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Research article

PGPR CONSORTIUM IN ALLEVIATING DOWNY MILDEW OF CUCUMBER

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ABSTRACT: PGPR strains *viz., Achromobacter sp.* F2feb.44, *Streptomyces sp.* Zapt10 and *Bacillus licheniformis* AE 6 alone and in all possible combinations were exploited to induce systemic resistance in cucumber against the foliar disease of downy mildew caused by the phytopathogen, *Pseudoperenospora cubensis* and to enhance yield improvement too. Seed priming coupled with foliar spray supplement at fortnight interval had probably ameliorated antagonism of phytopathogens by utilizing other disease suppressive traits of PGPR, in addition to induced systemic resistance. A relatively high biocontrol of the said pathogen was achieved with the take all strain mixture of F2feb.44 + Zapt10 + AE6, biocontrol efficiency for this treatment being 86.6 % with respect to untreated control. Besides the delayed onset of the disease symptoms on PGPR treated plants with respect to control along with the slow progress as evidenced from the disease severity index (highest disease severity recorded was 7.73 for F2feb.44 + Zapt10 and lowest 1.89 for F2feb.44 + Zapt10 + AE6 treatment) establishes induction of systemic resistance in cucumber cv. Baropata against the phytopathogen. Defense enzyme studies in such cucumber leaves have corresponded with the results of the field trials. Highest enzyme activities were obtained for F2feb.44 + Zapt10 + AE6 treatment. While Phenylalanine ammonia lyase (PAL) activity remained relatively higher immediately after bacterization till 15 DAP and prior to pathogen infestation whereas, Peroxidase (PO) and Polyphenol oxidase (PPO) the same treatments showed high activities after pathogen attack only and peaked between 5-8 days after the attack. Further Isoperoxidase native PAGE analysis indicated that the isomer forms induced by rhizobacteria were different from pathogen infected ones. The fruit yield with respect to size, number and weight/ plant was also significantly high indicating the biofertilizer potential of the consortium.
Key words: consortium, *Pseudoperenospora cubensis,* peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase
Abbreviations: DAP= days after planting, dpi= days post inoculation.

INTRODUCTION

The exploitation of plant growth promoting rhizobacteria for biological control of pathogenic fungi in cucumber dates back to 1992, when Wei *et al.,* [36] attempted biocontrol of a foliar pathogen, *Colletotrichum orbicularae* causing anthracnose using four PGPR strains *viz.,* 89B-61 (*Pseudomonas putida*), 90- 166 (*Serratia marcescens*), INR-5 (*Flavomonas oryzihabitans*), INR-7 (*Bacillus pumilis*). Zhou and Paulitz [37] demonstrated that *Pseudomonas* spp. was capable of inducing resistance in cucumber against soil- borne pathogen, *Pythium aphanidermatum* causing root rot. Subsequently, Liu *et al.,*[21] successfully induced resistance in the crop against *Fusarium* wilt disease using PGPR strain mixture of 89B- 27 (*Pseudomonas putida*) and 90- 166 (*Serratia marcescens*). Earlier works exploited single strain or strain mixtures in dual combinations only until Raupach and Kloepper, [28, 29] reported that three strain mixture comprising of *Curtobacterium floccumfaciens* ME1, *Bacillus pumilis* strain INR7 and *Bacillus subtilis* strain GB03 in all possible combinations were capable of successfully counteracting two foliar pathogens of cucumber, *Colletotrichum orbiculare* and *Pseudomonas syringae* pv. *lachrymans* (causing angular leaf spot). However in the latter study, such strain mixture was coupled with fumigation with Methyl bromide which is a harmful chemical pesticide, largely responsible for stratospheric depletion of ozone [8]. Due to such detrimental consequences, this being once a popularly used soil fumigant has now been banned due to its detrimental effects [25].

Downy mildew of cucumber caused by *Pseudoperenospora cubensis* (Berk. and Curt.) Rostovzev has been a major concern globally since 1980s [9] and its major outbreaks in India were reported by Mahrisi and Siradhana [22] followed by Singh and Sokhi [33]. Initially the disease was successfully controlled by host resistance but after 2004 this has no longer been effective. The phytopathogen has been combated effectively by foliar spray of chemical fungicides like copper oxychloride, mancozeb (Dithane M-45), (mefenoxam+ mancozeb) (Ridomil Gold 72 WP), Spinosyn A and D (Success 72 WP), and Aluminium tris (o- Ethyl Phosphonate) (Alliete) [6], where the disease incidence was recorded to be lowest for Success (9%), Ridomil (9 %) and still lower for Alleite (11%) compared to control (78%). The number of fruits per plant, length and yield of fruit was also significantly higher for such treatments. However the phytotoxicity of such fungicides coupled with prolonged persistence in tissues and huge residue accumulation in soil have resulted in dreadful consequences with respect to environment and human health.

