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In vitro antagonistic action of egg plant and sweet potato phylloplane bacteria to some parasitic fungi

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Summary. Bacteria were isolated from the phylloplane of two crop plants commonly cultivated in Egypt. Thirty-one bacteria isolated from *Solanum melongena*, and twenty from *Ipomoea batatas* cv. Mabrouka were screened for their antagonistic behavior against five foliar and soil-borne fungal pathogens. The antagonism data derived from the 51 isolates were subjected to cluster analysis. Four isolates, two from *S. melongena* (SM8, SM31) and two from *I. batatas* (IB2, IB18), were chosen for further identification as they showed the greatest inhibition to all the phytopathogens tested. The four antagonistic isolates were identified as *Erwinia herbicola,* (SM31, IB2), *Pseudomonas fluorescens* (SM8) and *Bacillus subtilis* (IB18).

Key words: *in vitro* antagonism, phylloplane bacteria, *Solanum melongena*, *Ipomea batatas*.

Introduction

The surfaces of aerial plant parts provide a habitat for epiphytic micro-organisms, many of which also influence the growth of pathogens. Bacteria are generally the predominant initial inhabitants of newly expanded leaves, while yeasts and filamentous fungi dominate later in the growing season (Kinkel *et al*., 1987). A large body of information has been accumulated regarding antagonism between bacteria and fungi on the leaf surface, and its possible role in the biological control of pathogenic fungi (Gowdu and Balasubramanian, 1988). Biological control may be an alternative to chemicals in the control of some pathogenic fungi, in order to reduce environmental pollution. Saprophytic organisms play an important part in reducing the incidence of foliar diseases from fungi and bacteria on crops in the field (Blakeman and Fokkema, 1982; Janisiewicz *et al*., 1991; Frommell and Pazos, 1993).

Four *Pseudomonas* strains were evaluated for their ability to control *Sclerotinia homeocarpa* and *Bipolaris sorokiniana* on the phylloplane of Kentucky bluegrass (Hodges *et al*., 1994). *Bacillus subtilis* was the best biocontrol agent for yam leaf spot caused by *Curvularia eragrostidis* (Michereff *et al*., 1994). Mew *et al*. (1998) reported that *B. subtilis* strain 916 successfully controlled rice sheath blight under field conditions. All the selected isolates, *Pseudomonas, Bacillus* and *Stenotrophomonas maltophilia,* showed antifungal activity against *Verticillium dahliae* var. *longisporum in vitro* and were evaluated as potential biocontrol agents by Berg *et al*. (1998). Rajappan and Ramaraj (1999) evaluated the efficacy *in vitro* of *P. fluorescens* and *B. subtilis* against the cauliflower wilt pathogen

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Fusarium moniliforme. Fluorescent pseudomonad strains found to be effective against *Sclerotium rolfsii* were evaluated by Patil *et al*. (1998) under greenhouse conditions for their effects on groundnut and on collar rot incidence. *Trichoderma viride* and *Pseudomonas* sp. controlled stalk rot (associated with *Pythium aphanidermatum* and *Fusarium graminearum*) at the seedling stage of maize (Chen *et al*., 1999). Jindal and Thind (1993) found that *Flavobacterium* sp. isolated from the phylloplane of cowpea (*Vigna unguiculata* L. Walp.) was more inhibitory against bacterial blight of cowpea than other bacteria isolated from the same plant. *Erwinia herbicola* protected mung bean foliage from the bacterial leaf spot pathogen *Xanthomonas campestris* pv. *vignaeradiatae* (Bora *et al*., 1993). Experiments *in vitro* and *in vivo* by Rozsnyay *et al*. (1992) showed that some strains of *P. fluorescens*, some epiphytic bacteria and some fungi inhibited canker and dieback diseases of apricot.

The objective of this study was to survey the occurrence of bacterial isolates and to assess their potential in the phylloplane of eggplant and sweet potato planted in Egypt, as biological control agents for some foliar and soil-borne pathogenic fungi causing damage to these and other crops.

