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Exploitation of Antagonistic Phylloplane Bacteria for Management of Bacterial Leaf Spot of Mungbean

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ABSTRACT. *Three isolates of Phylloplane Bacteria (Plb) (Bacillus spp.) from Mungbean leaves were selected based on their antogonistic potential against six races of Xanthomonas campestris pv. vignaeradiatae (Xcv), the incitant of bacterial leafspot in mungbean. Based on the growth of antagonists in four different liquid media, size of inhibition zone, amount of metabolites produced, colonising ability in leaf tissue, isolate Plb-3 was identified as the best antagonist against Xcv. All the Plb isolates harboured single plasmid in each of 45 kb. Curing and transformation experiments proved the role of 45 kb plasmid in the antibiosis. Of the various combinations tested, bio-control agent with talc in the ratio of 1:10 along with the sticker carboxy methyl cellulose at 10% of total weight of formulation was found effective even at 1 g/kg seed. The formulation is also effective against Xanthomonas oryzae pv. Oryzae, X. campestris pv. malvacearum, X.c. pv. mangiferaeindicae, X.c. pv. campestris and X. campestris pv. citri.*

INTRODUCTION

Mungbean (*Vigna radiata* (L.) Wilczek) is an important grain legume in India and other parts of South East Asia. It has been cultivated since time immemorial and occupies 3.08 million ha with an annual production of 1.31 million tons (Asthana and Chaturvedi, 1999). Being a rich source of protein (22-24%) and other essential vitamins it has been considered as an indispensable constituent of cereal based diets of vegetarian. Bacterial leafspot of greengram caused by *Xanthomonas campestris* pv. *vignaeradiatae* (Xcv) (Schaad *et al.*, 2000) is one of the most important diseases and causes yield losses upto 15% (Patel and Jindal, 1970).

The role of phylloplane antagonists in the management of bacterial diseases has been well recognised (Blackeman and Fokkema, 1982). However, very little actions have taken to produce antagonistic phylloplane bacteria as a commercial mixture. This failure lies in the lack of understanding of the mode of action of antagonists, their interaction with the pathogen, inadequate knowledge of production methods and formulation for commercial use. Therefore, the objectives of the present study were identify and develop suitable phylloplane and antagonist and its formulation to control bacterial leaf spot of mungbean..

MATERIALS AND METHODS

Isolation and establishment of pure cultures of Xcv and Plb

Mungbean leaves infected with Xcv were collected from fields and isolation made by the streak plate method on sucrose peptone agar (SPA) medium ($Na_2HPo_4.12H_2O$ – 2.0 g, $Ca(NO_3)_2.4H_2O$ – 0.5 g, $FeSO_4$ – 0.5 g, peptone 5.0 g, sucrose – 20.0 g, agar – 20.0 g, distilled water – 1000 ml, pH – 6.8). Pathogenicity was tested on a susceptible cultivar Pusa Baisakhi using injection – infiltration method (Klement, 1963).

The Phylloplane Bacteria (Plb) (*Bacillus* spp.) were isolated by using the wash method (Voznyakoyskaya and Khudyakov, 1960). Five grams of leaves were taken in a conical flask with 100 ml sterile water and stirred vigorously with a sterile glass rod for 10 min. Serial dilutions were prepared by transferring 1 ml of leaf washing to 9 ml sterile distilled water blanks. An aliquot of 0.1 ml of each dilution was spread with glass spatula on the surface of SPA medium containing 0.05 g cycloheximide per litre. Colonies of different shapes, sizes and colours were picked up on yeast glucose chalk agar (YGCA) slants.

Morphological and biochemical characters

Staining procedures for the study of morphology were followed according to "Manual of Microbiological Methods, 1957" whereas the media, test reagents, indicators and method employed in studies of biochemical characters were followed as given by Dye (1962).

Bacteria cultured *in vitro* was deposited on an electron microscope grid by placing a droplet of bacterial suspension on a hydrophilic (glow discharged) carbon-coated Formavar grid, allowing the bacterial cells to sediment over a period of 4 min, and drained and air dried. Preparations of bacterial cells that were briefly air-dried and inverted onto droplets of 2 ml uranyl acetate for 5-10 sec, drained and examined immediately in the electron microscope (JEOL – 100 CX – II transmission electron microscope).

