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STUDY OF ANTIFUNGAL EFFECT AND NATURE OF SUPPRESSIVE VOLATILE PRODUCED BY *BACILLUS SUBTILIS* BS 2924

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INTRODUCTION

Only a few works are devoted to studies of suppressive volatiles in bacilli. The inhibitive effects of volatiles from bacilli were observed on cyanobacteria (Wright et al., 1991) and some plant pathogenic fungi (Fiddaman and Rossal, 1993, 1994). A volatile with a broad spectrum of action upon bacteria, yeasts and fungi was found in *B. subtilis* BS 2924 (Sharga, 1999).

The tasks of the present study were: *i)* to determine the influence of the *B. subtilis* BS 2924 volatile upon the hyphal and colony growth of *Botrytis fabae*, the causal agent of chocolate spot on broad-beans, *ii*) to evaluate the ability of *B. subtilis* BS 2924 to produce the volatile on the roots of plants cultivated in-vitro, and *iii*) to study the nature of the volatile by use of mass spectrometry.

MATERIAL AND METHODS

The *B. fabae* MJW94 was gifted by Prof. J.W. Mansfield (London

University, UK). The *B. subtilis* BS 2924 was isolated in Ukraine. The seeds of broad-bean cv. Maris bed ('Plant Breeding International', Cambridge, UK) and grains of wheat cv. Mironvska (Subcarpathian Regional Seed Station, Gliboke, Ukraine) were surficially sterilised in solution of sodium hypochlorite (2% available chlorine), washed in sterile distilled water and germinated on wet filter paper. They were then soaked for 10 min in a suspension of *B. subtilis* BS 2924 at 10^{10} cfu/ml, prepared in a 5% solution of D-glucose or water and planted in autoclaved sand in 200 ml honey jars. The plant-growing substrates were wetted with the same suspensions (10 ml jar^{-1}).

The quarters of a cotton disc penetrated by the roots of two-week-old wheat or broad-bean plants (with aboveground parts cut) were turned root end up in a Petri dish and inoculated with *B. subtilis* BS 2924 at 10^{10} cfu ml^{-1} in sterile distilled water, or in a 5% solution of D-glucose.

Then plates or jars were hermetically sealed with the same plates or honey jars containing 1-day-old *B. fabae* colony grown on malt agar (8 mm dia malt agar disc) with young mycelium. The volatile production was investigated in substrates with or without D-glucose and plants (Table 20.1).

Table 20.1 Effect of substrate on production of *B. subtilis* BS 2924 volatile

Substrate	*B. fabae MJW 94 colony, r, mm*†	*B. subtilis BS 2924, mean cfu* g^{-1}
Sand+Beans, control	34.4 ± 0.36	—
Sand+BS	33.3 ± 0.61	10^9
Sand+BS+Beans	26.6 ± 0.1	5×10^9
Sand+BS+D	21.1 ± 0.09	7.2×10^{10}
Sand+BS+D+Beans	17.9 ± 0.2	4.3×10^{11}
Sand+Wheat, control	31.3 ± 0.13	—
Sand+BS+D+Wheat	22.5 ± 0.33	6.7×10^{11}
*Cotton+Bean roots, control	23.1 ± 0.19	—
*Cotton+BS	19.1 ± 0.21	1.5×10^9
*Cotton+BS+Beans	14.5 ± 0.3	6.8×10^9
*Cotton+BS+D	10.3 ± 0.43	8.5×10^{10}
*Cotton+ BS+D+Bean roots,	2.6 ± 0.23	1.2×10^{11}
*Cotton+Wheat roots, control	22.2 ± 0.29	—
*Cotton+BS+D+Wheat roots	1.07 ± 0.14	5.2×10^{11}

*Sealed Petri plates. BS: *B. subtilis* BS 2924; D: D-glucose; †Mean ± SE; n = 5.

To study the influence of volatiles on *B. fabae* colony morphology, D-glucose, starch or sucrose were utilised in the sealed plate method (Fiddaman and Rossal, 1993) at 3% concentration in oxoid nutrient agar.

To see the effect of volatiles on germ tubes, the suspension of *B. fabae* MJW 94 conidia was inoculated on a cellophane covered malt agar plate.

After 48 h of incubation at 28°C in sealed plates, the cellophane was observed under a light microscope for growth of germ tubes.

The nature of the *B. subtilis* BS 2924 volatile was studied by mass spectrometry (mass spectrometer MI1201, 'Electron', Sumi, Ukraine) of the headspace of the culture grown on 100 ml of Oxoid nutrient agar with or without 3% D-glucose in 500 ml Erlenmeyer flasks.

