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Implication of oil based formulation of *Beauveria bassiana* vuillemin in inducing defense related enzymes in onion plants

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Abstract
The foliar application of oil based formulation of *B.bassiana* (Bb 112) @ 10^8 spores ml^{-1} had significant influence on the induction of defense related enzymes against *Thrips tabaci* Lindeman infestation in onion. On four days after treatment (DAT), maximum activity of phenylalanine ammonia-lyase (0.63 µmol trans-cinnamic acid min^{-1} g^{-1} of fresh tissue) and total phenol content of 7.60 µg of catechol g^{-1} of fresh tissue was recorded. The maximum activity of lipoxygenase (0.21 min^{-1} g^{-1} of fresh tissue) was recorded on 6 DAT and on 10 DAT, peroxidase, polyphenol oxidase, catalase and super oxide dismutase (SOD) reached its maximum in thrips infested onion plants treated with foliar application of oil based formulation of Bb 112.

Keywords: Implication, oil based formulation, *Beauveria bassiana*, onion plants

Introduction
The evolutionary race between plants and insects has resulted in the development of an elegant defense system in plants that recognizes the non-self molecules or signals from the damaged plant parts/cells or the insect regurgitants and in turn activates the plant defense response against the herbivores (Howe and Jander, 2008; Karban, 2011; Smith and Clement, 2012) [17, 18, 31]. The oxidative state of the host plants results in the production of reactive oxygen species (ROS) and toxic secondary metabolites (Howe and Jander, 2008; Zhao *et al.*, 2009; Wu and Baldwin, 2010) [17, 41, 39].

Induced resistance is a plant-mediated biocontrol mechanism whereby the biocontrol agent and the phytopathogen do not make physical contact with one another. Plants react to the presence of a pathogen with a rapid expression of defense-related genes. The signaling mechanisms for this induced resistance are based on jasmonic acid (JA) and ethylene (Van Loon *et al.*, 1998; Van Wees *et al.*, 2008; Gutjahr and Paszkowski, 2009) [34, 35, 14]. Most of the studies have reported that wounding by plant pathogenic microbes, beneficial rhizobacteria, and/or insect feeding leads to release of linolenic acid from membrane lipids which was then converted enzymetically into Jasmonic acid (JA). JA, in turn, causes the transcriptional activation of genes encoding proteinase inhibitors and of enzymes involved in the production of volatile compounds and other defense related compounds (Boland *et al.*, 1999; Vega *et al.*, 2009) [5, 36].

Induction of systemic resistance via the JA/ ethylene signaling pathway has been reported primarily for plant growth-promoting bacteria, however, it is also operative for many mycorrhizal fungi (Gutjahr and Paszkowski, 2009) [14]. In onion, *T. tabaci* is a major constraint and some entomopathogenic fungi have been reported to control thrips effectively. Besides the fungal parasitism on insects, it was reported that the entomopathogenic fungi can induce resistance against herbivores in plants by colonization in plant vascular tissues. Karthiba *et al.* (2010) [19] recorded a greater accumulation of enzymes, lipoxygenase and chitinase activity against leaffolder insect in rice plants treated with Pf1 + AH1 + B2 (Pf1 and AH1 – *Pseudomonas flourescens* Migula strains; B2 – *B. bassiana*) combination under field condition. Also, the plants showed higher accumulation of defence enzymes *viz.*, peroxidase and polyphenol oxidase activity against sheath blight pathogen. Hence, the present investigation was carried out to understand the induction of defensive response if any, against *T. tabaci* in onion plants treated with oil based formulation of *B. bassiana* (Bb 112).

