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Antagonistic Activity of *Bacillus subtilis* Towards *Rhizopus nigricans**

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ABSTRACT

Antagonistic effect of seven isolates of *Bacillus subtilis* has been examined in dual culture tests against *Rhizopus nigricans*, the incitant of 'papaya' (*Carica papaya* L.) fruit-rot. Isolate AF 1 which caused maximum inhibition (9.6 mm) was grown in potato dextrose broth for 96 h and cell-free culture filtrate was used to study the inhibition. The concentrated extract of culture filtrate of the antagonist was diluted to indicate 0—40 per cent in potato dextrose agar and Richard's solution. There was 93% inhibition at 40 per cent in both the media. Germinated spores formed clumps in Richard's solution at and above 20 per cent concentration of the antagonistic culture filtrate, but failed to produce sporangia. Concentrated extract induced formation of bulbous structures in hyphae. Biological control of papaya fruit-rot was achieved by dipping the fruits in cell suspension of *B. subtilis* or in the culture filtrate, 24 h before treatment with *R. nigricans*.

Key Words: *Bacillus subtilis*, antagonism, *Rhizopus nigricans*, fruit rot, biological control

Post-harvest diseases of fruits have become a major problem for storage and marketting life of fruits. Although fungicides are known to control the post-harvest fruit-rot diseases, biocontrol would be a useful substitute. Wilson and Pusey (1985) envisaged that biological control may be an alternative to chemical control of post-harvest environment.

Cell suspensions of *Bacillus subtilis* have been successfully used in the biological control of fruit-rot diseases (Pusey and Wilson, 1984; Singh and Deverall, 1984). The purpose of our study was to investigate the potential of *B. subtilis* in the post-harvest control of papaya watery-rot caused by *Rhizopus nigricans*. Seven isolates of bacteria were screened for their *in vitro* antagonism against *R. nigricans* and the potential antagonistic isolate was used in further experiments.

MATERIALS AND METHODS

R. nigricans was isolated from infected papaya fruits obtained from the local markets. Three isolates of *B. subtilis* (AF 1, AF 2 and AF 3) were isolated from the soil, while BACT 1, BACT 2, AB 6 and AB 9 isolates were kindly supplied by Dr. R.S. Utkhede, Agriculture Canada, Research Station, British Columbia, Summerland, Canada. Inhibitory effect of various isolates of *B. subtilis* against *R. nigricans* was studied in dual culture test (Utkhede and Rahe, 1983) run in triplicate in potato dextrose agar (PDA) plates.

To study the effect of culture filtrate of AF 1 on the growth of *R. nigricans*, *B. subtilis* (AF 1) was grown in 2 litre flasks containing 1 litre of potato dextrose broth (PDB) and incubated on a reciprocating shaker (120 strokes/min) at 28 ± 2°C for 96 h. The PDB was then centrifuged at 15,000 rpm for 20 min and the supernatant was concentrated to 1/10th volume in a boiling water bath and passed through 0.45 μm millipore filters. This is referred to as concentrated cell-free culture filtrate (CCCF). CCCF was incorporated in PDA to the extent of 5, 10, 20 and 40 per cent and the pH was then adjusted to 5.5. Ten-fold concentrated fresh PDB diluted to indicate 40 per cent in PDA, served as control. PDA plates were inoculated with actively growing cultures of *R. nigricans* (10 mm plug) and incubated at 26 ± 2°C. Fungal growth was measured in terms of colony diameter at 12 h intervals upto 96 h. The experiment was run in triplicate.

CCCF was also incorporated (as mentioned above) in Richard's solution. Fifty ml quantity taken in 250 ml flasks, was inoculated with 0.5 ml of spore suspension containing 10^7 spores/ml. Dry weight of the mycelium was recorded after 96 h of growth. Morphological variations in the hyphae after 72h were recorded.

