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Induction of defence related biochemical responses by different treatments (elicitors, bioagents, antibiotics and chemicals) in citrus against *Xanthomonas axonopodis* pv. *citri*

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Abstract
The glasshouse and laboratory experiments were conducted to evaluate the enzymatic activity of the antioxident enzymes viz; Phenylalanine ammonia lyase (PAL), Catalase (CAT) and Peroxidase (PO) against *X. axonopodis* pv. *citri* for different treatments at, Department of Plant Pathology, College of Agriculture, G.B.P.U.A. &T., Pantnagar. Experiment was laid out in Completely Randomized Design (CRD) in glass house comprising ten treatment combinations replicated thrice, where the pretreatment of citrus plants with Pant bioagent 2, Pant bioagent 3, L-methionine, GABA and Salicylic acid induced the levels of plant defense and thus induced disease resistance against *X. axonopodis*pv.*citri*. These compounds can be used as effective alternative compounds to copper bactericides for the management of citrus canker.

Keywords: *Xanthomonas axonopodis* pv. *citri*, Citrus, phenylalanine ammonia lyase (PAL), catalase (CAT), peroxidase (PO)

1. Introduction
Citrus canker is one of the most devastating diseases caused by *Xanthomonas axonopodis* pv. *citri* and is of great economic importance all over the citrus growing area of the world including India, affecting fruit production, productivity and economic value of citrus crop. There are three types of citrus canker disease caused by different pathovars and variants of the bacteria viz; Canker A caused by group of *X. axonopodis* pv. *citri* strains originally found in Asia, Canker B caused by group of strains of *X. axonopodis* pv. *aurantifolli* strains originally found in South America and third is Canker C caused by the same form as Canker B *i.e. X. axonopodis* pv. *aurantifolli* originally found in Brazil (Gottwald *et al*., 2002) [8].

2. Material and methods
2.1. Experimental site
Experiment was conducted in glasshouse and biocontrol laboratory, Department of plant pathology, college of agriculture, G.B.P.U.A.&T., Pantnagar. For the greenhouse experiments, one-year-old lemon plants were used and they were maintained at 25–30 °C and 60% relative humidity. Lemon plants were foliarly sprayed with different treatments. Biocontrol agents used as treatments were procured from Biological control laboratory, Department of plant pathology, college of agriculture, G.B.P.U.A.&T., Pantnagar, Uttarakhand, India.
Immature leaves of plants were inoculated using injection with *X. axonopodis* pv.*citri* (Pantnagar isolate), 48 hours post treatment. Bacterial suspension was prepared from 24 hours old culture of *X. axonopodis* pv.*citri* and adjusted to a 7×10^8 (Cfu) by adding sterilized distilled water. Activity of antioxidant enzymes: catalase, peroxidase, and phenylalanine ammonia-lyase were investigated at 48 hours post treatment and 24, 48, and 72 hours post inoculation spectrophotometrically.

The treatments used in pot experiment under glass house conditions were as under.

S. No.	Treatments
T 1	L-arginine
T 2	L-methionine
T 3	(γ-aminobutyric acid) GABA
T 4	Salicylic acid
T 5	Streptocycline
T 6	Blitox-50
T 7	Pant bioagent 1(*Trichoderma harzianum*)
T 8	Pant bioagent 2 (*Pseudomonas fluorescens*)
T 9	Pant bioagent 3 (*Trichoderma harzianum* + *Pseudomonas fluorescens*)
T 10	Untreated / Check

2.2. Biochemical estimation of defense related parameters
Defense related compounds

2.3 Phenylalanine ammonia lyase (PAL) activity

Phenylalanine ammonia lyase activity was determined by the method given by Edward and Kessmann (1992) [6]. Homogenization buffer consisted of 25mM tris buffer (pH 8.8). Reaction mixture was prepared by adding 0.1 ml of enzyme extract and 0.4ml of 0.05 M tris buffer (pH 8.8) containing 0.2mM L-phenylalanine and was incubated in water bath at 37 ºC for 60 min. Reaction was stopped by adding 0.1 ml of 0.5 N HCl. The trans-cinnamic acid was extracted by adding 2ml of toluene. The absorbance was taken at 412nm and the enzyme activity was expressed in µmol trans-cinnamic acid $min^{-1}g^{-1}$ fresh weight.