In-vitro antagonism of Plb against Xcv

For testing antagonism against Xcv, 1 ml Xcv suspension (0.1 O.D) was mixed with 25 ml melted cooled (40-45°C) SPA and poured into a petriplate. The solidified plates were spot inoculated with the 24 h old solid growth of Plb isolates. After three days incubation at 27°C, the plates were examined for zones of inhibition. Plates without Plb served as control.

Estimation of amount of metabolite production

Liquid cultures of Plb's were prepared by inoculating sterilised 200 ml sucrose peptone broth contained in conical flasks (1 l) with 10 ml (0.1 O.D) culture suspension of Plb's. The flasks were incubated at 27°C for 4 days in a shaker incubator at 180 rpm. The liquid growth in 4 different media was collected everyday for four days for estimation of metabolite production. Each liquid culture was centrifuged at 6000×g for 15 min to separate bacterial cells. The supernatant was then extracted as the liquid portion with Ethylacetate. The organic layer was dried over anhydrous sodium sulphate and concentrated on a rotary evaporator at 40-50°C under reduced pressure. The residue was dissolved in 1 ml acetone and 50 µl of the samples were spotted on thin layer chromatography (TLC) plate (Silica gee 60 F_{234}Merck). These spotted TLC plates were developed in Hexane:Benzene (1:1) and Benzene solvent systems in case of crude metabolites of Plb's and visualised by Iodine vapours. The Rf (retardation factor) values were calculated by the following formula:

$$Rf = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

Colonising ability of Plb *in planta*

The trifoliates of three weeks old, green house grown mungbean plants (cv. Pusa Baisakhi) were used for the *in planta* assay. Cell suspension (about 10^7 cells/ml) of the three isolates of Plb were prepared using 2-day-old slant cultures. The leaflets of a trifoliate leaf were inoculated by an injection–infiltration technique as described by Klement (1963). The population development in the leaf tissue was observed until 6 days after inoculation.

To estimate the bacterial population size in the leaf tissue, leaf discs were cut with a cork borer (diameter 0.5 cm), homogenised in a mortar, and suspended in sterilised double distilled water. Appropriate tenfold dilutions were plated on SPA media and colonies were counted from three replicated plates.

Role of plasmids in antibiosis

The plasmids from three Plb isolates were isolated by the method of Birnboim and Doly (1979) and transformation studies were conducted based on the protocol given by Mandel and Higa (1980) to prove the role of plasmids in antibiosis.

Develoment of bioformulation

Based on the multiplication rate in leaf tissue, amount of metabolite production, size of inhibition zone the Plb–3 isolate (*Bacillus* spp.) was selected for development of bioformulation. The highly potential strain Plb-3 was inoculated in SPB at 27°C for 10 days. Then the growth was centrifuged at 10,000 rpm for 15 min. The pellet was suspended in 1/20 volume of 6% lactose and 4 volume of acetone. After complete stirring, the pellet was collected by removing the liquid phase with suction and

was dried in vacume dissicator (Dulmage *et al.*, 1970). The powder was mixed with carrier Talc in different combinations *i.e.*, 1:1, 1:2, 1:5, 1:10 and 1:15 in which carboxy methyl cellulose was added (10% of the total weight).

Viability and evaluation of bio-formulation

The bio-formulation was kept at different temperature ranges from 10-45°C and tested for antibiosis 7 days after incubation. Further, the bioformulation was also tested for antibiosis at different concentrations.

The healthy seeds of susceptible mungbean cv. Pusa Baisakhi were soaked in highly virulent Xcv culture suspension for 8 h. The seeds were treated with bioformulation and sown in 12" pots in three replicates at different doses *i.e.*, 1 g/kg to 10 g/kg in different combinations of Biocontrol agent power and carrier at 1:1, 1:2, 1:5, 1:8 and 1:10. Artifically Xcv infected seeds were used as a positive control. Untreated seeds used as a negative control whereas artificially Xcv inoculated seeds used as a positive control. The plants were observed for symptom expression from the cotyledenary stage onwards. The formulation was also tested against *Xanthomonas campestis* pv. *citri*, *X. c* pv. *mangiferae indicae*, *X. c.* pv. *malvacearum*, *X. c.* pv. *campestris*, *X. oryzae* pv. *oryzae*

RESULTS AND DISCUSSION

Characters of these Antagonistic Phylloplane Bacteria

Cultural, morphological and biochemical characters of these bacteria are summarised in Table 1 whilst Fig. 1 shows morphological features of these bacteria.