RESULTS AND DISCUSSION

Antifungal volatile activity was absent in sand inoculated with a water suspension of *B. subtilis* BS 2924. Only a slight antifungal volatile production by bacilli occurred in the presence of germinated broad-bean or wheat. Significant reduction in *B. fabae* MJW 94 colony size was detected after sand treatment with suspension of the bacterium prepared in 5% D-glucose solution. However, the best reduction in growth of the test fungus was seen in the presence of D-glucose and plants. These correlated with greater amounts of viable bacilli cells (Table 20.1). The addition of D-glucose to the growing substrates or presence of roots increased the production of inhibitive gaseous substances by bacilli. This was detected in both the sealed plates and sealed jars. The suppressive effect of *B. subtilis* BS 2924 volatiles was stronger in the volume of air in sealed plates. The volatile concentration was lower in sealed jars due to their larger volume. In a separate test, some reduction in plant growth was observed in the presence of D-glucose at 5% concentration. D-glucose suppressed the emergence of plants from the sand and outgrowth of cut aboveground parts of broad-bean and wheat in sealed plates and jars also. The non-inoculated beans in control developed severe symptoms of chocolate spot 10 days after sealing the jars. The wheat roots in control were colonised by mycelium of *B. fabae* MJW 94 during the same 10-day period.

A protective effect was observed when plants were treated by suspension of the *B. subtilis* BS 2924 in 5% D-glucose (Figures 20.1 and 20.2).

In all cases of the stronger antifungal effect, increased concentrations of the viable bacilli cells were isolated from substrates.

The main responses due to volatile action in macrocolonies were change in their morphology and colour, a fact not described in earlier works. Malformations, curling of hyphal tips and shortening of cells were observed in all experimented variants. Maximum inhibition was observed when *B. subtilis* BS 2924 was grown in the presence of D-glucose, (Figure 20.3:1). The shift to rhizomorphic growth occurred under the influence of the volatile produced by bacilli cells in the presence of

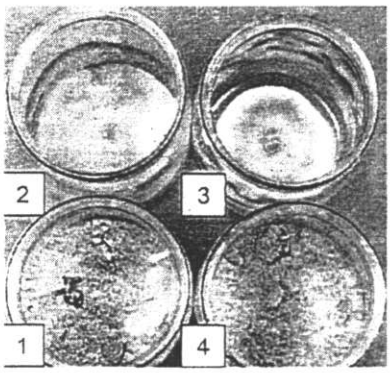

Fig. 20.1 Production of *B. subtilis* BS 2924 volatile at the presence of broad beans:
Left: Control. Young beans (1) infected by conidia shed from the above grown colony of *B. fabae* MJW 94(2).
Right: Suppression of *B. fabae* MJW 94 growth (3) by volatiles from germinated beans (4) inoculated by bacilli suspension in 3% solution of D-glucose at 10^{10} cfu/ml.

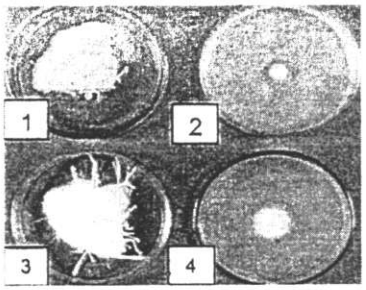

Fig. 20.2 Production of *B. subtilis* BS 2924 volatile on the roots of wheat:
1,2-suppression of *B. fabae* MJW 94 growth by the volatiles produced on wheat roots. The D-glucose retarded the regrowth of wheat leaves;
3,4-spreading of *B. fabae* MJW 94 mycelium onto wheat roots in control.

sucrose, (Figure 20.3:2). The volatile produced in the presence of starch induced concentric rings in the colony of *B. fabae* MJW 94—grey coloured in the centre of the colony and light brown at the periphery, (Figure 20.3:3). Both starch and sucrose induced a lower level of antifungal volatile production, due to slower utilisation of these complex carbohydrates. It seems possible that variation in fungal response reflected the differences in composition of biologically active components in the volatile mixtures produced on media with starch, sucrose or D-glucose.

