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B Anusha
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

G Thiribhuvanamala
Assistant Professor, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

K Prabakar
Director, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Corresponding Author:
B Anusha
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Induction of defense isoforms by hexanal and bacterial antagonists in mango

B Anusha, G Thiribhuvanamala and K Prabakar

Abstract
Induced resistance has been recognized as an attractive tool for plant disease management in modern agriculture. Bacterial antagonists such as *Pseudomonas fluorescens* and *Bacillus subtilis* are known to induce systemic resistance in plants. The present study was taken up to determine the ability of the volatile compound hexanal to induce systemc resistance in plants upon challenge inoculation with *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*, the causative agents of mango anthracnose and stem-end rot respectively. Glass house studies were undertaken in mango grafts with six treatments, comprising hexanal and bacterial biocontrol agents in order to examine the expression patterns of peroxidase, polyphenol oxidase and catalase enzymes. Native PAGE analysis showed that hexanal was able to induce defense enzymes in the grafts, upon challenge inoculation with the pathogen.

Keywords: Modern agriculture, bacterial antagonists, *Pseudomonas fluorescens*, *Bacillus subtilis*, plants

Introduction
Plants establish multiple layers of defense responses, including physical barriers such as the cuticle and cell wall, as well as chemical defenses such as secretion of antimicrobial or anti-insect compounds [1]. The term induced systemic resistance describes activation of the host plant's physical or chemical defenses by an inducing agent.

Many plant originated enzymes are involved in defense reactions against pathogens. These include oxidative enzymes such as peroxidase and polyphenol oxidase which catalyze the formation of lignin and other oxidative phenols that contribute to the formation of defense barriers by reinforcing cell wall structures [2]. Peroxidases have been implicated in the regulation of plant metabolic processes such as cell elongation, phenol oxidation, polysaccharide cross-linking, IAA oxidation, cross linking of extensin monomers, oxidation of hydroxyl-cinnamyl alcohols into free radical intermediates and wound healing [3] Peroxidases are involved in polymerization of proteins and lignin or suberin precursors into plant cell wall, thus constructing a barrier that prevents pathogen penetration of cell walls and movement through vessels [4]. Polyphenol oxidase usually accumulates in plants upon wounding. PPO can be induced *via* octadecanoid defence signal pathway [5].

Biocontrol agents are known to promote plant growth and improve the host's capacity to withstand against pathogen attack by causing competition, antibiosis and by inducing systemic resistance. The possibility of using the plant's own defense mechanisms induced by bacterial endophytes in the management of diseases is an area of current interest. Once expressed, ISR activates multiple potential defense mechanisms in plants, including increased activity of chitinases, β-1,3-glucanases, peroxidases [6], accumulation of low molecular weight antimicrobial substances, phytoalexins [7] and formation of protective biopolymers such as lignin, callose and hydroxyproline rich glycoproteins [8]. *Bacillus* strains consistently provided systemic protection against multiple diseases in various crops. A multifold increase in chitinase, β-1, 3 glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenol accumulation in plants treated with mixed formulation of PGPR Pf-1 + *B. subtilis* + neem + chitin has also been reported [9].

Several fruit aroma volatiles have been demonstrated to have antibacterial and antifungal activity. Hexanal ($C_6H_{12}O$), is a six-carbon aldehyde, produced in plants from linoleic acid, by the oxidative degradation of fatty acids via the lipoxygenase pathway [10]. Volatiles formed by this pathway in wounded plants have antifungal properties, as shown by early research. Hexanal contributes to the "green" taste of many fruit and vegetables species [11]. It is available commercially and has been approved as a food additive by the U.S. Food and Drug Administration with an ORL MAM LD 50 of 3700 mg/kg [12].

The compound of our interest, hexanal, has been reported to show antifungal properties against spoilage microorganisms *in vitro* and in real fruit storage systems.

