African Journal of Biotechnology Vol. 11(60), pp. 12389-12395, 26 July, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.023
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Role of antibiosis on suppression of bacterial common blight disease in French bean by *Paenibacillus polymyxa* strain HKA-15

Vellaichamy Mageshwaran[1]*, Kalyan Kumar Mondal[2], Upendra Kumar[3] and Kannepalli Annapurna[3]

[1]Chemical and Biochemical Processing Division, Central Institute for Research on Cotton Technology, Mumbai 400 019, India.
[2]Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012, India.
[3]Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110 012, India.

Accepted 1 March, 2012

***Paenibacillus polymyxa* strain HKA-15, a soybean bacterial endophyte showed strong antagonism against bacterial common blight pathogen *Xanthomonas campestris* pv. *phaseoli* strains M-5 and CP-1-1. In agar diffusion assay, the antibacterial metabolite from *P. polymyxa* HKA-15 showed a clear zone of inhibition against M-5 and CP-1-1. Under phytotron conditions, the biocontrol activity of *P. polymyxa* HKA-15 against bacterial common blight pathogen *X. campestris* pv. *phaseoli* M-5 was assessed. At four days after inoculation of M-5, lowest mean disease rate (MDR) (1.13) and percent disease incidence (PDI) (28.25) were recorded in streptomycin sulphate at 100 ppm as positive chemical treatment. The application of crude metabolite from *P. polymyxa* HKA-15 at 100 ppm was on par with positive chemical control in suppression of bacterial common blight disease in French bean plants.**

Key words: Antibacterial activity, biocontrol, French bean, *Paenibacillus polymyxa*, *Xanthomonas campestris* pv. *phaseoli*.

INTRODUCTION

The common bean or French bean (*Phaseolus vulgaris* L.) is one of the most important crops in both economic and nutritional aspects (Broughton et al., 2003). Common blight of bean caused by *Xanthomonas campestris* pv. *phaseoli* occur more frequently in both tropical and temperate climates (Gilbertson and Maxwell, 1992; Wallen and Galway, 1979). *X. campestris* pv. *phaseoli* causing bacterial common blight disease is an important pathogen in French bean which naturally infects the other legumes and is a pathogen hard to control (Zanatta et al., 2007). Increasing resistance of pathogen and detrimental effect of chemicals force us to search an alternative for better disease management.Of the different management strategies, biocontrol of the pathogens by plant growth promoting bacteria (PGPB) offers best alternative for current chemical control (Compant et al., 2005). Antagonistic mechanisms of *Paenibacillus*, *Bacillus* and their related genera include the production of hydrolytic enzymes, siderophore and antibiotics. They are also known to induce systemic resistance in host plant and competing out the phyto pathogens (Sturz et al., 1997). *Bacillus* sp. and its related genera are reported for production of wide range of cyclic lipopeptides active against various microorganisms (Kim et al., 2003). *Bacillus* and *Paenibacillus* have been identified as a potential biocontrol agent for the control of fungal

*Corresponding author. E-mail: mageshbioiari@gmail.com. Tel: 0222412727376. Fax: 02224130835.

Abbreviations: MDR, Mean disease rate; **PDI,** percent disease incidence.

pathogens such as *Leptosphaeria maculans* in canola (Beatty and Jensen, 2002), *Alternaria brassicae* in *Brassica napus* (Danielsson et al., 2006) etc. However, very few works has been carried out on biocontrol of bacterial pathogens such as *X. campestris* (Monteiro et al., 2005; Salerno and Sagardoy, 2003) and *Pseudomonas syringae* pv glycine (May et al., 1996). Earlier work done in our laboratory showed that *Paenibacillus* sp. HKA-15 was active against *Rhizoctonia bataticola* causing charcoal rot disease in soybean (Senthilkumar et al., 2007a). The antifungal property of this strain was found to be peptide antibiotics since it failed to produce siderophore, chitinase, protease and HCN. The necrotrophic effect on fungal hyphae by HKA-15 cells and its metabolites was examined under light and scanning electron microscope. Based on phylogenetic analysis, HKA-15 was identified as *Paenibacillus polymyxa* (Senthilkumar et al., 2009).

