*International Journal of Environment and Climate Change*

***Volume 13, Issue 10, Page 2277-2287, 2023; Article no.IJECC.104679***
***ISSN: 2581-8627***
*(Past name: British Journal of Environment & Climate Change, Past ISSN: 2231–4784)*

# Bioagents Induced Resistance to *Ceratocystis fimbriata* in Pomegranate

**Raja [a*], Gururaj Sunkad [b] and Amaresh, Y. S. [b]**

*[a] Department of Plant Pathology, Agriculture College - Bheemarayanagudi, UAS, Raichur, India.*
*[b] Department of Plant Pathology, Agriculture College - Raichur, UAS, Raichur, India.*

***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/IJECC/2023/v13i102892

**Open Peer Review History:**
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/104679

***Original Research Article***

***Received: 21/06/2023***
***Accepted: 26/08/2023***
***Published: 05/09/2023***

## ABSTRACT

Pomegranate is the most important fruit crop consumed in the world. In India the plant is cultivated in almost all agroecological areas, however, yields remain low due to attacks by various pathogens and insects. Among the pathogens, wilt caused by *Ceratocystis fimbriata* is an important disease and its soil-borne pathogen is difficult to manage. To contribute to the control of this microbial pathogen, the stimulatory effect of the Pomegranate defense system of bioagents in the pomegranate interaction was evaluated. Resistance-inducing rhizobacteria offer an excellent alternative in providing natural, effective, safe, persistent, 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 defense 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.

**Corresponding author: E-mail: rajarc8888@gmail.com;*

*Keywords: Pomegranate; fungal diseases; bioagents; induced resistance.*

## 1. 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" [1]. Area under pomegranate is increasing worldwide because of its hardy nature, wider adaptability, drought tolerance, higher yield levels with excellent keeping quality, and remunerative prices in domestic as well as export markets. It thrives well in dry tropics and sub-tropics and comes up very well in soils of low fertility status as well as in 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, the total area is 23,230 ha with production 2,61,820 metric tonnes (http://nhb.gov.in**)**.

"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 pomegranates in recent years is threatened by different pests 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 a faster rate. It was first noticed in some areas of Vijayapur districts of India in 1990. By 1993, the rapid spread of this disease was observed in the 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. The disease is characterized by initial symptoms of yellowing and wilting of leaves on one to several branches leading to the death of affected plants in a few weeks. Cross sections of diseased plants revealed brown discoloration in the outer xylem from the roots to the main trunk" [1].

"The disease is prevalent in parts of Maharashtra, Karnataka, Telangana, Gujarat, and Tamil Nadu states" [2]. "Despite many factors conducive to the high severity, seedlings' selection for planting, soil-borne nature, and also an 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, hence, emphasis given is on *C. fimbriata"* [3,4].

"In the modern era of organic fruit production, dependence on fungicides and other chemicals is reducing. In this context, the use of antagonists, as well as their combinations with fungicides to manage the disease, is receiving a lot of attention. Resistance-inducing rhizobacteria offer an excellent alternative in providing natural, effective, safe, persistent, 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 bacterium, *Pseudomonas fluorescens"* [5]. "*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" [6].

The aim of this study was to assess the activation of the pomegranate defense system against *Ceratocystis fimbriata* of some bioagents through the quantification of some resistance-inducing biomolecules like total phenols and the

quantification of the main phenylalanine ammonia-lyase (PAL), peroxidase (PO), and polyphenol oxidase (PPO) activities.

## 2. MATERIALS AND METHODS

The experiment was in a glass house at the Department of Plant Pathology of UAS Raichur. During Hastbahar 2015, disease-free plants were transported from the Raichur taluk's Ganjalli village. The popular variety Kesar, a three-month-old plant, was placed in a plot and maintained in a glass house for more investigation (average temperature: 27 °C). Pomegranate plants were utilized to carry out induced systemic resistance (ISR) by initially challenging the plants with a pathogen and then using bio-agents under pot conditions.

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

### 2.1 Treatments

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.

### 2.2 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 (T1, T2, T3, T4, T5)

### 2.3 Enzyme Extract

The leaves collected from different treatment combinations 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 the estimation of defense enzymes like peroxidise (PO) polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL).

### 2.4 Assay of Peroxidase (PO)

The peroxidase activity was assayed spectrophotometrically [7].

#### 2.4.1 Reagents

*2.4.1.1 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.

*2.4.1.2 Pyrogallol, 0.05 M*

6.3005 g of pyrogallol was dissolved in 100 ml of distilled water. The solution was prepared every time freshly.

*2.4.1.3 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 a small quantity of water and made up the volume to 1000 ml with distilled water.

**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)*

Solution B: 28.4 g of sodium phosphate dibasic ($Na_2HPO_4$, Mol Wt. 142 g) was dissolved in a 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.

*2.4.1.4 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.

*2.4.1.5 Assay*

The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of the enzyme 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 a change in the absorbance at 420 nm min/g/ on a fresh weight basis [8].

## 2.5 Assay of Polyphenol Oxidase (PPO)

The polyphenol oxidase activity was determined as per the procedure given by Mayer et al. [9].

