main chain hydrogen bonds, and a few hydrophobic interactions. Dislodging the mobile loop from the chaperonin-10 surface and preparing it for association with the chaperonin-60 tetradecamer might not therefore involve a large entropy barrier. Clearly, further structural work will be necessary for dissecting the role of the mobile loop in chaperonin-60: chaperonin-10 association.

1. Hartl, F. U., *Nature*, 1996, **381**, 571–580.
2. Houry, W. A., Frishman, D., Eckerskorn, C., Lottspeich, F. and Hartl, F. U., *Nature*, 1999, **402**, 147–154.
3. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L. and Sigler, P. B., *Nature*, 1994, **371**, 578–586.
4. Xu, Z., Horwich, A. L. and Sigler, P. B., *Nature*, 1997, **388**, 741–750.
5. Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L. and Deisenhofer, J., *Nature*, 1996, **379**, 37–45.
6. Mande, S. C., Mehra, V., Bloom, B. R. and Hol W. G. J., *Science*, 1996, **271**, 203–207.
7. Hunt, J. F., van der Vies, S. M., Henry, L. and Deisenhofer, J., *Cell*, 1997, **90**, 361–371.
8. Landry, S. J., Ryalls, J. Z., Fayet, O., Georgopoulos, C. and Gierasch, L. M., *Nature*, 1993, **364**, 255–258.
9. Landry, S. J., Taher, A., Georgopoulos, C. and van der Vies, S. M., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 11622–11627.
10. Cole, S. T. *et al.*, *Nature*, 1998, **393**, 537–544.
11. Taneja, B. and Mande S. C., *Protein Eng.*, 2001, **14** (in press).
12. Collaborative Computational Project, Number 4, *Acta Crystallogr. D*, 1994, **50**, 760–763.
13. Murshudov, G. N., Vagin, A. A. and Dodson, E. J., *Acta Crystallogr. D*, 1997, **53**, 240–255.
14. Brunger, A. T. *et al.*, *Acta Crystallogr. D*, 1998, **54**, 905–921.
15. Murzin, A., *Curr. Opin. Struct. Biol.*, 1996, **6**, 386–394.
16. Taneja, B. and Mande, S. C., *Protein Eng.*, 1999, **12**, 815–818.
17. Sibanda, B. L. and Thornton, J. M., *Methods Enzymol.*, 1991, **202**, 59–82.
18. Landry, S. J., Steede, N. K. and Maskos, K., *Biochemistry*, 1997, **36**, 10975–10986.
19. Esnouf, R. M., *Acta Crystallogr. D*, 1999, **55**, 938–940.
20. Kraulis, P. J., *J. Appl. Crystallogr.*, 1991, **24**, 946–950.

ACKNOWLEDGEMENTS. We thank Vijay Mehra and Barry Bloom for the generous gift of plasmid encoding *M. tuberculosis* chaperonin-10. We are grateful to C. R. Suri for overall help, Sagar Nimsadkar for technical help and Girish Sahni for access to chromatographic equipment. We also thank Sharmila Mande for stimulating discussions and C. M. Gupta, A. Ghosh and Wim Hol for encouragement. Financial support from the Council of Scientific and Industrial Research, and the Department of Biotechnology, New Delhi to S.C.M. is gratefully acknowledged. B.T. is a CSIR senior research fellow.

Received 18 June 2001; revised accepted 25 June 2001

Antibiosis-mediated necrotrophic effect of *Pseudomonas* GRC_2 against two fungal plant pathogens

C. P. Gupta, R. C. Dubey, S. C. Kang† and D. K. Maheshwari*

Department of Botany and Microbiology, Gurukul Kangri University, Haridwar 249 404, India
†Department of Biotechnology, Taegu University, Kyungsan, Kyungbuk 712-714, Korea

A fluorescent *Pseudomonas* GRC_2 isolated from rhizosphere of potato, showed necrotrophic antibiosis *in vitro* against two major plant pathogens, *Macrophomina phaseolina* and *Sclerotinia sclerotiorum*. After 5 days of incubation at 28 ± 1°C, this strain caused clear inhibition zones in dual culture, restricting the growth of *M. phaseolina* and *S. sclerotiorum* by 80.1% and 73.5%, respectively. Scanning electron photomicrographs from the zone of interaction showed loss of sclerotial integrity, hyphal shrivelling, mycelial and sclerotial deformities and hyphal lysis in *M. phaseolina*. Hyphal perforations, lysis and fragmentation were observed in case of *S. sclerotiorum*. Similar result was also observed when both the pathogens were grown on tryptic soy agar medium juxtaposed to 5-day-old culture filtrate of *Pseudomonas* GRC_2. These morphological abnormalities in fungal pathogens were due to production of antifungal secondary metabolites by *Pseudomonas* GRC_2.