Higher seed germination, vigour index and better growth were observed in soybean plants where seeds were treated with *P. polymyxa* HKA-15 (Mageshwaran et al., 2010). *P. polymyxa* HKA-15 grown in nutrient broth at 48 h of incubation showed strong antibacterial activity against *X. campestris* pv. *phaseoli* strains M-5 and CP-1-1 (Mageshwaran et al., 2011a). To better understand the effect of *P. polymyxa* HKA-15 and its antibacterial substance on the suppression of bacterial pathogen *X. campestris* pv. *phaseoli* under *in planta* conditions, the present investigation was taken up; (i) to study the effect of crude metabolite from *P. polymyxa* HKA-15 on *X. campestris* pv. *phaseoli* cells and (ii) to evaluate the efficacy of *P. polymyxa* HKA-15 and its antibacterial substance on suppression of bacterial common blight symptom in French bean under phytotron conditions.

MATERIALS AND METHODS

Microorganisms and culture conditions

Soybean bacterial endophyte *P. polymyxa* HKA-15 was obtained from Division of Microbiology, Indian Imperial Agricultural Research Institute (IARI), New Delhi. Bacterial phytopathogens viz., *X. campestris* pv. *phaseoli* M-5 and *X. campestris* pv. *phaseoli* CP-1-1 were obtained from Division of Plant Pathology, IARI, New Delhi. The bacterial strains were grown in nutrient broth (NB) at 30°C for overnight. The strains were cryopreserved in 40% (v/v) glycerol at -20°C.

Extraction of crude metabolite

P. polymyxa HKA-15 was grown in one liter of NB amended with 0.5% of glucose at 30°C for 48 h of incubation under shaking conditions (180 rpm) into which was added with equal volume of n-butanol. After vigorous shaking for 30 min, the contents were allowed to settle down. Butanol phase was separated and concentrated to dryness using vacuum evaporator. The dried compound (4 mg) was dissolved in one ml of high-performance liquid chromatography (HPLC) grade methanol.

Test for antibacterial activity of crude metabolite

The antibacterial activity of crude metabolite from *P. polymyxa* HKA-15 was tested against *X. campestris* pv. *phaseoli* using agar diffusion assay. In agar diffusion assay, nutrient agar (NA) plates were spread with 100 µl of 0.5 OD pathogenic cultures CP-1-1 and M-5. Wells of diameter 0.5 cm were made using sterile cork borer and the base of the wells was sealed with 0.8% plain agar. 30 µl of the crude metabolite of HKA-15 was placed into these wells. In one of the well, methanol alone was added for control. 10 µl of overnight grown HKA-15 cells was placed on the plate to find out any inhibition of pathogen by HKA-15 cells itself. The plates were then incubated at 30°C for 48 h.

Effect of crude metabolite on morphology of *X. campestris* pv. *phaseoli* cells

The effect of crude metabolite of *P. polymyxa* HKA-15 on the morphology of *X. campestris* pv. *phaseoli* M-5 and CP-1-1 was studied using phase contrast microscope (PCM) Leica DM 1000 and transmission electron microscope (TEM). 50 µl of overnight grown culture of M-5 and CP-1-1 was mixed with 50 µl of crude metabolite (4 mg/ ml) and microscopic observations were taken at 2 and 4 h of incubation. 50 µl of culture mixed with 50 µl of sterile water served as control.

In vivo biocontrol ability of *P. polymyxa* HKA-15 and its crude metabolite against bacterial common blight in French bean

There were six treatments viz., (1) inoculation of *X. campestris* pv. *phaseoli* M-5 (*X.c* pv. M-5) alone, (2) *X. campestris* pv. M-5 + seed treatment with HKA-15 cells (10^6 cfu/ml), (3) *X. campestris* pv. M-5 + seed treatment with streptomycin sulphate at 100 ppm, (4) co-inoculation of *X. campestris* pv. M-5 and HKA-15 culture (10^6 cfu/ml) on leaves, (5) co-inoculation of *X. campestris* pv. M-5 and crude metabolite at 100 ppm on leaves, and (6) co-inoculation of *X. campestris* pv. M-5 and Streptomycin sulphate at 100 ppm on leaves. Three replications were made. Surface sterilized (Vincent, 1970) seeds of French bean variety Varun were sown in sterile pots. The seed treatment of HKA-15 was carried out by submerging in double the volume of log phase inoculum of HKA-15 for 10 min. After incubation, excess inoculum was drained out and seeds were immediately sown. Plants were irrigated with Jensen's liquid nutrient broth (N+) on alternate days up to 30 days. Pots were kept under phytotron conditions where the temperature (20°C) and RH (80%) were maintained.