The antifungal properties of hexanal have been explored against many post-harvest fungal pathogens, *viz*., *Lasiodiplodia theobromae*, *Botrytis cinerea*, *Monilinia fructicola*, *Sclerotinia sclerotiorum* and *Alternaria alternata. Exposure to h*exanal vapour has been found to inhibit the mycelial growth and spore germination of *L. theobromae* in strawberry [13].

The present study was taken up to study the ability of hexanal to induce systemis resistance alone and in combination with bacterial antagonists, against *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* in mango.

Materials and methods

Sample collection

Glass house studies was carried out to study the induction of isoforms of defense enzymes in mango grafts due to hexanal and biocontrol agents spray and challenge inoculation with the pathogens. Mango grafts of the cultivar Neelum were selected such that they were uniform in size and free from pests and diseases and used for the study. Spray treaments involved individual and combination treatments, along with Carbendazim and pathogen-inoculated control, for comparison of expression of defense enzymes.

The treatments imposed were:

T1: Hexanal 0.04% spray
T2: *P. fluorescens* Pf-1 0.5%spray
T3: *B. subtilis* EPCO-16 0.5%spray
T4: Combination spray (T1+T2+T3)
T5: Carbendazim 0.1% spray
T6: Inoculated control
T7: Absolute control

Enzyme extraction

The treated saplings were challenge inoculated separately with *C. gloeosporiodes* and *L. theobromae*. Leaf samples were collected on 0, 3, 5 and 7 days after treatment, in order to study the induction of defense enzymes activated by the antagonist. The leaf samples were ground into fine powder using liquid nitrogen and extracted with the appropriate buffer for each enzyme at 4°C. The homogenate was centrifuged for 20 minutes at 10,000 rpm. The supernatant was used as the enzyme source for estimation of defense enzymes *viz*., peroxidase, polyphenol oxidase and catalase.

Native gel electrophoresis

To study the expression pattern of peroxidase isoforms in the treatments, native gel electrophoresis was carried out using resolving gel of 8 per cent and stacking gel of 4 per cent. Around 40 µl of the enzyme source was taken, to which 7 µl of sample buffer was added and mixed well. The samples were loaded into the gel and electrophorosed at 4°C.

Expression of peroxidase isoforms

After electrophoresis, the gel was incubated in a solution containing 0.05 % benzidine and 0.03 per cent hydrogen peroxide in acetate buffer (pH 4.2) for 30 minutes in dark condition [14]. Peroxidase isoforms were visualized as brown coloured bands, after which the gel was washed with distilled water.

Expression of polyphenol oxidase isoforms

After native electrophoresis, the gel was equilibrated for 30 minutes in 0.1 per cent *p*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) and 10 mM catechol. The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete bands [15].

Expression of catalase isoforms

After electrophoresis, the gel was incubated in staining solution containing 0.003 per cent hydrogen peroxide solution, 1 per cent (w/v) $FeCl_3$ and 1 per cent (w/v) $K_3Fe(CN_6)$ solution for 10 minutes after which greenish-blue coloured bands appeared [16].

Results

Isoform pattern of peroxidase

Two isoforms of peroxidase, PO1 and PO2 were obtained for all the treatments. However, Pf-1 induced an additional isoform of peroxidase, PO3. Likewise, a third isoform was also induced in the inoculated control grafts against *C. gloeosporioides*. In case of *L. theobromae* inoculated grafts, two isoforms of peroxidase were expressed. Among them, the intensity of the bands was higher with Pf-1, compared to the other treatments (Plates 1a and b).