IN recent years, fluorescent pseudomonads have drawn attention worldwide because of production of secondary metabolites such as siderophores[1], antibiotics[2,3], volatile compound HCN[4], enzymes[5] and phytohormones[2,3]. These have been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneously-induced plant growth. Biological control of plant diseases with bacterial antagonist is a potential alternative of chemical control, because chemical control is expensive and results in accumulation of hazardous compounds being toxic to soil biota.

Certain bacteria, including species of *Pseudomonas* have been reported to destroy the host cell after or slightly before invasion. They show necrotrophy and utilize nutrients from the dying or dead host. The invasion is often initiated by attack and lysis of hyphae or survival structures[4,6]. However, the mechanism by which *Pseudomonas* GRC_2 protects the plants/seedlings against soil-borne fungal pathogens is unknown. *Macrophomina phaseolina* Tassi Goid and *Sclerotinia sclerotiorum* Lim de Bery are important seed- and soil-borne plant pathogens distributed worldwide. They cause considerable economic loss in yield of crop plants[7,8]. Therefore, a study was undertaken to investigate the interaction between *Pseudomonas* GRC_2 and *M. phaseolina* and *S. sclerotiorum* which could be utilized for biocontrol of these pathogens.

A strain of fluorescent *Pseudomonas* GRC_2 was isolated from the rhizosphere of potato and was characterized according to Bergy's Manual of Determinative Bacteriology[9]. It was maintained on tryptic soy agar medium (TSM). Cultures of *M. phaseolina* and *S. sclerotiorum* were isolated from abnormal seeds of peanut by blotter technique[10]. The fungal pathogens were main-

*For correspondence. (e-mail: akataria@hwr.bhel.co.in)

