounds, respectively, were used to compare the nutrient utilization list of the antagonist with that of the pathogen. In contrast to the list developed for the pathogen, compounds were listed from most to least utilized by the antagonist. The first 10 compounds from the top of the list developed for the pathogen (compounds least utilized by pathogen) were marked on the antagonist utilization list. Compounds marked in the top seven and 10 positions for amino acids and carbohydrates, respectively, were used for further studies to enhance biocontrol on fruits.

Fruit tests. The antagonist was suspended in aqueous solutions of the compounds selected from in vitro tests. Concentrations of carbohydrates ranged from 0.05 to 12.8 mg/ml in 2× increments, and amino acid concentrations were 5, 20, or 80 mM. Each of the five concentrations of the antagonist (1.2, 3.0, 8.0, 17, 54×10^7 cfu/ml) was suspended in each concentration of a nutrient solution. The concentrations of the antagonist were adjusted with a PC 900 colorimeter. Golden Delicious apples were wounded by removing blocks of tissue 3 × 3 × 3 mm at two sites 2 cm apart along the calyx-stem axis. Twenty-five microliters of the antagonist suspensions was placed in each wound. Each wound was inoculated with 20 μl of 10^4 conidia per milliliter of *P. expansum* within 30 min after application of the antagonist. Fruits were placed on fruit trays in plastic boxes and incubated at 22 ± 2 C for 7 days. At that time the diameter of the lesions developing from the wounds was measured perpendicular to calyx-stem end axis. There were five fruits per treatment and treatments were replicated three times. Treatments were arranged in randomized block design. For the amino acids, each box contained a control and three concentrations of an amino acid at a single concentration of the antagonist. For carbohydrates, because of the larger number of concentrations tested, treatments were placed in blocks of three boxes. The experiment was repeated once. Less mature fruit (firmness 55.6 N), purchased locally, were used in the second study.

Recovery of the antagonist. Compounds selected from in vitro tests were evaluated for their effect on the population dynamics of the antagonist on the fruit at the wound site. Antagonist suspensions at 1.2×10^7 cfu/ml were prepared in 80 mM aqueous solutions of the test compounds. Fruit were wounded on the equator, and 20 μl of the antagonist suspension was applied to each wound. Fruit were placed on fruit trays in plastic boxes and incubated at 22 ± 2 C. Fruit samples were taken from the boxes to determine antagonist population 1 h after inoculation (time 0) and after 2, 4, 8, and 16 days. To recover the antagonist, wound tissue was removed with a cork borer (1 cm diameter × 1 cm deep). The resulting cylinder was placed in a mortar with 1 ml of 0.05 M phosphate buffer at pH 6.8 (11) and ground with a pestle. Serial 10-fold dilutions were made in phosphate buffer, and 0.1 ml of each dilution was plated on NYDA medium in triplicate. Plates were incubated at 24 ± 2 C for approximately 48 h, and the colonies were counted. There were three replicates of three fruit each per treatment. The treatments were arranged in a completely randomized design. In an exploratory experiment, effects of L-proline and L-asparagine were tested at different times, but in the second experiment they were tested concurrently. The results were similar and we report only on the second experiment.

Inhibition of biocontrol. To determine the critical period that determines the effectiveness of biocontrol, both antagonist and pathogen were applied to two wounds on each fruit (25 μl of antagonist at 5.4×10^8 cfu/ml and, within 30 min, 20 μl of 10^4 conidia per milliliter of *P. expansum* per wound) then 25 μl of an aqueous solution of streptomycin (100 mg/L) was applied to the wounds of the treated fruit at 0, 6, 24, and 48 h after inoculation of antagonist and pathogen. Fruit were incubated on fruit trays in plastic boxes at 22 ± 2 C. Number of infected wounds (visible rot development) was determined after 10 days. Firmness of the fruit was 55.6 N. There were three fruit per treatment and each treatment was replicated three times. Treatments were arranged in randomized block design. The experiment was repeated once.

Data analysis. Model for response surfaces of the effect of L-asparagine and L-proline on lesion size and percentage of wound infected was based on estimates of parameters from general linear

models (GLM) of a statistical analysis system (SAS), based on type III sums of squares of balanced linear model and randomized block design (22). An LSD test was conducted on fruit rot data from fruit protected and not protected with the antagonist and treated with streptomycin. Control not treated with streptomycin was included in analysis.

RESULTS

Effect of nutrients on antagonist. Fourteen out of 36 carbohydrates (Table 1), and seven out of 23 nitrogenous compounds (Table 2) were strongly utilized (increase in OD > 0.1) by the antagonist. The remaining compounds were not significantly utilized. As the concentration of the carbohydrate increased, utilization also increased, with the exception of D-trehalose dehydrate, where utilization declined from OD = 0.347 at 0.5 mg/ml to OD = 0.212 at 1 mg/ml concentration.

