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*RESEARCH PAPERS*

# Endophytic bacterial induction of defence enzymes against bacterial blight of cotton

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**Summary.** Bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) is a major yield constraint in cotton crops. The effect of inducing systemic resistance against *Xam* in cotton with a talc-based bioformulation of the endophytic *Bacillus* strains EPCO 102 and EPCO 16 and *Pseudomonas fluorescens* strain Pf1, with or without the addition of chitin, was tested under greenhouse conditions. The bioformulation, applied through seed, soil or foliar spray, significantly reduced disease incidence. The addition of chitin to the formulation reduced disease incidence still further. EPCO 102 with chitin led to the lowest bacterial blight incidence. The bacterial strains also caused higher levels of chitinase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and phenol in cotton, besides reduction of blight incidence. In addition, the endophytic *Bacillus* strains increased cotton yield under greenhouse conditions.

**Key words**: endophytes, induced systemic resistance, bioformulation, *Xanthomonas axonopodis* pv. *malvacearum*, *Bacillus*.

**Abbreviations**: PO, Peroxidase; PPO, Polyphenol oxidase; PAL, Phenylalanine ammonia lyase.

## Introduction

Cotton plays an important role in the economic and social affairs of the world, employing about 60 million people in its cultivation, trade or processing (Mayee *et al*., 2002). Being an ancient and, next to food crops, the most important commercial crop grown, it remains the backbone of the rural economy, particularly in dryland areas. It contributes nearly 70% of the raw material for the textile industry (Kairan, 1997). Cotton is cultivated in an area of some 88,000,000 ha in India, making it the first in area of cultivation in the world, and the fourth in volume of production (Verma and Jayaraman, 2002). Cotton is attacked by a number of pests and diseases, including a bacterial blight of cotton (BBC) caused by *Xanthomonas axonopodis* pv. *malvacearum* (Smith) Vauterin (*Xam*). *Xam* causes average losses of 30 to 35% in India (Sheoraj and Verma, 1988) and annual yield losses ranging from 5 to 25%, which may go up to 100% when the infection is severe (Verma, 1992). Though fungicides and insecticides exist to control *Xam*, they cannot be seen as a long-term solution because of

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concerns over exposure risk, health and environmental hazards, and residue persistence. Moreover, the frequent use of pesticides may lead to the development of tolerance in the target organism. As a result, in recent years the focus has shifted to the control of insect pests and diseases using biocontrol agents, which are a safe and promising alternative to synthetic pesticides. There is some evidence that endophytes can contribute to the control of plant diseases (Kloepper *et al*., 1992a). Endophytic bacteria are prokaryotes that colonize the internal tissues of healthy plants but do not cause any disease symptoms (Wilson, 1995). Bacterial endophytes promote plant growth and improve the host's capacity to withstand pathogen attack by causing organism competition, antibiosis and by inducing systemic resistance (ISR). The possibility of using the plant's own defence mechanisms induced by bacterial endophytes in the management of pests and diseases is a matter of current interest. Biotic and abiotic inducers have been reported to enhance the resistance of crop plants to various pests (Karban and Baldwin, 1997) and pathogens (Baker *et al*., 1997). The classical inducers of plant resistance include pathogens (Dalisay and Kuc, 1995), plant-growth promoting rhizobacteria (PGPR) (Leeman *et al*., 1995, Wei *et al*., 1996) chemicals (Ward *et al*., 1991) and plant products (Singh *et al*., 1990).

## Materials and methods

### Seed material, pathogen and endophytic bacterial strains

The experiments were conducted in the Rockefeller glasshouse, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. The blight-susceptible cotton cultivar LRA 5166, obtained from the Central Cotton Research Institute, Coimbatore, India, and the virulent isolate of *Xam* were used in all the experiments. Bacterial endophytes were isolated from different parts of cotton plants. For isolation, whole plants were manually uprooted and brought to the laboratory. Cotton roots and stems were cut into sections 2–3 cm long using a sterile scalpel. Root sections were taken just below the soil line for younger plants (14 days), and 5–10 cm below the soil line for older plants (21 days). Stem sections were taken 1–2 cm above the soil line in younger plants and 10 cm above the soil line in older plants. Stem sections were weighed and surface-sterilized with 1% sodium hypochlorite (NaOCl) in 0.05% triton X-100 for 10 min and rinsed four times in 0.02 M sterile potassium phosphate buffer (PB) pH 7.0. A 0.1 ml aliquot was taken from the final buffer wash and transferred to 9.9 ml tryptic soy broth (TSB) to serve as a sterility check. Samples were discarded if growth was detected in the sterility check samples (agitating samples in TSB, Hi Media Code No. M 011, at 28±2°C) within 48 h.