Antagonistic effect of three Plb's against Xcv

All the three isolates were tested against six races of Xcv to identify the antagonistic activity of Plb's. The Plb-3 isolate produced larger inhibition zones of (22 mm) (Fig. 2) when compared to the Plb–1 (21 mm) and Plb–2 (20 mm). They are antagonistic to all the 6 races of Xcv.

The three Plb isolates were screened for the quantity of metabolite production. The Plb-3 produced more (2.27 mg/l) metabolites than Plb-2 (2.02) and Plb-1 (1.97). The metabolites were tested for antagonistic activity against Xcv at different concentrations (100, 200, 500, 1000 and 2000 ppm). Increasing concentration of metabolites increased the inhibition zone (Fig. 3).

Table 1. Cultural, morphological and biochemical traits of three antagonistic phylloplane bacteria.

Character	Plb-1	Plb-2	Plb-3
Cultural			
Colony character	Creamy white, irregular margin smooth, flat, circular 9-13 mm in diameter	Creamy white irregular margin smooth, flat, irregular 17-22 mm in diameter	Creamy white irregular margin smooth, flat, irregular 22-24 mm in diameter
Morphological			
Shape	Short Rod	Short Rod	Short Rod
Size	1.5-2 × .5-1.0 μm	2 × 1.0 μm	1.5-2 × .5-1.0 μm
Gram reaction	+ve	+ve	+ve
Spore	+ve	+ve	+ve
Flagella	Flagellate	Peritrichous	Peritrichous
Envelope	Enveloped	Enveloped	Enveloped
Biochemical			
Ammonia production	+ve	+ve	+ve
Catalase reaction	+ve	+ve	+ve

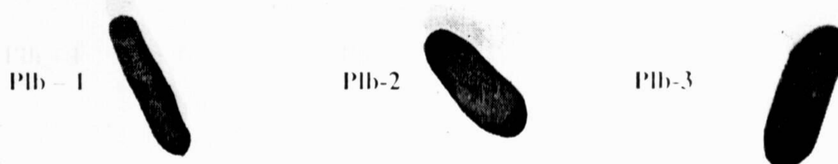


Fig. 1. Electron micrographs of Phylloplane bacteria.

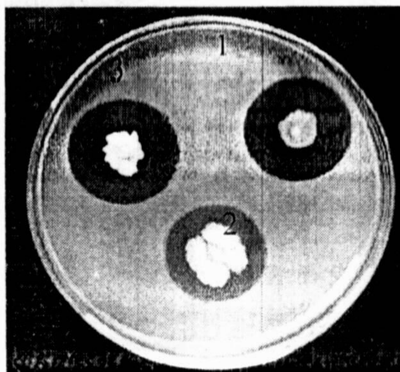


Fig. 2. Antagonistic effect of three isolates of Plb against Xcv.

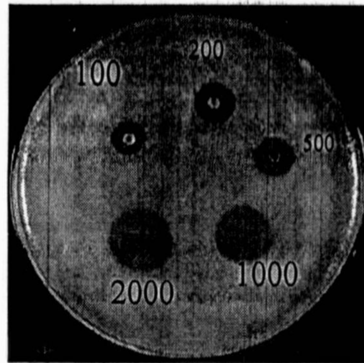


Fig. 3. Inhibition zone produced by metabolite at different concentrations (ppm) against Xcv.

Effect of different media on metabolite production

To identify the best media for metabolite production, out of four media tested [Luria broth (LB), Nutrient broth (NB), Nutrient sucrose broth (NSB) and Sucrose peptone broth (SPB)], SPB was found to be the best (Fig. 4). Maximum amount of metabolite production (2.27 mg/l) was recorded on fourth day (Table 2). Three Plb isolates (1, 2, 3) produced four compounds in each *viz.*, I. (Rf=.87), II. (Rf=.73), III. (Rf=.44), IV. (Rf=.28) (Fig. 5).