In general, as the concentration of the nitrogenous compounds increased from 1 to 20 mM, utilization by the antagonist also increased. However, at several higher concentrations, utilization declined, e.g., for L-leucine, OD was 0.137, 0.062, and 0.017 at 1, 5, and 20 mM concentrations, respectively, and for *trans*-4-hydroxy-L-proline OD declined from 0.156 at 5 mM to 0.056 at 20 mM concentration. L-Glutamic acid was better utilized at 5 mM (OD = 0.247) than at 1 or 20 mM (OD = 0.113 and 0.198, respectively). The most stimulatory amino acids and carbohydrates at 20 mM and 0.5 mg/ml concentrations, respectively, were utilized by the antagonist at a similar rate (similar OD values). Therefore, results from these concentrations were used to compare nutrients best utilized by the antagonist with the nutrients that least stimulated germination and growth of *P. expansum*.

Effect of nutrients on pathogen. Ranking of the compounds according to their effect on conidia germination and germ tube growth was similar; thus, only results from germination are reported. Germination of *P. expansum* conidia was not stimulated by 10 carbohydrates, and another 10 stimulated germination by less than 2%. All of these compounds and 10 carbohydrates that least stimulated radial growth were marked on an antagonist utilization list. Only two of these carbohydrates were among 10 best utilized by the antagonist (Table 1). Those compounds (*myo*-inositol and L-arabinose) were tested for enhancing biocontrol of *P. expansum* on apples.

All nitrogenous compounds affected germination and radial growth of *P. expansum*. The least stimulatory compounds were marked on the antagonist utilization list. The first seven top compounds that were marked were used in tests to enhance biocontrol of *P. expansum* on apples (Table 2).

Enhancement of biocontrol. Tests on fruit with the same carbohydrate concentrations that were used in in vitro tests did not affect the efficiency of the biocontrol; therefore, higher concentrations, up to 12.8 mg/ml of carbohydrates in the antagonist suspensions, were used. None of the carbohydrates enhanced biocontrol, and, at the higher concentration, significant reduction in biocontrol was observed (W. J. Janisiewicz, *unpublished data*). Out of seven amino acids tested, only L-asparagine and L-proline significantly reduced lesion size and percentage of wounds infected. In general, lesion size decreased with an increase in con-

TABLE 1. Effect of carbohydrates on radial growth and conidia germination of *Penicillium expansum* and their utilization by *Pseudomonas syringae*

P. expansum			*P. syringae*		
Compound	Radial Growth (mm)	Compound	Germination (%)	Compound	Optical Density
#[a] 2-Deoxy-D-glucose	10.7	*[a] α-D-Lactose	0.0	D-Glucose	0.406
# Melezitoze dihydrate	17.4	* β-D-Lactose	0.0	D-Fructose	0.376
# D-Arabinose	17.7	* D-Arabinose	0.0	* L-Arabinose	0.375
# D-Fucose	18.2	* D-Glycero D-gulo heptose	0.0	D-Sorbitol	0.365
# 3-*O*-Methl-D-glucopyranose	19.7	* D-Lyxose	0.0	D-Trehalose dihydrate	0.347
# D-Raffinose pentahydrate	19.7	* 2-Deoxy-D-galactose	0.0	D-Mannitol	0.310
# L-Lyxose	20.4	* 2-Deoxy-D-glucose	0.0	D-Ribose	0.305
# L-Mannose	20.4	* L-Lyxose	0.0	# *myo*-Inositol	0.302
# Palatinose	21.0	* L-Mannose	0.0	D-Galactose	0.270
# *myo*-Inositol	21.4	* 3-*O*-Methyl-D-glucopyranose	0.0	2-Deoxy-D-ribose	0.249
α-D-Melibiose hydrate	21.7	* D-Fucose	0.3	D-Xylose	0.212
D-Ribose	21.7	* L-Fucose	0.4	D-Mannose	0.156
2-Deoxy-D-ribose	22.0	* D-Cellobiose	0.4	Sucrose	0.127
L-Arabinose	22.0	* L-Arabinose	0.4	* D-Maltose monohydrate	0.101
β-D-Lactose	22.0	* α-D-Melibiose hydrate	0.6	# * D-Fucose	0.041
D-Glucose	22.2	* Stachyose tetrahydrate	0.8	* L-Fucose	0.033
D-Mannitol	22.4	* D-Raffinose pentahydrate	1.3	* 2-Deoxy-D-glucose	0.033
L-Xylose	22.7	* D-Maltose monohydrate	1.9	* D-Cellobiose	0.029
D-Maltose monohydrate	23.0	* Palitinose	1.9	# * D-Arabinose	0.025
Stachyose tetrahydrate	23.0	* Melezitose dihydrate	2.1	* L-Xylose	0.011
α-D-Lactose monohydrate	23.4	L-Ribose	2.4	# * 3-*O*-Methyl-D-glucopyranose	−0.003
D-Xylose	23.4	D-Ribose	2.5	L-Ribose	−0.003
D-Galactose	23.7	D-Mannose	3.3	# L-Lyxose	−0.005
D-Trehalose dihydrate	23.7	2-Deoxy-D-Ribose	4.2	* β-D-Lactose	−0.006
D-Sorbitol	24.4	D-Galactose	4.8	L-Rhamnose	−0.007
D-Glycero-D-gulo-heptose	24.7	L-Xylose	8.2	* 2-Deoxy-D-galactose	−0.007
D-Lyxose	25.4	Sucrose	10.6	* Stachyose tetrahydrate	−0.010
D-Fructose	25.7	*Myo*-Inositol	12.0	* D-Lyxose	−0.011
2-Deoxy-D-galactose	25.7	D-Trehalose dihydrate	13.9	L-Sorbose	−0.013
D-Mannose	26.7	D-Sorbitol	14.5	* D-Glycero-D-gulo-heptose	−0.019
L-Sorbose	28.0	L-Rhamnose	14.8	# * D-Raffinose pentahydrate	−0.020
L-Fucose	28.2	D-Mannitol	24.3	* α-D-Lactose monohydrate	−0.027
L-Ribose	28.7	L-Sorbose	45.2	# * Palatinose	−0.031
L-Rhamnose	29.0	D-Glucose	72.9	* α-D-Melibiose hydrate	−0.032
D-Cellobiose	29.7	D-Fructose	73.5	# * Melezitose dihydrate	−0.036
Sucrose	33.0	D-Xylose	85.4	# * L-Mannose	−0.045