Materials and methods

Plant material

Two species of crop plants were used in this study: eggplant [*Solanum melongena* (Solanaceae)] (SM) and sweet potato [*Ipomoea batatas* 'Mabrouka' (Convolvulaceae)] (IB). The plants were raised in the Botanical Gardens of the Faculty of Agriculture, Cairo University, Egypt .

Isolation of bacteria from plant material

The leaves used to isolate antagonistic bacteria were randomly collected from 3 plants of *S. melongena* and 3 plants of *I. batatas* (ten leaves per plant). Leaves were placed in 200 ml phosphate buffer (0.1M, pH 6.8) and agitated for 10 min at 100 rpm (Goodman and Shaffer, 1971). The buffer from the first washing was discarded, and the leaves were washed for a second time in an MSE (London, UK) ultrasonic disintegrator for 30 sec at the beginning of the second wash. The sonicated step was used to facilitate detachment of organisms from the leaf surface. Sonicated samples were plated directly on nutrient yeast-dextrose agar (NYDA) medium (0.1 ml per plate). After 24 h incubation at 28°C, single colonies were removed with a sterile needle and transferred to fresh NYDA medium. Macroscopic examination of the developed bacterial colonies and Gram stain were carried out in order to classify the isolates into groups for further identification.

Screening of potential antagonists

Bacterial isolates from the phylloplane of *S. melongena* and *I. batatas* were screened for their ability to antagonize five pathogenic fungi: *Macrophomina phaseolina, Helminthosporium tetramera, Alternaria tenuis, Fusarium solani* and *Sclerotium rolfsii*. A 0.6-cm fungal disc was inoculated onto sterilized potato-dextrose-agar plates (PDA) at one pole. A bacterial isolate was streaked on the opposite pole of the same plates except for the controls, which were inoculated only with the fungal disc. Three replicates were conducted for each trial. Plates were incubated at 28 °C for 7 days and, at the end of each period, the area of fungal growth was estimated in cm^2.

Identification of bacterial isolates

One fluorescent (SM8) and 3 nonfluorescent (SM31, IB2, IB18) isolates which showed strong antibiosis towards the five fungal pathogens used in the assays and which were highly represented on the leaf surfaces, were characterized on the basis of results of conventional bacteriological tests described in Bergey's Manual (1984 and 1986).

Statistical analysis

All data were subjected to statistical analysis, including calculation of the mean, standard error and F-test at a level of $P<0.05$, according to the method of Armitage (1971), for control and treatments. The results were statistically evaluated according to the F-value.

Cluster analysis

Similarity indexes between isolates were clustered (Joseph *et al*., 1992) by the average linked technique (unweighted pair-group method). The results were expressed as phenograms. Cluster analysis was performed with a computerized pro-

gram. In this analysis, clustering began with the fusion of the two most similar isolates and proceeded until all isolates were fused into clusters and/or all clusters fused. The clustering process was represented in the form of a phenogram (tree) in which the top branch indicated the highest fusion level, and so on. For reference purposes, the fusion levels were designated 1, 2, and so on, from top to bottom, respectively. Control treatments were not included in the phenograms.

Results

In vitro antibiosis

Numerous bacterial colonies were obtained from the washings of eggplant and sweet potato, of which 30 from eggplant and 17 from sweet potato were antagonistic against all five fungi used in the tests. Four isolates (SM2, IB3, IB12 and IB19) were antagonistic against four of the fungi but not against *S. rolfsii* (Tables 1 and 2).

Table 1. Inhibition of fungal pathogens by 31 bacterial isolates from the phylloplane of *Solanum melongena* (SM).