Experimental Methodology

Thrips infested and healthy onion plants were treated with oil based formulation of Bb 112 @ 10^8 spores ml^{-1} as foliar spray (@ 4 ml lit^{-1}), soil drenching (@ 4 ml lit^{-1}) and foliar + soil application (@ 2 ml lit^{-1}(foliar) + @ 2 ml lit^{-1} (soil drenching)) to assess the induction of defense related enzymes. Untreated control (without spray, but infested with thrips) and absolute control (without thrips infestation and healthy) were also maintained for comparison purpose. The seven parameters *viz.*, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, catalase, super oxide dismutase, lipoxygenase and total phenols were analyzed in above treatments.

(i) Peroxidase (PO)

Assay of PO activity was carried out as per the procedure described by Hammerschmidt *et al*. (1982) [15]. Samples from the treated, untreated (infested) and absolute control plants were collected on 1, 4, 6, 8 and 10 days and homogenized immediately with liquid nitrogen. One g of homogenized sample was extracted with 2 ml of 0.1 M phosphate buffer (pH 7.0) at 4 0C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 0C and the supernatant was used as enzyme source. For the assay of PO, 2.5 ml of a reaction mixture containing 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide was prepared. Enzyme extract (0.1 ml) was added to the above mixture to initiate the reaction, which was followed calorimetrically at 420 nm. The change in absorbance was recorded at 420 nm at 30 sec interval for three minutes from zero second of incubation at room temperature. The results were expressed as change in absorbance min^{-1} g^{-1} of fresh tissue.

(ii) Polyphenol oxidase (PPO)

The polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* (1965) [24]. Samples from the treated, untreated (infested) and absolute control plants were collected on 1, 4, 6, 8 and 10 days and homogenized immediately with liquid nitrogen. One g of homogenized sample was extracted with 2 ml of 0.1 M phosphate buffer (pH 7.0) at 4 ^{0}C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 0C and the supernatant was used as enzyme source. For the assay of PPO, 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) was prepared and to this, 200 □l of 0.01 M catechol, 200 □l of enzyme extract was added and the change in absorbance was recorded at 495 nm. The enzyme activity was expressed as $min^{-1}g^{-1}$ of protein.

(iii) Phenylalanine ammonia-lyase (PAL)

Activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Samples from the treated, untreated (infested) and absolute control plants were collected on 1, 4, 6, 8 and 10 days and homogenized immediately with liquid nitrogen. One g of homogenized sample was extracted with 2 ml of ice cold 0.1 M sodium borate buffer. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 0C and the supernatant was used as enzyme source. Sample containing 0.4 ml of enzyme extract was 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 ^{0}C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M-1cm-1 (Dickerson *et al.*, 1984) [11]. Enzyme activity was expressed in fresh weightbasis as µmol trans-cinnamic acid min-1 g-1 of sample.

(iv) Catalase (CAT)

CAT activity was assayed spectrophotometrically as described by Chaparro- Giraldo *et al.* (2000) [8]. Samples from the treated, untreated (infested) and absolute control plants were collected on 1, 4, 6, 8 and 10 days and homogenized immediately with liquid nitrogen. One g of homogenized sample was extracted with 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) at 4 ^{0}C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 ^{0}C and the supernatant was used as enzyme source. Three ml of assay mixture containing 100 mM potassium phosphate buffer (pH 7.5) and 2.5 mM H2O2 was prepared before use and added with 100 µl of enzyme extract. The enzyme activity was measured by monitoring the degradation of H2O2 using UV-Visible Spectrophotometer (Varian Cary 50) at 240 nm over 1 min. The decrease in H2O2 was followed as the decline in optical density at 240 nm and the enzyme activity was calculated using the extinction coefficient (ε240nm = 40 mM-1 cm-1) for H2O2 and expressed in □mol min-1 g-1 of sample.