Each sample (0.5 g) was ground with a sterile mortar and pestle in 9.5 ml of the final buffer wash. Serial dilutions up to $10^{10}$ of the triturate were made in PB. Each dilution of every sample was plated (0.1 ml) on three plates each containing one of three media; Tryptic soy agar (TSA-Hi Media, Code No. M290). Nutrient agar (NA; g $l^{-1}$ peptone 5, beef extract 2, and agar 20, pH 5.0) and King's B Medium (KB) (g $l^{-1}$ proteose peptone 20, $K_2HPO_4$ 1.5, $MgSO_4 \cdot 7H_2O$ 1.5; glycerol 20 ml, and agar 15, pH 7.2) (King *et al*., 1954). The dishes were incubated at 28±2°C for 48–72 h. At each sampling date and with each treatment one representative of each bacterium, identified by their colony type and morphology, was transferred to fresh KB plates to establish pure cultures.

*Pseudomonas fluorescens* isolate Pf1 (obtained from the Department of Plant Pathology, TNAU) and bacterial endophytes were cryopreserved at -80°C in 44% glycerol broth, and cells from stocks were first grown in KB. Inoculum was produced by transferring two loopfuls from the culture to 100 ml of KB in a 250 ml Erlenmeyer flask and incubating at room temperature (28±2°C) on a shaker at 100 ×*g* for 48 h. These strains were sub-cultured once a month and maintained until the end of the experiment in KB and NA slants at 4°C for further study.

### Preparation of bacterial inoculum

Endophytic bacteria were grown on KB with constant shaking at 100 ×*g* for 48 h at room temperature (28±2°C). Bacterial cells were harvested by centrifugation at 12,000 ×*g* for 15 min and bacterial cells were resuspended in PB (0.01 M, pH 7.0). The concentration was adjusted to approximately $10^8$ cfu $ml^{-1}$ ($OD_{595}$=0.3) with a spectrophotometer and used as bacterial inoculum (Thompson, 1996).

**Seed bacterization**

Cotton seeds (cv. LRA 5166) were surface-sterilized with 2% sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight under a sterile air stream. Endophytic bacterial strains, inoculated into their respective broths and bacterial suspension was prepared as mentioned above. The required quantity of seeds was soaked in bacterial suspension containing $3\times10^8$ bacteria $ml^{-1}$ for 2 h and dried under shade.

**Plant-growth promotion**

The plant-growth promoting activity of the bacterial endophytic strains was assessed on the basis of seedling vigour index as determined by the standard roll towel method (ISTA, 1993). Twenty seeds were kept on presoaked germination paper. The seeds were held in position with another presoaked germination paper strip on top of them and gently pressed. The polythene sheet along with the seeds was then rolled and incubated in a growth chamber for 14 days. Three replications were carried out for each treatment. The root and shoot length of individual seedlings was measured and seed germination percentage calculated.

The vigour index was calculated using the formula of Baki and Anderson (1973):

Vigour index = % germination × seedling length (shoot length + root length)

**Preparation of talc-based formulation**

A loopful of bacterium was inoculated into the KB and incubated in a rotary shaker at 100 ×*g* for 48 h at room temperature (28±2°C). After 48 h, the broth containing $9\times10^8$ cfu $ml^{-1}$ was used for the preparation of the talc-based formulation. To the 400 ml of bacterial suspension, 1 kg of purified talc powder (sterilized at 105°C for 12 h), 15 g calcium carbonate (to adjust the pH to neutral), and 10 g carboxymethyl cellulose (CMC) as an adhesive were mixed under sterile conditions, following the method described by Vidhyasekaran and Muthamilan (1995). After shade drying overnight the mixture was packed in a polypropylene bag and sealed. At the time of application, the population of the bacteria in the talc formulation was $2.5–3\times10^8$ cfu $g^{-1}$.

**Chitin amendments with talc-based formulations**

Five g of crab-shell chitin (Sigma, St. Louis, MO, USA) was slowly added to 100 ml of cold 0.25 N HCl with vigorous stirring and kept overnight at 4°C. The mixture was filtered through glasswool into 200 ml of ethanol at 4°C under rapid stirring. The resultant chitin suspension was centrifuged at 10,000 ×*g* for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral. The concentration was adjusted to 10 mg per ml and added to KB broth (1%, v:v). The broth, containing $9\times10^8$ cfu $ml^{-1}$ after 48 h of incubation, was used for the preparation of the talc-based formulation as described above.

**Greenhouse study**

The bacterial strains (with and without chitin) were assessed for their effectiveness in controlling BBC under greenhouse conditions. The trial was conducted in a completely randomized design. Twenty bacterized cotton seeds were sown in each pot and three replications were maintained for each treatment. Seeds were treated with plantomycin (100 ppm) as well as with a foliar spray at a 100 ppm concentration 30 days after planting. Control plants not receiving bacterial treatment were also maintained.