[a] Ten least stimulatory compounds for radial growth (#) and 20 for conidia germination (*) of *P. expansum*. The marks are placed by the same compounds along *P. syringae* utilization list. Compounds marked among the best utilized by *P. syringae* were selected for testing to enhance biocontrol in apples.

centration of the amino acids, and effects were more pronounced at the highest concentrations of the antagonist.

With increasing concentrations of L-asparagine at the highest concentration of the antagonist (5.4×10^8 cfu/ml), lesions were reduced from 8 to 0 mm (Fig. 1A). The greatest reduction in percentage of wounds infected (50%) also occurred at the highest concentration of the antagonist (Fig. 2A).

As concentrations of L-proline increased from 0 to 20 mM at the highest concentration of the antagonist, lesion size was reduced from 17 to 4 mm. However, at 80 mM concentration, no further reduction in lesion size occurred, and at the two highest concentrations of the antagonist a trend was observed toward larger lesion size (Fig. 3A). As the concentration of L-proline increased from 0 to 80 mM, infected wounds were reduced by 55% at the antagonist concentration of 5.4×10^8 cfu/ml. However, the reduction between 20 and 80 mM concentrations was only 6%, and at the antagonist concentration of 1.7×10^8 cfu/ml, the percentage of wounds infected increased by 17% (Fig. 4A).

Recovery of the antagonist. Population size of the antagonist at the wound site increased by more then two orders of magnitude for L-asparagine, slightly less for L-proline, but only by one order of magnitude for the control during the first 2 days after application (Fig. 5). During the next few days, populations stabilized and declined slightly from day 8 to 16 for all treatments.

Inhibition of biocontrol. Percentage of wounds infected dropped from 100 to 28% on fruit inoculated with antagonist and the pathogen, and treated with streptomycin at 24 and 48 h, respectively (Fig. 6). Protection was ongoing since no new lesions developed on the fruit after three additional days of incubation.

DISCUSSION

Compounds that least stimulated radial growth of *P. expansum* differed from those that least stimulated conidia germination in six out of 10 cases for carbohydrates and seven out of 10 cases for nitrogenous compounds. The amino acids that enhanced biocontrol also strongly stimulated conidial germination (L-proline 81.7% and L-asparagine 100%) but had little effect on radial growth of *P. expansum*. Thus, in this biocontrol system, inhibition of conidial germination may not be as important as inhibition of the subsequent stages of the pathogen development. This is consistent with observations on other nectrotrophic pathogens such as *Cochliobolus sativus, Septoria nodorum, Botrytis cinerea,* and *Alternaria* spp., which are usually antagonized at the mycelial phase of development (6,7). Saprophytic microflora of the phyllosphere has been shown to reduce not only conidial germination but also prepenetration mycelial growth of a number of nectrotrophic pathogens, resulting in reduced infection (3). Lack of biocontrol enhancing activity of carbohydrates was not unexpected because apples contain large amounts of soluble solids (24) that are readily available to the antagonist and the pathogen. In in vitro tests, carbohydrates that were utilized best by the antagonist, e.g., D-fructose, D-glucose, in general, were also utilized well by the pathogen. The two carbohydrates that were selected for testing on fruit after tests in vitro reduced biocontrol when applied to fruit (results not shown). This did not occur with nitrogen compounds where five out of the seven most utilized compounds by the antagonist were shown to have the least effect on radial growth of the pathogen and two of them enhanced biocontrol.