(v) Super oxide dismutase (SOD)

Samples from the treated, untreated (infested) and absolute control plants were collected on 1, 4, 6, 8 and 10 days and homogenized immediately with liquid nitrogen. One g of homogenized sample was extracted with 2 ml of 0.2 M citrate phosphate buffer (pH 6.5) at 4 0C. The homogenate was centrifuged at 10,000 rpm at 4 ^{0}C for 20 min. The supernatant served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of Nitroblue Tetrazolium (NBT) (Giannospolitis and Ries, 1977). The assay mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA (Ethylenediaminetetraacetic acid) and 100 µl of the enzyme extract and 2 µM riboflavin was added at the end. Tubes were shaken and placed under a 40-W fluorescent lamp at 25 ^{0}C. The reaction was initiated and terminated by turning the light on and off, respectively. The absorbance at 560 nm was measured against identical non-illuminated sample tubes as blank. Each reading was subtracted from the blank and mathematical difference was then divided by blank and multiplied by 100 to obtain the percentage inhibition of photo-reduction of NBT.

The SOD activity was expressed in SOD units g-1 tissue (50% NBT inhibition = 1 unit)
(Belid El-Moshaty *et al.*, 1993) [3].

(vi) Lipoxygenase (LOX)

The spectrophotometeric assay of LOX was carried out through the procedure developed by Kermasha and Metche (1986) [20]. Samples from the treated, untreated (infested) and absolute control plants were collected on 1, 4, 6, 8 and 10 days, then cooled in a dry ice bath (-30 ^{0}C) and macerated to a fine powder and defatted with acetone and diethyl ether. One g of defatted material suspended in 3 volumes of Tris HCl buffer with mechanical stirring for 16 h and centrifuged at 4,800 g for 30 min. The supernatant was used for enzyme assay and stock solution was prepared using linoleic acid as substrate (A: 1% linoleic acid (w/v) in absolute ethanol; B: 7.1 ml of solution A with 0.25 ml of Tween 20 and ethanol evaporated using rotary vacuum evaporator. Finally the residue dissolved in 100 ml of Na2HPO4 and pH adjusted to 9.0 using 1 N

NaOH). Stock solution was diluted ten times with 0.2 M citrate-phosphate buffer and used for further reactions. To 2.4 ml of substrate solution, 0.1 ml of enzyme extract was taken and absorbance was measured at 490 nm. Instead of sample, 0.1 ml of deionized water served as blank. Rate of increase in optical density from the initial linear portion of the graph was taken and the enzyme activity was expressed as OD234 $min^{-1}g^{-1}$ of protein.

(vii) Total phenol content

Phenol content was estimated as per the procedure given by Zieslin and Ben- Zaken (1993) [43]. Samples collected from the treated, untreated (infested) and absolute control plants were collected on 1, 4, 6, 8 and 10 days and homogenized immediately with liquid nitrogen. One g of homogenized sample was extracted with 10 ml of 80 per cent methanol using pestle and mortar and agitated for 15 min at 70 ^{0}C. One ml of the methanolic extract was added to 5 ml of distilled water and 250 □l of Folin Ciocalteau reagent (1 N) and the solution was kept at 25 ^{0}C. After 3 min, one ml of saturated solution of sodium carbonate and one ml of distilled water was added and the reaction mixture was incubated for 1 h at 25 ^{0}C. The absorption of the developed blue colour was measured using UV-Visible Spectrophotometer (Varian Cary 50, Victoria, Australia) at 660 nm. The total soluble phenol content was calculated based on a standard curve obtained from the reaction of Folin-Ciocalteau reagent with a phenol solution (C6H6O) and expressed as mg of catechol g^{-1} tissue weight.

Results and Discussion

The results on the assay of defense related enzymes disclosed that the oil based formulation of *B.bassiana* (Bb 112) had significant influence on the induction of defense related enzymes and components against thrips infestation. It was observed that the foliar application of oil based formulation of *B. bassiana* (Bb 112) influenced defensive response and had significant influence on the levels of enzymes *viz.*, PO, PPO, LOX, SOD, PAL, CAT and also total phenols which in turn resulted in reduced thrips population. Also, in thrips infested untreated control, marginal increase in defense related enzymes were noted as a result of herbivore induced damage The literatures pertaining to the induction of systemic resistance by EPF against onion thrips is not available and hence, the results are discussed with available literatures on other plants damaged by sucking or chewing pests.

