Studies on management of pomegranate wilt (*Ceratocystis fimbriata*) with bioagents enhanced defensive capacity in plants

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Abstracts:

Abstract: Pomegranate wilt caused by *Ceratocystis fimbriata* is an important disease and its soil borne pathogen difficult to manage. In the modern era of organic fruit production, dependence on fungicides and other chemicals is reducing. In this context, use of antagonists as well as their combinations with fungicides to manage disease is receiving lot of attention. Resistance inducing rhizobacteria offer an excellent alternative in providing natural, effective, safe, persistence and durable protection. Plants have endogenous defense mechanisms that can be induced in response to the pathogen and bio-agents. The increased activities of the defence enzymes, *viz.* peroxidase (PO), polyphenol oxidase (PPO), phenylalamine ammonia lyase (PAL) and phenolic compounds in the bio-agents treated plants of pomegranate challenged with *C. fimbriata* were recorded in the present studies. The maximum activity of defense enzymes *viz.,* peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and total phenol was recorded in diamond (*T. viride*) followed by *T. harzianum* (Th-R) and Platinum (*P. fluorescens*) indicating the role of bio-agents in increasing the role of defense enzymes in suppression of wilt.

Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit that belongs to the family lythraceae. Pomegranate is native to Iran, where it was first cultivated in about 2000 BC and spread to the Mediterranean countries. It is cultivated in India, Iran, China, Turkey, USA, Spain, Azerbaijan, Armenia, Afghanistan, Uzbekistan, the Middle East, Pakistan, Tunisia, Israel, dry regions of Southeast Asia, Peninsular Malaysia, the East Indies and tropical Africa. Area under pomegranate is increasing worldwide because of its hardy nature, wider adaptability, and drought tolerance, higher yield levels with excellent keeping quality and remunerative prices in domestic as well as export market. It thrives well in dry tropics and sub-tropics and comes up very well in soils of low fertility status as well as on saline soils. India is the world's leading country in pomegranate production.

It is one of the most adaptable subtropical fruit crops. In India it is regarded as a "vital cash crop", extensively grown in Maharashtra, Karnataka, Andra Pradesh, Telangana and Gujarat and is picking up fast in Himachal Pradesh, Rajasthan and Madhya Pradesh.

Small areas are under cultivation in Tamil Nadu, Mizoram, Odisha, Nagaland, Lakshadweep, Jharkhand and Jammu Kashmir. total area under pomegranate in India is 1,80,640 ha out of which 1,28,650 ha is in Maharashtra only. The total production in India is 17,89,310 metric tons and 11,97,710 metric tons in Maharashtra. In Karnataka total area is 23,230 ha with production 2,61,820 metric tonnes.

In Karnataka, the crop has spread across different districts *viz.,* Vijayapura, Bagalkot, Koppal, Yadgir, Raichur, Ballari, Chitradurga, Tumakuru and Hassan. The most popular varieties suitable for processing and table use are Ganesh, Mridula, Arakta, Bhagwa (Kesar), G-137 and Khandar. Successful cultivation of pomegranate in recent years is threatened with different pest and diseases. Bacterial blight, wilt, anthracnose, leaf spot and root knot nematode are important diseases. Among them, wilt caused by *Ceratocystis fimbriata* Ell. and Halst. is an emerging threat. At present the crop is severely affected by wilt pathogen and day by day the wilting severity is increasing at faster rate. It was first noticed in some areas of Vijayapur districts of India during 1990. By 1993, rapid spread of this disease was observed in entire Vijayapura district. The cause was not identified until 1995; however in 1996 the fungus *C. fimbriata* was isolated from discolored stem, root and branch tissues on wilting plants. Disease is characterized by initial symptoms of yellowing and wilting of leaves on one to several branches leading to death of affected plants in a few weeks. Cross sections of diseased plants revealed brown discoloration in the outer xylem from roots to the main trunk (Somasekhara and Walli, 2000).