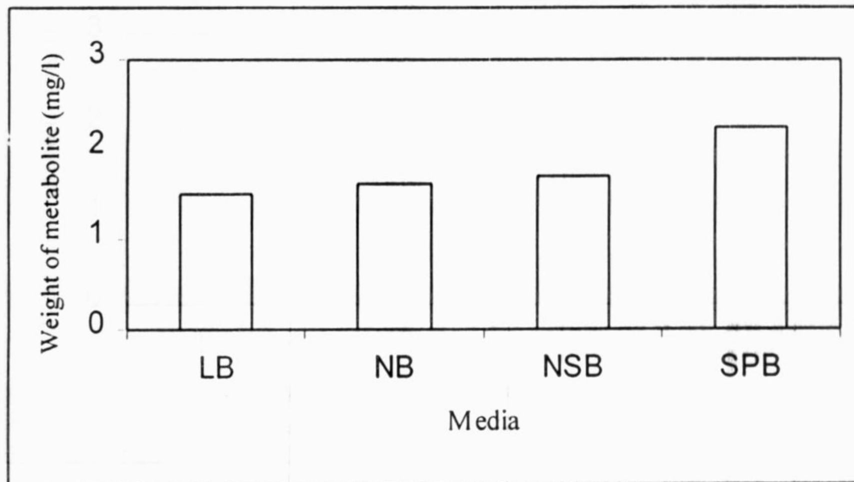


Fig. 4. Effect of different media on the production of metabolites in Plb-3.
[Note: LB-Luria broth; NB-Nutrient broth; NSB-Nutrient sucrose broth; SPB-Sucrose peptone broth].

Table 2. **Metabolite production by Plb during first four days of incubation.**

Incubation period (days)	Metabolite production (mg/l)
1	1.02
2	1.52
3	1.96
4	2.27

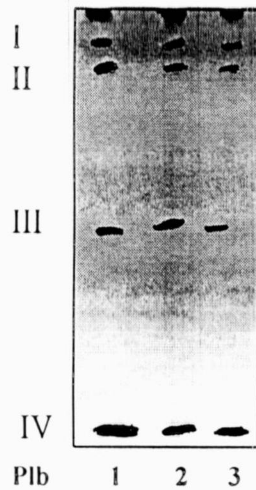


Fig. 5. Thin layer chromatography plate showing compounds produced by three Plb isolates.

Colonising ability of Plb *in planta*

The three isolates of Plb were inoculated into a susceptible mungbean cv. Pusa Baisakhi by the injection–infiltration method and spray method. In both methods *i.e.*, leaf tissue and on the leaf surface maximum colonising ability was recorded by Plb-3 followed by Plb-1 and Plb-2 on the fourth day (Fig. 6 and 7) and then population levels declined.

Role of plasmid in antibiosis

The three isolates of Plb harboured single plasmids, each of 45 kb (Fig. 8). The colonies of the cured strain lost antibiosis. *In vitro*, antibiosis of the wild and cured strains were tested against Xcv by the dual culture method and it was found that the wild

strain gave a clear halo zone depicting antibiosis, while the cured strain could not exhibit a similar property (Fig. 9). Transformed cells restored the plasmid of 45 kb and showed antibiosis property which confirmed the role of plasmids in antibiosis (Fig. 10).

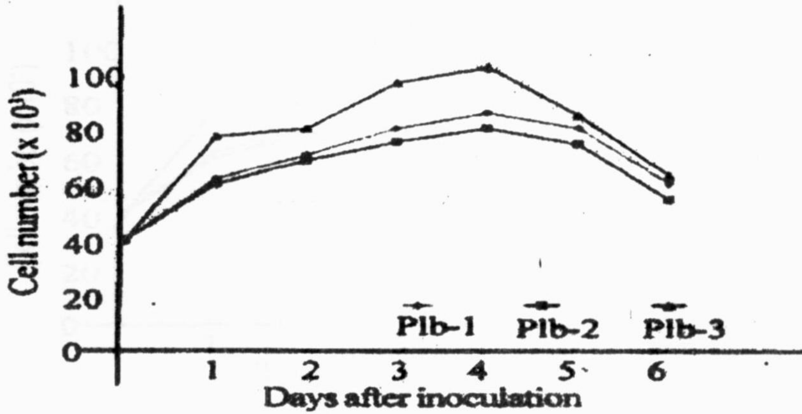


Fig. 6. Change in cell number of three Plb isolates in mungbean leaf tissue.