**Assessment of *Xam***

Cotton leaves were inoculated with *Xam* by the sand paper method. Leaves of 30-day-old plants were kept between sheets of sand paper and a gentle pressure was given. Then the bacterial suspension ($10^8$ cfu $ml^{-1}$) was sprayed with a hand sprayer and symptom expression was recorded 20 days after inoculation (Salah, 2002)

Fifty leaves were collected randomly from each pot and they were assessed for bacterial blight symptoms. The % disease index (PDI) was scored on a 7-point scale: (Santhanam, 1967), 0 immune; 1–3 resistant; 4–5 moderately susceptible and 6–7 susceptible.

The PDI was calculated using the formula (Mc Kinney, 1923)

$$PDI = \frac{\text{Sum of all numerical ratings}}{\text{Total No. of leaves scored}} \times \frac{100}{\text{Maximum score}}$$

**Sample collection, enzyme extraction, assay of PR proteins**

Leaves from inoculated and uninoculated plants maintained under the same conditions were collected at 0 h, 24 h, 48 h, 72 h, 96 h, 120 h

and 7-day intervals. The leaves and roots of plants subjected to bacterial infection and collected at the different intervals as mentioned above were stored at -70° C.

One g of powdered sample was extracted with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 ×*g*. The supernatant was used as crude enzyme extract to assay chitinase activity. Sodium phosphate buffer 0.1 M (pH 7.0) was used for the extraction of PO, PPO and PAL enzymes (Ramamoorthy *et al*., 2002a). The changes in chitinase and peroxidase activity were determined by colorimetric assay according to Boller and Mauch (1988).

#### Native gel electrophoresis

#### Peroxidase (PO)

Activity gel electrophoresis was carried out to study the expression pattern of different isoforms of peroxidases with the various treatments. For native anionic polyacrylamide gel electrophoresis, a resolving gel of 8% acrylamide and a stacking gel of 4% acrylamide were prepared. After electrophoresis, the gels were incubated for 30 min in the dark in a solution containing 0.15% benzidine in 6% $NH_4Cl$. Then drops of 30% $H_2O_2$ were added under constant shaking until the bands appeared. After staining, the gel was washed with distilled water and photographed (Sindhu *et al*., 1984).

#### Polyphenol oxidase (PPO)

PPO was extracted by homogenizing 1 g of tissue in 0.01 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000 ×*g*, 4°C for 15 min in a centrifuge and the supernatant was used as the enzyme source. After native electrophoresis the gel was equilibrated for 30 min in 0.1% *p*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in the appearance of dark brown discrete protein bands.

#### Yield assessment

All the treatments were maintained until the boll bursting stage. The number of bolls per plant and kapas yield was determined for all the treatments.

#### Statistical analysis

The data were statistically analysed (Rangaswamy, 1995) and treatment means were compared with Duncan's Multiple Range Test (DMRT). The IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics Unit, Philippines, was used for analysis.

## Results

#### Effect of bacterial endophytic strains on plant-growth promotion

One hundred and three endophytic bacteria were isolated from the healthy roots, stems, leaves and seeds of cotton plants. Endophytic *Bacillus* isolates EPCO 102 (leaf isolate) and EPCO 16 (root isolate) were found to increase the vigour index of cotton seedlings significantly, with a maximum vigour index of 1404.55 for cotton seedlings treated with EPCO 102 suspension, compared with a vigour index of 226.4 with the untreated controls (Table 1). No external symptoms appeared after endophytic bacteria treatment.

#### Effect of endophytes on BBC

Endophytic bacterial strains were tested for their effectiveness against *Xam* in potted cotton plants along with plantomycin as a chemical check. With plantomycin at 100 ppm the lowest incidence (8.38 PDI) of BBC was recorded 60 days after sowing, followed by EPCO 102 + chitin (14.853 PDI). EPCO 16 and *Pseudomonas fluorescens* Pf1 were similar in their effectiveness against *Xam*. Plants without any endophytic bacteria had the highest BBC incidence (40.56) (Table 2).

#### Induction of defence-related enzymes and proteins

Peroxidase activity was significantly higher in cotton leaves inoculated with *Xam* than in leaves without challenge inoculation. In general, PO activity remained constant over time in bacterized but uninoculated plants. Although PO activity increased in *Xam*-inoculated control plants, it became still greater when they were inoculated to inoculated plus bacterized plants. Plants bacterized with EPCO 102, EPCO 16 or Pf1 with chitin and challenged had significantly greater PO activity than the other plants. (Fig. 1a, b)

Upon pathogen challenge, PPO activity became

Table 1. Effect of bacterial endophytes on cotton seedling growth.