Number of (SM) isolate	Fungal growth area (cm^2)				
	Fungi causing foliar disease			Soil-borne fungi	
	Macrophomina phaseolina	*Helminthosporium tetramera*	*Alternaria tenuis*	*Fusarium solani*	*Sclerotium rolfsii*
Control	63.64 a	63.64 a	18.50 a	22.00 a	13.67 a
1	5.00 j-l	9.17 g	12.50 g-i	14.83 ij	7.30 jk
2	1.76 no	8.50 g	11.17 jk	19.83 b-d	10.17 f-h
3	1.00 o	14.67 b	13.50 e-h	13.83 jk	7.33 jk
4	15.67 b	11.67 c-e	14.00 ef	15.33 i	11.67 b-d
5	8.25 gh	8.17 g	11.00 kl	12.50 lm	10.83 d-f
6	1.25 o	14.33 b	10.00 lm	20.50 b	12.33 b
7	5.50 jk	10.83 ef	11.83 i-k	17.50 gh	11.17 c-e
8	9.92 ef	3.67 kl	9.00 m	7.17 p	8.67 i
9	13.33 cd	4.17 k	11.33 i-k	7.17 p	6.50 k
10	1.83 no	7.00 h	11.83 i-k	18.67 d-f	7.83 j
11	2.33 no	12.80 c	17.17 b	19.83 b-d	9.83 gh
12	2.33 no	11.33 d-f	16.00 bc	11.50 m	11.50 b-e
13	4.08 lm	12.00 c-e	2.33 p	14.67 ij	6.83 k
14	8.17 h	15.00 b	2.50 p	10.33 n	3.42 l
15	2.92 mn	8.67 g	16.17 bc	12.17 lm	12.00 bc
16	9.50 e-g	4.17 k	14.67 de	19.50 b-e	10.67 e-g
17	9.33 e-h	14.25 b	15.50 cd	17.17 h	10.17 f-h
18	2.83 n	4.33 k	16.67 bc	15.17 i	10.67 e-g
19	6.92 i	4.75 jk	5.67 n	16.83 h	12.33 b
20	14.33 c	6.17 hi	13.33 f-h	20.00 bc	11.67 b-d
21	5.33 j-l	14.25 b	15.67 cd	19.17 c-e	12.25 b
22	10.17 e	10.33 f	15.83 cd	13.17kl	12.17 b
23	9.17 e-h	12.25 cd	9.50 m	17.33 h	10.67 e-g
24	6.00 ij	6.00 hi	9.67 m	18.83 c-f	13.50 a
25	9.67 ef	5.50 ij	11.50 i-k	19.67 b-d	9.50 h
26	8.75 f-h	3.67 kl	13.67 e-g	13.83 jk	12.08 b
27	15.67 b	10.83 ef	12.33 h-j	18.50 e-g	8.17 ij
28	5.67 j	12.00 c-e	9.83 lm	19.50 b-e	11.67 b-d
29	12.67 d	8.33 g	9.83 lm	17.83 f-h	9.67 h
30	4.33 kl	3.00 lm	12.00 i-k	9.83 no	1.50 m
31	1.42 o	2.25 m	3.83 o	8.83 o	1.33 m

Differences between means followed by the same letter are not significant.

Table 2. Inhibition of fungal pathogens by 20 bacterial isolates from the phylloplane of *Ipomoea batatas* (IB).

Number of (IB) isolate	Fungal growth area (cm^2)				
	Fungi causing foliar disease			Soil-borne fungi	
	Macrophomina phaseolina	*Helminthosporium tetramera*	*Alternaria tenuis*	*Fusarium solani*	*Sclerotium rolfsii*
Control	63.64 a	63.64 a	18.50 a	22.00 a	13.67 a
1	12.67 c	6.92 j	16.17 b	19.50 bc	11.83 b
2	4.83 l	9.50 gh	1.33 h	15.00 gh	13.32 a
3	9.67 e	14.17 d	12.83 d	18.83 bc	9.83 c-e
4	6.50 ij	15.50 c	14.50 c	15.50 fg	8.83 ef
5	3.92 m	17.17 b	14.33 c	14.17 h	10.25 cd
6	7.83 f-h	4.00 l	12.41 d	14.83 gh	7.50 g
7	8.50 f	8.00 ij	15.83 b	20.00 b	9.17 d-f
8	1.83 o	1.92 m	12.17 de	9.83 l	9.50 d-f
9	2.92 n	9.17 hi	15.17 bc	18.33 cd	10.00 c-e
10	6.50 ij	10.83 f	10.83 f	17.42 de	11.50 b
11	18.33 b	9.50 gh	12.33 de	16.33 ef	11.67 b
12	3.83 m	4.33 l	1.92 h	12.67 ij	13.33 a
13	8.33 f	5.67 k	2.42 h	12.17 j	9.83 c-e
14	7.33 g-i	12.00 e	11.17 ef	11.83 jk	9.00 d-f
15	8.17 fg	10.67 fg	10.00 fg	10.67 kl	10.83 bc
16	7.00 h-j	9.33 h	9.50 g	10.17 l	9.67 c-e
17	6.33 jk	14.00 d	11.17 ef	12.17 j	8.33 fg
18	5.50 kl	2.67 m	2.50 h	10.67 kl	8.33 fg
19	10.67 d	7.33 j	10.17 fg	16.00 fg	13.33 a
20	13.17 c	8.83 hi	13.17 d	13.83 hi	10.83 bc