Apples are not a rich source of nitrogen and most of the nitrogen is bound in proteins and only a small portion exists as free amino acids (4,13). L-Asparagine and L-proline are the dominant amino acids in apple and pear, respectively (4). Considering the high concentration of carbohydrates in apple, perhaps nitrogen is the main growth limiting factor for the antagonist. When biocontrol systems with saprophytic microorganisms from the phylloplane were investigated under conditions of limited nutrients, amino acids became the limiting nutrient before carbohydrates, indicating that competition for amino acids is the major mechanism of antagonistic interaction in the phyllosphere with the nectrotrophic pathogen *Botrytis cinerea* (1,2). The amino acids in our system were utilized readily by the antagonist but poorly by the pathogen. These amino acids provided needed nutrients and reduced the amount of energy normally expended by the antagonist for synthesis of those compounds. This resulted in an increased growth of the antagonist and shifted the balance of the antagonist-

TABLE 2. Effect of nitrogen-containing compounds on radial growth and conidia germination of *Penicillium expansum* and their utilization by *Pseudomonas syringae*

P. expansum			*P. syringae*		
Compound	Radial Growth (mm)	Compound	Germination (%)	Compound	Optical Density
#[a] L-Aspartic acid	17.0	*[a] L-Cysteine	4.2	# * L-Valine	0.380
# L-Threononine	17.7	* L-Valine	7.1	* L-Cysteine	0.338
# L-Proline	17.7	* L-Isoleucine	18.1	# L-Asparagine	0.320
# Glycine	19.7	* L-Leucine	25.8	# L-Proline	0.237
# L-Isoleucine	20.0	* L-Methionine	32.6	# L-Glutamic acid	0.198
# L-Valine	20.0	* L-Lysine monohydrochloride	37.0	# Glycine	0.162
# L-Asparagine	20.0	* L-Histidine monohydrochloride	38.9	* L-Methionine	0.101
				L-Gutamine	0.095
# L-Leucine	20.4	* L-Serine	41.1	# * L-Isoleucine	0.086
# L-Glutamic acid	20.7	* L-Threonine	41.5	# *trans*-4-Hydroxy-L-proline	0.056
# L-Tryptophane	21.4			L-Lysine monohydrochloride	0.054
# *trans*-4-Hydroxy-L-proline	21.2	* L-Tryptophane	53.8	$NaNO_3$	0.054
L-Methionine	21.4	$(NH_4)_2SO_4$	64.3	* L-Tryptophane	0.032
NH_4Cl	21.7	*trans*-4-Hydroxy-L-proline	64.6	NH_4CL	0.031
L-Lysine monohydrochloride	21.7	L-Arginine	67.5	L-Arginine	0.027
L-Arginine	21.7	NH_4Cl	68.0		
$(NH_4)_2SO_4$	22.0	$NaNO_3$	70.8	L-Alanine	0.019
L-Phenylalanine	22.0	Glycine	72.7	# L-Leucine	0.017
L-Alanine	22.4	L-Alanine	75.6	* L-Histidine monohydrochloride	0.013
$NaNO_3$	22.4	L-Proline	81.7	$(NH_4)_2SO_4$	0.009
L-Cysteine	22.7	L-Glutamine	85.3	# * L-Threonine	−0.011
L-Serine	22.7	L-Asparatic acid	98.3	* L-Serine	−0.012
L-Histidine monohydrochloride	23.0	L-Glutamic acid	98.4	L-Phenylalanine	−0.033
L-Glutamine	26.4	L-Asparagine	100.0	# L-Aspartic acid	−0.152

[a] Ten least stimulatory compounds for radial growth (#) and 10 for conidia germination (*) of *P. expansum*. The marks are placed by the same compounds along *Pseudomonas syringae* utilization list. Compounds marked among the seven best utilized by *P. syringae* were selected for testing to enhance biocontrol in apples.

pathogen system to favor the antagonist, resulting in enhanced biocontrol. These results are consistent with observations in various biocontrol systems on fruit, where a strict quantitative relationship exists between the concentration of antagonist, pathogen and the level of biocontrol (15,16,18).

Streptomycin applied 48 h after fruit were inoculated with antagonist and pathogen, had significantly less effect in reducing biocontrol than earlier application. This indicated that the first 48 h after application of the antagonist, to a great extent, determined the outcome of biocontrol under the conditions in this study. Thus, it was not surprising that L-asparagine and L-proline, which stimulate an exponential population growth of the antagonist on fruit during that period, also greatly enhanced biocontrol. Cessation of exponential growth after 48 h indicated that nutrients became limited for the antagonist and, most likely, for the pathogen. Enhancement of biocontrol by L-asparagine and L-proline was greatest at the highest concentrations of the antagonist. This could result from the exponential growth pattern of the antagonist

