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Antagonism of Lactic Acid Bacteria against Phytopathogenic Bacteria

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A variety of lactic acid bacteria, isolated from plant surfaces and plant-associated products, were found to be antagonistic to test strains of the phytopathogens *Xanthomonas campestris*, *Erwinia carotovora*, and *Pseudomonas syringae*. Effective "in vitro" inhibition was found both on agar plates and in broth cultures. In pot trials, treatment of bean plants with a *Lactobacillus plantarum* strain before inoculation with *P. syringae* caused a significant reduction of the disease incidence.

Several members of the lactic acid bacteria are known to produce antibacterial substances. The antibacterial effect has been ascribed to the production of antibiotics or antibiotic-like substances such as acidophilin and lactocidin produced by *Lactobacillus acidophilus* (32; J. R. Vakil and K. M. Shahani, Bacteriol. Proc., p. 9, 1965) or lactolin produced by *Lactobacillus plantarum* (11) or nisin produced by *Streptococcus lactis* (8). Wheater et al. (33, 34) and Gilliland and Speck (5) ascribed the effect to hydrogen peroxide production, while Kao and Frazier (9) and Tramer (31) reported lactic acid to be the antibacterial substance. In a heterogeneous population nutrient depletion and a decrease in the reduction-oxidation potential may cause competitive antagonism.

The interactions of lactic acid bacteria with other bacteria have been widely researched in food products and especially in fermented foods (2, 27–29) and silages (10, 12, 30). However, information on the occurrence of lactobacilli on living plants is scarce, and no information is available on the interactions of plant-associated lactic acid bacteria with phytopathogenic bacteria. Reports have been made on the isolation of atypical streptobacteria and betabacteria, as well as on the following *Lactobacillus* species from plants: *L. plantarum*, *L. fermentum*, and small numbers of *L. brevis*, *L. casei*, *L. viridescens*, *L. cellobiosus*, and *L. salivarius* (17, 25, 30). Although some authors (17) do not consider plants to be a natural reservoir of lactobacilli, this scarcity might rather be ascribed to the antibacterial effect of some extracts of higher plants, often due to 1,4-naphthaquinone derivates (25). On cut or bruised plant tissue, lactobacilli become more prevalent (30).

In the present study, preliminary tests were conducted to investigate possible antagonism between plant-associated lactic acid bacteria and some phytophatogenic bacteria. The ultimate aim would be the implementation of lactic acid bacteria for the biological control of bacterial plant disease.

MATERIALS AND METHODS

Organisms. (i) Lactic acid bacteria. Authentic cultures of *L. plantarum* were obtained from the German Culture Collection (DSM 20205) and the American Type Culture Collection (ATCC 8014). In our laboratories 41 isolates of lactic acid bacteria were obtained from a wide variety of plants, including haricot beans (*Phaseolus vulgaris*), gherkins (*Cucumis sativus*), and several plants and flowers indigenous to southern Africa as well as plant-associated products such as Mageu (a drink produced from fermented maize) and coffee extract.

(ii) Phytopathogenic bacteria. *Pseudomonas mangiferaeindicae* Ps1 (culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria) was originally isolated from mangoes. The name was subsequently changed to *Xanthomonas campestris* (15, 21). *P. syringae* Ps2, pathogenic to bean plants, was obtained from the S. A. National Institute for Plant Protection and *P. syringae* var. *capsici* Ps3 was from the German Culture Collection (DSM 50336). *Erwinia carotovora* Erw was isolated in our Department and *X. campestris* pv. *mangiferaeindicae* Xan was received from the British National Collection of Plant Pathogenic Bacteria (NCPPB 490).

Media. Lactic acid bacteria were isolated on Rogosa agar (E. Merck AG) (22) containing 0.1% (wt/vol) cycloheximide (Calbiochem-Behring) and were mainted in MRS broth (Merck) (4). Plant pathogenic bacteria were grown at 25°C on nutrient broth-yeast extract agar (NBY) described by Schaad (23).