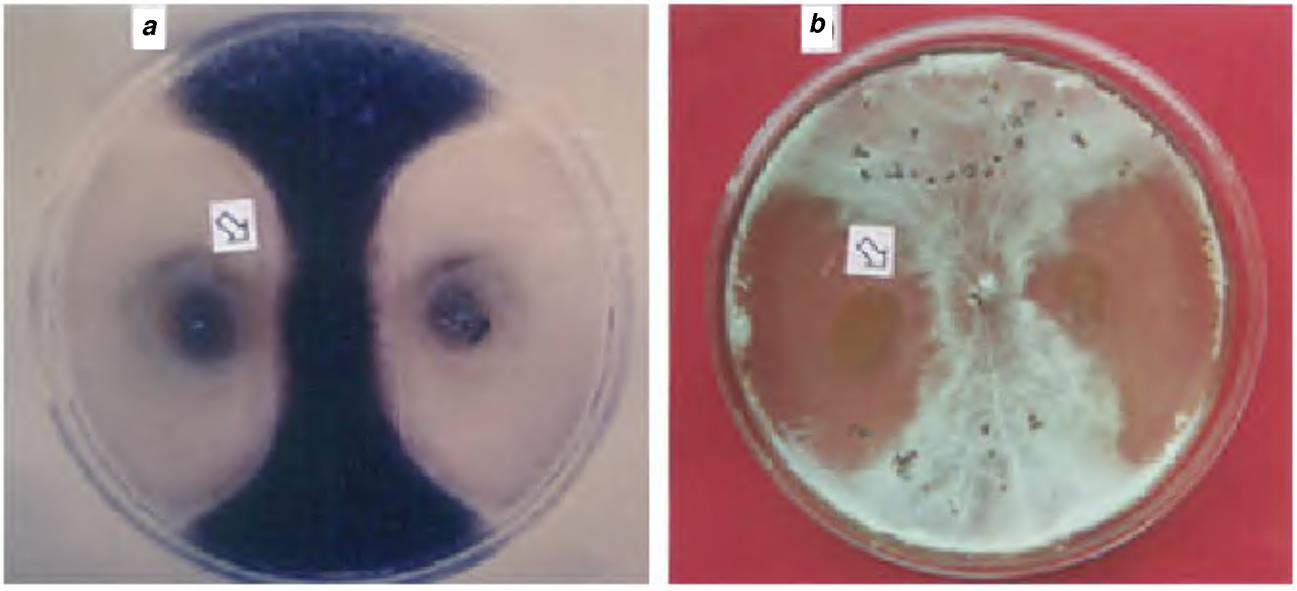

Figure 1. Dual culture showing colony interaction. ***a***, *Pseudomonas* GRC_2 vs *M. phaseolina;* ***b***, *Pseudomonas* GRC_2 vs *S. sclerotiorum.* Arrows indicate the zone of inhibition.

tained on Czapek–Dox agar slants at 4°C temperature for further study. The antagonistic properties of *Pseudomonas* GRC_2 were tested against *M. phaseolina* and *S. sclerotiorum* on TSM plates using dual culture technique[11]. An agar block (5 mm dia) of 5-day-old culture of each pathogen was placed separately in the centre of plates containing TSM. A loopful 24-h-old culture of fluorescent *Pseudomonas* GRC_2 was inoculated at 2 cm juxtaposed to the pathogens. The fungal pathogens inoculated centrally on TSM plates, but uninoculated by fluorescent *Pseudomonas* GRC_2, served as control. The inoculated plates were incubated at 28 ± 1°C for 5 days and inhibition of the colony growth was measured. Fungal mycelia growing towards the zone of interaction were processed for scanning electron microscopic (SEM) study following the procedure of Weidenborner *et al.*[12]. Agar discs of 1 mm thickness were cut from interaction zone and placed on cover glasses. These were treated with 2% osmium tetraoxide for 24 h at 20°C. The samples transferred to copper stubs over double adhesive tape were coated with gold in POLARON, AU/PD sputter coater and scanned by SEM (Philips 505, Holland) at 30 kV.

In a separate set of experiments, antifungal effect of culture filtrate of *Pseudomonas* GRC_2 on the growth of pathogens was assessed by agar-well method. TSM broth inoculated with *Pseudomonas* GRC_2 was incubated at 28 ± 1°C for 5 days. It was then centrifuged at 7000 rpm for 15 min and was finally passed through a millipore filter paper (0.2 µm porosity) to get cell-free culture filtrate. Two wells (5 mm dia) were prepared with the help of a sterile cork borer on TSM agar plates in one radius, 2 cm away from the centre. An actively growing mycelial disc was inoculated in the centre of each plate. The culture filtrate (1.5 ml) was pipetted in each well. The plates were incubated as described earlier and growth inhibition in the colonies of the pathogens was recorded after 5 days.

Table 1. Per cent inhibition of radial growth of *M. phaseolina* and *S. sclerotiorum* due to *Pseudomonas* GRC_2 and its culture filtrate after five day of incubation at 28 ± 1°C

	Antagonistic effect of *Pseudomonas* GRC_2	
Pathogen	In dual culture	In culture filtrate
Macrophomina phaseolina	80.1	73.4
Sclerotinia sclerotiorum	73.5	71.2

In dual culture, growth inhibition of *M. phaseolina* and *S. sclerotiorum* occurred due to fluorescent *Pseudomonas* GRC_2, which was evident with a clear zone of inhibition. Inhibition in sclerotial formation by the pathogens was observed in the zone of inhibition (Figure 1 *a* and *b*). Maximum growth inhibition in *M. phaseolina* and *S. sclerotiorum* due to presence of the bacterial strain after 5 days of incubation was 80.1% and 73.5%, respectively (Table 1). Increase in incubation time corresponded with increase in the zone of inhibition up to 5 days only. After this, the mycelia growing towards the interaction zone stopped and the sclerotia/mycelia gradually lost vigour. Scanning electron photomicrographs from the dual culture of *M. phaseolina* and fluorescent *Pseudomonas* GRC_2 showed loss in structural integrity of sclerotia produced in the zone of interaction (Figure 2 *a*), possessed hyphal shrivelling (Figure 2 *b*) and mycelial and sclerotial deformities (Figure 2 *c*), finally resulting into lysis (Figure 2 *d*). In case of *S. sclerotiorum*, *Pseudomonas* GRC_2 caused hyphal perforations (Figure 3 *a* and *b*), hyphal lysis (Figure 3 *c*) and fragmentation (Figure 3 *d* and *e*). Similar inhibitory effect of culture filtrate of

Pseudomonas GRC_2 against both the pathogens was also observed (Table 1).