Germination count was taken at 10 days after sowing. Pathogen inoculation on leaves was done at 30 days after sowing by pin prick method in which 2.5 µl of bacterial pathogens was inoculated in damage surface. In each treatment 15 pathogenic inoculations were made (five inoculations per replication). Hence 15 leaves were analyzed for the occurrence of disease symptom in each treatment. To study the efficacy of biocontrol potential of crude metabolite (100 ppm) and HKA-15 cells (10^6 cfu / ml), 2.5 µl of these were inoculated in the area of pathogen treated surface. Observations were made four days after inoculation and numerical disease rating was assigned as follows: 0, healthy leaf; 1, yellowish brown lesion with narrow margin; 2, dark brown lesion with broad margin; 3, dark brown lesion with half of the leaf surface; 4, damage to the whole surface.

$$MDR = (ax0) + (bx1) + (cx2) + (dx3) + (ex4) / (a+b+c+d+e)$$

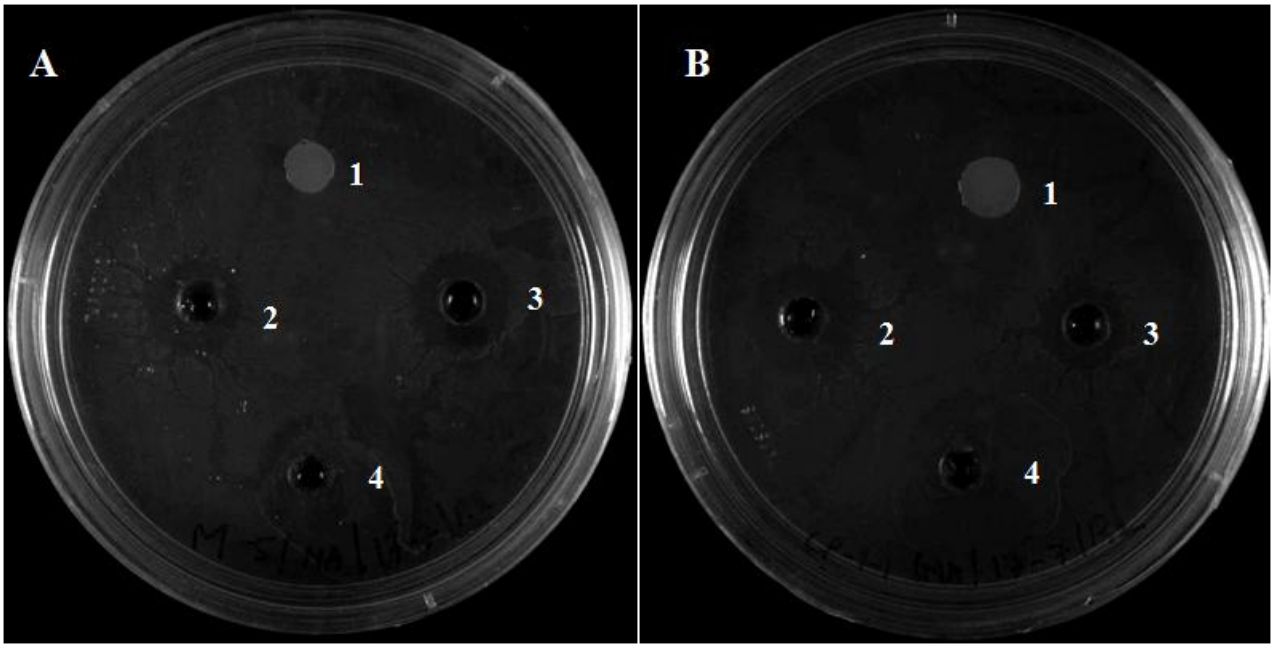

Figure 1. Agar diffusion assay of crude metabolite showing zone of inhibition against *X. campestris* pv. *phaseoli* strains M-5 and CP-1-1; A, M-5 plate; B, CP-1-1 plate (1, HKA-15 cells; 2, 3, crude metabolite showing zone of inhibition (indicated by arrow); 4, control (methanol).

Where a, b, c, d and e are the number of plants with the disease rating of 0, 1, 2, 3 and 4, respectively.