(i) Peroxidase

The results of PO activity in *T. tabaci* infested onion leaves applied with oil based formulation of *B. bassiana* (Bb 112) exhibited significant difference among the treatments. The activity of PO increased with the increase in the days after treatment and reached the maximum at 10 days after treatment (DAT). The highest PO activity was recorded in the treatment involving foliar application of onion plants infested with thrips (0.63 $min^{-1}g^{-1}$ of fresh tissue) followed by foliar application + soil drenching (0.62 $min^{-1}g^{-1}$ of fresh tissue) and soil drenching alone (0.47 $min^{-1}g^{-1}$ of fresh tissue). (Fig 1a)

The role of PO in the production of semiquinone free radicals and subsequent formation of quinones has been attributed to its direct post ingestive toxicity against insects (ZhuSalzman *et al.*, 2008; Barbehenn *et al.*, 2010) [42, 2]. In addition, it also mediates the oxidation of hydroxylcinnamyl alcohols into free radical intermediates, oxidation of phenols, cross-linking of polysaccharides and monomers, lignifications and suberization (Zhang *et al.*, 2008; Chen *et al.*, 2009) [40, 41], which in turn lead to the production of antinutritive compounds (Gulsen *et al.*, 2010; He *et al.*, 2011) [13, 16].

(ii) Polyphenol oxidase

The significant variation in PPO activity in various treatment was observed. Activity of polyphenol oxidase was higher in thrips infested onion plants treated with foliar application of oil based formulation of *B. bassiana* (Bb 112). PPO activity reached the maximum at 10 DAT with foliar application of oil based formulation (1.68 $min^{-1}g^{-1}$ of fresh tissue) followed by foliar application + soil drenching (1.62 $min^{-1}g^{-1}$ of fresh tissue) and soil drenching alone (1.51 $min^{-1}g^{-1}$ of fresh tissue). Untreated control plants infested with thrips recorded the least PPO activity with a meagre increase throughout the study period (Fig 1b).

The PPO plays an important role in plant defense against insect herbivory as an antinutritional enzyme and reduces the food quality (Mahanil *et al.*, 2008; Bhonwong *et al.*, 2009) [23, 4]. It oxidizes phenols to highly reactive and toxic quinones that interact with the nucleophilic side chain of amino acids, leading to cross-linking of proteins and thereby, reducing their availability to insect pests (Zhang *et al.*, 2008; Bhonwong *et al.*, 2009) [40, 4]. In addition to their role in digestibility and palatability of plant tissues, melanin formation by PPOs increases the cell wall resistance to insects and pathogens (Zhao *et al.*, 2009) [41].

(iii) Phenylalanine ammonia-lyase

PAL activity reached the maximum on 4 DAT and declined thereafter. Foliar application of oil based formulation recorded the maximum activity of PAL (0.63 µmol trans-cinnamic acid min^{-1} g^{-1} of fresh tissue) followed by foliar application + soil drenching (0.57 µmol trans-cinnamic acid $min^{-1}g^{-1}$ of fresh tissue) and soil drenching alone (0.42 µmol trans-cinnamic acid $min^{-1}g^{-1}$ of fresh tissue) after 4 days of treatment (Fig 1c).

The *de novo* synthesis and increased activity of PAL is an initial plant defensive response to insect damage (Campos Vargas and Saltveit, 2002) [7], which leads to accumulation of phenolic compounds in plants that are sequestered in cell vacuole (Zhao *et al.*, 2009) [41] and forms toxic compounds upon oxidation (Bhonwong *et al.*, 2009) [4].