Differences between means followed by the same letter are not significant.

Table 3. Maximum and minimum percentage of reduction of fungal growth caused by bacterial isolates from phylloplane of eggplant (SM) and sweet potato (IB) plants in relation to control values.

Pathogen	Percentage antagonistic activity of isolates	
	maximum	minimum
M. phaseolina	98.4 SM[a] 97.0 IB[b]	75.4 SM 71.0 IB
H. tetramera	96.5 SM 97.0 IB	76.4 SM 73.0 IB
A. tenuis	86.5 SM 92.0 IB	9.9 SM 12.6 IB
F. solani	67.4 SM 55.3 IB	6.8 SM 9.1 IB
S. rolfsii	90.1 SM 45.1 IB	1.2 SM 2.5 IB

[a] SM, *Solanum melongena*.
[b] IB, *Ipomea batata*.

Growth of *M. phaseolina* was greatly restricted by isolates SM12, SM16, SM17 and IB7 (Tables 1 and 2). Optimum antagonistic activity among isolates from both plants against *A. tenuis* was exhibited by SM13 and IB2 (Table 1 and 2), which is also shown in representative plate cultures in Figure 1, in comparison with some selected isolates.

Isolate SM8 reduced growth of *F. solani* by 67.4% compared to control values (Tables 1 and 3). That same isolate reduced growth of *S. rolfsii* by 63.4% (Tables 1 and 3). Isolates SM9, SM14, SM30 and SM31 and IB13 formed inhibition zones around *S. rolfsii*.

Cluster analysis of the 31 isolates from eggplant and the 20 isolates from sweet potato against each pathogen provided a quick overview of the clustering process, showing which isolate was found in each cluster and which was most important for the antagonistic activity against each of the pathogens tested.

