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The use of *Bacillus subtilis* and *Debaromyces hansenii* in controlling postharvest decay of sweet orange fruits

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ABSTRACT: *Bacillus subtilis* and *Debaromyces hansenii* isolated from fruit washings of sweet oranges that did not decay after four weeks of bulk storage were tested for antagonism towards three rot fungi of oranges namely: *Aspergillus niger, Botryodiplodia theobromae* and *Penicillium italicum.* Spore suspension (approx. 105 spores/ml) of each rot fumgus was introduced into wound sites on oranges before, simultaneously or after inoculation of cell suspension (5.2 x 107 cfu/ml) of *B. subtilis* or *D. hansenii*. The two microbes significantly ($P < 0.05$) reduced the number of wound sites developing infections. The antagonists were more effective when applied before than after inoculation of pathogen. The antagonists significantly ($P < 0.05$) reduced the rate of natural infection of sweet orange fruits over a three weeks storage period, and their efficacy was comparable to that of fungicide (Tecto) treatment. The efficacy of *B. subtilis* in controlling postharvest rot of sweet orange fruits was higher than that of *D. hansenii.*

Key words: Biocontrol, postharvest rot, sweet orange, *Bacillus subtilis, Debaromyces hansenii.*

Introduction

Sweet orange fruits often decayu in transit and during storage due to microbial attack. The fungi causing decay of sweet orange fruits in Nigeria are mainly *Botryodiplodia theobromae, Aspergillus niger* and *Penicillium italicum* (1, 2).

Fungicide treatments are often recommended for use in controlling postharvest rot of fruits (3,4). However, investigation into finding alternatives to chemical treatment is now gaining popularity. Our reason for this is the increasing concern over the health of consumers by consuming fruits with chemical residues (5). Further, the high cost of fungicides has made them unaffordable to the average African farmers.

Elsewhere, there are reports in literature on the use of microbial agents in controlling postharvest decay in fruits (6,7). However, there is yet to be any serious investigation on the biocontrol of postharvest disease of any Nigerian crop and the few reports available have been on the control of field diseases. The present study thus reports the efficacy of two microbial isolates *Bacillus subtilis* and *Debaromyces hansenii* isolated from fruit washings in controlling three rot fungi of orange fruits.

Materials and Methods

Sweet orange *(Citrus sinensis)* fruits that did not decay after four weeks bulk storage were selected for the isolation of surface microflora. The fruits were rinsed in sterile distilled water and the aliquots plated on nutrient agar and malt extract agar plus 60µg/ml of cloramphenicol to suppress bacterial growth. The plates were incubated at room temperature (28 ± 2°C) for 48 - 72 hours and the emerging yeasts and bacteria sub-cultured until pure culture of each isolate was established.

The three rot fungi of orange fruits namely *Aspergillus niger, Botryodyplodia theobromae and Penicillium italicum* were isolated from infected orange fruits as previously reported (2). The three rot fungi were maintained on potato dextrose agar and subcultured every two weeks.

The in vitro antagonistic activities of isolated microbes were tested by the dual culture method (8). The rot fungi were separately inoculated at one edge of PDA plates while the isolated microbes were individually inoculated at the other edge of the plate. The dual culture plates were incubated at 28 ± 2°C and observed daily for interactions. The two microbes that were most inhibitory on agar plates were identified by carrying out morphological, physiological and biochemical tests to be *Bacillus subtilis* and *Debaromyces hansenii*.

Mature sweet orange fruits of uniform size were rinsed with tap water and then with sterile distilled water. The fruits were injured to a depth of 3 x 3 mm in diameter at four points equidistant from the centre using a sterile dissecting needle protruding 5 mm through a cork. The two antagonists were grown on nutrient-yeast extract dextrose broth (9) and suspensions contained at least 5.2 x 107 colony forming units (cfu) per ml. A few drops (0.5 ml) of each antagonist was then applied to each wound site. The third treatment consisted of fungicide application. Tecto (Thiabendazole; 50%e.c.; Merk, Shap & Dohme) at the rate of 500 ppm while sterile NYDB was applied to the control. Spore suspension (105 cfu/ml), 0.5ml of each rot fungus was applied into the wound site simultaneously with antagonists or at 6 h, 12 h, 18 h, or 24 h before and after antangonists. Twenty fruits were used for each treatment and the experiment was carried out in triplicate. The fruits were separately packaged in unsealed plastic bags according to treatments and kept at 28 ± 2°C and > 85% relative humidity. The number of inoculation sites that developed decay was counted after 7 days.

The antagonists were also evaluated for their ability to control natural infection of sweet orange fruits. The fruits were wounded at four points as previously describved and dipped in cell suspension of antagonists for one hour, dried at room temperature for 12 hrs. packed in plastic bags at 50 per bag and stored at room temperature. One hundred fruits were used for each treatment thus giving 400 wounds per treatment. For the check fruits were dipped in 500 ppm of Tecto. The fruits were examined at weekly intervals for three weeks and the percentage decay recorded.