| Bacterial isolate | Vigour index [a] | | Bacterial isolate | Vigour index [a] | |
|---|---|---|---|---|---|
| | Roll towel | Pot study | | Roll towel | Pot study |
| EPCO1 | 435.0 c-j | 334.7 b-o | EPCO53 | 405.0 c-i | 509.5 c-u |
| EPCO2 | 2115.0 x-B | 607.2 e-x | EPCO54 | 1029.8 l-s | 547.3 d-x |
| EPCO3 | 1939.0 v-B | 746.1 i-A | EPCO55 | 375.0 c-f | 519.8 d-w |
| EPCO4 | 1369.0 q-x | 715.0 i-A | EPCO56 | 555.0 c-m | 499.8 d-w |
| EPCO5 | 2169.7 x-B | 715.0 i-A | EPCO57 | 719.2 d-p | 587.5 e-x |
| EPCO6 | 2379.8 A-D | 802.5 o-B | EPCO58 | 969.6 k-s | 799.6 n-B |
| EPCO7 | 614.8 c-m | 566.8 d-x | EPCO59 | 545.0 c-l | 484.8 d-v |
| EPCO8 | 824.8 f-r | 437.5 c-t | EPCO60 | 1309.4 p-v | 689.7 g-A |
| EPCO9 | 280.0 bc | 382.2 b-r | EPCO61 | 1764.6 t-v | 407.4 b-r |
| EPCO10 | 2256.0 z-D | 762.2 i-B | EPCO62 | 879.0 j-s | 484.8 d-v |
| EPCO11 | 2770.0 BCD | 660.0 g-z | EPCO63 | 315.0 cd | 267.4 b-h |
| EPCO12 | 340.0 cd | 377.5 b-p | EPCO64 | 515.0 c-k | 612.2 e-x |
| EPCO13 | 2104.0 w-B | 684.8 g-A | EPCO65 | 1196.7 n-u | 307.5 b-k |
| EPCO14 | 1962.0 v-B | 1092.1 x-C | EPCO66 | 160.0 ab | 392.4 b-r |
| EPCO15 | 495.0 c-k | 667.3 g-z | EPCO67 | 2034.6 v-B | 1052.3 w-C |
| EPCO16 | 2710.0 BCD | 1218.7 y-C | EPCO68 | 0.5 a | 369.9 b-r |
| EPCO17 | 866.2 i-s | 677.4 g-y | EPCO69 | 0.5 a | 0.5 a |
| EPCO18 | 508.0 c-k | 694.2 g-y | EPCO70 | 2405.0 A-D | 945.0 t-C |
| EPCO19 | 577.4 c-l | 709.5 g-A | EPCO71 | 1999.7 v-B | 1549.5 C |
| EPCO20 | 789.6 e-q | 699.3 h-A | EPCO72 | 0.5 a | 735.0 i-A |
| EPCO21 | 669.6 c-o | 425.0 b-s | EPCO73 | 2502.8 A-D | 547.2 d-x |
| EPCO22 | 290.0 bc | 335.0 b-m | EPCO74 | 2750.0 BCD | 1582.0 C |
| EPCO23 | 1341.2 q-w | 429.8 c-t | EPCO75 | 0.5 a | 342.5 b-p |
| EPCO24 | 902.4 j-s | 924.7 t-C | EPCO76 | 0.5 a | 819.7 p-B |
| EPCO25 | 2286.0 z-D | 0.5 a | EPCO77 | 1484.8 s-z | 899.9 s-C |
| EPCO26 | 2152.0 x-C | 709.9 i-A | EPCO78 | 2497.8 A-D | 307.5 b-l |
| EPCO27 | 1970.0 v-B | 627.2 f-y | EPCO79 | 0.5 a | 795.0 o-B |
| EPCO28 | 0.5 a | 919.8 t-C | EPCO80 | 2177.6 y-C | 439.8 c-t |
| EPCO29 | 2384.8 A-D | 1296.9 ABC | EPCO81 | 0.5 a | 559.8 d-x |
| EPCO30 | 889.5 j-s | 952.29 t-C | EPCO82 | 0.5 a | 222.5 b-e |
| EPCO31 | 2294.4 z-D | 937.2 t-C | EPCO83 | 0.5 a | 847.4 r-B |
| EPCO32 | 2720.0 BCD | 0.5 a | EPCO84 | 0.5 a | 434.7 c-t |
| EPCO33 | 709.8 d-p | 667.1 g-z | EPCO85 | 2116.8 w-B | 837.3 q-B |
| EPCO34 | 854.4 h-r | 595.0 e-x | EPCO86 | 1049.9 l-s | 742.5 j-A |
| EPCO35 | 709.8 d-o | 590.0 e-x | EPCO87 | 1972.9 v-B | 772.5 m-B |
| EPCO36 | 1079.7 m-t | 709.5 g-A | EPCO88 | 0.5 a | 367.4 b-q |
| EPCO37 | 405.0 cde | 365.0 b-p | EPCO89 | 1972.7 v-B | 449.7 c-u |
| EPCO38 | 145.0 ab | 264.9 b-g | EPCO90 | 482.0 c-j | 312.3 b-j |
| EPCO39 | 1217.4 o-u | 687.3 g-A | EPCO91 | 2109.6 w-B | 627.3 f-y |
| EPCO40 | 865.0 i-s | 482.4 c-v | EPCO92 | 2780.0 BCD | 845.0 r-B |
| EPCO41 | 1084.0 m-t | 352.3 b-q | EPCO93 | 3090.0 CD | 1012.4 v-C |
| EPCO42 | 350.0 cd | 289.8 b-i | EPCO94 | 2388.0 A-D | 1282.0 z-C |
| EPCO43 | 400.0 c-h | 477.1 c-t | EPCO95 | 0.5 a | 979.5 u-C |
| EPCO44 | 829.8 g-r | 332.4 b-n | EPCO96 | 2740.0 BCD | 1234.5 y-C |
| EPCO45 | 1785.0 u-A | 197.5 bcd | EPCO97 | 2420.0 A-D | 832.3 q-B |
| EPCO46 | 1419.2 r-y | 140.0 bc | EPCO98 | 2170.0 y-C | 1010.5 v-C |
| EPCO47 | 380.0 c-g | 354.7 b-q | EPCO99 | 0.5 a | 178.0 b |
| EPCO48 | 840.0 h-r | 364.6 b-r | EPCO100 | 2580.0 A-D | 684.8 g-A |
| EPCO49 | 639.6 c-n | 322.4 b-l | EPCO101 | 2172.8 y-D | 997.2 v-C |
| EPCO50 | 719.8 d-p | 587.1 d-x | EPCO102 | 3130.0 D | 1404.5 BC |
| EPCO51 | 619.9 c-m | 574.7 d-w | EPCO103 | 2363.0 A-D | 755.0 k-B |
| EPCO52 | 75.0 a | 529.9 d-x | Control | 40.0 a | 226.4 b-f |