Cluster analysis in Figure 2A demonstrated that

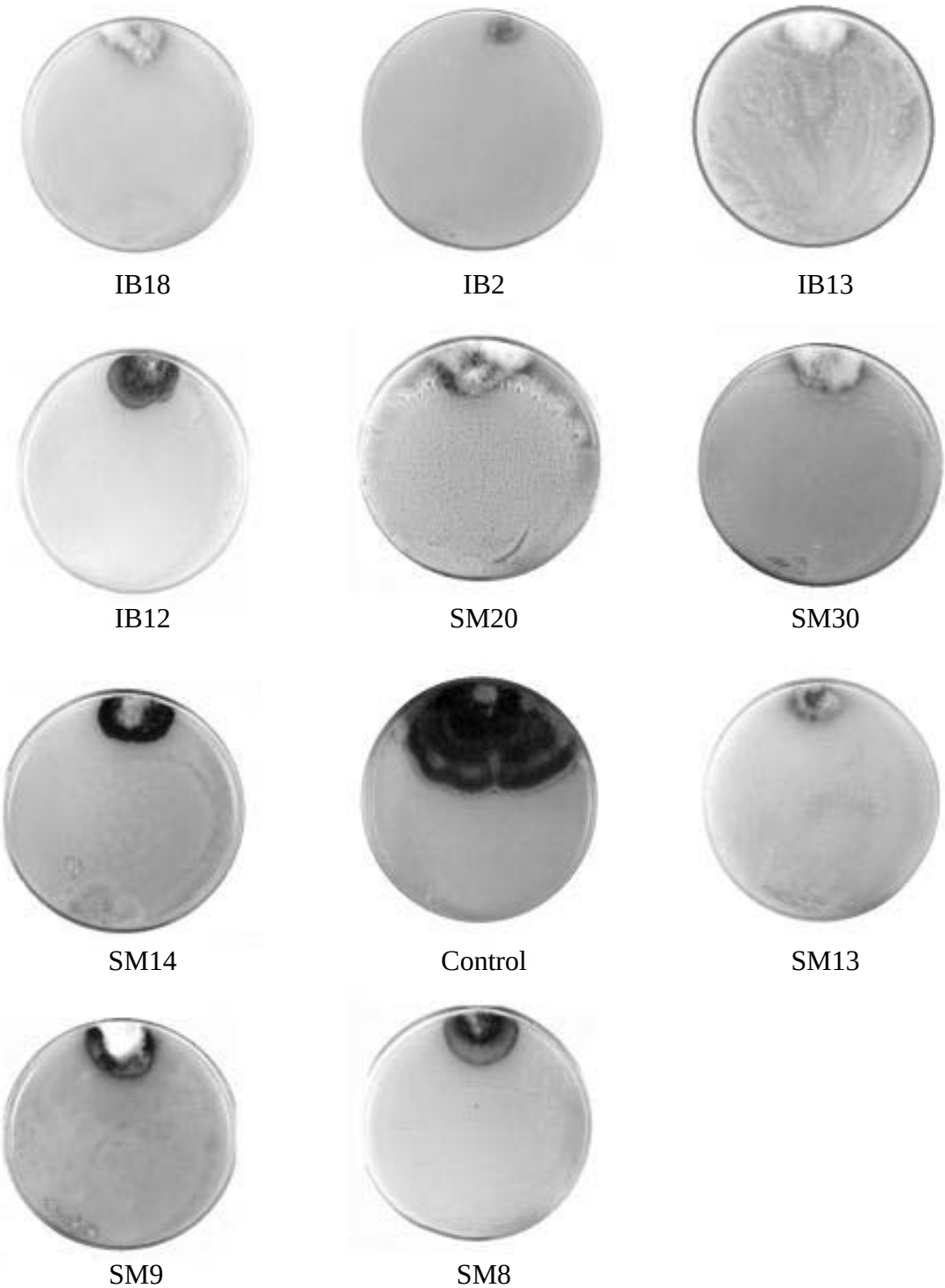

Fig. 1. An example of the antagonistic effect of some of the bacterial isolates used in this study against the fungal pathogens tested. Top of Petri plate: *A. tenuis*; bottom, different bacterial strains isolated from eggplant (SM) or sweet potato (IB).