The disease is prevalent in parts of Maharashtra, Karnataka, Telangana, Gujarat and Tamil Nadu states (Jadhav and Sharma, 2009). Despite many factors conducive for the high severity, seedlings selection for planting, soil borne nature and also association with shot hole borer and plant parasitic nematodes is noticed. This might be the reason for the current rampant spread of the disease in south Indian states. Several agents are known to cause wilt in pomegranate, but *C. fimbriata* is the major cause (Sharma, 2009 and Sharma *et al.*, 2010), hence, emphasis given be on *C. fimbriata*.

In the modern era of organic fruit production, dependence on fungicides and other chemicals is reducing. In this context, use of antagonists as well as their combinations with fungicides to manage disease is receiving lot of attention. Resistance inducing rhizobacteria offer an excellent alternative in providing natural, effective, safe, persistence and durable protection. Plants have endogenous defense mechanisms that can be induced in response to the pathogen and bio-agents. One classical biotic inducer is the plant growth promoting

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bacterium, *Pseudomonas fluorescens* (Iavicoli *et al*., 2003). *Trichoderma* spp. can reduce the severity of plant diseases by inhibiting plant pathogens in the soil through its highly potent antagonistic and mycoparasitic activity. Moreover, as revealed by research in recent decades, some *Trichoderma* strains can interact directly with roots, increasing plant growth potential, resistance to disease and tolerance to abiotic stresses (Rosa Hermosa *et al*., 2012).

Material and Methods

Induced systemic resistance (ISR) was carried out in pomegranate plant by challenge inoculation of the plants with pathogen followed by application of with bio-agents under pot condition.

Plants which were inoculated with distilled water served as control. Four plants were inoculated to maintain four replications under glass house condition. The inoculated plants were kept in glass house (average temperature of 27 °C) for further studies. The following six treatments were formulated for the study.

Treatments

Chart 1: List of treatments used for the study

Treatment	Treatment details
T_1	Platinum (0.7 g/l) – Platinum (0.7 g/l) – Platinum (0.7 g/l)*
T_2	Diamond (0.7 g/l) – Diamond (0.7 g/l) – Diamond (0.7 g/l)
T_3	*T. harzianum* (Th-R) (5 g/l) - *T. harzianum*(Th-R) (5 g/l) – *T. harzianum* (Th-R) (5 g/l)
T_4	*P. fluorescens* (RP-46) (5 g/l) - *P. fluorescens* (RP-46) (5 g/l) – *P. fluorescens* (RP-46) (5 g/l)
T_5	*C. fimbriata*
T_6	Control

* Application of bio-agents three times at an interval of 15 days as soil drench @ 2 lit/plant.

Platinum - (*P. fluorescens*), Diamond- (*T. viride*)

The activities of enzymes such as Phenylalanine ammonia-lyase (PAL), Polyphenol oxidase (PPO) and Peroxidase (PO) sampling were done at different intervals (0, 15, 30, 60 and 90 days after imposing of treatment). The assay of induced systemic resistance related enzymes was carried out as follows.

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Sample collection:

Samples were collected from individual treatments to study the induction of defense enzymes in response to pathogen attack in pomegranate leaf from different treatments.

Enzyme extract:

The leaves collected from different treatment combination of pomegranate plants were collected from immediately homogenized with liquid nitrogen. 1 g of powdered sample was extracted with 2 ml of sodium phosphate buffer, 0.1M (pH 7.0) at 4 ºC. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extracts prepared from pomegranate tissues were used for estimation of defense enzymes like peroxidise (PO) polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL).

Assay of peroxidase (PO)

The peroxidase activity was assayed spectrophotometrically (Hartee, 1955).

Reagents:

Hydrogen peroxide solution: 1 ml of H_2O_2 was mixed with 99.00 ml of distilled water to get 100ml of 1% H_2O_2 solution. The solution was prepared every time freshly.

Pyrogallol, 0.05 M: 6.3005 g of pyrogallol was dissolved in 100 ml of distilled water. The solution was prepared every time freshly.

Phosphate buffer, 01 M, 6.5 pH: Solution A: 27.6 g of sodium phosphate monobasic ($NaH_2PO_4.2H_2O$, Mol. wt. - 156.01) was dissolved in small quantity of water and made up the volume to 1000 ml with distilled water.