Very little effort has been made globally to achieve biological control of the phytopathogen. The only existing biofungicide that has been known to suppress downy mildew pathogen in cucumber is Azoxystrobin (Amistar 25 SC), obtained from secondary metabolite of *Strobilurus tenacellus*, a small mushroom, which when sprayed at an optimum dose of 125g a.s./ ha could efficiently arrest disease progress upto an extent of 90.08% though the concerned study has not presented convincing evidence on the defense enzyme changes during the period [2]. Prior state of art in this area has also questioned the efficiency of the fungicide for a long period due to the resistance exhibited by *Cercospora beticola* against the molecule [19]. Recently, *Pseudomonas fluorescens* (10g l^{-1}) has only been exploited to reduce disease severity in conjunction with Azoxystrobin at a concentration of 0.25, 0.5 and 1.0 ml l^{-1} by eliciting defense enzymes against the pathogen in the host [1]. Results showed that *Pseudomonas fluorescens*. strain was a relatively weak inducer of induced systemic resistance compared to Azoxystrobin but the disadvantage of the latter lies in the persistence in tissues upto seven days after spraying though the residue added to soil was below detectable level and the fungicide exhibited least phytotoxicity. Mode of application of PGPR has played a crucial role in governing the efficacy of biocontrol agents in curbing plant diseases as the efficiency of the latter depends on the fluctuations in the micro- climate of the tissue. Review of the previous works have revealed that foliar diseases caused by fungal pathogens like *Botrytis cinrea, Cladosporium herbarum* and *Phoma betae* on beet leaf, *Colletotrichum gloeosporoides* on mango leaf can be effectively controlled by foliar spray of talc based formulations of *Pseudomonas fluorescens* supplemented with chitin in mango at a concentration of 5g/l [5, 35] which also improves fruit production. The dosage and frequency of application vary for different crops and this has to be standardized for our experiment.

Appreciating the gaps in designing a suitable biocontrol agent for appropriate management of the target disease from the previous works, the present study has aimed at investigating the biocontrol potential of a PGPR consortium comprising of three bacterial strains, *viz. Achromobacter sp.* (F2feb.44), *Streptomyce sp.* (Zapt10) and *Bacillus licheniformis* (AE 6) in suppressing downy mildew of cucumber when applied in all possible combinations. Moreover the objectives of this research is to determine if the PGPR strains in question were capable of inducing systemic resistance against the foliar disease of cucumber, *viz*. downy mildew caused by *Pseudoperenospora cubensis.*

MATERIALS AND METHODS

Bacterial cultures

Three selected PGPR strains, *viz., Achromobacter* sp. (F2feb.44), *Streptomyces* sp Zapt10 and *Bacillus licheniformis* strain AE6 that were isolated from cucumber rhizosphere and demonstrated ISR in field trials of cucumber against different fungal pathogens (K. Sen, unpublished data) were used. For long term storage, these bacteria were maintained in nutrient broth (NB) (Himedia Laboratories, Mumbai, India).

For experimental use, bacteria were transferred onto nutrient agar (NA), (Himedia) plates and incubated at 28° - 30° C for 24- 48 h. The bacterial cells were harvested and suspended in 20 ml of sterile water containing 0.02 M potassium phosphate buffer, pH 7.0. The concentration was adjusted to 10^9 CFU/ ml for seed priming with single strain and 10^9-10^{10} CFU/ ml for strain mixtures. Strain mixtures were prepared by mixing equal volumes of individual strain suspensions.

Cucumber cultivar and seed priming with bacterial strains

Cucumis sativus L. cv. Baropata susceptible to Downy mildew was used in this experiment. Seeds treated with bacterial suspension for 24 hours were mixed with 2% methyl cellulose and air dried over night in laminar flow hood, then primed by incubating the treated seeds for 20 hours at 25° C over sterile sand moistened with sterile water.

Field trials

The field trials were conducted from February- May of three consecutive years, 2011- 2013 at the Horticulture Station of the Department of Botany, University of Kalyani, Nadia, India.

The trials relied on natural disease infestation and cucumber cv. Baropata was planted in randomized complete block design. There were eight treatments each replicated six times and eleven plants per replication. Treatments included seed bacterization with three individual PGPR strains (F2feb.44, Zapt10 and AE6), all possible strain combinations and a non bacterized control. Seeds were hand planted and cross contamination was avoided by using disposable latex gloves, which were changed between treatments. Foliar application of the three strain mixture at a concentration of 1Kg talc based formulation/ ha was done at 15, 30, 45 and 60 DAP. Cultural practices like rogueing, irrigation and addition of manure and above strains as soil irrigation for enhancement of fertility was done. Disease severity was monitored from the onset of the disease three weeks after planting till 57 DAP at seven days intervals and yield promotion at 45 and 60 DAP using a visual disease rating scale from 0- 5, in which 0= no lesion; 1= 0-10%; 2= 10.1-15%; 3= 15.1-25%; 4= 25.1-50% and 5= > 50% leaf area covered with mildew growth. Leaf samples were collected on alternate days for 10 days at post infection stage.

Enzyme activity assay

Fresh cucumber leaves were washed in running tap water after sampling and homogenized with liquid nitrogen. The homogenized tissue was rinsed with the same volume of 10 mM sodium phosphate buffer (pH 6±0) at 4°C. The tissue extracts were centrifuged at 12000 g for 20 min at 4°C. The supernatant to be used for the enzyme assays was transferred to a 1.5 ml vial and stored at -80°C. A colorimetric assay for enzymatic activity was performed with a CECIL CE 7200 Aquarius 7000 series Spectrophotometer (Milton Technical Centre, Cambridge, UK.). The reaction rates were linear and proportional to the enzyme or protein concentration added. The standard Bradford assay was employed to test the protein concentration of leaf extracts in each sample. The isoperoxidase assay was performed through a native polyacrylamide gel electrophoresis (PAGE).