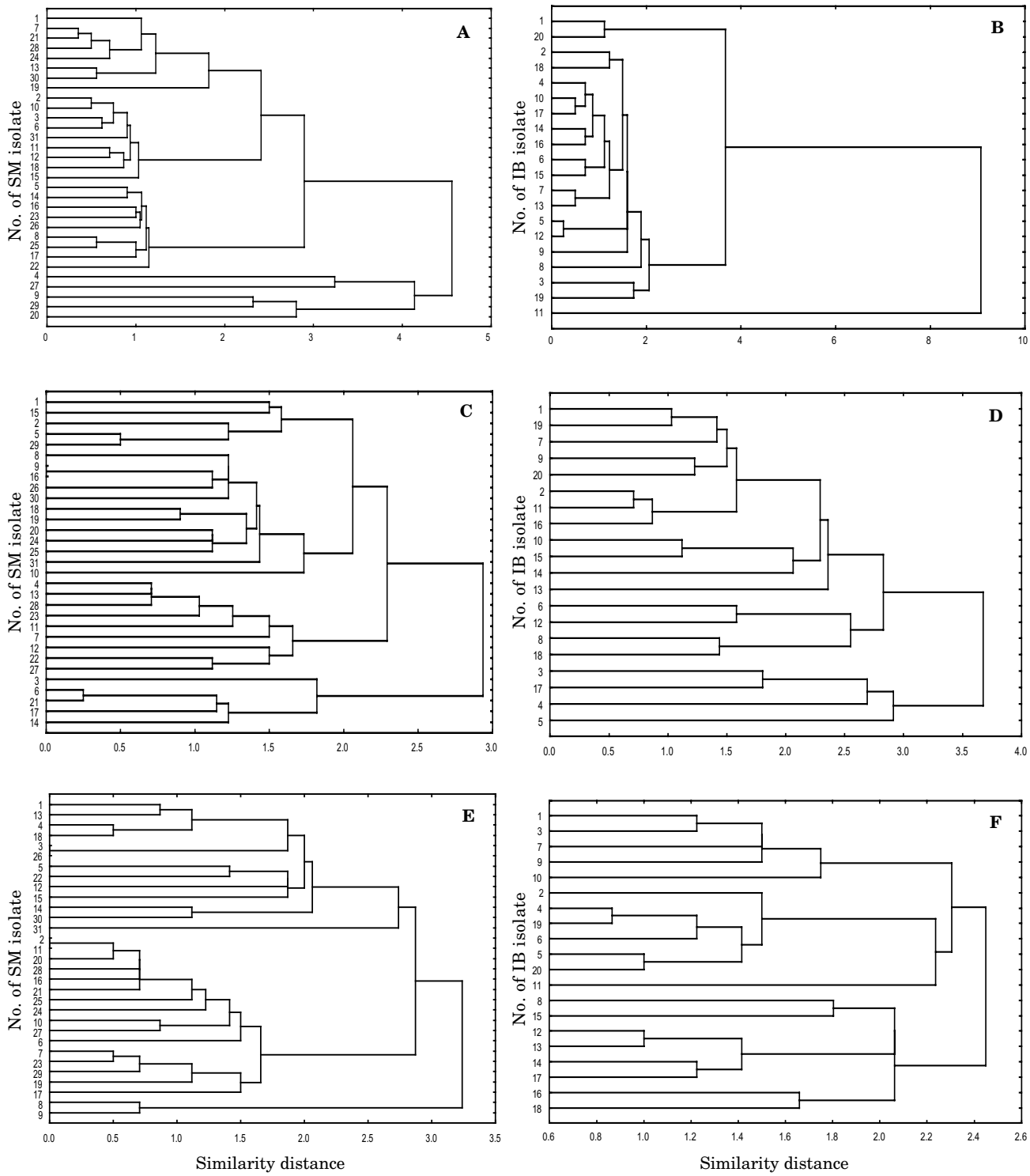

Fig. 2. Phenograms based on average linkage cluster analysis of 31 bacterial isolates from eggplant (SM) and 20 bacterial isolates from sweet potato (IB) against *Macrophomina phaseolina* (A, B); *Helminthosporium tetramera* (C, D); *Alternaria tenuis* (E, F) (this page); *Fusarium solani* (G, H); *Sclerotium rolfsii* (I, J) (next page).