Isolation and identification of lactic acid bacteria. One gram of plant material was vortexed for ca. 30 s in 9 ml of quarter-strength Ringer solution (Merck) containing glass beads (diameter = 2 mm). A dilution series was made in Ringer solution and plated onto Rogosa agar containing 0.1% (wt/vol) cycloheximide to inhibit possible fungal contamination. Colonies which developed within 48 h at 30°C under anaerobic conditions were tested for catalase activity with 4% H_2O_2 (7), and catalase-negative colonies were transferred to MRS agar. The colony and cell morphologies of the pure cultures were examined, and the following tests were done to identify the isolates: pseudo-catalase activity (35); lactic acid configuration (1), using D- and L-lactate dehydrogenases (Boehringer Mannheim Biochemicals); presence of *meso*-diaminopimelic acid in cell walls (6); growth at 4, 15, 19, and 45°C; and growth in the presence of 10% (wt/vol) NaCl and at pH 3.9. Arginine hydrolysis was tested according to the Nessler method (7). The formation of gas from glucose and slime from 10% sucrose (24) served as additional criteria for classification. Motility, gelatinase activity, and nitrate reduction were tested in a semisolid

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TABLE 1. Lactic acid bacterial species isolated from plants with indication of the degree of in vitro antagonism found within each group of species against five strains of phytopathogenic bacteria

Species of lactic acid bacteria	Origin	No. of isolates	Degree of antagonism[a]
Lactobacillus plantarum	Indigenous plants	10	+ + to + + +
	Coffee extract	3	+ + to + + +
	DSM 20205	1	+ + +
	ATCC 8014	1	+ +
L. brevis	Indigenous plants	3	+ + to + + +
	Gherkins	6	+ +
L. vaccinostercus	Indigenous plants	2	+ + to + + +
L. bavaricus	Indigenous plants	1	+ + +
L. hilgardii	Gherkins	2	+ to + +
L. sake	Beans	4	+ to + + +
L. casei subsp. *rhamnosus*	Gherkins	1	+ + +
Heterofermentative lactobacilli	Gherkins	1	+ +
	Mageu	1	−
Leuconostoc mesenteroides	Indigenous plants	1	+ +
	Beans	1	−
	Mageu	4	− to + +
Leuconostoc paramesenteroides	Gherkins	1	+ +

[a] Determined by measuring the average diameter of clear zones surrounding agar disks cut from lactic acid bacterial cultures. −, No inhibition; +, weak; + +, mild; + + +, strong inhibition.

medium described by Reuter (20) and, finally, the sugar fermentation pattern of the isolates was determined (24–26).

Determination of "in vitro" inhibition. An agar disk technique was used to determine whether the lactic acid bacteria were capable of inhibiting the plant pathogens in vitro. Pour plates were made of the lactic acid bacteria by mixing 1 ml of a 36-h broth culture in ca. 15 ml of MRS agar. After incubation at 30°C for 48 h, disks with a diameter of 7 mm were stabbed from the agar. The disks were placed on NBY agar covered with suspensions of 48-h cultures of the plant pathogens in Ringer solution. Sterile MRS agar disks were used as a control. After an incubation period of 36 h at 25°C, the diameter of clear zones surrounding the disks was measured. The experiment was done in triplicate to ensure repeatability.

In a separate experiment, the growth curves of the plant pathogens were compared with their growth curves in the presence of lactic acid bacteria. The five plant pathogens were each inoculated into two flasks containing 100 ml of NBY broth. Into one of each pair of flasks, a strain of *L. plantarum* was inoculated before incubation at 25°C. Samples were taken from the cultures at 12-h intervals for 84 h. Serial dilutions of the samples were plated onto NBY agar and incubated at 25°C under aerobic conditions, and the colonies of phytopathogenic bacteria were counted after 48 h.

Determination of antagonism in pot trials. The effect of an *L. plantarum* isolate (L 292) on the pathogenicity of *P. syringae* Ps2 was tested by spraying suspensions of the organisms onto the leaves of young haricot beans. The suspensions were prepared of 48-h cultures in sterile distilled water containing 1 ml of Tween 80 (Merck) per liter. The plants were germinated in the greenhouse (25 to 30°C), and as soon as the first two primary leaves were fully grown, 50 plants were sprayed with strain L292 only, 50 were sprayed with Ps2 only, and 50 were sprayed with Ps2 24 h after being sprayed with the L292 suspension. The symptoms of halo blight (black water-soaked lesions with yellow haloes and the curling of infected leaves) developed ca. 14 days after inoculation with the plant pathogen. The number of lesions per plant, total number of leaves per plant, and number of dead leaves per plant were counted, and the dry mass of each plant was determined.

Statistical analysis. Results were analyzed by variance and covariance analysis, using the SAS statistical package (19).

RESULTS AND DISCUSSION

Identification of lactic acid bacteria. The lactic acid bacteria isolated from the plants and plant-associated products are shown in Table 1, as are their antagonistic activities, which were determined as described below.