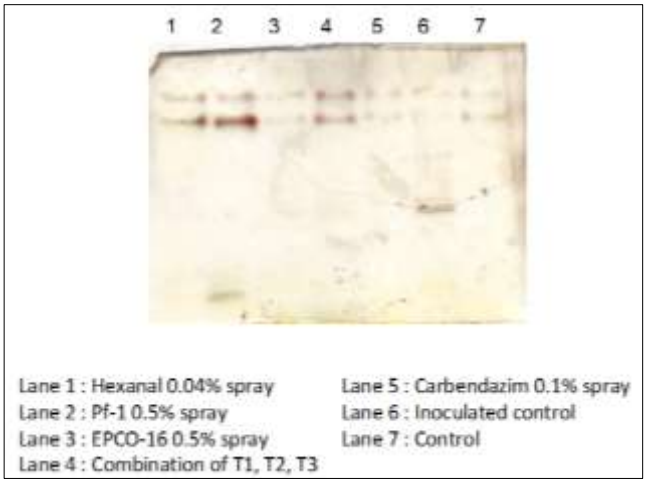

Plate 1a: Induction of PO isoforms against *C. gloeosporioides*

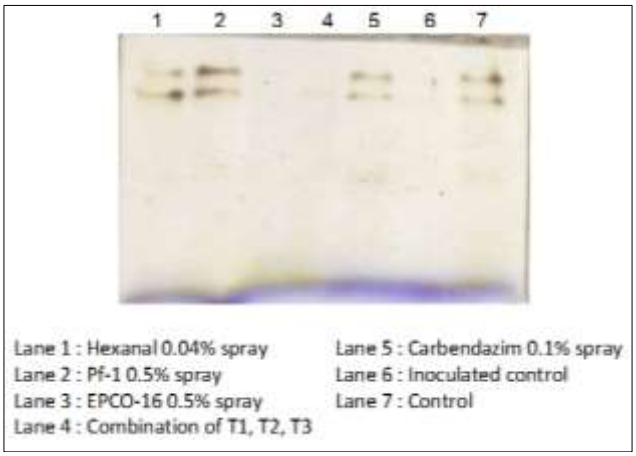

Plate 1b: Induction of PO isoforms against *L. theobromae*

Isoform pattern of polyphenol oxidase

Upon inoculation of *C. gloeosporioides* into mango grftas subjected to various treatments, four isoforms of PPO, namely PPO1, PPO2, PPO3 and PPO4 were induced in all the treatments. The band corresponding to the third isoform was more intense in hexanal treatment, than the other treatments. When the treated grafts were inoculated with *L. theobromae*, five isoforms of PPO, namely, PPO1, PPO2, PPO3, PPO4 and PPO5 were expressed in all the treatments. However, the band intensity corresponding to PPO4 was more intense with the EPCO-16 treatment, followed by hexanal treatment (Plates 2a and b).