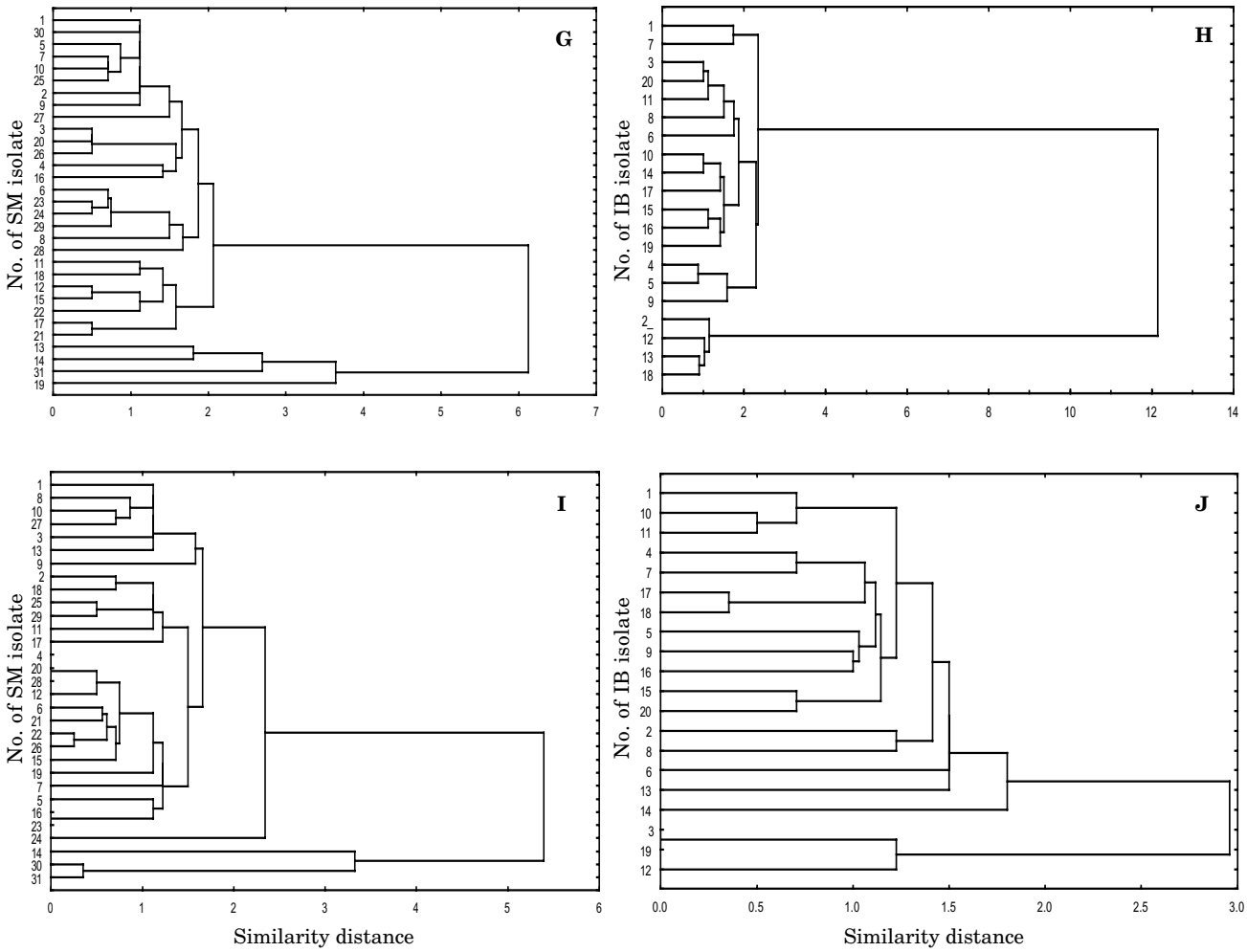

Fig. 2. (*continued from the preceding page*)

some isolates against *M. phaseolina* were very similar (SM22 and up), while others clustered at lower levels of similarity (SM4, 27, 9, 29 and 20). In the phenogram of Figure 2B, showing antagonism to *M. phaseolina* from sweet potato isolates, all isolates were more closely related to each other than to IB11, which stood apart. In the phenogram showing the isolates of eggplant against *H. tetramera* (Fig. 2C) there were two main clusters at an average distance of about 2.9 i.e. clustered at lower levels of similarity. The first group contained isolates SM3, 6, 21, 17 and 14, the second the remaining isolates, so that SM8, 9, 30 and 31 were in the same cluster.

Figure 2D shows the phenogram of the 20 isolates from sweet potato against *H. tetramera*. The cluster fell into two groups, the first containing isolates IB3, 17, 4 and 5, which clustered at a lower level of similarity, the second being in turn divided into subclusters, with isolates IB2 and IB18 in different subclusters.