Phenylalanine ammonia-lyase (PAL). One hundred µl of crude leaf extracts were mixed with 900 µl of 6 µM L-phenylalanine and 0.5 M Tris-HCl buffer solution. The mixture was placed in a water bath at 37°C for 70 min. PAL activity was determined as described by Dickerson *et al.*,[11]. The amount of trans-cinnamic acid formed from L-phenylalanine was measured spectrophotometrically at a wavelength (k) of 290 nm (u.v.). Rhodotorula glutinis PAL (Sigma, St. Louis, MO, U.S.A.) was used as the standard and the results were expressed as units of PAL activity gram protein^{-1}. One unit in the commercial standard sample used in this study was deaminated as 1.0 µmol of L-phenylalanine to trans-cinnamate and NH_3 per min at pH 8.5 at 30°C.

Peroxidase (PO). The leaf tissue extracts were diluted 10-fold with the homogenization buffer. One-hundred ul of the diluted extracts were added to 792 µl of 5mM sodium phosphate buffer (pH 6±0), and mixed with 7.5 µl guaiacol (60 mM in the mixture). The reaction was started by the addition of 100 µl 0.6 M H_2O_2 to the mixture, and the initial rate of increase in absorbency at λ 470 nm was measured over 1 min. Horseradish peroxidase (Sigma-Aldrich, St. Louis, MO. USA) was used as the standard and PO activity was expressed as units of PO mg protein^{-1} in each sample. One unit is defined as the amount that will form 1.0 mg purpurogallin (PPG) from pyrogallol in 20 sec at pH 6.0 at 20°C [4]

Polyphenoloxidase (PPO). Two-hundred µl of the leaf extracts used for PPO (catechol oxidase) assay were mixed with 700 µl of homogenization buffer. The rate of increase in absorbency at λ 495 nm was measured for 1 min after the addition of 100 µl 0.2 M catechol. Mushroom PPO (Sigma, St. Louis, MO, U.S.A.) was used as the standard, and the result was expressed as lg PPO mg protein^{-1} in each sample. The standard sample (PPO content of 4.4 units µg solid^{-1}) used to express PPO activity contained 4x 10^5units mg^{-1} protein, and the units were defined as an increase of 10^{-3} optical density (OD) values at λ 280 nm per min in a 3 ml reaction mixture containing catechol and ascorbic acid at pH 6.5 at 25°C.

Isoenzyme activity examined by native PAGE. Anodic enzymes were resolved at pH8.8. Anodic enzyme (acidic) slab gels were prepared (80 x 70 x .75 mm) were prepared at 37.5:1 ratio of acrylamide to bisacrylamide and made upto 12 % for separating and 5 % for stacking. For the acidic protein assay, Tris glycine (pH 8.8) was used as electrophoresis buffer which contained 25 mM and 192mM, respectively. Bromophenol blue 0.01 % was used as a tracking dye. Electrophoresis buffer for the low pH gel system was made upto 0.14 M. Native PAGE was done using mini vertical slab gel electrophoresis system (Biotech mini gel apparatus Cat # 05-03) and powerpack (Biotech economy model mini pack 50-250 V DC)

Samples contained 5 µg proteins per lane for enzyme activity analyses. The gel for isoperoxidase activity was examined by a staining reagent containing 50 mM H_2O_2 and 50 mM sodium acetate (pH 5.0) for 20 min. and then the gel was washed with deionized distilled water. **Statistical Analysis:** Analysis of variance was performed for each experiment using the general linear models (GLM) procedure, and treatment means were separated by the least significant difference (LSD) test at $P = 0.05$ with SPSS 21 software (SPSS statistics, IBM, NC, USA).

RESULTS AND DISCUSSION

This study deviates from the previous works in exploiting an absolutely unique PGPR consortium comprising of three strains *viz., Achromobacter* sp (F2feb.44), *Streptomyces* sp. (Zapt 10) *Bacillus licheniformis* (AE6) and all possible combinations of the strains for achieving biocontrol of downy mildew of cucumber without integrating with chemical control, host resistance breeding or chemical elicitors that are known to induce systemic resistance in plants but at the same time has been proven to be phytotoxic. Besides the consortium showed significant biofertilization ability too. Unlike earlier works where bacteria were inoculated and supplemented by seed treatment and root irrigation mostly, we have used seed priming by coating with talc based formulation at the rate of 2g/ Kg seeds (10^9 cfu/ g) with a boost up of foliar spray at 15, 30, 45 and 60 days respectively in addition to bacterial inoculation and soil drench.