[a] Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P$=0.05. Values are means of three replications.

Table 2. Effectiveness of bacterial endophytes against bacterial blight of cotton under greenhouse conditions.

| Treatment | Percent disease index [a] | | |
|---|---|---|---|
| | 40 DAS[b] | 50 DAS | 60 DAS |
| EPCO 102 | 16.570(24.018) c | 18.280(25.310) d | 19.040(25.868) de |
| EPCO 16 | 16.570(24.018) c | 18.470(25.445) d | 20.186(26.695) d |
| Pf1 | 14.470(22.346)[c] d | 16.946(24.291) de | 18.850(25.730) de |
| EPCO 102 + C | 8.571(17.017) e | 10.750(19.130) f | 14.853(22.646) g |
| EPCO 16 + C | 13.712(21.731) d | 16.190(23.725) e | 17.710(24.885) ef |
| Pf1 + C | 12.950(21.078) d | 15.996(23.566) e | 16.566(24.009) f |
| Chitin | 25.090(30.052) b | 28.806(32.459) b | 29.376(32.818) b |
| Plantomycin | 5.520(13.560) f | 7.806(16.208) g | 8.380(16.824) h |
| Control | 29.520(32.907) a | 32.570(34.798) a | 40.566(39.560) a |

[a] Values are means of three replications.
[b] DAS, days after spraying.
[c] Values in parentheses are arcsine transformed.
Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P$=0.05.

significantly greater in the bacterized cotton plants than in the control. PPO activity peaked 48 h after inoculation. Pf1, EPCO 102, EPCO 16 with chitin significantly increased PPO activity in cotton for 48 h, then it declined again compared to other treatments. PPO activity was greatest in plants treated with Pf1, followed by plants treated with EPCO 102 and EPCO 16 plus chitin. (Fig. 1c, d).

PAL activity began to increase in cotton plants 24 h after inoculation with *Xam*. This increase in activity was however only temporary in the leaves, and it returned to control-plant levels 72 h after inoculation. PAL activity was much the same with and without chitin. In the leaves of bacterized cotton plants inoculated with *Xam*, PAL activity was at least twice as high as in the control plants (Fig. 1e, f).

Chitinase activity was low in healthy leaf samples. But it increased markedly in *Xam*-inoculated leaves. Activity of this hydrolytic enzyme was more pronounced in cotton treated with endophytes. The bacterial strains EPCO 102, EPCO 16 and Pf1 produced the same level of chitinase in the leaves after challenge with *Xam*. The results indicated that the endophytic bacterial strain stimulated chitinase activity locally and systemically in the leaves after the inoculation with *Xam* (Fig. 1g, h).

Levels of phenols were highest in plants inoculated with *Xam* and treated with Pf1 plus chitin, followed by *Xam* inoculated plants treated with EPCO 102 and EPCO 16 plus chitin. With all these treatments phenol levels were significantly different from those in the control plants. Higher phenol levels occurred for up to 3 days in Pf1-treated plants after which they again declined. Endophytic bacterial strains with or without chitin showed similar increases in phenol level (Fig. 1i, j).

**Native PAGE analysis of defense enzymes**

Native gel electrophoretic separation of enzyme extract from plants treated with endophytic bacteria after challenge inoculation with *Xam* showed that there were four isoforms, PO1, PO2, PO3 and PO4, whereas in the control plants only three isoforms, PO2, PO3 and PO4 were found (Fig. 2).