The phenogram representing the 31 isolates from eggplant against *A. tenuis* is shown in Figure 2E. This phenogram fell into two main groups which clustered at a very low level of similarity, with an average distance of 3.3. The first group contained isolates 8 and 9. The second group was divided into two subclusters. In one of these subclusters isolates 30 and 31 were contained in the same groups, indicating a high level of similarity. In Figure 2F, representing the 20 isolates from sweet potato against *A. tenuis,* isolates 2 and 18

were in two different subclusters and the similarity between these isolates was very low.

In Figure 2G the phenogram of the 31 isolates from eggplant against *F. solani* showed that isolates 31, 8, 9 and 30 were in different groups. By contrast, isolates 2 and 18 from sweet potato were in one and the same group (Fig. 2H), thus showing a high similarity level in their reaction to the pathogen. A high similarity was evident between isolates 8 and 9 from eggplant against *S. rolfsii* (Fig. 2I) because both were in the same group. Also similar were isolates 30 and 31 in another group. Isolates 2 and 18 from sweet potato tested against *S. rolfsii* fell into two subclusters with low similarity (Fig. 2J).

Identification

Among the four isolates which were considered the most efficient antagonists against *M. phaseolina, H. tetramera, A. tenuis, F. solani* and *S. rolfsii* the fluorescent isolate SM8 was identified as *Pseudomonas fluorescens* (Palleroni, 1984), the nonfluorescent isolates SM31 and IB2 as *Erwinia herbicola* (Lelliott and Dickey, 1984), and IB18 as *Bacillus subtilis* (Claus and Barkeley, 1986).

Discussion

The results of this study showed that antagonistic bacterial communities on the leaf surfaces of *S. melongena* and *I. batatas* belonged mainly to three species: *B. subtilis* on the phylloplane of sweet potato, *P. fluorescens* on eggplant and *E. herbicola* on both. These are the bacterial species best known and most commonly used in tests for the microbiological control of a range of plant diseases. Loeffler and co-workers (1986) found that *B. subtilis* gave good control of *R. solani* in many crops. The bacterium produces bacilysin and fengymycin A and B, which are composed of a $C_{15} - C_{18}$ lipid moiety and a peptide moiety of eight amino acid residues and it is thought that these substances account for the antagonistic activity of this bacterium. Janisiewicz and Roitman (1988) reported that blue mold and grey mold of apples and pears could be controlled by *Pseudomonas* (*Burkholderia*) *cepacia* Burkh and by the antifungal compounds this bacterium produces. The bacterium strongly inhibited fungal growth of *Penicillium expansum* during *in vitro* screening on NYDA medium. Studies by Thomashow and Weller (1990) reviewed the importance of the antibiotic phenazine-1-carboxylic acid, produced by strain 2-29 of *P. fluorescens*, to suppress take-all disease of wheat caused by *Gaeumannomyces graminis* f. sp. *tritici*. Leaf application of *P. fluorescens* effectively conferred resistance against leaf pathogens (Hoffland *et al*., 1996).

E. herbicola was frequently isolated from samples of many plants. Wilson *et al*. (1992) and Kearns and Hale (1996) suggested that the inhibition of pathogens by *E. herbicola* was by colonization. Goodman (1965), Riggle and Klos (1972), Hsieh and Buddenhagen (1974) and Beer *et al*. (1984) on the other hand stated that inhibition was due to acid conditions produced by the bacterium. Other explanations include competition for nutrients (Slade and Tiffin, 1984), production of bacteriostatic substances (Erskine and Lopatecki, 1975), of bacteriocin (Beer and Vidavet, 1978), of herbicolin (Ishimaru *et al*., 1988; Kempf *et al*., 1994) or of an antibiotic (Vanneste *et al*., 1992; Kearns and Hale, 1996).

Whatever the actual mechanism of inhibitory action may be, these bacteria have been shown to inhibit or delay the onset of many diseases of cultivated plants, and compounds to apply them in the field have been developed and are commercially available in some countries.

The strains isolated in the present study will now be used in field trials to test their effectiveness in countering soil-borne diseases of eggplant and sweet potato.

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