Field trials: Angular yellow coloured spots developed on the upper surface of the leaves, mostly restricted to the veins after natural contraction of the disease. On the lower surface, these spots appeared as purplish, downy growth. However in plants treated with PGPR, the lesions on leaves appeared much later and the disease progress was very slow and did not proceed to the extent of wilting or fruits were least affected. Significant biocontrol could be achieved with the three individual strains aswell as the strain mixtures (three 2 strain combinations and take all combination) at ($P \leq 0.05$) with respect to uninoculated control Table 1. Treatments where mixtures of PGPR were applied provided better disease suppression than individual strain. Lowest disease severity and highest biocontrol efficiency was recorded for F2feb.44 + Zapt10 + AE6 combination having values of 10.6% and 86.6 % with respect to control where the disease severity was 79.6 % at 57 DAP. Similarly the strain mixture promoted highest fruit yield with respect to number, size and weight being 18.8/ plant, 20 cm and 3.21 kg/plant respectively. Strain mixture of Zapt10 + AE6 only showed relatively lower biocontrol traits as well as yield promotion compared to individual strain treatments with F2feb.44 and AE6 respectively.

From the three consecutive field trials carried out from 2011- 2013, it is clearly evident that the PGPR mediated induced systemic resistance in local cucumber variety alone could suppress the phytopathogen, *Pseudoperenospora cubensis* without the use of chemical pesticides and protect the plant against such natural disease incidence. This has by far been a major breakthrough in PGPR research [17] as several fungicides which had been previously used to manage foliar diseases, like methyl bromide [29], ridomil [6], have been banned due to their phytotoxicity and the environmental degradation caused by them [31]. This development also seems to be prospective for organic crop industry.

From the field studies it could be inferred that with respect to the biocontrol efficiency, the strain mixtures showed better performance compared to single strains. Plants treated with the dual and triple combinations of PGPR showed reduced disease severity and maximum growth. Earlier works have revealed similar instances. Jetiyanon and Kloepper [16] observed that 11 combinations of mixtures of various strains of *Bacillus amyloliquefaciens, B. pumilis* and *B. sphaericus* in double combinations were more effective in reducing disease severity of cucumber mosaic virus than individual strain. Similar observations were reported for *Colletotrichum orbicularae, Pseudomonas syringae* pathogen attack to cucumber using dual combinations of INR7 (*Bacillus pumilis*), ME1 *(Curtobacterium flaccumfaciens)* and GB03 *(Bacillus subtilis)* where the least disease severity of 33.5 % was recorded for INR 7 + GB03 but the treatment comprising of all three, i.e. INR 7 + ME1 + GB03 showed unsatisfactory performance (disease severity = 69.8 %) [28]. Since the double and triple strain mixtures are more conducive to soil as they are better capable of facing competition and diverse environmental changes during the growing season, they are more potent in suppressing phytopathogens by various mechanisms. This is consistent with the observations made by Raupach and Kloepper, [29]; Liu *et al.*,[21]. In contrast to the above, the strain mixtures did not always show higher defense enzyme activities compared to single strains which may be due to the weak competence of either of the individuals.

The success of our field trials may be attributed to the mode and frequency of application of the PGPR. Report by Raupach and Kloepper [28] clearly demonstrated that PGPR strains applied as seed treatment yielded convincing results comparable to chemical inducers like Actigard in the field trial of angular leaf spot disease, 1996 and in a mixed infection of angular leaf spot and anthracnose, 1997, under conditions of high inoculums potential, but two strains, IN 26 (*Burkholderia gladioli*) and INR 7 (*Bacillus pumilis*) applied as foliar spray, significantly suppressed disease by induction of ISR. Biopriming of seeds for successful establishment of PGPR upto 100% in the rhizosphere has been established by Vidyasekharan and Muthamilan, [34] in case of chick pea by priming with *P. fl.* formulation. In the light of this observation, it may be concluded that targeted application of seed treatment is more encouraged as it allows earlier protection than could be provided by foliar spray. Though foliar spray requires several applications and favourable weather conditions, it is indispensible in foliar diseases to cope up with rapid disease spread by arresting spore germination by producing antibiotics or cell wall degrading enzymes [3, 23].

In contrast to the above studies, a significant number of previous and contemporary works also reported the biocontrol ability of individual strains too [27, 7].

The PGPR strain mixture in our investigation was equally capable of enhancing fruit yield. The highest performance was recorded for the take all combination (the number of fruits/ plant being 18, fruit length 20 cm and the weight of the fruit/ plant being 3.21 kg), this being neared by *Achromobacter- Bacillus* combination having values of 15.5 fruits/ plant, 18 cm 2.93 kg/ plant respectively. Even the individual strain of *Achromobacter* had considerably high values. *Achromobacter* which has been identified as a biocontrol agent of *Fusarium* wilt of tomato [24] is also known to promote plant growth by phosphate solubilization [32]. A close investigation by Gopalakrishnan *et al.,* [13] using five strain of *Streptomyces* (CAI- 24, CAI- 121, CAI- 127, KAI- 32 and KAI- 90) that were earlier reported as potent biocontrol agents against *Fusarium* wilt of chickpea, has revealed that they were capable of significantly improving yield of rice and sorghum; in case of paddy the stover yield being enhanced upto 25 %, grain yield by 10 % and total dry matter upto 18 %, root length, root volume and dry weight upto 15 %, 36 % and 55 % respectively with respect to control. Although *Bacillus* in association with *Achromobacter* and *Streptomyces* have not been reported to improve growth and yield but different strains of *Bacillus viz., Bacillus sphaericus* strain SE56 and *B. pumilis* strains INR7, SE34, SE49, SE52 significantly enhanced the germination rate and growth of shoot of loblolly pine *(Pinus taeda)* and slash pine *(Pinus elliottii).*