Morphological abnormalities in hyphae and sclerotia of the fungal pathogens by *Pseudomonas* GRC_2, which was mediated by antibiosis, were clearly observed under SEM. Such abnormalities occurred in secondary metabolites and diffusable lytic substances produced by the bacterium. In an earlier study, it was observed that *Pseudomonas* GRC_2 produced siderophores, antibiotic substances (unidentified), volatile compound HCN, chitinase and IAA (unpublished data). Several other workers have also reported production of antifungal compounds by fluorescent pseudomonads that were responsible for suppression of plant pathogenic fungi[1–5]. SEM studies showed no sign of the presence of cells of *Pseudomonas* GRC_2 on the surface of the fungal hyphae and/or sclerotia. Involvement of secondary metabolites (antifungal compounds) in these morphological abnormalities may be a possibility as the phological abnormalities may be a possibility as the metabolites inoculated in the well also caused these abnormalities. Similarly, Upadhyay and Jayaswal[6] also reported mycelial deformities and inhibition of conidiation in phytopathogenic fungi by *Burkholderia cepacia* through the production of antifungal metabolites *in vitro*. On the other hand, Amer *et al.*[13] reported that bacterial cells attached and penetrated the hyphae of *Fusarium oxysporum* and *Rhizoctonia solani*, subsequently resulting in their lysis and deformities. However, they did not find direct involvement of bacterial metabolites in antagonism. Our study demonstrates that the mycelial/sclerotial abnormalities occurred due to production of antifungal metabolites by *Pseudomonas* GRC_2.

Therefore, on the basis of the study we conclude that one of the mechanisms by which fluorescent *Pseudomonas* GRC_2 antagonizes the fungal pathogens is due to