For testing the biological control efficiency of *B. subtilis* on papaya fruit, bacterial sus-

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TABLE 1. Antagonistic activity of *R. nigricans* against *B. subtilis*

Isolates of *B. subtilis*	Inhibition zone (mm)	Non-sporulation zone of mycelium around inhibition zone (mm)
AF 1	9.6	2.7
AF 2	7.4	0.8
AF 3	8.6	0.8
BACT 1	8.0	1.0
BACT 2	6.6	0.5
AB 6	6.8	0.2
AB 9	9.4	3.1

(Mean of three replicates)

pension in sterile distilled water was prepared from 5-day old cultures on PDA (10^6 cells/ml). Batches of 10 fruits were dipped in bacterial suspension or CCCF, air-dried and after 24 h, the fruits were dipped in a spore suspension of *R. nigricans* (10^3/ml.). Fruits dipped in sterile distilled water served as controls. Individual fruits were put in polythene bags and incubated at 25°C for 5 days. The experiment was run in triplicate.

RESULTS AND DISCUSSION

In dual culture test, all the seven isolates of *B. subtilis* proved inhibitory to the growth of *R. nigricans* (Table 1). However, isolate AF 1, showed the maximum inhibition zone of 9.6 mm followed by AB 9 (9.4 mm). There was a zone of only vegetative hyphae around the inhibition zone. Since AF 1 showed the widest inhibition zone, it was used in further experiments.

R. nigricans failed to grow in PDA that contained 40 per cent concentration of the 10-fold concentrated extract (Table 2). Significant reduction in radial growth occurred at 20 per cent concentration as compared to control (78% inhibition). Up to 24 h, even at 5 per cent concentration, inhibition was 53%, although, *R. nigricans* grew unaffected at 5 per cent concentration by 60 h.

A meagre stimulation of mycelial dry weight was recorded at 5 per cent concentration (2.3%). However, there was a significant reduction in dry weight proportional to CCCF concentration, with a maximum of 93 per cent at 40 per cent concentration (Table 3). Germinated spores gave rise to hyphal clumps at 20 per cent or higher concentration as compared to cottony mycelium of the control.

Variation in the hyphae of *R. nigricans* was evident when grown on CCCF-amended Richard's solution. The changes observed were, absence of sporangia in 20 per cent concentration (Fig. 1A & B) and formation of swellings in the hyphae (looking like bulbous structures) (Fig. 1 A, B & C). Black sporangia, which were found luxuriantly in the control (Fig. 1 D) were scant when the fungus was grown in the presence of 5 per cent concentration and none at 40% concentration (Fig. 1 B).

Dipping the papaya fruits in *B. subtilis* AF 1 cell suspension before 24 h of *R. nigricans*-inoculation protected the fruits from infection over a period of 72 h. During that period,

TABLE 2. Effect of concentrated cell-free culture filtrate of *B. subtilis* on *R. nigricans*

Concentration (%)	Radial growth (mm) of *R. nigricans* h after							
	12	24	36	48	60	72	84	96
Control	35	90	90a	90a	90a	90a	90a	90a
5	18(49)	43(53)	75(17)	86(4)	90	90	90	90
10	12(93)	28(69)	47(48)	61(32)	70(22)	76(16)	78(13)	78(13)
20	0(100)	12(93)	14(84)	17(81)	20(78)	20(78)	20(78)	20(78)
40	0(100)	0(100)	12(93)	12(93)	12(93)	12(93)	12(93)	12(93)

a = luxuriant growth, mycelium compactly packed in the plate.
Figures in parenthesis represent per cent inhibition over control