Table 1. Disease severity, biocontrol efficiency against downy mildew pathogen and yield improvement by PGPR[a]

Treatment [e]	Disease severity [b] (%)	Percent disease index [c] (PDI)	Biocontrol efficiency over control[d] (%)	No. of fruits/ plant	Length of fruit (cm)	Weight of fruit/plant (kg)
F2feb.44	24.4e [f]	4.02e	69.3c	13.4b	14.3b	2.10c
Zapt10	32.3c	6.01d	59.3f	9.3d	10.5d	0.75f
AE6	27.6e	5.02e	65.3d	12.5c	11.6c	1.03e
F2feb.44 + Zapt10	35.7b	7.73b	55.1g	7.5d	10.2d	0.79f
Zapt10 + AE6	30.1d	7.12c	62.1e	11.0c	11.0c	1.51d
F2feb.44 + AE6	18.5f	2.21f	76.7b	15.5b	18.0a	2.93b
F2feb.44 + Zapt10 + AE6	10.6g	1.89g	86.6a	18.8a	20.0a	3.21a
Control	79.6a	9.12a	-	5.0e	10.0d	0.65f
S. E. m (±)	2.12	0.17	0.12	0.45	1.23	0.31
LSD (0.05)	1.09	0.32	2.67	2.37	2.20	0.54

[a]Results are the means of 11 replications and three years field trials. Field experiments were conducted consecutively with cucumber cv Baro pata during February- May, 2011 – 2013 respectively

[b] Disease severity was recorded at 7 days interval after first appearance of symptoms as per the formula

$$\text{Disease Severity} = \frac{\text{No. of infected plants}}{\text{Total No of Plants assessed}} \times 100$$

[c] Percent disease index (PDI) = $\dfrac{n_1 \times 1 + n_2 \times 2 + n_3 \times 3 + n_4 \times 4 + n_5 \times 5}{\text{Total number of seedlingss observed} \times \text{Maximum grade}} \times 100$

Where, n_1 to n_5 represent total number of seedlings falling under grade 1-5 of the scale respectively.

The severity of downy mildew was rated on a 0-5 scale: 0= no infection; 1= 0-10%; 2= 10.1- 15%; 3= 15.1-25%; 4= 25.1-50% and 5= > 50% leaf area covered with mildew growth

[d]Biocontrol efficiency (%) = $\dfrac{\text{Disease incidence in untreated control} - \text{Disease incidence in PGPR treatment}}{\text{Disease incidence in untreated control}}$

[e] PGPR strain identification: F2feb.44 = *Achromobacter* sp Zapt10 = *Streptomyces* sp and AE6 = *Bacillus licheniformis* Bacteria were applied as seed priming aswell as foliar sprays.

[f] Means with different letters are significantly different at P =0.05 according to LSD test procedure using SPSS.

Defense enzyme activities in cucumber leaves

Phenylalanine ammonia lyase (PAL) activity was activated in cucumber leaves immediately after bacterization and remained significantly high for about 15 days in absence of pathogen challenge (Fig 1a, b). Defense enzymes *viz*., PAL, PO and PPO peaks studied from leaves elicited by the strain or its mixtures corresponded with the biocontrol results, i.e. F2feb.44 + Zapt10 + AE6 showed highest peak for the three enzymes after pathogen challenge and disease establishment respectively at 30 dpi, though the enzyme activity for the dual combination of Zapt 10 + AE6 contradicted field results for F2feb.44 + AE6 nearing F2feb.44 + Zapt10 + AE6 with respect to biocontrol activity. For PO and PPO, the pathogen challenged sets exhibited higher values than PGPR controls where high values were noted for 15 dpi (Fig 2a, b; Fig 3a, b) but in contrast to these, for PAL, the PGPR controls maintained relatively high peaks immediately after bacterizaton and throughout, but the peaks gradually declined after pathogen attack when the peak of untreated control was still noted to be relatively higher (Fig 1 a, b).

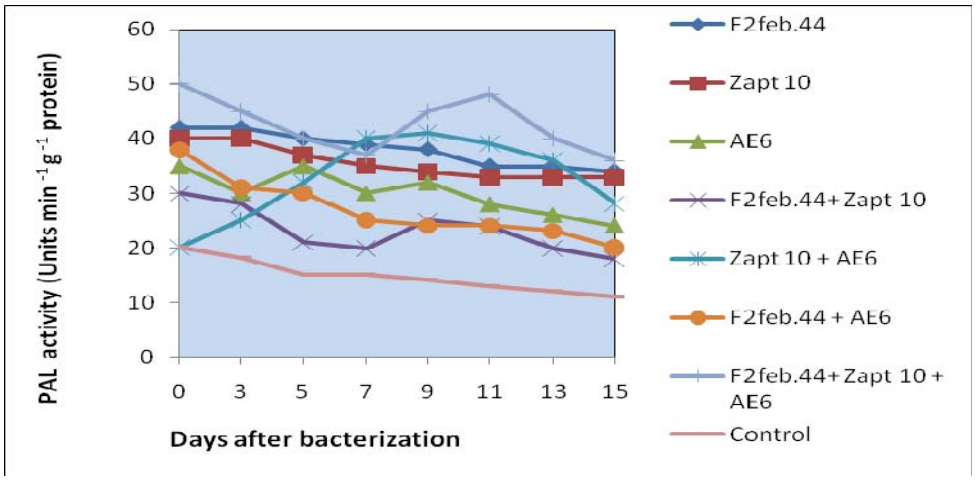

Fig 1a. Phenylalanine ammonia Lyase activity in cucumber leaves bacterised with PGPR