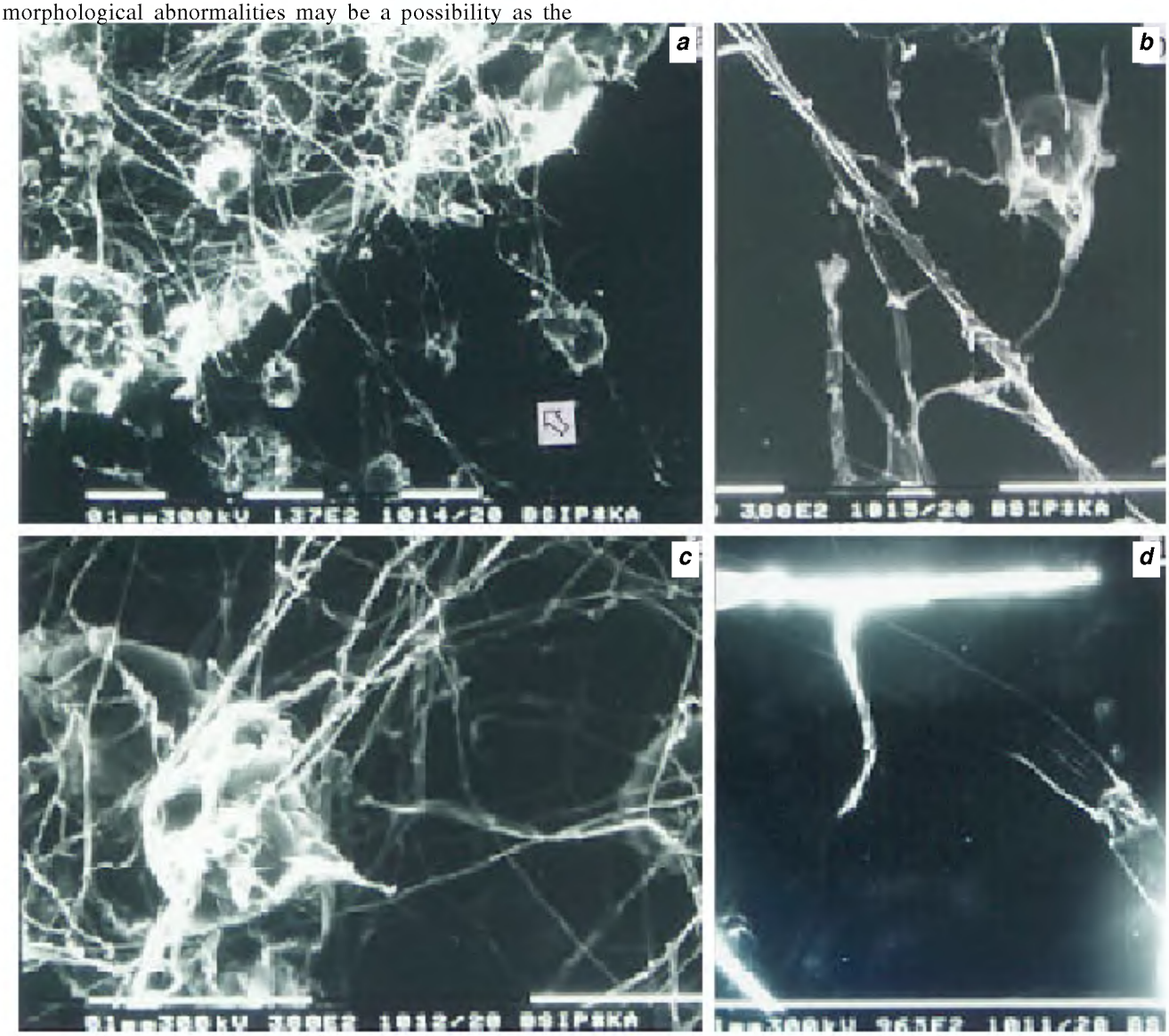

Figure 2. Scanning electron photomicrographs of post-interaction events in *M. phaseolina* due to *Pseudomonas* GRC_2. Loss of the structural integrity of sclerotia (***a***), hyphal shrivelling (***b***), hyphal and sclerotial deformities (***c***), and hyphal lysis (***d***). Arrows indicate the interaction zone where bacterial metabolites gradually diffused.