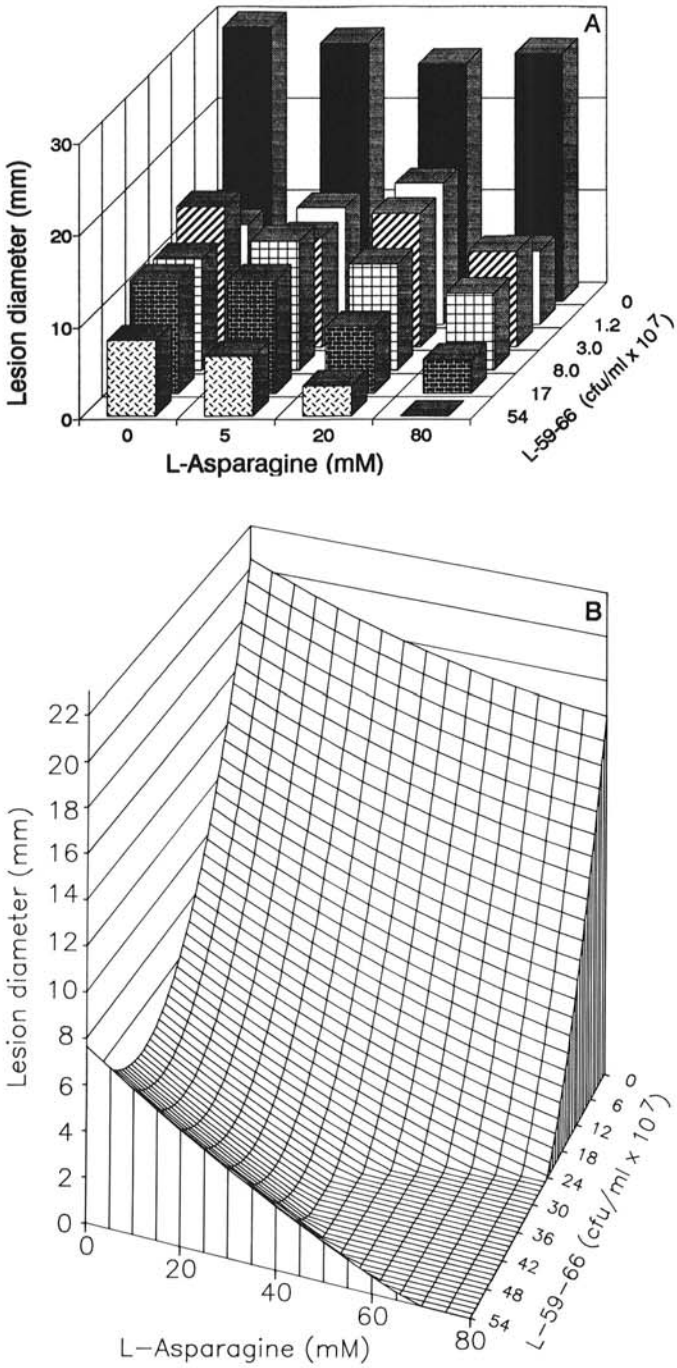

Fig. 1. Effect of L-asparagine and antagonist (*Pseudomonas syringae* strain L-59-66) concentrations on **A,** observed and **B,** predicted lesion diameter of blue mold on Golden Delicious apple. Antagonist was suspended in one of four concentrations of L-aspargine. Fruit were wounded, inoculated with antagonist-L-asparagine suspension, challenged with conidia of *Penicillium expansum,* and incubated for 7 days at 22 C. Response surface (B) was generated using the equation: $Y = 20.43793 - 0.084257A - 0.88627L + 0.0004226A^2 - 0.00103AL + 0.01202L^2$ with $R^2 = 0.60$; where Y indicates lesion diameter, A concentration of L-asparagine, and L concentration of antagonist.