Solution B: 28.4 g of sodium phosphate dibasic (Na_2HPO_4, Mol Wt. 142 g) was dissolved in small quantity of water and made up the volume to 1000 ml with distilled water

265 ml of solution A was mixed with 735 ml of solution B. finally pH was adjusted with NaOH.

Preparation of enzyme extract: One gram of plant sample was homogenized in 3 ml of 0.1 M phosphate buffer, pH 6.5 at 4 °C. This mixture was filtered through 4 layered muslin cloth. The filtrate was centrifuged at 12000 rpm at 4 °C for 20 minutes. The supernatant was collected and used for estimation of peroxidase activity.

Assay: The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of the enzyme

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extract and 0.5 ml of one per cent H_2O_2. The reaction mixture was incubated at room temperature (28 ± 10 °C). The change in absorbance was recorded at 470 nm at a time interval of 30 sec. up to 3 min in Hitachi U-2900 spectrophotometer. The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance at 420 nm min/g/ on fresh weight basis (Hammerschmidt *et al.*, 1982).

Assay of polyphenol oxidase (PPO)

The polyphenol oxidase activity was determined as per the procedure given by Mayer *et al*. (1965).

Reagents:

Phosphate buffer, 0.1 M, 7.0 pH: Solution A: 27.6 g of sodium phosphate monobasic (NaH_2PO_4. $2H_2O$ Mol. Wt.-156.01) was dissolved in small quantity of water and made up the volume to 1000 ml with distilled water.

Solution B: 28.4 g of sodium phosphate dibasic (Na_2HPO_4Mol Wt. 142 g) was dissolved in small quantity of water and made up the volume to 1000 ml with distilled water

610 ml of solution A was mixed with 390 ml of solution B. finally pH was adjusted with NaOH. The buffer is stored under refrigerated condition.

Catechol, 0.1M (Mol. Wt. 111.011g): 11.011 g of catechol was dissolved in small quantity of water and volume was made to 1000 ml with distilled water.

Preparation of enzyme extract: One gram of plant sample was homogenized in 5 ml of 0.1M phosphate buffer, pH 7.0 at 4 °C. This mixture was filtered through 4 layered muslin cloth. The filtrate was centrifuged at 10000 rpm at 4 °C for 20 minutes. The supernatant was collected and used for estimation of polyphenol oxidase activity.

Assay: One gram of leaf and roots were used for phenol oxidase estimation: the reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer (pH 7.0) and 500 µl of the enzyme extracts. To start the reaction, 500 µl of 0.01 M catechol was added. The change in absorbance was recorded at 495 nm at a time interval of 30 sec. Up to 3 min in Hitachi U-2900 spectrophotometer. The polyphenol oxidase activity was expressed as changes in absorbance at 495 nm/min/g fresh weight of tissue.

Assay of Phenylalanine Ammonia Lyase (PAL)

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PAL activity was determined as the rate of conversion of L-phenyl alanine to trans-cinnamic acid at 290 nm as per the method described by Ross and Sederoff (1992).

Reagents:

Borate buffer, 0.1M, 8.8 pH: 6.183 g of boric acid and 1 g of NaOH was dissolved in 800 ml of water and volume was made to 900 ml. To this solution 0.1 g of polyvinyl pyrrolidine (PVP) was added.

Substrate solution: L-Phenyl alanine, 12 mM: 1.98 g of L- phenylalanine was dissolved in 1000 ml of distilled water. The solution was prepared freshly.

Trans-cinnamic acid: 29.64 mg of trans-cinnamic acid was dissolved in 10 ml of acetone. 1 ml of this solution was diluted to 10 ml with borate buffer to obtain 2 moles trans-cinnamic acid/ml working standard solution. The buffer is stored under refrigerated condition.

Trichlro acetic acid (TCA, Mol. Wt. 163.39 g), 1 M: 16.339 g of TCA was dissolved in 100 ml of water.

Preparation of enzyme extract: one gram of sample was homogenised with 5 ml of 0.1 M ice cold sodium borate buffer (pH 8.8). The homogenate was filtered through 4 layered muslin cloth. The filtrate was centrifuged at 15000 rpm at 4 °C for 20 min. The supernatant was collected and used for estimation of PAL activity.