Native PAGE analysis of plants treated with endophytes after *Xam* inoculation showed a comparatively lower induction of the PPO1 isoform than did the uninoculated bacterized plants. The three isoforms PPO1, PPO2 and PPO3 were conspicuous in plants inoculated with *Xam* and treated with EPCO 16 plus chitin. Isoforms PPO1 and PPO2 were not visible in the control plants even after *Xam* inoculation (Fig. 3).

**Effect of bacterial endophytes on cotton yield**

Bacterized plants in the greenhouse had a significantly higher yield than untreated and plantomycin-treated plants. The number of bolls and

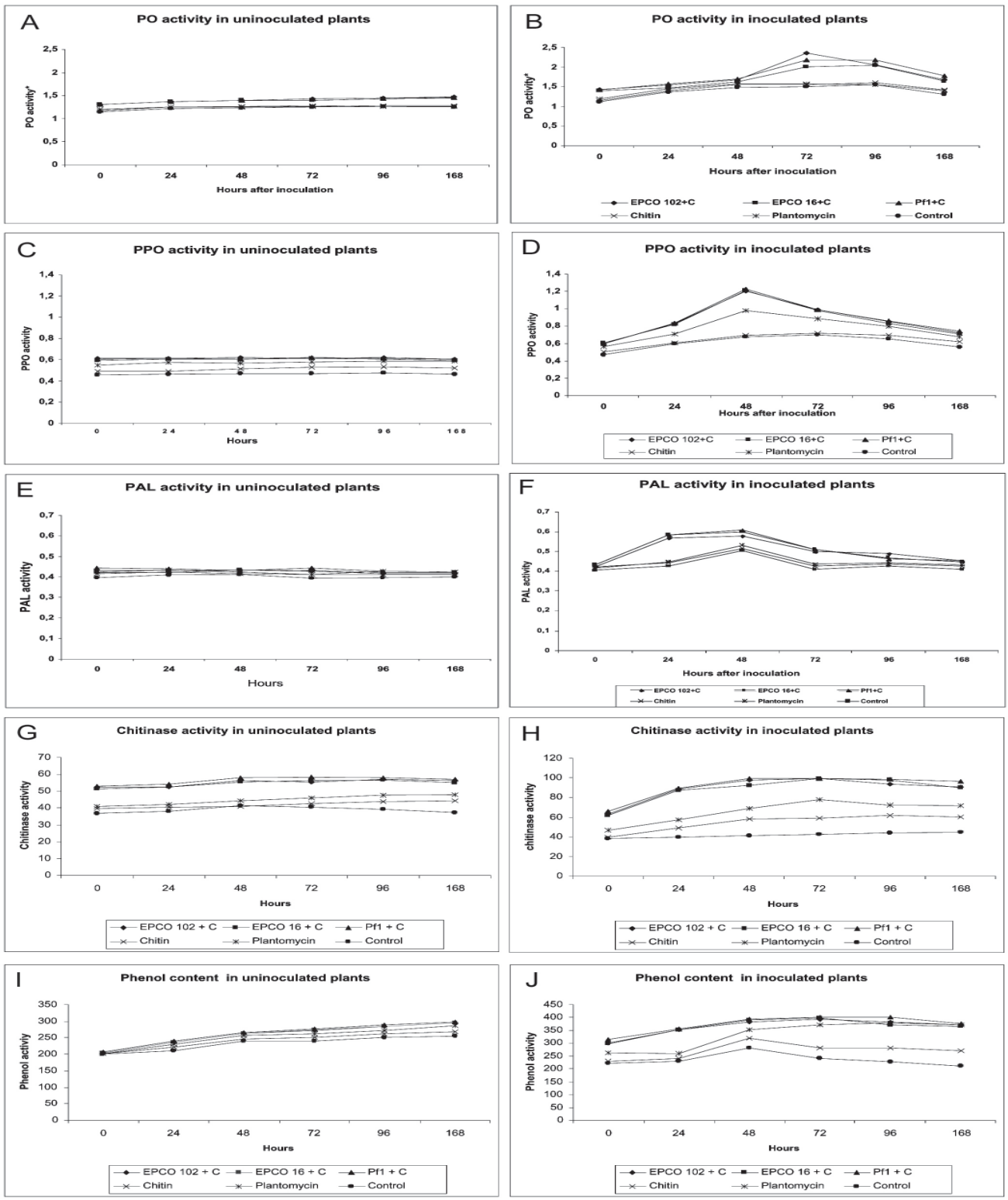
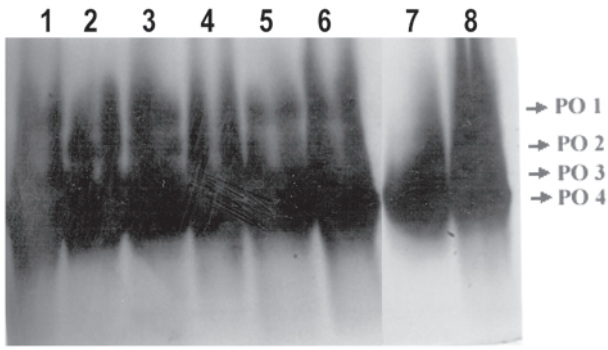
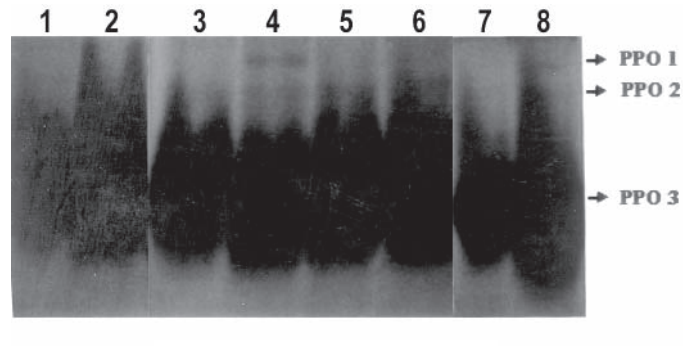