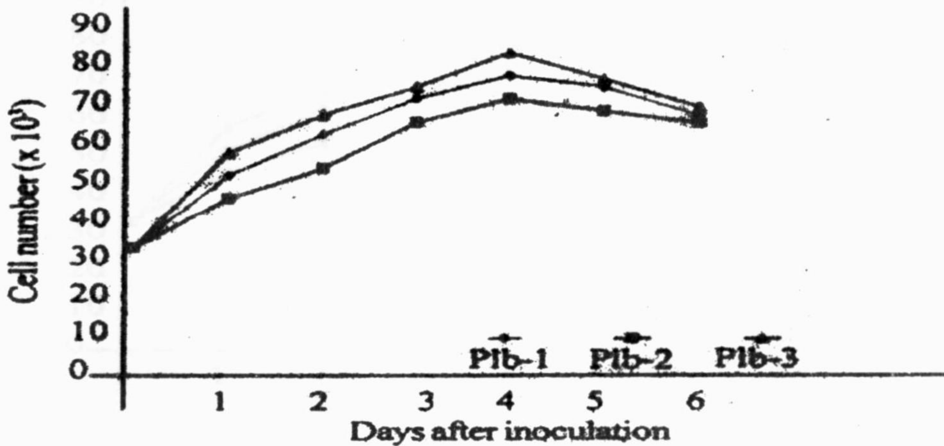


Fig. 7. Change in cell number of three Plb isolates in mungbean leaf surface.

In vivo antibiosis was also checked against the same pathogen and it was found that the wild type strain (in the presence of Xcv) gave protection, which was exhibited by a hypersensitive reaction (HR). The cured strain did not protect the mungbean plants from Xcv infection by showing susceptible reaction (SR

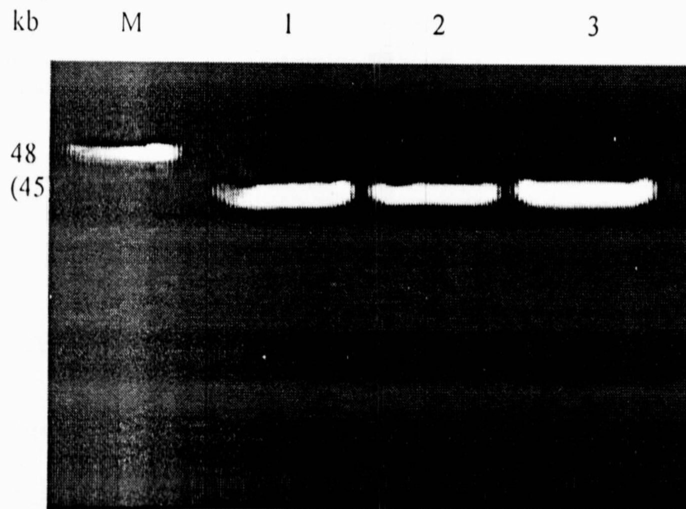


Fig. 8. **Plasmid profile of three Plb isolates.**
[Note: M–Marker; 1–Plb-1; 2–Plb-2; 3–Plb-3].

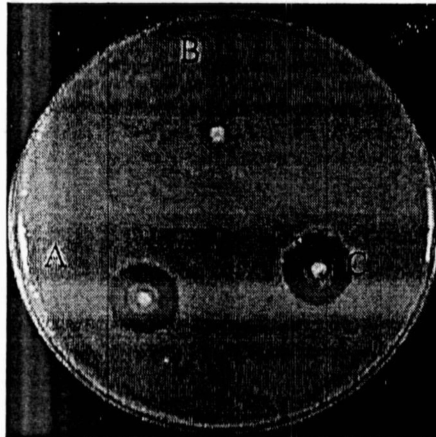


Fig. 9. **Inhibitory effect of wild, cured and transformed cells of Plb-3 against Xcv.**
[Note: A–wild; B–cured; C–transformed].

Viability of bacterial formulation

The spore powder mixed with a carrier and sticker was kept at different temperature ranges from 10-45°C. The bioformulation was found effective even at 45°C indicating its efficacy under tropical conditions (Fig. 11). The bioformulation remained viable for 6 months at room temperature.

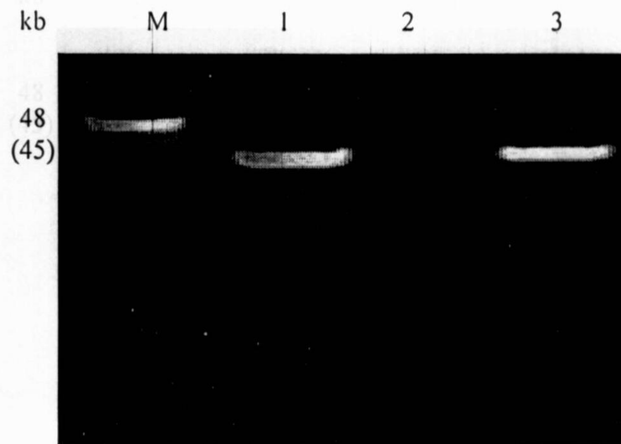


Fig. 10. Plasmid profile of wild, cured and transformed cells of Plb-3.
[Note: M–Marker; 1–wild; 2–cured; 3–transformed].