PDI = (MDR x100) / maximum grade

Statistical methods

Data from the completely randomized design experiment on *in vivo* biocontrol activity were arc sine transformed prior to statistical analysis. Results were analyzed using one –way analysis of variance (ANOVA) (AGRES ANOVA package version 7.01; TNAU, India).

RESULTS AND DISCUSSION

Antibacterial activity of *P. polymyxa* HKA-15

Paenibacillus and its related genera are common soil bacteria producing wide range of antibiotics with biological activity (Beatty and Jensen, 2002; Pichard et al., 1995; Pueyo et al., 2009, Selim et al., 2005; Senthilkumar et al., 2007b). The crude metabolite extracted from 48 h old culture of *P. polymyxa* HKA-15 was tested for antibacterial activity against *X. campestris* pv. *phaseoli*. In agar diffusion assay, a clear zone of inhibition was observed around the well containing crude metabolite in NA plates spread with *X. campestris* pv. *phaseoli* strains CP-1-1 (Figure 1B2 and B3) and M-5 (Figure 1A2 and A3). However no inhibition zone was observed around the well containing methanol (control) (Figure 1A4 and B4) and HKA-15 culture growth (Figure 1A1and B1). The antagonism of metabolite against major fungal pathogens are well reported (Beatty and Jensen, 2002; Danielsson et al., 2006). However Monteiro et al. (2005) reported that lipopeptides produced by *Bacillus* sp. showed antagonism against nine strains of *X. campestris*, causual agent of crucifers black rot. The results obtained in this experiment show the antagonism of *P. polymyxa* HKA-15 against bacterial common blight pathogen *X campestris* pv. *phaseoli* under *in vitro* conditions.

Effect of crude metabolite on morphology of *X. campestris* pv. *phaseoli* cells

It is important to understand the mode of action of crude metabolite against M-5 and CP-1-1. Even though many reports on light microscopic studies on the effect of these metabolites on fungal hyphal morphology have been published (Senthilkumar et al., 2007a; Hassanein et al., 2009) the effect on bacterial cell morphology has been less reported (Hashizume et al., 1996; Nakao et al., 1981). PCM observations at 1000 X magnification showed lysis of M-5 and CP-1-1 and many of the cells fragmented to two or three after 2 h of incubation (Figures 2b and e) whereas, observation after 4 h of incubation showed the cells become disintegrated into many fragments and become small round structures