Assay: Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C. The reaction was arrested by adding 0.5 ml of 1M TCA and incubated at 37 °C for 5 min. The blank contains 0.4 ml of crude enzyme extract and 2.7 ml of 0.1M borate buffer (pH 8.8) and absorbance was measured at 290 nm in Hitachi U-2900 spectrophotometer. Standard curve was drawn with graded amounts of cinnamic acid dissolved in acetone. The enzyme activity was expressed as µM of trans-cinnamic acid/ min/g fresh weight of tissue.

Total phenol

The total phenol present in plant sample was estimated by following Folin-Ciocalteau reagent (Bray and Thorpe, 1954).

Reagents: 1. Folin-Ciocalteau (FCR, 1 N)

2. Sodium carbonate (2%)

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Procedure

One ml of each alcoholic extract was taken in a test to which one ml of Folin-Ciocalteau reagent was added following by two ml of sodium carbonate solution (2%). The tubes were shake well and incubated to heat in a hot water bath for exactly one minute and then cooled under running tap water. The colour developed was diluted to 25 ml distilled water and its absorbance was read at 650 nm in spectrophotometer. The amount of phenols present in sample was calculated from a standard curve prepared from catechol. The total phenols activity was expressed as (mg/g fresh wt) fresh weight of tissue.

Result

The studies on induced systemic resistance (ISR) were carried out in pomegranate by challenge inoculation of the plants with the pathogen followed by applying effective bio-agents under pot condition as explained in 'Material and Methods'. The assay of defense related enzymes, *viz.,* peroxidase (PO), polyphenol oxidase (PPO), phenyl alanine ammonia lyase (PAL) and total phenols was carried out in the present investigation and results are presented in Table 1,Table 2, Table 3, Table 4, Plate 1a and Plate 1b.

Induced systemic resistance as inferred biochemical analysis revealed the increased activities of the enzymes, *viz.*, peroxidase (PO), polyphenol oxidase (PPO), phenylalamine ammonia lyase (PAL) and phenolic compounds in the bio-agents treated plants in pomegranate challenged with *C. fimbriata* pathogen. In general, the expression of defense enzymes and other compounds in bio-agents treated plants upon inoculation with pathogen and their interaction in pomegranate was comparatively increased considerably with the increase in the period of inoculation as compared to the control set.

Peroxidase (PO)

Assay of peroxidase activity in pomegranate plants inoculated with bio-agents showed differences among the various treatments. Increased activity of PO was observed in all treatments at different day's intervals upon challenge inoculation with pathogen, when compared to untreated control (Table 1).

In general, the PO activity was gradually increased from 0 to 60 DAI and later it was decreased at 90 DAI in all the treatments. The maximum activity of PO was recorded in Diamond (*T. viride*) (0.157) followed by *T. harzianum* (Th-R) (0.129) and Platinum

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(*P. fluorescens*) (0.085). While, the least activity was noticed in plants treated with *C. fimbriata* (0.016) indicating the role of bio-agents in increasing the activity of peroxidase. The healthy control treatment recorded lesser activity (0.038) of peroxidase activity.

Plate 1 A. Experimental view of induced systemic resistance against *C. fimbriata* in glass house

B. Induced systemic resistance treatments:

T_1 – Platinum T_2 – Diamond T_3 – *T. harzianum*
T_4 – *P. fluorescens* T_5 – *C. fimbriata* T_6 – Control

Polyphenol oxidase (PPO)

Assay of polyphenol oxidase activity in pomegranate plants inoculated with bio-agents showed differences among the various treatments. Increased activity of PPO was observed in all the treatments at different day's intervals upon challenge inoculation with

pathogen, when compared to untreated control (Table 2).

The PPO activity was gradually increased from 0 to 60 DAI in all the treatments and later it was decreased at 90 DAI in all the treatments. The bio-agent formulation, Diamond (*T. viride*) recorded highest activity of PPO (0.154) followed by *T. harzianum* (Th-R) (0.130) and Platinum (*P. fluorescens*) (0.101). The least activity was in plants treated with pathogen alone (0.016) which indicated the role of bio-agents in increasing the activity of polyphenol oxidase. The healthy control treatment recorded less activity of 0.045 polyphenol oxidase.