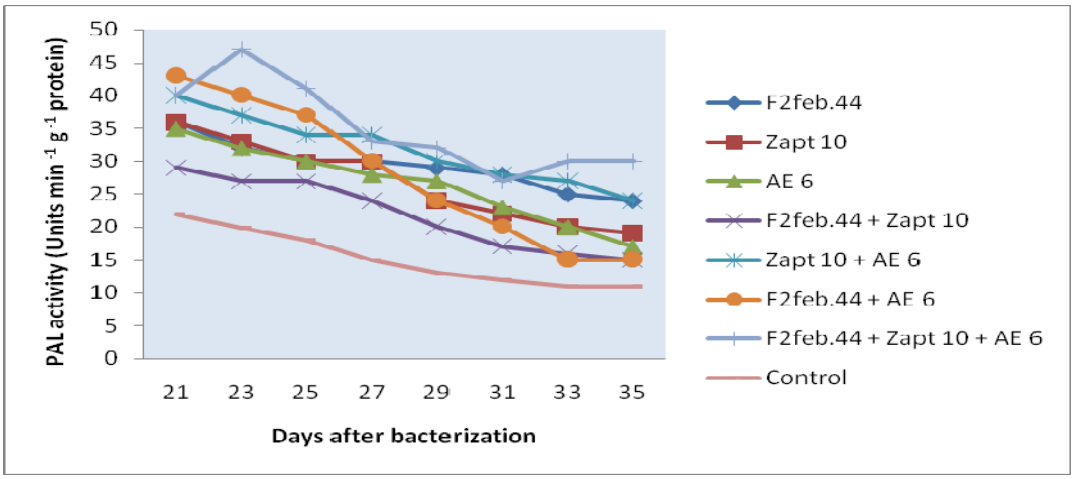

Fig 1b. Phenylalanine ammonia Lyase activity in cucumber leaves bacterised with PGPR and contracted with *Pseudoperenospora cubensis* 21 days after bacterization

Native PAGE assay of downy mildew affected plants indicated that cucumber leaves naturally contained acidic isomers of peroxidase (Fig 4.). Anodic isoperoxidase in cucumber leaves could be activated by PGPR or pathogen (*Pseudoperenospora cubensis*). Five anodic isomers designated as A1-A5 (R*f* = .28, .31, .35, .39, .43 respectively) were observed in cucumber leaves obtained from different treatments. A1 (Rf = .28) isomer weakly appeared in bacterised treatment (F2feb.44 + AE 6), in F2feb.44 + AE 6 and F2feb.44 + Zapt10 + AE 6 treated plants challenged with pathogen as well as in uninoculated control (C). A2 was abundant in all bacterised (F2feb.44 + AE 6, F2feb.44 + Zapt10 + AE 6 and F2feb.44) as well as pathogen challenged leaves but absent in control. A3 and A4 were found in F2feb.44 + AE 6 and F2feb.44 + Zapt10 + AE 6 treated pathogen challenged plants only and A5 was only found in F2feb.44 + AE 6 plants challenged by pathogen.

By colorimetric assay and PAGE, no peroxidase activity was detected from bacterial cells or cell free cultures. Previously similar studies with fungal mycelia have shown that such peroxidase activity is neither detected here i.e. they do not produce any isomer of peroxidase at pI 4.5- 8.8. *Pseudoperenospora cubensis* being an absolute biotroph, evidence had to be cited from previous works. No basic isomer of peroxidase from cucumber leaves and experimental strains were detected with cathodic electrophoresis.