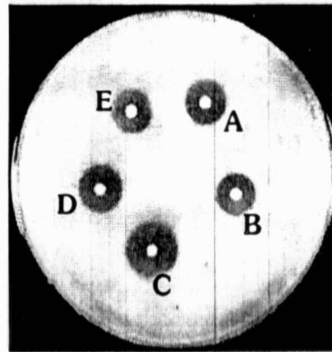


Fig. 11. Inhibitory effect of biocontrol agent after exposing to different temperatures for seven days.
[Note: A-10°C; B-20°C; C-30°C; D-40°C; E-45°C].

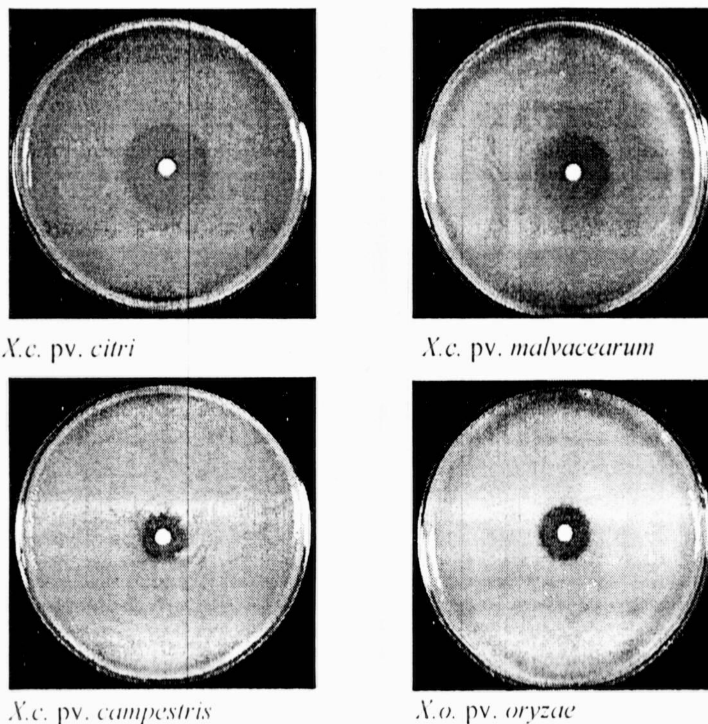
Field experiments for leafspot disease suppression

Among five different ratios (1:1, 1:2, 1:5, 1:8, 1:10 and 1:15) of biocontrol agent powder and talc, the 1:10 ratio was the best on the basis of its ability to prevent the seed from early seed borne infection even at 1 g/kg seed (Table 3). The formulations developed by Kloepper and Schroth (1981); Bashan (1986); Dandurand *et al.* (1994); Vidhyasekharan and Muthamilan (1995) and Krishnamurthy and Gnanamanikam (1998), by using *Pseudomonas* spp. produced encouraging results in the management of bacterial diseases by seed mixing. The formulation was also found effective against *Xanthomonas campestis* pv. *citri*, *X. c* pv. *mangiferae indicae*, *X. c.* pv. *malvacearum*, *X. c.* pv. *campestris*, *X. oryzae* pv. *oryzae* (Fig. 12).

Table 3. **Effect of different combinations and doses of bioformulations on disease management through seed treatment.**

Dose of bio-formulation (g/kg seed)	Ratio of BCA and carrier (Talc)				
	1:1	1:2	1:5	1:10	1:15
1	-	-	-	-	+
2	-	-	-	-	+
4	-	-	-	-	+
8	-	-	-	-	+
10	-	-	-	-	+

Positive control - Artificially Xcv treated seeds; Negative control - Healthy seeds;
+ - Disease development; – - No disease development.



X.c. pv. *citri* *X.c.* pv. *malvacearum*

X.c. pv. *campestris* *X.o.* pv. *oryzae*

Fig. 12. *In vitro* antagonistic effect of formulation against xanthomonads.

CONCLUSIONS

Based on size of inhibition zone amount of metabolite production in different media, colonising ability in leaf tissue, the isolate Plb 3 (*Bacillus* spp.) was found to be the best and was employed in developing the formulation. The formulation was effective in eliminating seed borne infection of Xcv even at 1 g/kg of seed.

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