Thus from the present study, it is clearly evident that, the higher activity of defense related enzymes *viz.*, PO, PPO and PAL was recorded in thrips infested onion. Plants treated with foliar application of *B. bassiana* (Bb 112) compared with untreated control (plants infested with thrips alone) and absolute control. A similar finding was reported by Bandi and Sivasubramanian (2012) [1] who demonstrated the enhanced activity of PO, PPO and PAL activities against *T. tabaci* in onion through foliar application of *P. fluorescens* (Pf1). Elicitation of PO, PPO and PAL activity was also depicted in okra against *Earias vitella* (Fabricius) infested plants through application of Pf1 (*P. flourescens*) + B2 (*B. bassiana*) (Thiruveni *et al.*, 2012) [33]. Further, in tomato, Prabhukarthikeyan *et al.* (2014) [27] also reported the increased activity of PO and PPO in B2 (*B. bassiana*) + EPC 8 (*Bacillus subtilis*) treated plants against *H. armigera* and *Fusarium oxysporum* f. sp. *lycopersici* Sacc. (Fol).

(iv) Catalase and super oxide dismutase

The steep increase in catalase activity was observed in all the treatments throughout study period except untreated and absolute control. Among the treatments, the highest catalase

activity was recorded in the treatment involving foliar application of *B. bassiana* (Bb 112) on 10 DAT (0.93 µmol of H2O2 consumed $min^{-1}g^{-1}$ of fresh tissue) followed by foliar application + soil drenching and soil drenching alone which recorded 0.91 and 0.84 µmol of H2O2 consumed $min^{-1}g^{-1}$ of fresh tissue, respectively. Untreated control plants infested with thrips also recorded the catalase activity with the least increase throughout the experimental period (Fig 2a).

The consistent increase in SOD activity was noted with increase in days after treatment and reached the maximum on 10 days after treatment (DAT). The maximum enzyme activity was observed in the treatment involving foliar application of oil based formulation of Bb 112 on onion plants infested with thrips at 10 DAT (0.96 unit min^{-1} $g^{-1}$of fresh tissue). Next in the order were foliar application + soil drenching (0.82 unit min-1 g-1of fresh tissue) and soil drenching alone (0.75 unit $min^{-1}g^{-1}$ of fresh tissue) (Fig 2b).

Plants produce active oxygen species (AOS) such as superoxide anion (O2-), hydrogen peroxide (H2O2) and hydroxyl radical as one of the earliest responses to biotic stress (Grant and Loake, 2000) [12]. CAT and SOD are of particular interest because of their role as scavengers of free radicals produced during biological injury (Khattab and Khattab, 2005) [21]. Increased CAT activity in plants increases cell wall resistance and also acts as a signal for the induction of defensive genes (Chen *et al.*, 1993) [10]. With regards to SOD, it acts as the first line of defense by catalyzing the dismutation of superoxide into oxygen and H2O2 (Raychaudhuri and Deng, 2000) [29].

Findings of Senthilraja (2008) [30] depicted higher activities of CAT and SOD in leaf miner insect and collar rot pathogen infested groundnut plants treated with Pf1 (*P. flourescens*) + B2 (*B. bassiana*) bioformulation. Similarly, Prabhukarthikeyan (2012) [27] observed higher CAT and SOD activity in *H. armigera* and *F. oxysporum* f. sp. *lycopersici* infested tomato plants treated with Pf1 (*P. flourescens*) + B2 (*B. bassiana*) bioformulation.

(v) Lipoxigenase

The LOX activity reached its maximum at 6 DAT in foliar application of oil based formulation (0.21 min^{-1} g^{-1} of fresh tissue). This was followed by foliar application + soil drenching (0.19 min^{-1} $g^{-1}$of fresh tissue) and soil drenching alone (0.15 $min^{-1}g^{-1}$ of fresh tissue) (Fig 2c).