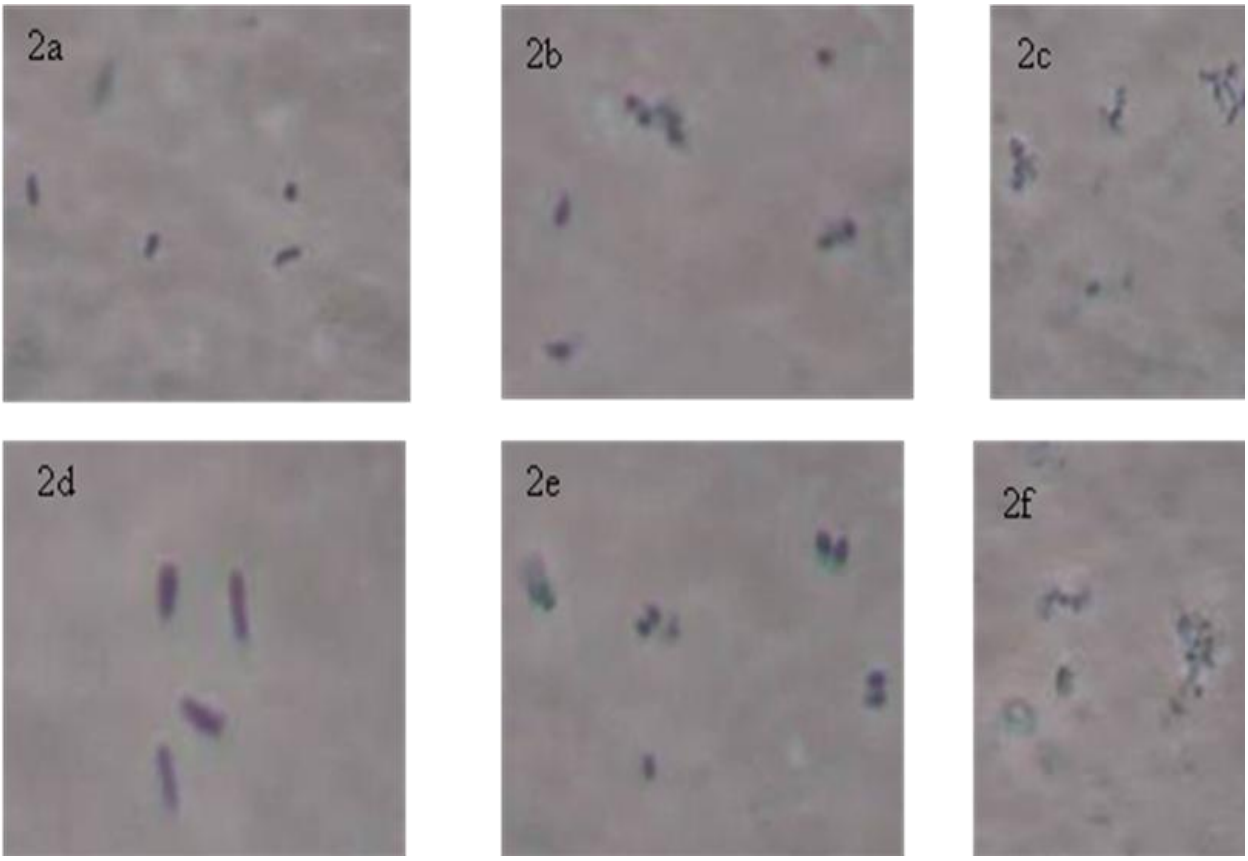

Figure 2. Phase contrast microscopic observations (1000 X) on the effect of crude metabolite on *X. campestris* pv. *phaseoli* cells showing fragmentation and disintegration at 2 and 4 h of contact. a, Control M-5 cells, b, 2 h contact with M-5 cells, c, 4 h contact with M-5 cells; d, control CP-1-1 cells; e, 2 h contact with CP-1-1 cells; f, 4 h contact with CP-1-1 cells.

(Figures 2c and f). However cells mixed with sterile water were found intact after 4 h of incubation (Figures 2a and d). The disruption of cell wall and cell membrane after 2 h of incubation (Figures 3b and e) and complete disintegration of *X. campestris* pv. *phaseoli* cells into many fragments was observed after 4 h of incubation (Figures 3c and f) under TEM.

The observations on the effect of crude metaboliote on cell morphology of CP-1-1 and M-5 using PCM and TEM revealed the bactericidal effect on *X. campestris* pv. *phaseoli* cells. In our previous work, the characterization of antibacterial metabolite produced by *P. polymyxa* HKA-15 revealed the lipopeptide nature of metabolite having strong antagonism against phytopathogen *X. campestris* pv. *phaseoli* (Mageshwaran et al., 2011b). Pueyo et al. (2009) reported that *Bacillus* sp. and related genera have been identified as potential biocontrol agents as they produce wide range of lipopeptides active against various microorganisms. The bactericidal nature of the metabolite could be due to biosurfactant property. Many cyclic lipopeptides have the property of biosurfactant which reduce the surface tension of the medium and results in killing of bacterial cells (Neilsen et al., 2002).

Biocontrol of bacterial common blight disease in French bean

As shown in Table 1, higher seed germination (90%) was recorded in treatment when French bean seeds were treated with HKA-15 cells (10^6 cfu/ml). After four days of pathogen inoculation, the highest mean disease rate (MDR, 3.53) and percent disease incidence (PDI, 88.25) were recorded in negative control in which typical symptoms were noticed that is, brown lesion in entire leaves. The lowest MDR (1.13) and PDI (28.25) were recorded in positive chemical control. The suppression of blight symptom and statistically on par biocontrol ability with chemical control was recorded in treatment where leaves were treated with crude metabolite at 100 ppm simultaneously with pathogen treatment. The stimulatory