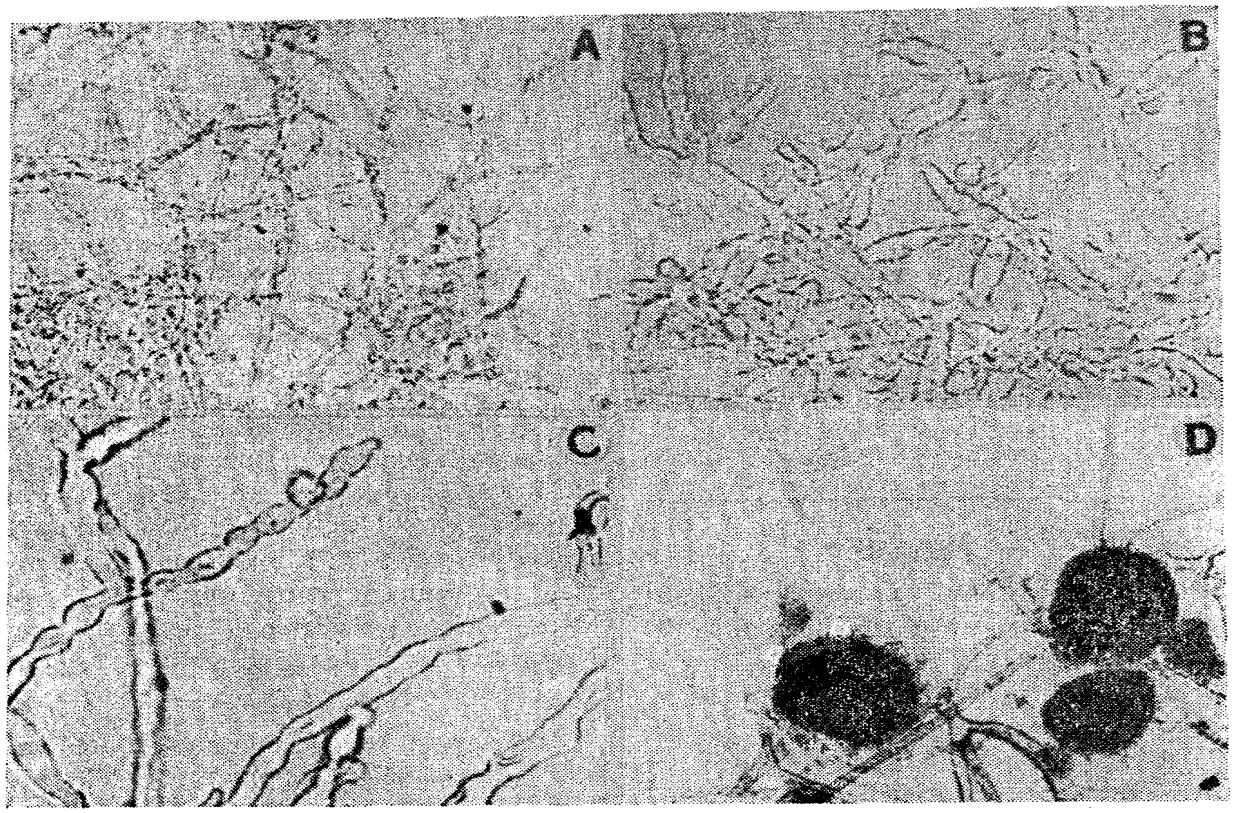

Fig. 1 A — D. Effect of concentrated extract of *B. subtilis* on the morphology of *R. nigricans* after 72 h of growth.
A. at 20 per cent of CCCF (150 X)
B. at 40 per cent of CCCF (375 X)
C. at 40 per cent of CCCF (450 X)
D. control (150 X)

TABLE 3. Effect of concentrated cell-free culture filtrate of *B. subtilis* (AF 1) on the mycelial dry weight of *R. nigricans*

% concentration	Dry weight (mg)	% inhibition*
Control	349	—
5	357	2**
10	328	6
20	188	46
40	26	93

*% inhibition;
(**% stimulation)
(Mean of three determinations)

80 per cent of fruits dipped in sterile distilled water developed watery-rot symptoms (Table 4). CCCF also protected fruits from infection and 40 per cent control was recorded after 120 h.

TABLE 4. Effect of *B. subtilis* AF 1 treatment on the development of papaya fruit rot symptoms.

	No. of fruits (out of 10) showing symptoms*-hours after				
	24	48	72	96	102
Control	5	6	8	8	8
Treated with *B. subtilis*	0	0	2	4	4
Treated with CCCF	0	3	4	6	6

*average of triplicates

Antagonism of *B. subtilis* against post-harvest fungal plant pathogens has led to its use for biological control of these pathogens (Pusey and Wilson, 1984; Singh and Deverall, 1984). The inhibitory effect of *B. subtilis* may be persumably due to antibiotics secreted into the medium (Baker *et al.*, 1983; Podile *et al.*, 1987; Singh and Deverall, 1984). CCCF of *B. subtilis* is inhibitory to the growth of *R. nigricans*. Long ago an antifungal compound bulbiformin was reported in the culture filtrate of *B. subtilis* (Vasudeva *et al.*,

1958). These authors have reported that conidia of *Fusarium udum* proliferate as bulbous structures, when treated with culture filtrate of *B. subtilis*. Development of similar bulbous structures in the mycelium of *R. nigricans* in liquid medium could be due to some bulbiformin-like compound. Formation of such bulbous structures has been observed in many wilt fungi (Podile, 1986) with CCCF of *B. subtilis*.