The dioxygenation of polyunsaturated fatty acid by LOX in response to insects feeding leads to the formation of highly reactive LOX products [Hydroperoxyoctadecatrienoic acid (HPOTE), Hydroperoxy octadecadienoic acid (HPODE)]. These are subsequently transformed into jasmonates which are involved in signalling events, regulate plant defence gene expression and synthesis hydroperoxide lyase products which behave as volatile phytoalexins (Thaler *et al.*, 2002) [32]. Lipid peroxidation end products act as insect repellents (Bruinsma *et al.*, 2009) [6], which are directly toxic to insect pests (Maffei *et al.*, 2007; Bhonwong *et al.*, 2009) [22, 4]. Besides, enhanced accumulation, regulation of LOX pathway contributes for the induction of phytoalexins against pathogens and attracts natural enemies for the predation of insect pests. Karthiba *et al.* (2010) [19] reported that the application of a Pf1 (*P. flourescens*) + AH1 (*P. flourescens*) + B2 (*B. bassiana*) mixture induced higher LOX and chitinase activity against leaffolder in rice compared to individual treatments and untreated control. Similarly, Prabhukarthikeyan *et al.* (2014) [27] reported that the application of B2 (*B. bassiana*) + EPC8 (*B. subtilis*) mixture induced higher LOX activity against *F. oxysporum* and *H. armigera* in tomato plants compared with individual treatments and untreated control.

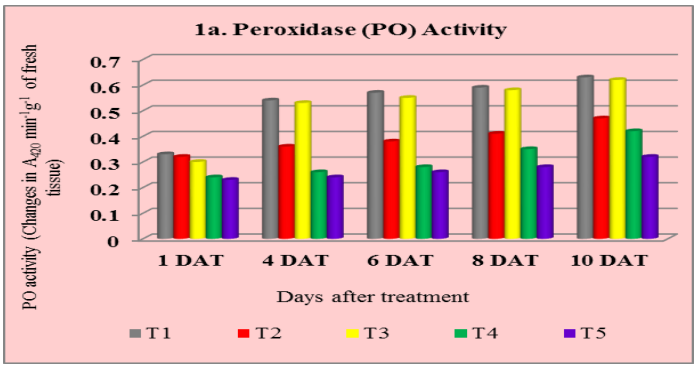
(vi) Total phenol content

The application of oil based formulation of *B. bassiana* (Bb 112) in *T. tabaci* infested onion leaves resulted in increased phenol content than untreated control. Unlike other defense related enzymes, phenol content reached the maximum on 4 DAT in all the treatments and thereafter started to decline. On 4 DAT, the highest accumulation of phenol was recorded in the treatment involving foliar application of onion plants infested with thrips (7.60 µg of catechol g^{-1} of fresh tissue) followed by foliar application + soil drenching (6.70 µg of catechol g-1 of fresh tissue) and soil drenching alone (4.80 µg of catechol g^{-1} of fresh tissue). The least phenol accumulation was recorded in untreated control infested with thrips at 4 DAT (3.70 µg of catechol g^{-1} of fresh tissue) (Fig 2d).

Phenolic compounds are either directly toxic to insects (Walling, 2000; Bhonwong *et al.*, 2009) [38, 4] or indirectly mediate various transduction pathways, which in turn, produce toxic secondary metabolites and activates the defensive enzymes (Walling, 2000; Maffei *et al.*, 2007; Bhonwong *et al.*, 2009) [38, 22, 4]. Quinones formed by oxidation of phenols bind covalently to leaf proteins and inhibit protein digestion in herbivores (Bhonwong *et al.*, 2009) [4].

The result is accordance with the findings of Prasad (2013) [28] who reported increased phenol content in onion plants treated with *B. bassiana* against *T. tabaci* in onion. Vinale *et al.* (2008) [37] reported that the plants infected with hypocrealean fungi do not have the complex structures associated with mycorrhizal infection, they can occupy a nutritional niche in or on the plant and develop an active cross talk with their plant hosts that results in induced resistance.