### 2.5.1 Reagents

*2.5.1.1 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 ($Na_2HPO_4$Mol 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.

*2.5.1.2 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.

*2.5.1.3 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.

*2.5.1.4 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.

## 2.6 Assay of Phenylalanine Ammonia Lyase (PAL)

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 [10].

### 2.6.1 Reagents

*2.6.1.1 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 the volume was made to 900 ml. To this solution, 0.1 g of polyvinyl pyrrolidine (PVP) was added.

*2.6.1.2 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.

*2.6.1.3 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.

*2.6.1.4 Trichlro acetic acid*

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

*2.6.1.5 Preparation of enzyme extract*

one gram of the sample was homogenized 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 the estimation of PAL activity.

*2.6.1.6 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. A 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 tissue weight.

## 2.7 Total Phenol

The total phenol present in the plant sample was estimated by following Folin-Ciocalteau reagent (Bray and Thorpe, [11]).

### 2.7.1 Reagents

1. Folin-Ciocalteau (FCR, 1 N)
2. Sodium carbonate (2%)

### 2.7.2 Procedure

One ml of each alcoholic extract was taken in a test to which one ml of Folin-Ciocalteau reagent was added followed by two ml of sodium carbonate solution (2%). The tubes were shaken well and incubated to heat in a hot water bath for exactly one minute and then cooled under running tap water. The color developed was diluted to 25 ml distilled water and its absorbance was read at 650 nm in a spectrophotometer. The number of phenols present in the 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.

## 3. RESULTS

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 conditions as explained in 'Material and Methods'. The assay of defense related enzymes, *viz.,* peroxidase (PO) Table 1, 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) Table 1, polyphenol oxidase (PPO) Table 2, phenylalamine ammonia lyase (PAL) Table 3 and phenolic compounds Table 4 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 the pathogen and their interaction in pomegranate was comparatively increased considerably with the increase in the period of inoculation compared to the control set.

## 3.1 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 intervals upon challenge inoculation with pathogen, when compared to untreated control (Table 1).

In general, the PO activity gradually increased from 0 to 60 Days After Inoculation (DAI) and later it decreased to 90 DAI in all the treatments. The maximum activity of PO was recorded in Diamond (*T. viride*) (0.157 absorbance @ 470 nm/min/mg protein) followed by *T. harzianum* (Th-R) (0.129 absorbance @ 470 nm/min/mg protein) and Platinum (*P. fluorescens*) (0.085 absorbance @ 470 nm/min/mg protein). While the least activity was noticed in plants treated with *C. fimbriata* (0.016 absorbance @ 470 nm/min/mg protein) indicating the role of bio-agents in increasing the activity of peroxidase. The healthy control treatment recorded lesser

activity (0.038 absorbance @ 470 nm/min/mg protein) of peroxidase activity.

### 3.2 Polyphenol Oxidase (PPO)

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

The PPO activity gradually increased from 0 to 60 Days After Inoculation (DAI) in all the treatments and later it decreased at 90 DAI in all the treatments. The bio-agent formulation, Diamond (*T. viride*) recorded the highest activity of PPO (0.154 absorbance @ 470 nm/min/mg protein) followed by *T. harzianum* (Th-R) (0.130 absorbance @ 470 nm/min/mg protein) and Platinum (*P. fluorescens*) (0.101 absorbance @ 470 nm/min/mg protein). The least activity was in plants treated with pathogen alone (0.016 absorbance @ 470 nm/min/mg protein) which indicated the role of bio-agents in increasing the activity of polyphenol oxidase. The healthy control treatment recorded less activity of 0.045 (absorbance @ 470 nm/min/mg protein) polyphenol oxidase.

### 3.3 Phenyalanine Ammonialyase (PAL)

In general, the PAL activity (Table 3) was gradually increased from 0 to 60 Days After Inoculation (DAI) and later it decreased to 90 DAI in all the treatments as was noticed in PO and PPO. The maximum activity of PAL was recorded in Diamond (*T. viride*) (0.094 absorbance @ 470 nm/min/mg protein) followed by *T. harzianum* (Th-R) (0.081) and Platinum (*P. fluorescens*) (0.077 absorbance @ 470 nm/min/mg protein). While, the least activity was noticed in plants treated with *C. fimbriata* (0.009 absorbance @ 470 nm/min/mg protein) indicating the role of bio-agents in increasing the activity of phenyalanine ammonialyase. The healthy control treatment recorded 0.034 (absorbance @ 470 nm/min/mg protein) phenyalanine ammonialyase activity.