Phenyalanine ammonialyase (PAL)

In general, the PAL activity (Table 3) was gradually increased from 0 to 60 DAI and later it was decreased at 90 DAI in the all the treatments as it was noticed in PO and PPO. The maximum activity of PAL was recorded in Diamond (*T. viride*) (0.094) followed by *T. harzianum* (Th-R) (0.081) and Platinum (*P. fluorescens*) (0.077). While, the least activity was noticed in plants treated with *C. fimbriata* (0.009) indicating the role of bio-agents in increasing the activity of phenyalanine ammonialyase. The healthy control treatment recorded 0.034 phenyalanine ammonialyase activity.

Total phenols

The results (Table 4) for assay of total phenols activity in pomegranate plants inoculated with bio-agents showed that, enzyme activity was gradually increased from 0 to 90 DAI in all the treatments unlike PO, PPO and PAL wherein the activity was reduced at 90DAI. The maximum activity of total phenols was recorded in Diamond (*T. viride*) (7.797) followed by *T. harzianum* (Th-R) (7.513) and Platinum (*P. fluorescens*) (7.105). While, the least activity was noticed in plants treated with *C. fimbriata* (2.343) indicating the role of bio-agents in increasing the activity of total phenols. The healthy control treatment recorded 6.003 total phenols activity.

DISCUSSION

Induced resistance is a state of enhanced defensive capacity against broad spectrum of pests and pathogens developed by a plant when appropriately stimulated (Van Loon *et al.*, 1998). The resulting elevated resistance due to biotic agents is referred to as induced systemic

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resistance (ISR) whereas that by other than biological control agents is called systemic acquired resistance (SAR) (Zhu- Salzaman *et al.*, 2005). In our study, we concentrated on biotic (Diamond (*T. viride*), Platinum (*P. fluorescens*), *T, harzianum* (Th-R) and *P. fluorescens* (RP-46)) inducers for inducing the defense molecules challenged with *C. fimbriata* in pomegranate plants followed by applying bio-agents under pot condition. The ISR in this study was primarily focused for the defense related proteins, *viz.* PO, PPO, PAL and phenols. The results of the present study revealed that there was significant increase in the activity of PO, PPO, PAL and total phenolic contents in pomegranate plants treated with Diamond (*T. viride*), Platinum (*P. fluorescens*), *T, harzianum* (Th-R) and *P. fluorescens* (RP-46) (Table. 1, 2, 3 & 4). Similar studies, which showed an increase in PO, PPO, PAL and Phenols activity were reported by Shanti and Rajendran (2006) who concluded that application of *Pseudomonas fluorescens* @ 20 g/plant significantly increased the activities of PO, PPO and PAL enzymes in banana *cv*. Robusta under field conditions. Further, PO, PAL and total phenolic content both in *T. viride* applied and *F. oxysporum* f.sp. *cubense* challenge inoculated plants revealed that these were significantly higher compared to control plants and inoculated with the pathogen (*F. oxysporum* f.sp. *cubense*) plants alone (Thangavelu and Mustaffa, 2010). Mallesh and Lingaraju, (2015) reported that fluourescent pseudomonads isolates were found effective, systemically induce resistance against wilt complex pathogens of coleus and ashwagandha by the accumulation of battery of enzymes in response to pathogens infection. The present findings are also similar to that supported by several other workers (Akila *et al*., 2011; Jain *et al*., 2012 and Pushpavathi *et al*., 2016).

PAL is the first enzyme in phenylpropanoid metabolism involved in the production of phenolics and phytoalexins that prevent establishment of the pathogens (Daayf *et al.*, 1997). The increase in PAL activity in plants is presumably related to the lignifications process and also plays a role in plant defense system. Peroxidases is a key enzyme in plant detoxification system and is involved in scavenging reactive oxygen species and lignifications of vascular tissues, might important in the defense of plants against vascular wilt pathogens (Pomar *et al*., 2004). PO catalyses H_2O_2-dependent condensation of phenolics into lignin in responses to plant-pathogen interaction and creates a physical barrier to limit the pathogen invasion in host tissues, playing specific role in hypersensitive containment of the pathogen (Saravanan *et al*., 2004)

