Implication of oil based formulation of *Beauveria bassiana* vullemin 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) @ 10⁹ 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 lipoygenase (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 enzymatically 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, lipoygenase 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, lipoygenase 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 °C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C 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) [28]. 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 °C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C 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 μl of 0.01 M catechol, 200 μl 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 °C 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 °C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1}cm^{-1} (Dickerson et al., 1984) [111]. 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 °C. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °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 μmol 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 °C. The homogenate was centrifuged at 10,000 rpm at 4 °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 °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) Lipoygenase (LOX)

The spectrophotometric 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 °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⁻¹g⁻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 °C. One ml of the methanolic extract was added to 5 ml of distilled water and 250 μl of Folin Ciocalteau reagent (1 N) and the solution was kept at 25 °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 °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⁻¹g⁻¹ of fresh tissue) followed by foliar application + soil drenching (0.62 min⁻¹g⁻¹ of fresh tissue) and soil drenching alone (0.47 min⁻¹g⁻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 digestive toxicity against insects (Zhu et al., 2008; Barbehenn et al., 2010) [42, 2]. In addition, it also mediates the oxidation of hydroxycinnamyl 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⁻¹g⁻¹ of fresh tissue) followed by foliar application + soil drenching (1.62 min⁻¹g⁻¹ of fresh tissue) and soil drenching alone (1.51 min⁻¹g⁻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) [46, 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⁻¹g⁻¹ of fresh tissue) followed by foliar application + soil drenching (0.57 μmol trans-cinnamic acid min⁻¹g⁻¹ of fresh tissue) and soil drenching alone (0.42 μmol trans-cinnamic acid min⁻¹g⁻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) [13], 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) [11] 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. fluorescens) + B2 (B. bassiana) (Thiruveni et al., 2012) [33]. Further, in tomato, Prabhukarthyikan 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 thrips infested onion leaves treated with foliar application of B. bassiana (Bb 112) + soil drenching (Fig 1d).
activity was recorded in the treatment involving foliar application of \textit{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$^{-1}$of 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, 2006) [28].


(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) 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).

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 \textit{B. bassiana} against \textit{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 \textit{Trichoderma} can be applied to other fungal entomopathogens (Owlen et al., 2010) [25] which can also be corroborated with our present findings.
Fig 1: Induction of PO, PPO and 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)
References


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