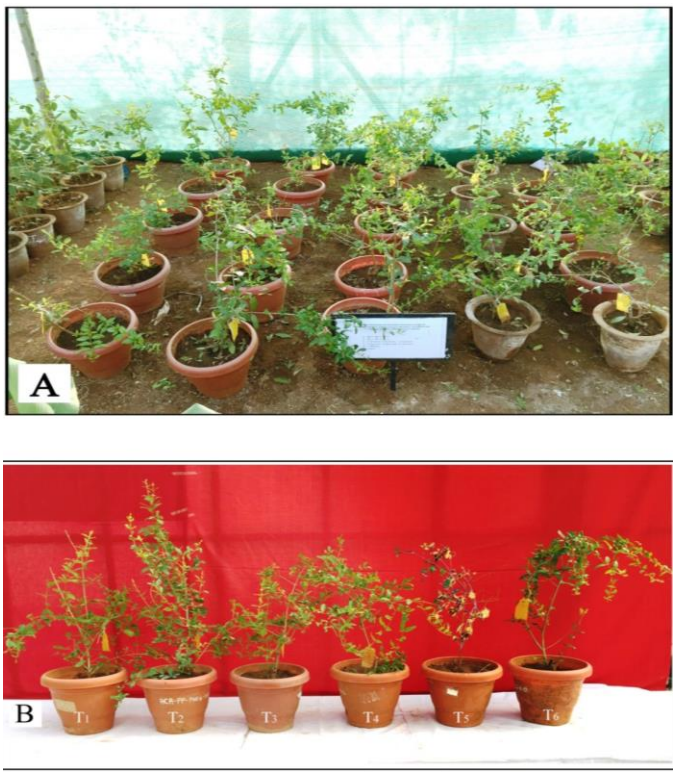


**Plate 1 A. Experimental view of induced systemic resistance against *C. fimbriata* in glass house; B. Induced systemic resistance treatments**

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

### 3.4 Total Phenols

The results (Table 4) for the assay of total phenols activity in pomegranate plants inoculated with bio-agents showed that enzyme activity was gradually increased from 0 to 90 Days After Inoculation (DAI) in all the treatments, unlike PO, PPO, and PAL wherein the activity was reduced at 90 DAI. The maximum activity of total phenols was recorded in Diamond (*T. viride*) (7.797 absorbance @ 470 nm/min/mg protein) followed by *T. harzianum* (Th-R) (7.513 absorbance @ 470 nm/min/mg protein) and Platinum (*P. fluorescens*) (7.105 absorbance @ 470 nm/min/mg protein). While the least activity was noticed in plants treated with *C. fimbriata* (2.343 absorbance @ 470 nm/min/mg protein) indicating the role of bio-agents in increasing the activity of total phenols. The healthy control treatment recorded 6.003 (absorbance @ 470 nm/min/mg protein) total phenols activity.

## 4. DISCUSSION

Induced resistance is a state of enhanced defensive capacity against a broad spectrum of pests and pathogens developed by a plant when appropriately stimulated [12]. The resulting elevated resistance due to biotic agents is referred to as induced systemic resistance (ISR) whereas that by other than biological control agents is called systemic acquired resistance (SAR) [13]. 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 on the defense-related proteins, *viz.* PO, PPO, PAL, and phenols. The results of the present study revealed that there was a 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 [14] 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 [15]. Mallesh and Lingaraju, [16] reported that fluourescent pseudomonads isolates were found effective, systemically inducing resistance against wilt complex pathogens of coleus and ashwagandha by the accumulation of a battery of enzymes in response to pathogens infection. The present findings are also similar to that supported by several other workers [17,18,19].

PAL is the first enzyme in phenylpropanoid metabolism involved in the production of phenolics and phytoalexins that prevent the establishment of pathogens [20]. The increase in PAL activity in plants is presumably related to the lignification process and also plays a role in plant defense systems. Peroxidases are a key enzyme in the plant detoxification system and are involved in scavenging reactive oxygen species and lignifications of vascular tissues, which might be important in the defense of plants against vascular wilt pathogens [21]. PO catalyses $H_2O_2$-dependent condensation of phenolics into lignin in response to plant-pathogen interaction and creates a physical barrier to limit the pathogen invasion in host tissues, playing a specific role in the hypersensitive containment of the pathogen [22].

Defense responses are characterized by the early accumulation of phenolic compounds at the infection site, which slows down the development of phenolic compounds at the infection site, which slows down the development of the pathogen occurring as a result of rapid cell death [23]. 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 a high level of vascular lignifications and suberisation of endodermal cells. This might restrict xylem invasion by fungal pathogens and prevents the 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 the oxidative process than secondary metabolic pathways of cell wall fortification.

**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 the complete establishment of plants in earthen pots under glasshouse condition uniformly*
*DAI: Days After Inoculation*

**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 the complete establishment of plants in earthen pots under glasshouse condition uniformly*

*DAI: Days After Inoculation*

**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 the complete establishment of plants in earthen pots under glasshouse condition uniformly*
*DAI: Days after Inoculation*

**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 the complete establishment of plants in earthen pots under glasshouse condition uniformly*
*DAI: Days After Inoculation*

## 5. CONCLUSIONS

In conclusion treatment of bioagents the 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. These bioagents can be used to stimulate the pomegranate defence system against *C. fimbriata.*

## ACKNOWLEDGEMENT

The authors wish to thank financial contributions from the University Grants Commission-Rajiv Gandhi National Fellowship (UGC-RGNF) and the University for Agricultural Sciences, Raichur (UASR).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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