In the present work, the fungus failed to sporulate in the periphery of the inhibition zone in the dual culture tests, at 40 per cent concentration in radial growth experiment and 20 per cent concentration in liquid culture. Cell-free culture filtrate of *B. subtilis* was found active even after autoclaving which was also reported by Singh and Deverall (1984).

The antifungal activity of *B. subtilis* on the surface of the fruits might be due to the bacterial growth as well as antibiotic activity. However, some toxic metabolites (other than antibiotics) such as NH_3 was considered to cause some inhibition (Pusey and Wilson, 1984). A low molecular weight, acidic and autoclavable antifungal compound, showing biological activity against *R. nigricans* was isolated from culture filtrate of *B. subtilis* (Podile *et al.*, 1987). Considering that cell-free culture filtrate was also effective, the involvement of antibiotics could be presumed. Apparently, the mode of action of AF 1 on the fruit rot development is by inhibiting the growth of *R. nigricans* and further by reducing the formation of sporangia.

B. subtilis was compatible with commercial fruit waxes, dicloran and simulated cold storage conditions (Pusey *et al.*, 1986) and the strain B-3 or its antibiotic could potentially be incorporated for brown rot control in commercial packing by the same methods currently used to apply fungicides. This laboratory demonstration of *B. subtilis* against the fruit rot, need further testing to establish the actual commercial application.

Results presented above clearly indicate that *B. subtilis* is a good natural fungal antagonist which can be tried in controlling the diseases incited by *R. nigricans*. Some factors which need consideration before the large scale use of *B. subtilis* as control agent for post-harvest diseases of fruits are the possible biological problems, economics of its use and conceiveable harmful effects on foodstuffs.

REFERENCES

Baker, C.J., Stavely, J.R., Thomas, C.A., Sasser, M. and MacFall, J.S. 1983. Inhibitory effect of *Bacillus subtilis* on *Uromyces phaseoli* and on development of rust pustules on bean leaves. *Phytopathology*, **73**, 1148-1152.

Podile, A.R. 1986. Biochemical determinants of pathogenesis and biological control with special reference to vascular wilt pathogens. Ph.D. Thesis. Sardar Patel University, Vallabh Vidyanagar.

Podile, A.R., Prasad, G.S. and Dube, H.C. 1987. Partial characterization of antagonistic principle of *Bacillus subtilis* AF 1. *J. Biol. Control*, **1**, 60-65.

Pusey, P.L., Wilson, C.L., Hotchkiss, M.W., and Franklin, J.D. 1986. Compatibility of *Bacillus subtilis* for post-harvest control of peach brown rot with commercial fruit waxes, dicloran and cold-storage conditions. *Plant Dis.*, **7** , 587-590.

Pusey, P.L. and Wilson, C.L. 1984. Post harvest biological control of stone fruit brown rot by *Bacillus subtilis*. *Plant Dis.*, **68**, 753-756.

Singh, V. and Deverall, B.J. 1984. *Bacillus subtilis* as a control agent against fungal pathogens of citrus fruit. *Trans. Br. Mycol. Soc.*, **83**, 487-490.

Utkhede, R.S. and Rahe, J.E. 1983. Interaction of antagonist and pathogen in biological control of white rot. *Phytopathology*, **73**, 890-893.

Vasudeva, R.S., Subbaiah, T.V., Sastry, M.L.N., Rangaswamy, G. and Iyengar, M.R.S. 1950. '*Bulbiformin*' an antibiotic produced by *Bacillus subtilis*. *Ann. appl. Biol.*, **46**, 336 - 345.

Wilson, C.L. and Pusey, P.L. 1985. Potential for biological control of post-harvest plant diseases. *Plant Dis.*, **69**, 375-378.