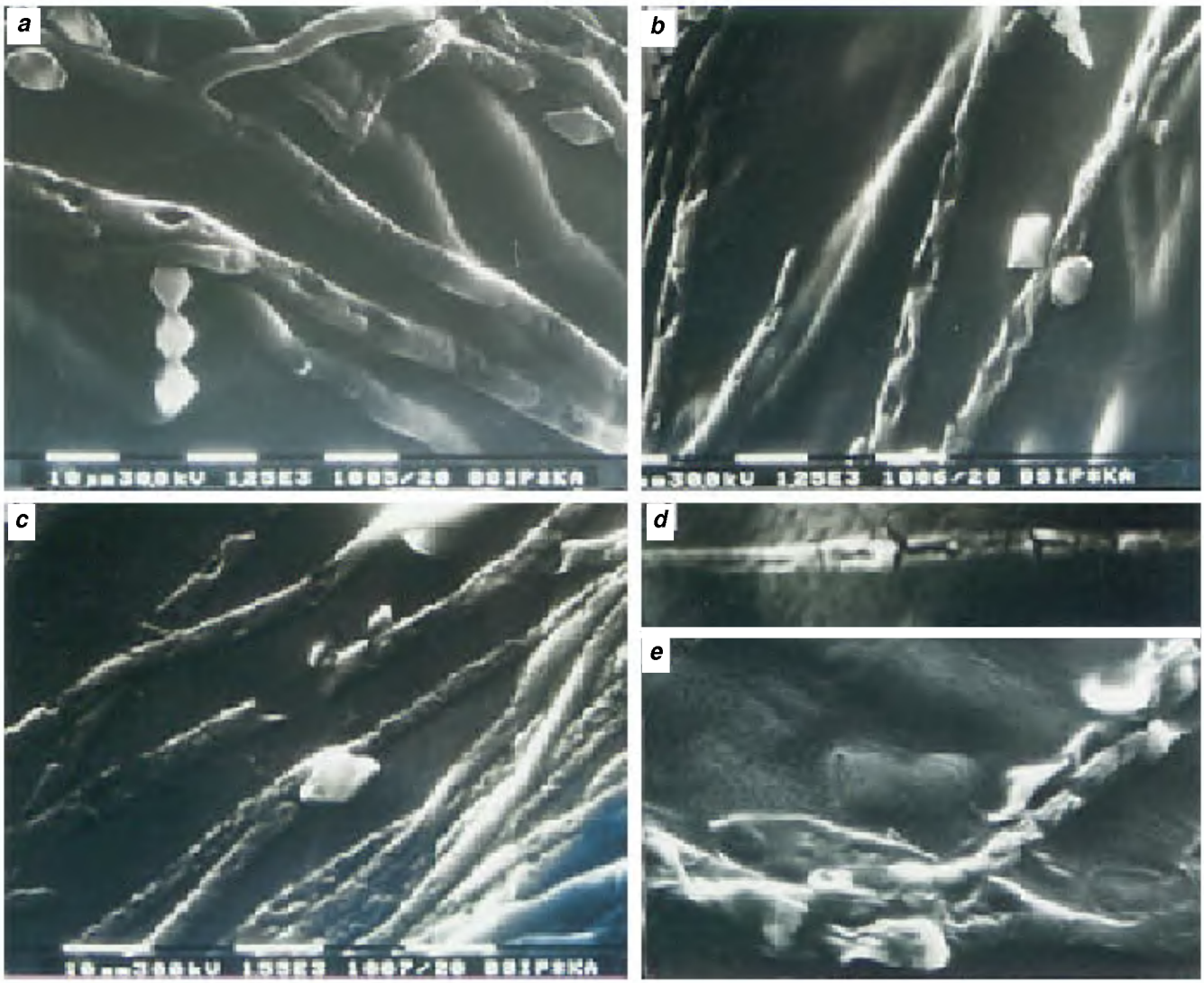

Figure 3. Scanning electron photomicrographs of post-interaction events in *S. sclerotiorum* due to *Pseudomonas* GRC_2, hyphal perforations during early stage of growth (***a***) and during late stage of growth (***b***), hyphal lysis (***c***), and hyphal fragmentation (***d***, ***e***).

production of deleterious antifungal compound which caused inhibition in growth and sclerotial formation, besides morphological deformities. These observations are significant in light of the attractive role of fluorescent pseudomonads in biological control of plant pathogens[2–5,12].

1. Hamdram, H., Weller, D. M. and Thomashow, L. S., *Appl. Environ. Microbiol.*, 1991, **57**, 3270–3277.
2. Keel, C. *et al.*, *Mol. Plant Microb. Inter.*, 1992, **5**, 4–13.
3. O'Sullivan, D. J. and O'Gara, F., *Microbiol. Rev.*, 1992, **56**, 662–676.
4. Defago, G. and Haas, D., *Soil Biochem.*, 1990, **6**, 249–291.
5. Lee, E. T. and Kim, S. D., *Acta Chem. Biotechnol.*, 1999, **42**, 7–11.
6. Upadhyay, R. S. and Jayaswal, R. K., *Curr. Microbiol.*, 1992, **24**, 181–187.
7. Sinclair, J. B. (ed.), in *Compendium of Soybean Diseases*, American Phytopathological Society, St Paul, MN, 1984, pp. 19–25.
8. Sandys-Winsch, D. C., Whipps, J. M., Fenlon, J. S. and Lynch, J. M., *Biocont. Sci. Technol.*, 1994, **4**, 269–277.
9. Holt, J. G., Krieg., N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T., *Bergey's Manual of Determinative Bacteriology*, Williams and Wilkins Press, London, 1994, pp. 151–157.
10. de Tempe, J., *Proc. Int. Seed Test. Assoc.*, 1963, **28**, 1933.
11. Skidmore, A. M. and Dickinson, C. H., *Trans. Br. Mycol. Soc.*, 1976, **66**, 57–74.
12. Weidenborner, M., Uziel, M., Hamacher, J., Hindorf, H. and Weltzien, H. C., *J. Phytopathol.*,1989, **126**, 1–6.
13. Amer, G. A., Aggarwal, R., Singh, D. V. and Srivastava, K. D., *Curr. Sci.*, 1997, **3**, 284–286.

ACKNOWLEDGEMENTS. We thank Dr K. Ambani, Birbal Sahni Institute of Palaeobotany, Lucknow for providing SEM facilities. Financial assistance to D.K.M. by TMOP–CSIR is gratefully acknowledged.

Received 30 December 2000; revised accepted 17 March 2001