The data obtained were analysed by the analysis of variance and the means separated according to Duncan's multiple range test (10). Percentage values were arc-sine transformed before analysis.

Results and Discussion

Several isolates among the yeasts and bacteria isolated from orange fruit washings were found to inhibit the growth of fruit rot fungi in culture plates. The two most inhibitory isolates were identified to be *Bacillus subtilis* and *Debaromyces hansenii*. The isolates of filamentous fungi isolated from fruit surface such as *Aspergillus, Penicillium* and *Botryodiplodia theobromae* are pathogenic to orange frits (1), and were thus not considered as candidates for antagonism.

Table 1 shows that the number of wound sites that developed rots were significantly ($P < 0.05$) reduced in *B. subtilis* and *D. hansenii* treated fruits compared to the control. Highest reduction in infection was obtained when antagonists were inoculated 24 hrs. before rot fungi. The efficacy of the antagonists thereafter declined with decreased time intervals between inoculation of antagonists and rot fungi. The ability of the antagonists to reduce infection of orange fruit was significantly reduced when introduced after the rot fungi had been inoculated.

Table 1: Postharvest control of three fruit rot fungi by *Bacillus subtilis* (Bs), *Dabaromyces hansenii* (Dh) and standard fungicide (Tecto) treatment.

Treatment	Infection rate (%)*					
	An		Bt		Pi	
	Bs	Dh	Bs	Dh	Bs	Dh
Antagonist applied 24h before pathogen	6.2a	8.0a	6.6a	7.7a	7.5a	8.3a
18h before pathogen	6.8a	8.5a	6.9a	8.1a	7.9a	8.8a
12h before pathogen	7.2a	9.2a	7.5a	8.9a	8.4a	9.9a
6h before pathogen	7.3a	9.5a	7.7a	9.7a	9.2a	11.2a
0h before pathogen	8.7a	10.9a	8.6a	11.3a	10.2a	15.5a
Antagonist applied 6h after pathogen	17.7b	24.9b	23.5b	28.7b	25.0b	3.4b
12h after pathogen	52.6c	41.6c	48.3c	68.3c	49.7c	73.3c
18h after pathogen	72.7d	100d	60.7d	100d	79.0d	100d
24h after pathogen	88.3e	100d	83.0e	100d	95.2e	100d
Fungicide (Tecto)	7.3a	–	6.4a	–	8.2a	–
Control	100f	–	100f	–	100e	–

*Figures represent percentage of wound sites that developed rot symptoms.
An = *Aspergillus niger*, Bt = *Botrydiplodia theobromae*, Pi = *Penicillium italicum.*
Values within the same column followed by different letter(s) are significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

The data in Table 2 shows that the antagonists were effective in inhibiting natural infection of orange fruits. Whereas infection rate increased to 55.3% in the control, it remained below 10% and 15% for *B. subtilis* and *D. hansenii*, respectively, after three weeks storage. The efficacy of *B. subtilis* in inhibiting natural infection of orange fruits was even higher than that of fungicide (Tecto) treatment.

The results of the present study indicate that *B. subtilis* and *D. hansenii* could effectively control rots of sweet orange fruits if antagonists are present on the wound surfaces in advance of invasion by pathogen. Fruit injuries occur mainly during the harvesting and post-harvesting operations, thus making it easy to apply antagonists before wounding is likely to occur.

Table 2: Postharvest control of natural infection in injured sweet orange fruits by *Bacillus subtilis, Debaromyces hansenii* and standard fungicidal (tecto) treatment.

Treatment	Storage period (in weeks)/infection rate (%)*		
	1	2	3
B. subtilis	11.2a	4.6a	9.4a
D. hansenii	3.4a	7.2a	13.8a
Fungicide (Tecto)	1.0a	4.8a	11.2a
Control	10.6b	23.9b	55.3b

*Figures represent percentage of wound sites that developed rot symptoms.
Values within the same column followed by different letter(s) are significantly different ($P < 0.05$ according to Duncan's Multiple Range Test.

Before recommendations could be made on the use of these antagonists to control rots of orange fruits, further studies are required to assess the compatibility of antagonists with fungicidal treatments and fruit additives such as mineral oils and paraffin used to reduce dehydration and improve the quality of fruits. Information is also needed on the toxicological aspect of these antagonists. For instance, Wilson and Wisniewski (7) reported that some strains of *Pseudomonas cepacia* are good biocontrol agents of post-harvest rots of apples and pears. However, some strains of same organism have been reported as opportunitsic pathogen associated with cystic fibrosis in man (11).

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