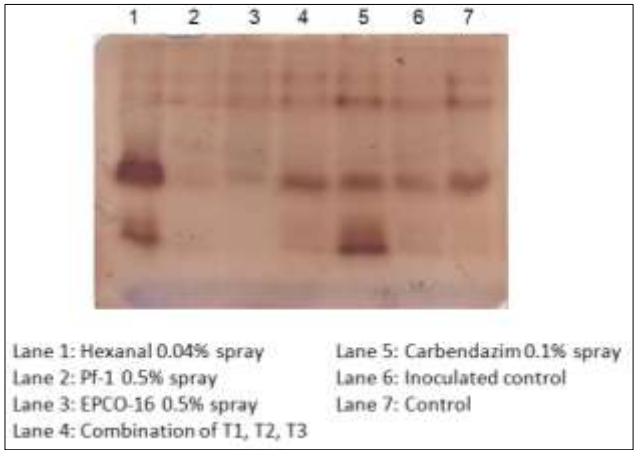

Plate 2a: Induction of PPO isoforms against *C. gloeosporioides*

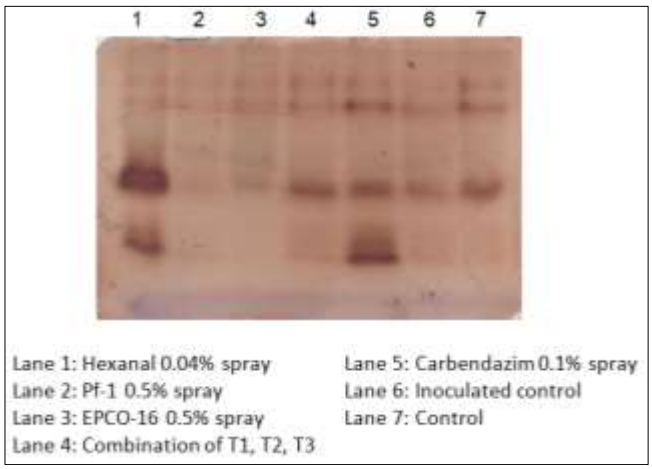

Plate 2b: Induction of PPO isoforms against *L. theobromae*

Isoform pattern of catalase

Five catalase isoforms *viz.*, CAT1, CAT2, CAT3, CAT4 and CAT5 were expressed in all the treatments. However, the intensity of the expression of isoform CAT1 was higher in the treatments of hexanal, Pf-1 and in *C. gloeosporioides* inoculated grafts. The expression of the remaining isoforms was similar in all the treatments. In *L. theobromae* inoculated grafts, uniform expression of one isoform was observed in the treatments of hexanal, Pf-1, EPCO-16 and the combination treatment. In the inoculated control plants, three isoforms were expressed, *viz*., CAT1, CAT2 and CAT3 (Plates 3a and b).

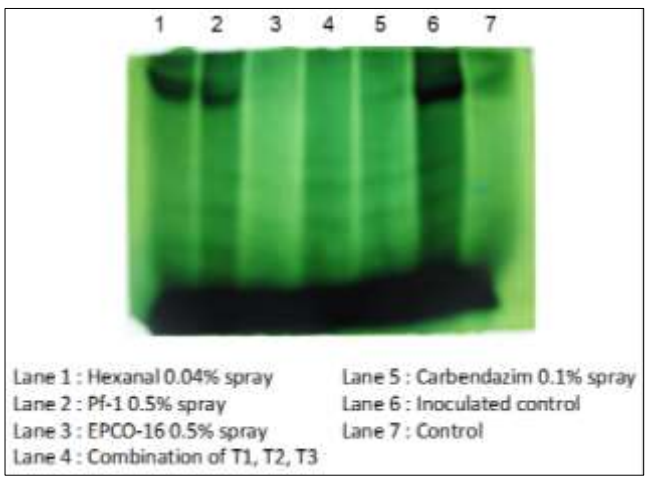

Plate 3a: Induction of CAT isoforms against *C. gloeosporioides*

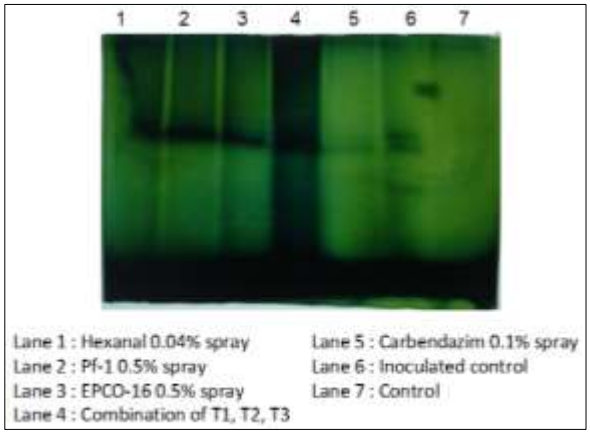

Plate 3b: Induction of CAT isoforms against *L. theobromae*

Discussion

Induced resistance is a strategy that provides a broad-spectrum disease control utilizing the inherent defense mechanism of the plant. ISR once expressed, activates multiple potential defense mechanisms in plants including increased activity of enzymes *viz*., peroxidases, polyphenoloxidases, lipoxygenase, superoxide dismutase, phenylalanine ammonia lyase, β-1,3-glucanase and chitinases. Peroxidases have been implicated in several physiological and biochemical processes that contribute to resistance in plants, such as exudation of hydroxyl cinnamyl alcohol into free radical intermediates, phenol oxidation and polysaccharide cross-linking. Enhanced peroxidase activity is often associated with resistance phenomena such as lignification and deposition of phenols in cell walls. In the present study, two isoforms of peroxidase, PO1 and PO2 were obtained for all the treatments, while Pf-1, showed an additional isoform, PO3. It was interesting to note that the mango grafts sprayed with hexanal and inoculated with *C. gloeosporioides* also expressed two isoforms of PO as faint bands. Similarly, in case of *L. theobromae* inoculated grafts, two isoforms of peroxidase were expressed.