Fig. 1. Bacterial blight infection and induced systemic resistance in cotton plants.




Lane 1. EPCO 102 + Chitin
Lane 2. EPCO 102 + Chitin + *Xam*
Lane 3. EPCO 16 + Chitin
Lane 4. EPCO 16 + Chitin + *Xam*
Lane 5. Pf1 + Chitin
Lane 6. Pf1 + Chitin + *Xam*
Lane 7. Healthy control
Lane 8. Control inoculated with *Xam*

Fig. 2. Native PAGE of peroxidase in cotton plants treated with endophytic bacterial strains against *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*).




Lane 1. EPCO 102 + Chitin
Lane 2. EPCO 102 + Chitin + *Xam*
Lane 3. EPCO 16 + Chitin
Lane 4. EPCO 16 + Chitin + *Xam*
Lane 5. Pf1 + Chitin
Lane 6. Pf1 + Chitin + *Xam*
Lane 7. Healthy control
Lane 8. Control inoculated with *Xam*

Fig. 3. Native PAGE of polyphenol oxidase in cotton plants treated with endophytic bacterial strains against *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*).

the kapas yield were higher in the bacterized plants. A maximum kapas yield of 8.12 g was produced with Pf1+chitin treated plants. The yield of plants bacterized with EPCO 102 and EPCO 16 was also significantly greater than that of the untreated control plants. Interestingly, Pf-1 and EPCO 102-treated plants had a significantly higher kapas yield than plants treated with antibiotic or untreated control plants. The kapas yield of plants receiving chitin alone was 3.51 g (Table 3).

## Discussion and conclusions

Bacterial blight of cotton is a very serious disease causing severe loss to cotton production. Management of BBC with cultural practices and toxic chemicals has both advantages and disadvantages. Late managing this disease by biological methods has become increasingly important. Among biological control methods, endophytic bacteria are an alternative to chemical pesticides

Table 3. Effect of bacterial endophytic strains on cotton yield.

| Treatment | No. of bolls / plant[a] | Kapas yield (g) [a] |
|---|---|---|
| EPCO 102 | 3.30 bc | 6.80 gh |
| EPCO 16 | 3.01 bc | 5.95 e |
| Pf1 | 3.60 c | 7.91 i |
| EPCO 102 + C | 3.40 bc | 6.85 h |
| EPCO 16 + C | 3.01 bc | 6.08 f |
| Pf1 + C | 3.67 c | 8.12 j |
| Chitin | 1.67 a | 3.51 b |
| Plantomycin | 3.30 bc | 6.76 g |
| Control | 1.00 a | 1.65 a |

[a] Values are means of three replications.
Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P$=0.05.