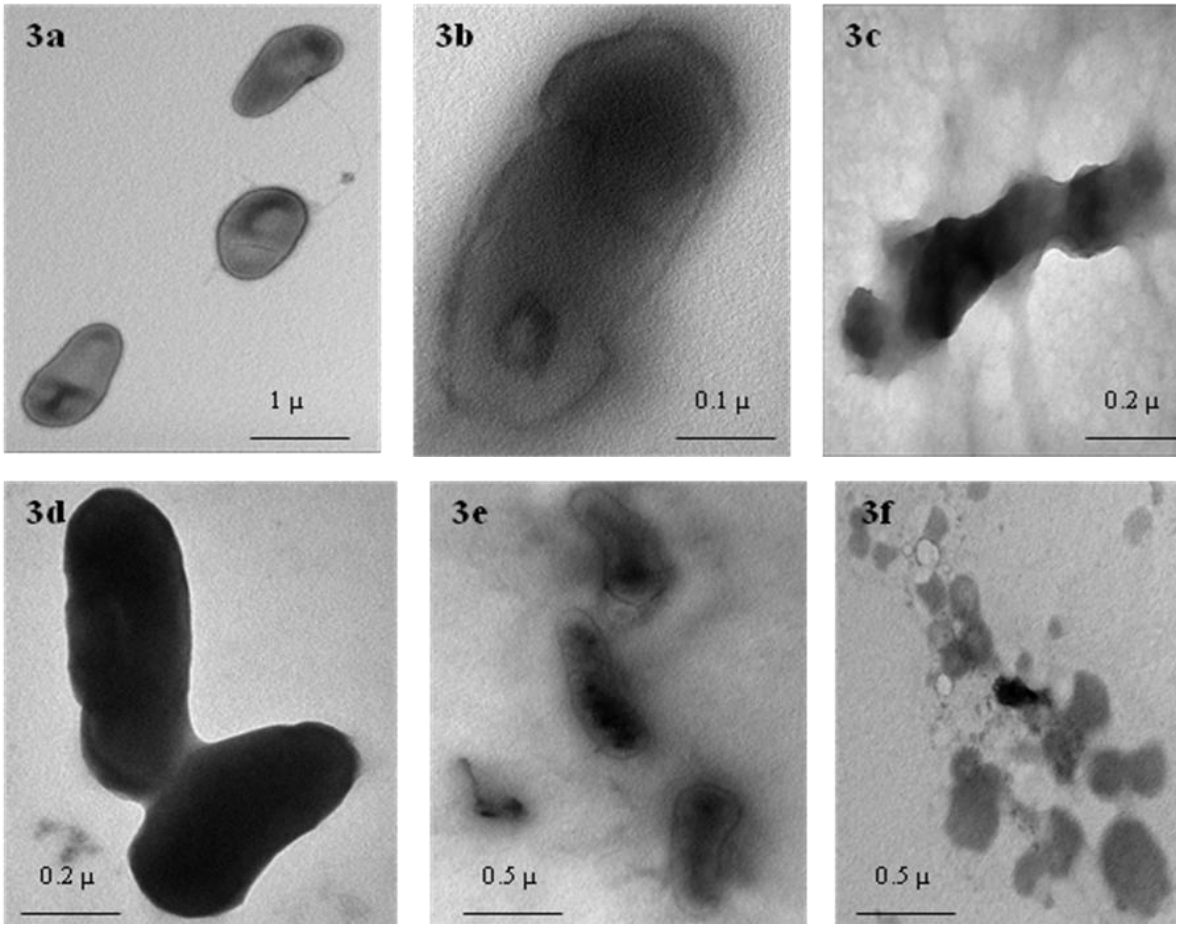

Figure 3. Transmission electron microscopic observations on the effect of crude metabolite on *X. campestris* pv. *phaseoli* cells showing disruption and disintegration at 2 and 4 h of contact. a, Control M-5 cells; b, 2 h contact with M-5 cells; c, 4 h contact with M-5 cells; d, control CP-1-1 cells; e, 2 h contact with CP-1-1 cells; f, 4 h contact with CP-1-1 cells.

effect of *P. polymyxa* HKA-15 cells on seed germination, growth and efficient control of blight pathogen *X. campestris* under *in vivo* conditions makes it a good candidate for biocontrol. Similar results were obtained when soybean seeds were treated with *Bacillus subtilis* cells and its metabolites showed higher seed germination percentage and growth parameters compared to control (Araujo et al., 2005).