Defense responses are characterized by the early accumulation of phenolic

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compounds at the infection site, that slowdown the development of phenolic compounds at the infection site, that slowdown the development of the pathogen occurring as a result of rapid cell death (Mace, 1963). The increased phenolic undergoes esterification and modifies the cell wall polysaccharides to resist the action of lytic enzymes produced by fungal pathogens. These increased phenols lead to the high level of vascular lignifications and suberisation of endodermal cells. This might restricts xylem invasion by fungal pathogens and prevents multiplication of the pathogen in the vascular tissues. The early and substantial increase in the PO, PPO and PAL in the plants indicates that a strong oxidative burst occurred and that the defense responses of the plant might involve lignin production and cell wall fortification. The drop-off in the production after the early response may imply that the defense response of the plants is more heavily on oxidative process than secondary metabolic pathways of cell wall fortification.

SUMMARY AND CONCLUSIONS

The increased activities of the defence enzymes, *viz.* peroxidase (PO), polyphenol oxidase (PPO), phenylalamine ammonia lyase (PAL) and phenolic compounds in the bio-agents treated plants of pomegranate challenged with *C. fimbriata* were recorded in the present studies. The maximum activity of defense enzymes *viz.,* peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and total phenol was recorded in diamond (*T. viride*) followed by *T. harzianum* (Th-R) and Platinum (*P. fluorescens*) indicating the role of bio-agents in increasing the role of defense enzymes in suppression of wilt.

Higher activity of defense enzymes *viz.,* peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and total phenol was recorded in diamond (*T. viride*) followed by *T. harzianum* (Th-R) and Platinum (*P. fluorescens*) indicating the role of bio-agents in increasing the activity of defense enzymes.

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Table 1. Induction of peroxidase activity in pomegranate plants treated with bio-agents and challenge inoculation of *C. fimbriata*

Sl. No.	Treatment	Peroxidase (Change in absorbance 470 nm/min/mg protein)					
		0 DAI	**15 DAI**	**30 DAI**	**60 DAI**	**90 DAI**	**Mean**
1	T_1 – Platinum (0.7 g/l) - Platinum (0.7 g/l) - Platinum (0.7 g/l)*	0.052	0.067	0.106	0.108	0.094	0.085
2	T_2 – Diamond (0.7 g/l) - Diamond (0.7 g/l) - Diamond (0.7 g/l)	0.055	0.085	0.180	0.266	0.197	0.157
3	T_3 – *T. harzianum* (Th-R) (5 g/l) - *T. harzianum* (Th-R) (5 g/l) – *T. harzianum* (Th-R) (5 g/l)	0.036	0.074	0.123	0.243	0.171	0.129
4	T_4 – *P. fluorescens* (RP-46) (5 g/l) - *P. fluorescens* (RP-46) (5 g/l) – *P. fluorescens* (RP-46) (5 g/l)	0.049	0.063	0.096	0.097	0.085	0.078
5	T_5 – *C. fimbriata*	0.024	0.055	0.000	0.000	0.000	0.016
6	T_6 – Control	0.020	0.024	0.039	0.057	0.048	0.038
	S. Em. ±	0.007	0.0006	0.004	0.001	0.001	-
	CD at 1%	0.031	0.0024	0.019	0.004	0.006	-

*Application of treatments three times at an interval of 15 days after complete establishment of plants earthen pots under glass house condition uniformly

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Table 2. Induction of polyphenol oxidase activity in pomegranate plants treated with bio-agents and challenge inoculation of *C. fimbriata*