Mechanisms for induced resistance by hypocrealean fungi are scant. However, much information available on the mechanisms of induced resistance by *Trichoderma* can be applied to other fungal entomopathogens (Ownley *et al.*, 2010) [25] which can also be corroborated with our present findings.



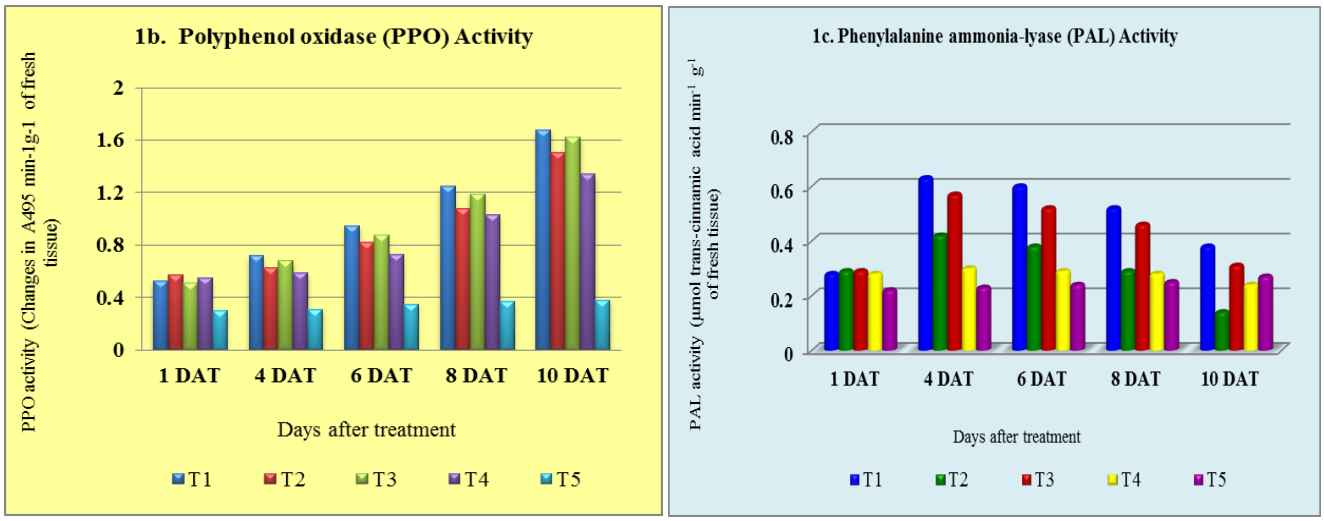


Fig 1: Induction of PO, PPOand PAL activity in onion plants applied with oil based formulation of *B. bassiana* (Bb 112) **T1 - *B. bassiana***(Bb 112) @ 10^8 Spores ml^{-1} (oil based formulation – foliar application) **T2 - *B. bassiana***(Bb 112) @ 10^8 Spores ml^{-1} (oil based formulation – soil drenching) **T3 - *B. bassiana***(Bb 112) @ 10^8 Spores ml^{-1} (oil based formulation – foliar application + soil drenching) **T4 -** Untreated control (without spray but infested with thrips) T5 - Absolute control (without thrips infestation and healthy)