In a similar experiment bacterial isolates compatible with *Rhizobium leguminosarum* bv. *phaseoli* were selected among 596 isolates. The selected bacterial isolates were tested for bioassay for selection of bio-control bacteria against bean common blight (Zannata et al., 2007). The correlation between antimicrobial activity and lipopeptides production indicated the role of lipopeptides produced by *Bacillus* sp. on the control of *X. campestris* pv. *campestris* (Monteiro et al., 2005). The present study results show the disintegration of *X. campestris* pv. *phaseoli* cells by antibacterial metabolite under PCM and TEM observations which supports our previous results (Mageshwaran et al., 2011a, b) on antibacterial activity of metabolite produced by *P. polymyxa* HKA-15. Also this study reveals the role of metabolite produced by *P. polymyxa* HKA-15 in suppressing the growth of *X. campestris* pv. *phaseoli* thereby preventing the formation of common blight disease in French bean under phytotron conditions. However, further work is needed on evaluation of metabolite under field conditions for sustainable control of *X. campestris* pv. *phaseoli* in beans.

ACKNOWLEDGMENTS

The authors thank Jawaharlal Nehru Memorial Fund, New Delhi, India and Indian Agricultural Research Institute, New Delhi, India for the financial assistance in the form of PhD scholarship for this study. Authors

Table 1. *In vivo* biocontrol activity of *P. polymyxa* HKA-15 and its crude metabolite against bacterial common blight pathogen in French bean.

Treatment	Seed germination (%)	4 days after inoculation	
		MDR	PDI
X.c pv. M-5	80 (63.43)[b]	(3.53)[d]	88.25(75.30)[c]
X. c pv. M-5 + ST at 10^6/ml	90 (71.56)[a]	(1.6)[b]	40.00(39.20)[a,b]
X. c pv. M-5 +ST with SS at 100 ppm)	85 (67.21)[b]	(2.13)[c]	53.25(46.91)[b]
X. c pv. M-5 + CL at 10^6/ml)	82 (64.89)[b]	(2.2)[c]	55.00(46.89)[b]
X. c pv. M-5+ CM at 100 ppm)	75 (60.00)[b]	(1.2)[a,b]	30.00(33.16)[a]
X. c pv. M-5+ SS at 100 ppm)	79 (62.72)[b]	(1.13)[a]	28.25(32.14)[a]

X.c pv. M-5 (*X. campestris* pv. *phaseoli* M-5) ST, seed treatment; CL, culture; CM, crude metabolite; SS, streptomycin sulphate; MDR, mean disease ratio; PDI, percent disease incidence. Figures in parentheses are arc sine transformed values. Treatment values followed by same alphabet do not differ significantly at P=0.05.

extend thanks to Dr. Jasveer, Division of Plant Pathology, IARI, New Delhi for his help rendered during TEM observations in this study.

REFERENCES

Araujo FF, Henning AA, Hungria, M (2005) Phytohormones and antibiotics produced by *Bacillus subtilis* and their effects on seed pathogenic fungi and on soybean root development. World J. Microbiol. Biotechnol. 21: 1639-1645.

Beatty PH, Jensen SE (2002) *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agent of blackleg disease of canola. Can. J. Microbiol. 48: 159-169.

Broughton WJ, Henandez G, Blair M, Beebe S, Gepts P, Vanderleyden, J (2003) Beans (*Phaseolus* sp.) model food legumes. Plant Soil. 252: 55-128.

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant disease: Principles, Mechanisms of Action and Future prospects. Appl. Environ. Microbiol. 71: 4951-4959.

Danielsson J, Reva O, Meijer J (2006) Protection of oilseed rape (*Brassica napus*) toward fungal pathogens by strains of plant-associated *Bacillus amyloliquefaciens*. Microb. Ecol. 54: 134-140.

Gilbertson RL, Maxwell DP (1992). Common bacterial blight of bean. In: Chaube, H.S., Kumar, J (Ed.), Plant disease of International importance, Vol II. Diseases of vegetables and oil seed crop. Prentice Hall, Englewood Cliffs, NJ, pp. 18-39.

Hashizume T, Nakamura K, Nakagawa S (1996) Affinities of BO-2727 for bacterial penicillin-binding proteins and morphological change of gram negative rods. J. Antibiotics, 50: 139-142.

Hassanein WA, Awny NM, El-Mougith AA, Salah El-Dien SH (2009) Characterization and antagonistic activities of metabolite produced by *Pseudomonas aeruginosa* Sha8. J Appl. Sci. Res. 5: 392-403.

Kim H S, Park J, Choi SW, Choi KH, Lee GP, Ban SJ, Lee CH, Kim CS (2003) Isolation and Characterization of *Bacillus* strains for biological control. J. Microbiol. 41: p. 196.