that can be more reliable and ecologically as well as economically sustainable. Cotton seed bacterization with the bacterial endophyte EPCO 102 increased seedling growth under greenhouse conditions. Similarly, Bhowmik *et al*. (2002) reported that cotton seed bacterization with the endophyte Endo PR8 was highly effective in reducing cotyledonary infection with *Xam*. Bacterized grapevines had a greater fresh weight of the shoots and roots, and faster growth with more lignin deposits (Barka *et al*., 2002). Endophytic bacteria from cotton tissues led to better seed germination and better control of cotton wilt caused by *V. dahliae* (Fu *et al*., 1999). Mondal (1999) found that five strains of *Pseudomonas* inhibited *Xam,* increased cotton seed germination by 12.8%, and improved normal seedling growth by 22.4%. Endophytic bacteria may have a role in triggering the plant defence mechanism (Benhamou *et al*., 1996). Many plant-growth promoting endophytic (PGPE) strains have been isolated from the internal tissues of various crops and tested against diseases by several researchers (Barka *et al*., 2002; Sabaratnam and Beattie, 2003). As a result of a greater ISR in plants, growth was enhanced and disease reduced in many crops (Adhikari *et al*., 2001; Bacon and Hinton, 2002). The endophytic bacterium *B. amyloliquefaciens* produces surfactin, iturin, bacillomucine and azalomycin F; *B. subtilis* produces surfactin and arthrobactin, and *B. pumilus* produces surfactin, amphomycin, arthrobactin and valinomycin, which are effective against black rot of crucifers caused by *X. campestris* pv. *campestris* (Wulff *et al*., 2002). The endophytic strain EPCO 102 with chitin led to the lowest BBC incidence under greenhouse conditions. Similar results were reported by Lafontaine and Benhamou (1996), who reported on the unique biological properties of chitin oligomers and their role in eliciting antifungal chemicals on various plant pathogenic fungi like *Fusarium oxysporum* f. sp. *radicis lycopersici* and *Pythium aphanidermatum*. Yuen *et al*. (2001) found that the incorporation of chitin into the broth increased the bacterial population and improved the efficiency of PGPE strains in reducing the severity of rust in bean plants. Treatment with the endophytes EPCO 102 and EPCO 16 with chitin played a dual role, both promoting plant growth and reducing disease severity as compared with other treatments. Endophytic bacteria enhance plant growth by producing plant growth regulators such as gibberellins, cytokinins and indole acetic acid, which directly or indirectly promote plant growth and development (Holland, 1997; Barka *et al*., 2002). Our study showed that early and increased expression of PO, PPO and chitinase enzymes led to a significant reduction in the severity of BBC. The roles of chitinases and peroxidases against various pathogens in plants have been reported by Kandan *et al*. (2002), Chen *et al*. (2000) and Ramamoorthy *et al*. (2002b) with their direct or indirect role in inducing ISR (Dalisay and Kuc, 1995). The addition of chitin to a talc-based formulation can enhance the ISR. That chitin induces systemic resistance when applied alone or in combination with biocontrol agents has been reported for tomato by Benhamou *et al*. (1998). Higher levels of PO have been correlated with enhanced ISR in several plants (Kandan *et al*., 2002; Ramamoorthy *et al*., 2002b). In the present study, cotton plants treated with the bioformulation containing endophytic bacteria and challenged with *Xam* showed higher levels of PO. Increased levels of PO1, PO2, PO3 and PO4 occurred in cotton plants treated with the endophytes and challenge-inoculated with *Xam*. PAL activity was triggered by the interaction of *Xam* and the fungal elicitors (Ramanathan *et al*., 2000). In the present study, PAL activity increased in plants treated with all the PGPE strains, with or without chitin. Similarly, PAL increased in cucumber treated with the fluorescent pseudomonad to protect it against *P. aphanidermatum,* and this increase was related to enhanced resistance (Chen *et al*., 2000). The present study also indicated that EPCO 102 and EPCO 16 with chitin enhanced PPO activity and increased levels of three isozymes of PPO. Radjacommare (2000) reported that strain Pf1 raised levels of PPO isozymes in rice against sheath blight and leaffolder. The chitinases and the β-1,3-glucanases (which are classified under the PR-3 and PR-2 groups of the PR proteins respectively) are reported to be associated with greater resistance in plants against pests and diseases (Maurhofer *et al*., 1994; van Loon, 1997). In the present study, elevated levels of chitinase were produced in plants treated with the bioformulations containing endophytic bacteria with chitin against *Xam*. Viswanathan and Samiyappan

(1999) reported that an ISR by fluorescent pseudomonads was associated with the production of chitinase, which appeared to be a promising means to manage red rot of sugarcane. Phenolic compounds enhanced the mechanical strength of the host cell walls and also inhibited the invading *Xam*. Seed treatment with *P. fluorescens* 63 caused levels of phenolics to rise in tomato root tissue (M'Piga *et al*., 1997). In the present study, higher levels of phenolics occurred in cotton plants treated with endophytes with chitin against *Xam*. Benhamou *et al*. (2000) reported that the endophytic bacterium *Serratia plymuthica* raised levels of phenolics in cucumber roots, affording protection against *Pythium ultimum*. In the present study, amendment with chitin considerably increased the biocontrol activity of the endophytes against *Xam*. Chitin, applied alone or in combination with biocontrol agents, enhanced systemic resistance in tomato (Benhamou *et al.,* 1998), mango (Vivekananthan *et al.,* 2004) and in cucumber (El-Ghaouth *et al.,* 1994).

Increasing the levels of defence related proteins and chemicals by PGPE strains with chitin is a promising new way to manage BBC. In the present study, a timely increase in such defence-related proteins by pretreatment with PGPE strains prevented infection with *Xam* in cotton under greenhouse conditions. Among the PGPE formulations evaluated, Pf1 followed by EPCO102 and EPCO16 with chitin significantly controlled BBC and enhanced yield.

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