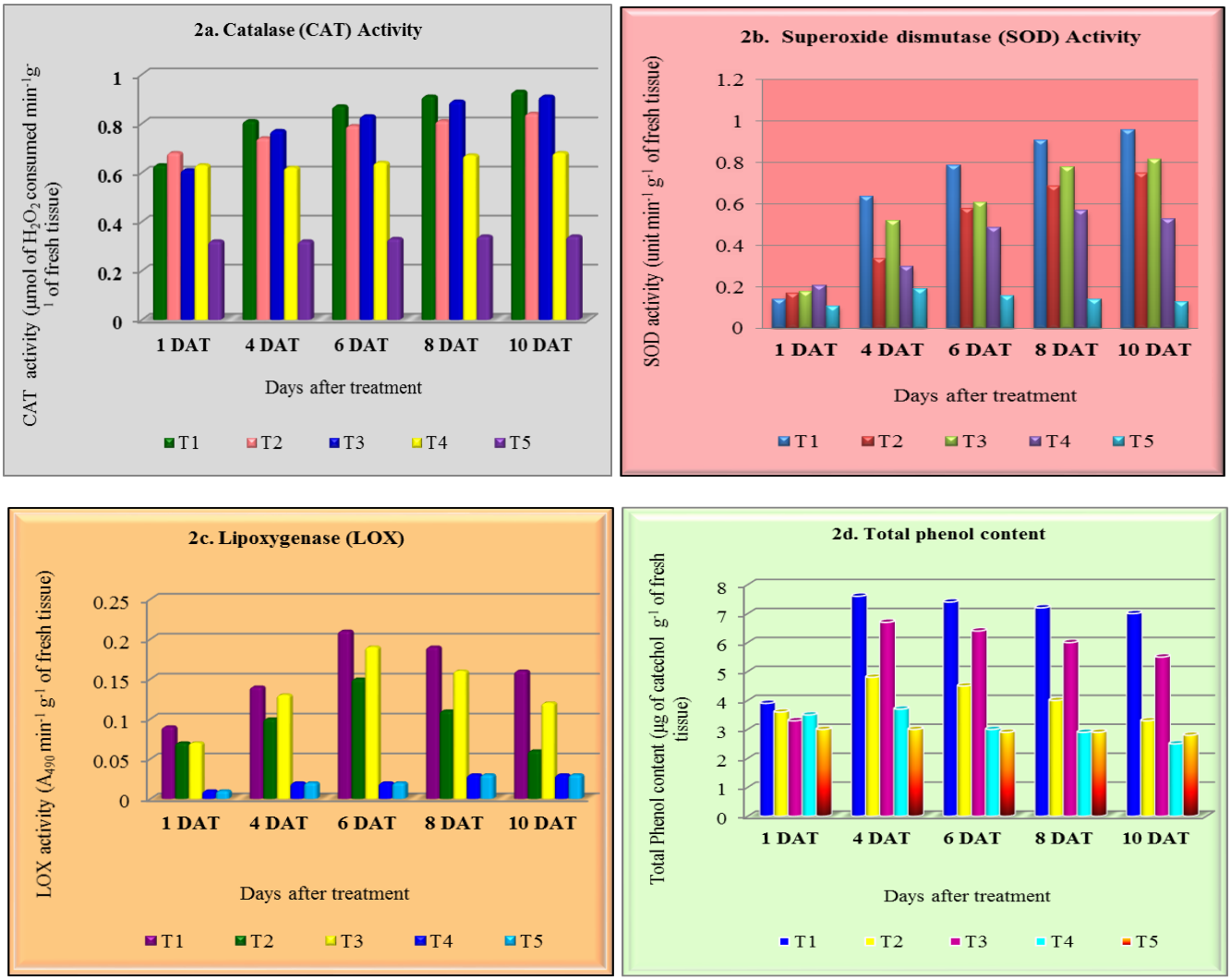


Fig 2: Induction of CAT, SOD, LOX activity and total phenol in onion plants applied with oil based formulation of *B. bassiana* (Bb 112) **T1 - *B. bassiana***(Bb 112) @ 10^8 Spores ml^{-1} (oil based formulation – foliar application) **T2 - *B. bassiana***(Bb 112) @ 10^8 Spores ml^{-1} (oil based formulation – soil drenching) **T3 - *B. bassiana***(Bb 112) @ 10^8 Spores ml^{-1} (oil based formulation – foliar application + soil drenching) **T4 -** Untreated control (without spray but infested with thrips) **T5 -** Absolute control (without thrips infestation and healthy)

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