Mageshwaran V, Gupta A, Kumar U, Padaria J, Annapurna K (2010) Effect of seed treatment with *Paenibacillus polymyxa* HKA-15 on suppression of charcoal rot disease and growth promotion of soybean. Pusa Agric. Sci. 33: 14-19.

Mageshwaran V, Walia S, Govindasamy V, Annapurna K (2011a) Antibacterial activity of metabolite produced by *Paenibacillus polymyxa* strain HKA-15 against *Xanthomonas campestris* pv. *phaseoli*. Indian J. Exp. Biol. 49: 229- 233.

Mageshwaran V, Suresh W, Annapurna K (2011b) Isolation and partial characterization of antibacterial lipopeptide produced by *Paenibacillus polymyxa* HKA-15 against phytopathogen *Xanthomonas campestris* pv. *phaseoli* M-5. World J. Microbiol. Biotechnol. 28: 909-917.

May R, Volksh B, Kampmann G (1996) Antagonistic activities of epiphytic bacteria from soybean leaves against *Pseudomonas syringae* pv. *glycinea in vitro* and *in planta*. Microb. Ecol. 34: 118-124.

Monteiro L, Mariano R, De LR, Souto-Maior (2005) Antagonism of *Bacillus* sp. against *Xanthomonas campestris* pv. *campestris*. Braz. Arch. Biol. Technol. 48: 23-29.

Nakao M, Kondo M, Tsuchiya K (1981) Light and Electron Microscopy of the morphological response of *Escherichia coli* and *Serratia marescens* to Cefmenoxime (SCE-1365). A new broad spectrum Cephalosprorin. J Antibiotics, 34: 1046-1054.

Nielsen TH, Sorensen D, Tobiasen C, Andersen JB, Christerophersen C, Givskov M, Sorensen J (2002) Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* sp. from the sugar beet rhizosphere. Appl. Environ. Microbiol. 68: 3416-3423.

Pichard B, Larue J-P, Thouvenot (1995). Gavesrin and saltavalin, new peptide antibiotics produced by *Bacillus polymyxa*. FEMS Microbiol. Lett. 133: 215-218.

Pueyo MT, Bloch CJ, Carmona-Ribeiro AM, Masico P (2009) Lipopeptides produced by a soil *Bacillus megaterium* strain. Microb. Ecol. 57: 367-378.

Salerno CM, Sagardoy (2003) Antagonistic activity by *Bacillus subtilis* against *Xanthomonas campestris* pv. *glycines* under controlled conditions. Spanish J Agric. Res. 1: 55-58.

Selim S, Negrel J, Govaerts C, Gianinazzi S, Tuinen D (2005). Isolation and partial characterization of antagonistic peptides produced by *Paenibacillus* sp. Strain B2 isolated from the sorghum mycorhizosphere. Appl. Environ. Microbiol. 71: 6501-6507.

Senthilkumar M, Govindasamy V, Annapurna K (2007a). Role of antibiosis in suppression of charcoal rot disease by soybean endophyte *Paenibacillus* sp. HKA-15. Curr. Microbiol. 55: 25-29.

Senthilkumar M, Govindasamy V, Dureja P, Annapurna K (2007b). Purification and partial characterization of antifungal peptides from soybean endophyte-*Paenibacillus* sp strain HKA-15. J. Plant Biochem. Biotech. 16: 131-134.

Senthilkumar M, Swarnalakshmi K, Govindasamy V, Lee YK, Annapurna K (2009) Biocontrol potential of soybean bacterial endophytes against charcoal rot fungus, *Rhizoctonia bataticola*. Curr. Microbiol. 58: 288-293.

Sturz AV, Christie BR, Matheson BG, Nowak J (1997) Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biol. Fertil. Soils, 25: 13-19.

Vincent (1970) A manual for the practical study of the root nodule bacteria. Blackwell Scientific Publications. Oxford, Edinburgh. Pp1-13.

Wallen VR, Galway DA (1979) Effective management of bacterial blight of beans in Ontario- a 10 year program. Can. J Plant Pathol. 1: 42-46.

Zanatta ZGCN, Moura AB, Maia LC, Santos AS (2007) Bioassay for selection of biocontroller bacteria against bean common blight (*Xanthomonas axonopodis* pv. *phaseoli*). Braz. J. Microbiol. 38: 511-515.