Higher induction of peroxidases in mango treated with *P. fluorescens* (FP-7) amended with chitin bioformulation against anthracnose infection caused by *C. gloeosporioides* [17] has been reported earlier. Enhanced levels of PO were noticed in fluorescent pseudomonads-treated sugarcane in response to infection by *C. falcatum* [18]. Higher levels of PO was reported in *P. fluorescens* strain Pf1 treated tomato plants challenged with *Fusarium oxysporum* f.sp. *lycopersici* [19].

Polyphenol oxidases are enzymes which use molecular oxygen to catalyze the oxidation of monophenolic and orthodiphenolic compounds. In the current study, four isoforms of PPO, namely PPO1, PPO2, PPO3 and PPO4 were induced in all the treatments and challenged with *C. gloeosporioides*. When the treated grafts were inoculated with *L. theobromae*, five isoforms of PPO, namely, PPO1, PPO2, PPO3, PPO4 and PPO5 were expressed in all the treatments. However, the band intensity was more prominent with the EPCO-16, followed by hexanal treatment.

Similar observations of enhanced PPO expression by bacterial antagonists have been reported earlier. *P. fluorescens* induced a two-fold increase in PPO in banana roots against *Fusarium* wilt disease [20]. Three isoforms of PPO in mango fruits treated with *P. fluorescens* strain FP-7+chitin against *C. gloeosporioides* [21]. The combined application of *P. fluorescens* Pf-1 + *B. subtilis* Bs-16 + *T. viride* Tv-1 + $ZnSO_4$ + neem cake + FYM induced higher levels of PPO in physic nut against *L. theobromae* [22]. Three isoforms of PPO (PPO1, PPO2 and PPO3) were induced in

green gram plants treated with *P. fluorescens* Pf-1+ chitin against *Macrophomina phaseolina* [23].

Plants produce reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) as one of the earliest responses to infection by pathogen [24]. Scavengers of reactive oxygen species like catalase catalyze the decomposition of H_2O_2, suppress the oxidative burst and inhibit tissue necrotization. With respect to catalase expression, in thepresent study, five isoforms *viz.*, CAT1, CAT2, CAT3, CAT4 and CAT5 were expressed in all the treatments, with the hghest band intensity being for hexanal, Pf-1 and pathogen inoculated grafts. The expression of the remaining isoforms was similar in all the treatments. In *L. theobromae* inoculated grafts, a uniform expression of one isoform was observed in the treatments of hexanal, Pf-1, EPCO-16 and the combination treatment.

Induction of catalase isoforms by biocontrol agents have been made earlier too. Two isoforms of catalase, CAT1 and CAT2 were induced in mango fruits due to treatment of the bioformulation of *P. fluorescens* strain FP-7 + chitin + *Saccharomyces cerevisiae.* Induction of CAT2 was observed for the treatment combination of FP-7 + *B. subtilis* + chitin, which were absent in control fruits [21]. The catalase activity was maximum in the treatment combination *P. fluorescens* Pf-1 + *B. subtilis* Bs-16+ *T. viride* Tv-1 + $ZnSO_4$ + neem cake + FYM in physic nut against *L. theobromae* [22].

Conclusion

It is evident from the above study that treatment of hexanal alone and incombination with bacterial antagonists, followed by subsequent inoculation of the pathogens could express defense enzymes namely, peroxidase, polyphenol oxidase and catalase. Hence hexanal could be successfully integrated as one of the treatment strategies against *C. gloeosporioides* and *L. theobromae* pathogens in mango.

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