Sl. No.	Treatment	Polyphenol oxidase (Change in absorbance 420 nm/min/mg protein)					
		0 DAI	**15 DAI**	**30 DAI**	**60 DAI**	**90 DAI**	**Mean**
1	T_1 – Platinum (0.7 g/l) - Platinum (0.7 g/l) - Platinum (0.7 g/l)*	0.050	0.078	0.093	0.145	0.138	0.101
2	T_2 – Diamond (0.7 g/l) - Diamond (0.7 g/l) - Diamond (0.7 g/l)	0.057	0.095	0.177	0.257	0.185	0.154
3	T_3 – *T. harzianum*(Th-R) (5 g/l) - *T. harzianum* (Th-R) (5 g/l) – *T. harzianum* (Th-R) (5 g/l)	0.052	0.087	0.127	0.223	0.161	0.130
4	T_4 – *P. fluorescens* (RP-46) (5 g/l) - *P. fluorescens* (RP-46) (5 g/l) – *P. fluorescens* (RP-46) (5 g/l)	0.044	0.070	0.090	0.121	0.119	0.089
5	T_5 – *C. fimbriata*	0.033	0.045	0.000	0.000	0.000	0.016
6	T_6 – Control	0.030	0.035	0.048	0.056	0.058	0.045
	S. Em. ±	0.001	0.001	0.003	0.005	0.005	-
	CD at 1%	0.004	0.006	0.013	0.022	0.023	-

*Application of treatments three times at an interval of 15 days after complete establishment of plants earthen pots under glass house condition uniformly

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Table 3. Induction of phenylalanine ammonia lyase activity in pomegranate plants treated with bio-agents and challenge inoculation of *C. fimbriata*

Sl. No.	Treatment	Phenylalanine ammonia lyase (nmol trans-cinnamic acid/hr/mg protein)					
		0 DAI	15 DAI	30 DAI	60 DAI	90 DAI	Mean
1	T_1 – Platinum (0.7 g/l) - Platinum (0.7 g/l) - Platinum (0.7 g/l)*	0.029	0.052	0.094	0.113	0.099	0.077
2	T_2 – Diamond (0.7 g/l) - Diamond (0.7 g/l) - Diamond (0.7 g/l)	0.030	0.066	0.107	0.152	0.116	0.094
3	T_3 – *T. harzianum* (Th-R) (5 g/l) - *T. harzianum* (Th-R) (5 g/l) – *T. harzianum* (Th-R) (5 g/l)	0.028	0.054	0.099	0.123	0.103	0.081
4	T_4 – *P. fluorescens* (RP-46) (5 g/l) - *P. fluorescens* (RP-46) (5 g/l) – *P. fluorescens* (RP-46) (5 g/l)	0.026	0.048	0.081	0.109	0.089	0.071
5	T_5 – *C. fimbriata*	0.020	0.024	0.000	0.000	0.000	0.009
6	T_6 – Control	0.016	0.021	0.038	0.043	0.052	0.034
	S. Em. ±	0.001	0.001	0.021	0.002	0.0005	-
	CD at 1%	0.003	0.005	0.090	0.011	0.002	-

*Application of treatments three times at an interval of 15 days after complete establishment of plants earthen pots under glass house condition uniformly

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Table 4. Induction of total phenols activity in pomegranate plants treated with bio-agents and challenge inoculation of *C. fimbriata*

Sl. No.	Treatment	Total Phenols (mg/g fresh wt)					
		0 DAI	15 DAI	30 DAI	60 DAI	90 DAI	Mean
1	T_1 – Platinum (0.7 g/l) - Platinum (0.7 g/l) - Platinum (0.7 g/l)*	5.400	6.770	7.317	7.830	8.207	7.105
2	T_2 – Diamond (0.7 g/l) - Diamond (0.7 g/l) - Diamond (0.7 g/l)	5.630	7.217	8.290	8.800	9.050	7.797
3	T_3 – *T. harzianum* (Th-R) (5 g/l) - *T. harzianum* (Th-R) (5 g/l) – *T. harzianum* (Th-R) (5 g/l)	5.283	6.733	8.123	8.633	8.794	7.513
4	T_4 – *P. fluorescens* (RP-46) (5 g/l) - *P. fluorescens* (RP-46) (5g/l) – *P. fluorescens* (RP-46) (5 g/l)	5.433	6.587	6.737	7.157	7.800	6.743
5	T_5 – *C. fimbriata*	5.217	6.500	0.000	0.000	0.000	2.343
6	T_6 – Control	5.137	5.333	6.400	6.347	6.800	6.003
	S. Em. ±	0.72	0.091	0.055	0.052	0.047	-
	CD at 1%	3.11	0.396	0.239	0.228	0.206	-

*Application of treatments three times at an interval of 15 days after complete establishment of plants earthen pots under glass house condition uniformly

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