Determination of the antagonistic effect on vegetative cells. In vitro tests showed agar disk inhibition zones on all five plant pathogenic bacteria produced by 37 of the 43 lactic acid bacteria (Fig. 1). One isolate from gherkins, *L. hilgardii* (L2501), inhibited four of the five pathogens, and isolate L2525 (*L. sake*) from beans inhibited only three of the pathogens. The four isolates which were not inhibitory (three strains of *Leuconostoc mesenteroides* and a heterofermentative *Lactobacillus* sp.) grew very weakly in MRS agar pour plates. However, most of the lactic acid bacteria had a wide range of inhibition against the pathogens. The average inhibition zones of the 15 most antagonistic lactic acid bacteria are presented in Table 2. Growth of Ps1 was stimulated around the inhibition zones (Fig. 1), possibly because of an increase in available nutrients from the zone of no growth or as a result of growth factors released by the lactobacilli. No inhibition or stimulation zones could be detected around the sterile MRS agar disks used as the control.

Effect of lactobacilli on plant pathogens in broth cultures. Broth cultures of *X. campestris* and *E. carotovora* were completely killed by isolates of *L. plantarum* within 36 h, *P.*

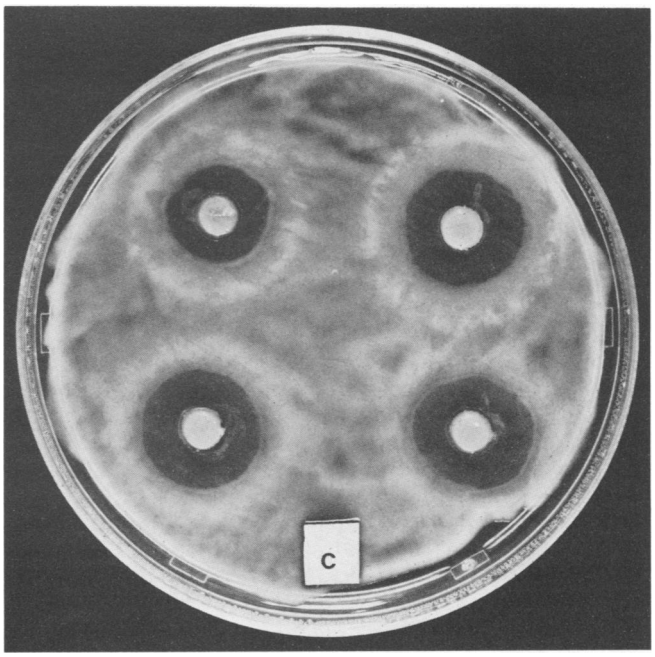

FIG. 1. Inhibition and stimulation zones surrounding disks of lactic acid bacterial cultures placed on a spread plate of *X. campestris* Ps1 on NBY agar.

TABLE 2. Diameter of zones formed on NBY agar by the 15 most antagonistic lactic bacterial isolates against test strains of phytopathogenic bacteria

Lactic acid bacteria	Isolate no.	Zone diam (mm)[a]				
		Ps1	Ps2	Ps3	Erw	Xan
L. plantarum	L1515A	20.2	25.5	22.0	21.3	33.1
L. plantarum	L379	18.2	26.5	22.4	21.1	32.9
L. plantarum	L1518	17.5	24.9	22.6	21.7	33.8
L. plantarum	L392A	18.2	27.0	21.7	23.5	30.2
L. vaccinostercus	L1506	20.5	26.4	21.5	19.3	31.0
L. sake	L2522	18.7	22.8	20.7	20.4	33.9
L. sake	L2521	19.9	25.0	18.7	20.1	32.2
L. plantarum	L1056	18.6	22.4	21.7	20.9	31.9
L. plantarum	L292	17.6	26.2	20.4	20.3	29.6
L. casei subsp. *rhamnosus*	L2506	17.1	24.5	19.7	21.0	29.9
L. bavaricus	L1553	17.4	25.8	20.7	17.7	28.0
L. plantarum	L491	18.3	23.9	19.7	17.4	29.8
L. plantarum	L2602	17.5	22.4	19.2	18.5	28.8
L. plantarum	DSM 20205	16.4	22.4	20.0	18.7	27.1
L. brevis	L1084	17.5	19.8	18.0	17.8	29.7

[a] Average of triplicate readings. Ps1, *X. campestris* (mango isolate); Ps2, *P. syringae* (bean pathogen); Ps3, *P. syringae* var. *capsici* (DSM 50336); Erw, *E. carotovora* (isolate); Xan, *X. campestris* pv. *mangiferaeindicae* (NCPPB 490).

syringae and *P. syringae* var. *capsici* (Fig. 2) were killed within 24 h, and *X. campestris* pv. *mangiferaeindicae* isolates were killed within 12 h. Thus, even in competition with the pathogens, under conditions favorable to the pathogens, effective inhibition occurred.