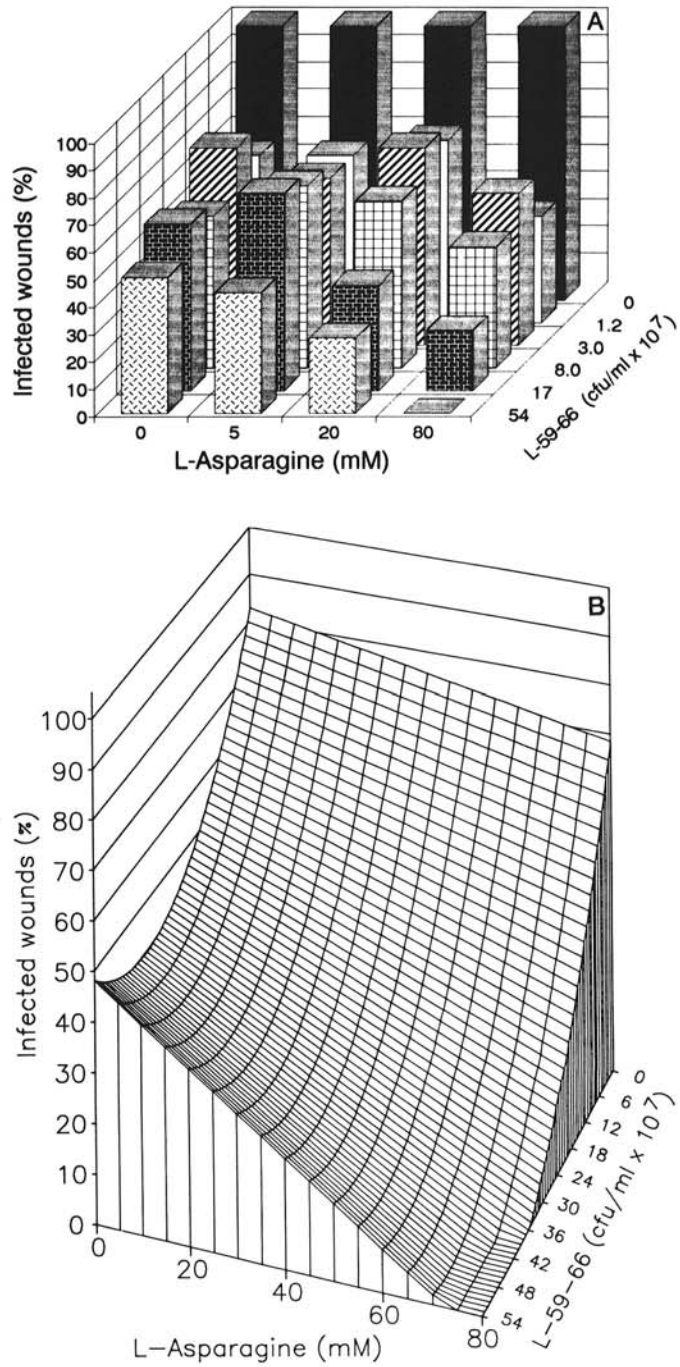

Fig. 2. Effect of L-asparagine and antagonist (*Pseudomonas syringae* strain L-59-66) concentrations on **A,** observed and **B,** predicted percent of infected wounds on Golden Delicious apple. Antagonist was suspended in one of four concentrations of L-asparagine. Fruit were wounded, inoculated with antagonist-L-asparagine suspension, challenged with conidia of *Penicillium expansum* and incubated for 7 days at 22 C. Response surface (B) was generated using the equation: $Y = 82.79148 - 0.18445A - 2.30461L - 0.00885AL + 0.000061A^2 + 0.03082L^2$ with $R^2 = 0.69$; where Y indicates percentage of infected wounds, A concentration of L-asparagine, and L concentration of antagonist.

on the fruit. Higher initial populations allowed for faster buildup to a threshold necessary for effective biocontrol before the critical time had passed. A similar effect was observed in a study of the competitive role of saprophytic microflora of wheat against *Septoria nodorum* and *Cochliobolus sativus* in the presence of honeydew, where the stimulatory effect of honeydew on disease severity was reduced only when higher concentrations of the saprophytes were added (10). Although the possibility of some negative effect of L-asparagine or L-proline on *P. expansum* may not be totally excluded (23), the major effect of these amino acids on the antagonist was clear. The antagonist utilized both amino acids rapidly and increased its population by more than one order of magnitude, which enhanced biocontrol.

Our research demonstrates that nutrients can enhance biocontrol of postharvest disease on ripe fruit. The probability of successfully finding stimulatory compound(s) can be increased by a rational selection of the compounds in in vitro tests before testing on fruits. Thus, under postharvest conditions where the