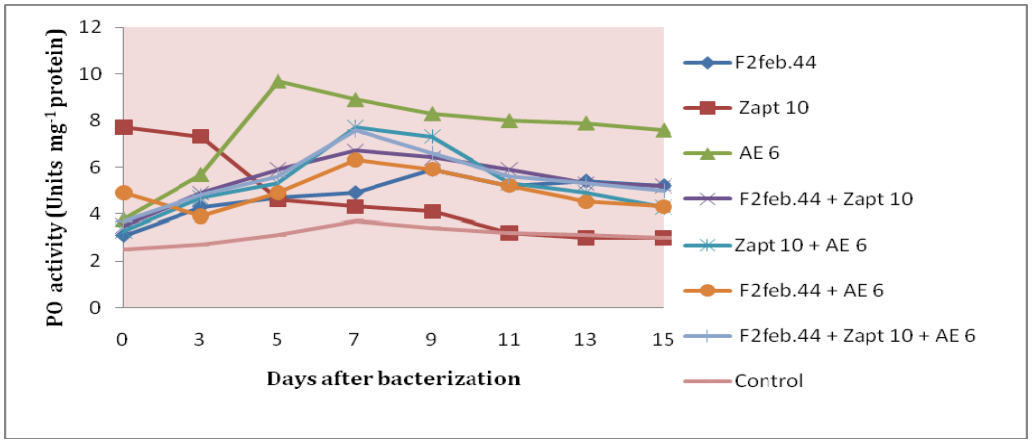

Fig 2a. Peroxidase activity over time in cucumber leaves bacterised with PGPR

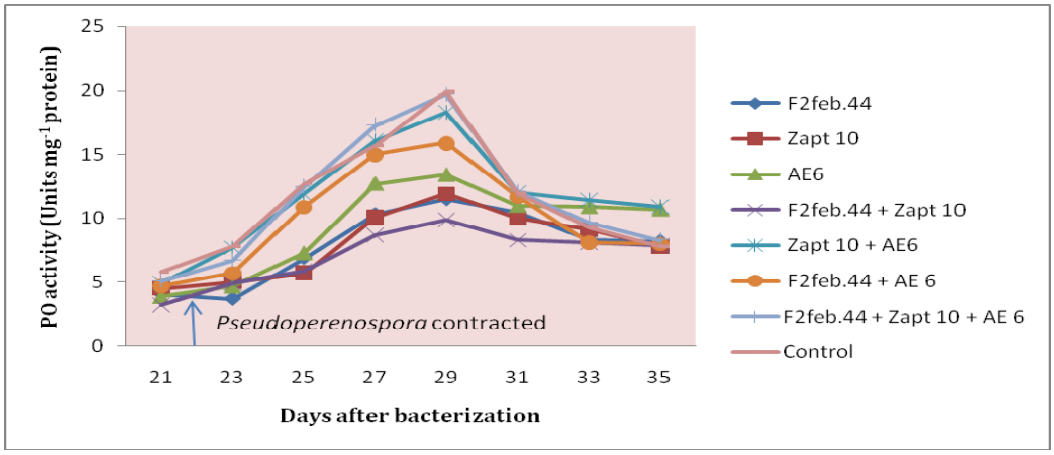

Fig 2b. Peroxidase activity in cucumber leaves bacterised with PGPR and contracted with *Pseudoperenospora cubensis* 21 days after bacterization

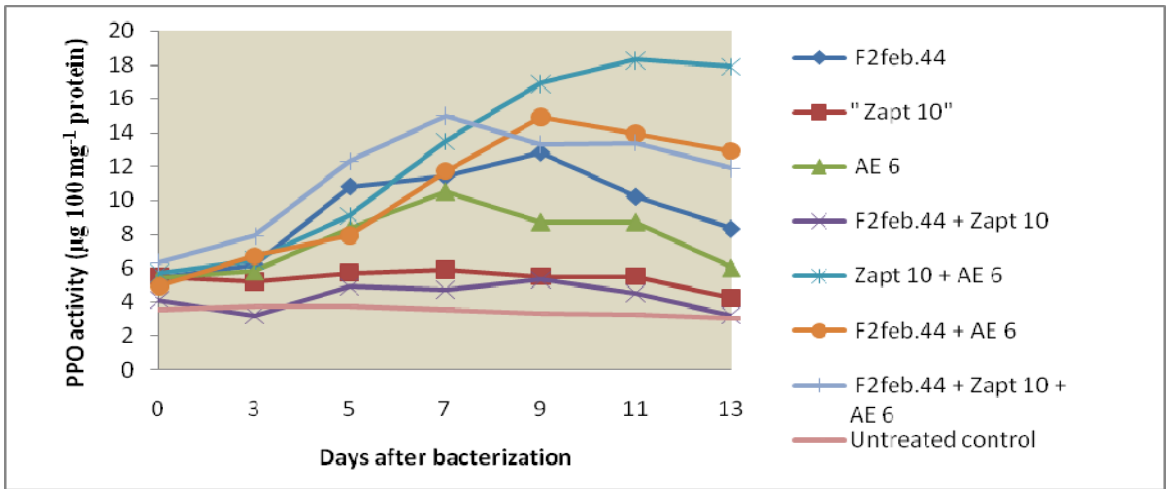

Fig 3a. Polyphenol oxidase activity over time in cucumber leaves bacterised with PGPR