Microscopic examination of excised pieces of the cellophane membrane revealed distinct differences in germ tube growth compared with control. About 64% of the *B. fabae* MJW 94 conidia failed to germinate. The germ tube lengths on the receiver plate were on average

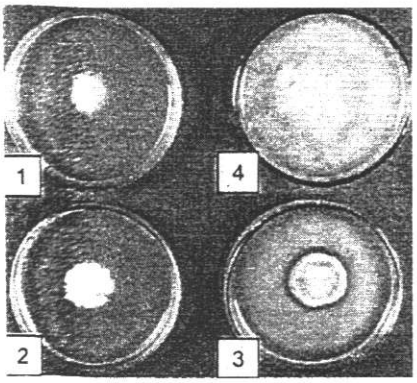

Fig. 20.3 Changes in *B. fabae* MJW 94 colony induced by volatiles of *B. subtilis* BS 2924: 1,2,3-colonies grown in sealed plate method above the lawns of *B. subtilis* BS 2924, cultivated on nutrient agar with 3% of D-glucose, sucrose or starch, respectively; 4-control.

6.2 times less than in control. Some of the germ tubes developed swellings, Figure 20.4(1). The young hyphae on the receiver plate were much less developed and some of them likewise had swellings. Most often extensive vacuolation was observed, Figure 20.4(2).

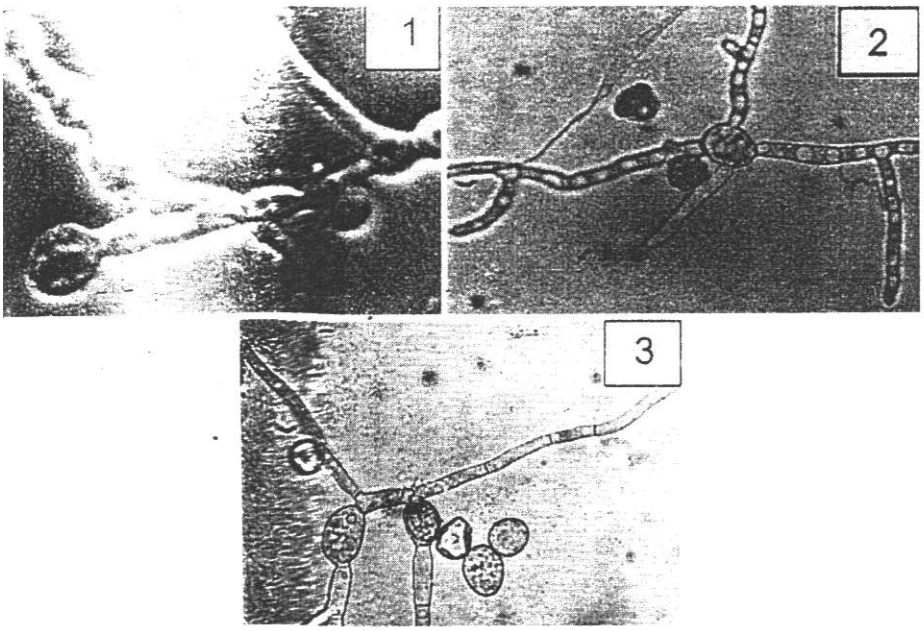

Fig. 20.4 Effect of volatiles on germ tube growth of *B. fabae* MJW 94

The mass spectrum of the air above the lawn of *B. subtilis* BS 2924 grown on nutrient agar with 5% D-glucose contained ion peaks with masses 44, 29, 12 and 2 a. u. m., which were 7.45, 3.87, 4.8 and 11 times respectively, larger than peaks of these ions in the mass spectrum of headspace of the lawn grown on nutrient agar without D-glucose (Table 20.2).

Table. 20.2 Results of mass spectrometry headspace analysis of nutrient agar with 5% D-glucose and headspace of *B. subtilis* BS 2924 lawn grown on nutrient agar with or without 5% D-glucose

Ion mass, a. u. m.	*Detected ions*	*Ion content in headspace of samples*		
		agar, 5% D-glucose	*B. subtilis BS 2924, 5% D-glucose agar*	*B. subtilis BS2924, agar without 5% D-glucose*
2	H_2^+	—	11.55×10^{-2}	1.55×10^{-2}
12	C^+	3.8×10^{-2}	2.4×10^{-1}	5×10^{-2}
14	N^+	1.92	1.94	2.25
16	O^+, CH_4^+	2.23×10^{-1}	6.59×10^{-1}	6.17×10^{-1}
17	OH^+	1.08×10^{-1}	3.9×10^{-2}	3.33×10^{-2}
18	H_2O^+	4.08×10^{-1}	3.5×10^{-1}	3.4×10^{-1}
20	Ne^+	5.08×10^{-2}	4.7×10^{-2}	4×10^{-2}
22	Ne^+	—	6.2×10^{-2}	10^{-2}
28	N_2^+	64.6	55	62.3
29	N_2^+	4.1×10^{-1}	3.87×10^{-1}	4.5×10^{-1}
31	OCH_3^+, CH_2OH^+	—	1.61×10^{-2}	—
32	O_2^+	13.1	1.6	8.7
34	H_2S	5×10^{-2}	1.78×10^{-2}	3.33×10^{-2}
40	Ar	1	1	1
44	CO_2^+, $CH_2CHO^+ + H^+$,	2.6×10^{-1}	11.6	1.33
45	CO_2^+, $COOH^+$, CH_3CHOH^+, $CH_3CHO^+ + H^+$, $(CH_2)_2OH^+$	2.31×10^{-2}	1.16×10^{-1}	2.33×10^{-2}
46	CO_2^+	—	5.04×10^{-2}	10^{-2}