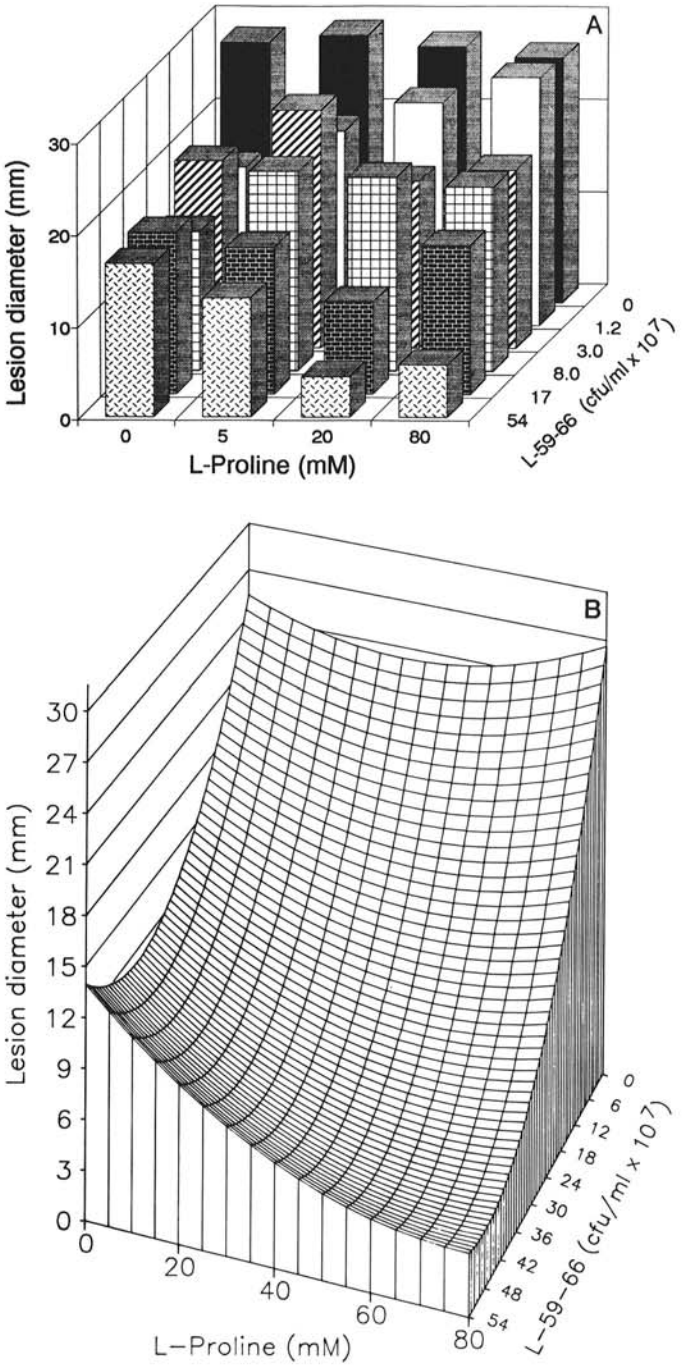

Fig. 3. Effect of L-proline and antagonist (*Pseudomonas syringae* strain L-59-66) concentrations on **A,** observed and **B,** predicted lesion diameter of blue mold on Golden Delicious apple. Antagonist was suspended in one of four concentrations of L-proline. Fruit were wounded, inoculated with antagonist-L-proline suspension, challenged with conidia of *Penicillium expansum* and incubated for 7 days at 22 C. Response surface (B) was generated using the equation: $Y = 25.39505 - 0.11741P - 0.75307L - 0.00265PL + 0.00166P^2 + 0.0100L^2$ with $R^2 = 0.78$; where Y indicates lesion diameter, P concentration of L-proline, and L concentration of antagonist.