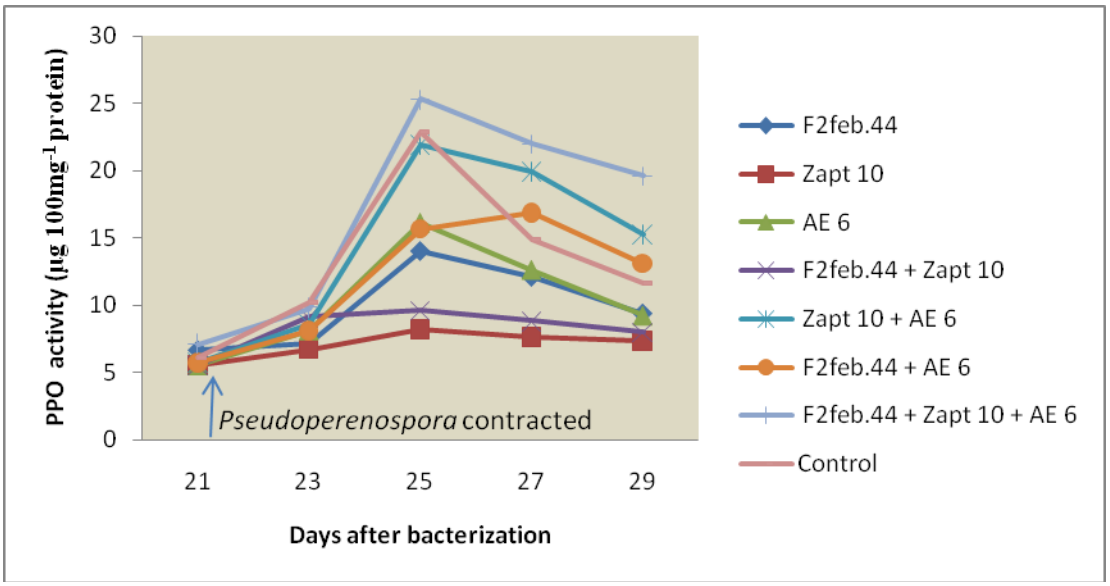

Fig 3b. Polyphenol oxidase activity in cucumber leaves bacterised with PGPR and contracted with *Pseudoperenospora cubensis* 21 days after bacterization

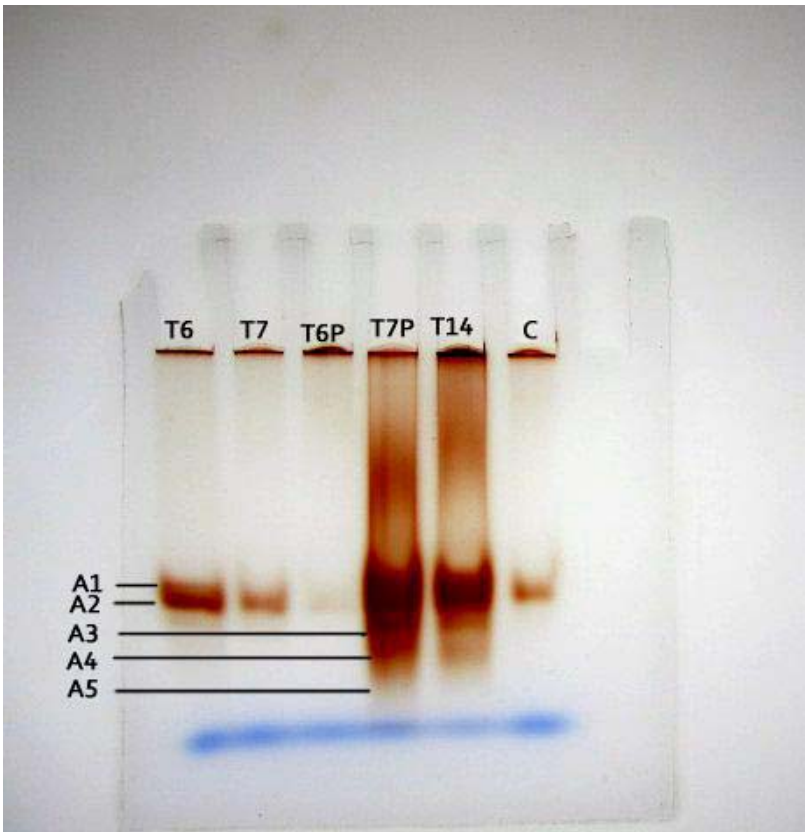

Fig 4. Native PAGE of Isoperoxidase from cucumber leaves treated with LSB and contracted with downy mildew.

Lane T6 =F2feb.44 + Zapt 10, T7 = F2feb.44 + Zapt 10 + AE6, T6P = F2feb.44 + Zapt 10 + *Pseudoperenospora* challenged, T7P = F2feb.44 + Zapt 10 + AE6 + *Pseudoperenospora* challenged, T14 = Pathogen control, C= Untreated control.

A1, A2, A3, A4 and A5 are the five isomers with Rf= .28, .31, .35, .39, .43 respectively

The role of plant defense related enzymes, PAL, POX, PPO and total phenols in disease resistance have been reported earlier [26, 18, 20] reported that the increased enzyme activities of PAL, POX, PPO and superoxide dismutase was responsible for switching on defense mediated by *Bacillus subtilis* AR- 12 against bacterial wilt of tomato, thus reducing the disease intensity. Such an outburst of enzyme activity, chiefly PAL and POX was also reported by Jayaraj *et al.,* [16], by foliar application of *B. subtilis* AUB S1 in sheath blight disease incidence of rice under green house condition. Reuveni *et al.,* [30] reviewed the role of peroxidase in fortifying defense mechanism by cross-linking of cell wall components and polymerization of lignin and suberin monomers. Earlier several workers established that PO was involved in lignin biosynthesis and in the production of toxic quinones and phytoalexins at the onset of resistance [12, 14, 10].

CONCLUSIONS

It may be concluded from the present study that although *Achromobacter* sp. *Streptomyces* and *Bacillus licheniformis* had previously been known to combat phytopathogens in various crops and stimulate plant growth as well, nevertheless, the wonderful combination of the respective strains involved in our study had a incredible potential to suppress downy mildew pathogen along with promoting plant growth and yield in cucumber with assured least phytotoxicity and long survivality in soil.

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