Note: Ion contents were estimated as means (n = 3) in relation to $^{40}Ar^+$ content.

Most probably, the H_2S estimated in similar quantities in all samples, including control, was introduced into the flasks from the laboratory air. The minimum amount of O_2 was found in culture grown in the presence of D-glucose. The levels of Ne and H_2O were close between all samples. As the ratios OH^+/H_2O^+ were very close in all mass spectra, the OH^+ ions originated from molecules of water. The amounts of N^+ were almost the same and levels of N_2^+ were close in air from all the flasks during mass spectrometry, indicating no production of nitrogen-containing compounds by bacterial lawns.

The quantities of C^+ and H_2^+ ions were about one order higher in ionised air from flasks with *B. subtilis* BS 2924 grown on agar with D-glucose than in ionised air from the flask with the culture consuming no D-glucose. This is clear evidence of carbon and hydrogen-containing compound production. The minimum ratio $O_2^+ / O^+ = 2.43$ was recorded in the mass spectrum of headspace of culture grown in D-glucose presence. It was about 5.8 times less than this ratio for culture grown without D-glucose and 24.2 times less than in the mass spectrum of control. Thus the presence of methane-ion (CH_4^+) is evident.

The ions of 31 a. u. m. (presumably, OCH_3^+, CH_2OH^+) were found only in the mass spectrum of headspace of the lawn utilising D-glucose.

The Voges-Proskauer test was positive for *B. subtilis* BS 2924, detecting acetoin (precursor of butanediol) presence. For the microbes known as mixed acid fermenters this test was negative. Mixed acid fermenters acidify a nutrient medium to a pH below 4.4 (Prescott et al., 1993); however, this was not observed for *B. subtilis* BS 2924, suggesting no mixed acid fermentation. Butanediol fermenters produce an excess of CO_2 at a molecular ratio CO_2/H_2 close to 5:1 (Prescott et al., 1993). The ratio CO_2^+/H_2^+ in the mass spectra of the headspace of *B. subtilis* BS 2924 lawns was very close to 100:1, indicating the presence not only of CO_2^+, but also $CH_2CHO^+ + H^+$ ions.

The ions of 45 a. u. m. in mass spectra of headspace of culture grown on nutrient agar with D-glucose were not only CO_2^+, but also $COOH^+$, $CH_3CHO^+ + H$, CH_3CHOH^+, $CH_3CHO^+ + H^+$ or $(CH_2)_2OH^+$. There is no reasonable explanation of the origin of these ions (31, 44, 45 a. u. m.) any more than for ionisation of CO_2 and ionising dissociation of ethanol (C_2H_5OH), acetone CH_3COCH_3, acetic (CH_3COOH), lactic ($CH_3CHOHCOOH$), succinic ($COOHCH_2CH_2COOH$) and formic acid ($HCOOH$) molecules, the products of 2,3-butanediol ($CH_3CHOH-CHOHCH_3$) fermentation of glucose.

Wright and colleagues (1991) discovered that some *Bacillus* strains produced 3-methyl-1-butanol, active against cyanobacteria. Fiddaman and Rossal (1994) revealed over 100 compounds in volatile produced by *B. subtilis*. They were tentatively identified as alcohols, aldehydes, ketones and esters. The authors suggested a similarity in types of volatiles produced from most of the substrates utilised by *B. subtilis* strain.

All things considered, the volatile of *B. subtilis* BS 2924 consisted of a mixture of organic compounds which may be produced by the bacterium on nutrient agar or in amended plant growing substrates in-vitro. Further studies should answer the question of the possible use

of this microbe as a biocontrol agent in the greenhouse for suppression of soil-borne plant pathogens.

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