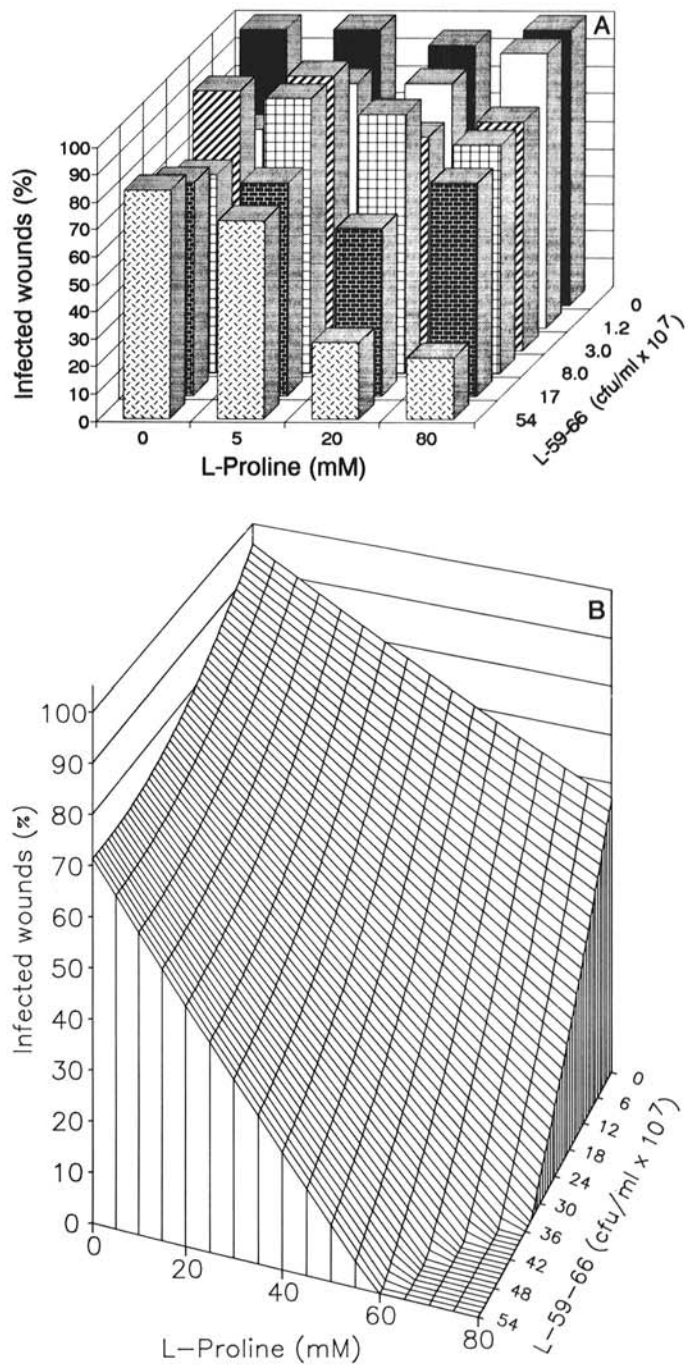

Fig. 4. Effect of L-proline and antagonist (*Pseudomonas syringae* strain L-59-66) concentrations on **A,** observed and **B,** predicted percentage of infected wounds on Golden Delicious apple. Antagonist was suspended in one of four concentrations of L-proline. Fruit were wounded, inoculated with antagonist-L-proline suspension, challenged with conidia of *Penicillium expansum* and incubated for 7 days at 22 C. Response surface (B) was generated using the equation: $Y = 95.60343 - 0.54538P - 0.97261L - 0.01321PL + 0.00758P^2 + 0.00973L^2$ with $R^2 = 0.75$; where Y indicates percentage of wounds infected, P concentration of L-proline, and L concentration of antagonist.

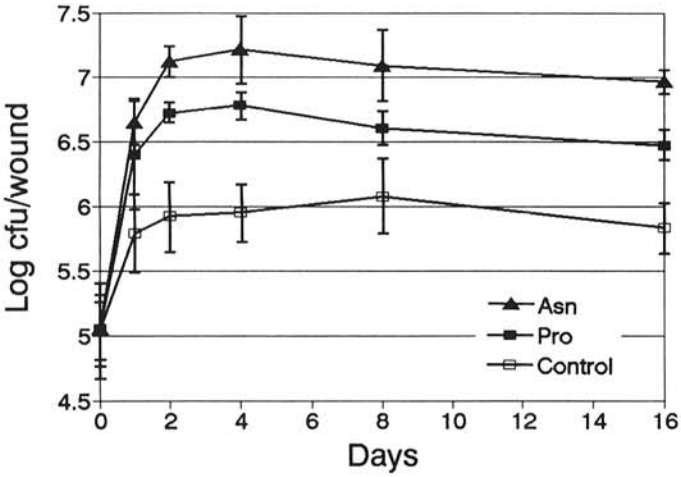

Fig. 5. Effect of L-asparagine (Asn) and L-proline (Pro), both at 80 mM concentration, on population dynamics of the antagonist (*Pseudomonas syringae* strain L-59-66) in wounds of Golden Delicious apple. Fruit were wounded, inoculated with the antagonist resuspended in water (control), L-asparagine or L-proline and incubated at 22 C. Fruit samples were removed at various times to recover antagonist from the wounds. Bars represent ± one standard error.

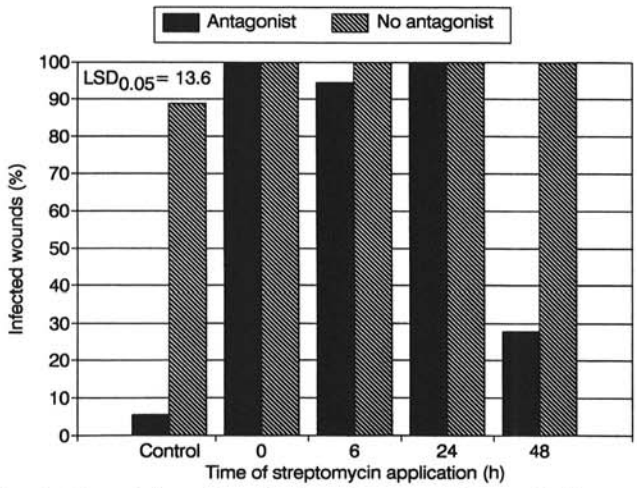

Fig. 6. Effect of time of application of streptomycin on effectiveness of biocontrol on Golden Delicious apple. Fruit were wounded, treated with the antagonist (*Pseudomonas syringae* strain L-59-66), challenged with the conidia of *Penicillium expansum* and treated with streptomycin at various times. Control = no streptomycin.

physical environment is controlled, another critical factor, the chemical environment, might be manipulated to enhance biocontrol by the antagonist. However, nutrients used to enhance biocontrol of one pathogen must not stimulate other pathogens. In our study both nutrients also enhanced biocontrol of *B. cinerea*, but the effects were stronger with *P. expansum* (17). Manipulation of the chemical environment resulted in control of the disease on ripe fruit without further increasing the concentration of the antagonist applied, and made this method of control more reliable and economically attractive. This approach could succeed in enhancing biocontrol of postharvest diseases of fruit and vegetables in other pathosystems. Use of a mixture of nutrients to further enhance biocontrol should be explored as well as screening for new antagonists based on the ability to utilize specific nutrients.

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