Effect of lactic acid bacteria on pathogenicity of *P. syringae* Ps2 to bean plants. Plants treated with lactic acid bacteria (isolate L292) before inoculation with *P. syringae* showed significantly fewer symptoms than those treated only with the pathogen. Plants treated only with lactobacilli showed none of these symptoms. The following parameters differed significantly ($P < 0.05$) between the treatments: (i) average number of lesions per leaf (ii) average number of lesions on leaves with lesions, and (iii) percentage of dead leaves (Table 3). The average dry mass of the plants in the three different treatments did not differ significantly ($P > 0.05$).

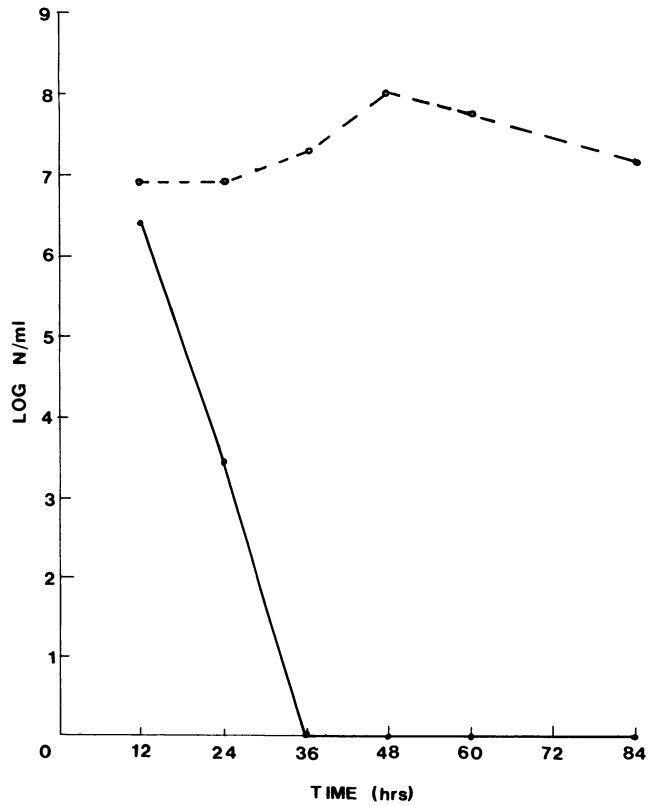

FIG. 2. Effect of lactobacilli on *E. carotovora* in NBY broth cultures. Symbols: (-----) *E. carotovora* alone; (——) *E. carotovora* in the presence of *L. plantarum* L392B.

Pot trials showed an isolate of *L. plantarum* (L292) to be effectively antagonistic against the bean pathogen *P. syringae*. However, the effectiveness of the interaction has yet to be proved under field conditions where factors such as rain, fluctuations in temperature and relative humidity, and a greater variety of competitive microorganisms will play a role.

Biocontrol of postharvest plant diseases of fruit and vegetables by lactic acid bacteria also seems an exceptionally exciting area to be explored. Limitations such as environmental conditions in the field, the targeting of biocontrol agents to the effective site, and the economical feasibility of control procedures under field conditions may be overcome under storage conditions (3, 36). Leben and Daft (13) reported that an epiphytic bacterium (isolate A180) from cucumber leaves reduced cucumber anthracnose, early blight of tomato, and northern leaf blight of corn when cultures or washed cells of the bacterium were applied as protectant sprays to seedlings in the greenhouse. Subse-

TABLE 3. Effect of lactic acid bacteria on pathogenicity of *P. syringae* Ps2 to haricot bean plants[a]

Treatment	Avg no. of lesions per leaf	Avg no. of lesions on leaves with lesions	% Dead leaves	Dry mass (g)
Pathogen	2.0^{a}	$4,7^{c}$	20.7^{f}	1.9^{i}
Pathogen + lactic acid bacteria	0.4^{b}	$1,6^{d}$	16.1^{g}	0.8^{i}
Lactic acid bacteria	0.0^{b}	$0,0^{e}$	1.6^{h}	0.8^{i}

[a] Means followed by the same letter do not differ significantly ($P > 0.05$) according to the least-squares test.

quently, Leben et al. (14) have demonstrated that isolate A180 was not effective under field conditions, probably as a result of its sensitivity to drying and UV rays.

Genetic manipulation could also be applied to produce effective antagonists that are ecologically adapted to the infection site. The incorporation of genes involved in the mode of action of an antagonist into the host plant itself is another possibility that deserves special attention (36).

The survival pattern of the lactic acid bacteria on the phyloplane and the mechanism of antagonism are at present being investigated in our laboratories.

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