2.4 Catalase (CAT) activity

Leaves were homogenized in common extraction buffer (Phosphate buffer). This homogenised leave extract is enzyme extract. Catalase activity was determined spectrophotometrically according to method described by Dihindsa *et al*. (1981). The reaction mixture consisted of 50 mM phosphate buffer (pH 7), 15 mM of H_2O_2, and 100 µl diluted enzyme extract in a total volume of 2 ml. The decomposition of H_2O_2 was followed by a decline in absorbance at 415 nm. Catalase activity was expressed as ΔA_{415}/min/mg protein.

2.5 Peroxidase (PO) activity

Peroxidase activity was determined using the method as described by Tatiana *et al*. (1999) [17]. The reaction mixture contained 0.05 M phosphate buffer (pH 5.5), 2 per cent H_2O_2, 0.05 M guaiacol and 0.1 ml enzyme extract in a final volume of 5ml. The reaction was started after the addition of enzyme extract. The formation of tetraguaiacol was measured at 470 nm. One unit of enzyme was defined as the amount of enzyme to decompose 1 µmol of H_2O_2 min^{-1} at 25 ºC. Peroxidase activity was expressed as ΔA_{470}/min/mg protein.

3. Result

The enzymatic activity of the antioxident enzymes viz; Phenylalanine ammonia lyase (PAL), Catalase (CAT) and Peroxidase (PO) against *X. axonopodis* pv.*citri* for different treatments was evaluated and the present study revealed that these can induce defense in citrus plant. A large number of studies on various species indicated that stress alters the amount of the defense related compounds and the activities of enzymes involved in scavenging of reactive oxygen species (Hernandez *et al*., 2002; Gosset *et al*., 1994 and Gueta *et al*., 1997) [12, 14, 9].

3.1 Phenylalanine ammonia lyase (PAL) activity

In glasshouse study, an increase in Phenylalanine ammonia-lyase activity in citrus plants was observed in all the treatments including control after pathogen inoculation. Treatments L-methionine (10.68) and Pant bioagent 3 (8.24) increased the PAL activity (µg cinnamic acid/mg /min) to a higher level as compared to control (1.92) before inoculation of the pathogen. While maximum PAL activity (µg cinnamic acid/mg /min) at 72 hours post inoculation was observed in Pant bioagent 3(24.64) followed by Pant bioagent 2 (24.37), L-methionine (23.95), GABA (23.26), Salicylic acid (13.42) and Streptocycline (12.56) compared to control (3.45) having significantly minimum PAL activity. Maximum fold increase in PAL activity was observed in GABA (11.54) followed by Pant bioagent 2(8.06), and Pant bioagent 1(5.67) at 72h hours post inoculation over before inoculation of pathogen (Fig.1).

Phenylalanine ammonia-lyase is the first enzyme in the metabolic pathways of phenylpropanoids in plants that catalyzes the conversion of phenylanine to trans-cinnamic acid which act as precursor for flavonoids, phytoalexin, and lignins that contribute to plant defense systems (Hahlbrock and Scheel, 1989) [10]. Phenylalanine ammonia-lyase also participates in the biosynthesis of the defense hormone salicylic acid, which is required for both local and systemic acquired resistance in plants (Dixon and Paiva 1995) [3]. In the present investigation the induction of phenylalanine ammonia-lyase activities increased significantly in plants treated with bioagents, L-methionine, GABA and Salicylic acid. This result is in agreement with previous findings that phenylalanine ammonia-lyase is involved in increasing resistance and significantly increases in response to the stimulation of different resistance elicitors in citrus fruit (Droby *et al*. 2009; Ballester *et al*. 2010) [5, 1]. In plants, GABA accumulation was observed in response to biotic and abiotic stresses (Shelp *et al.*, 1999; Roberts, 2007) [16]. Sharifi-Sirchi *et al.* (2011) [15] reported that BABA and GABA might have an important role in turning on defense signaling pathway against *X. citri* subsp. *citri* infection.

The results are in accordance with the finding of Hasabi *et al.*, 2014 [11], who observed that amino acid L-methionine significantly increased phenylalanine ammonia-lyase activity in the citrus plants and thus induced resistance.

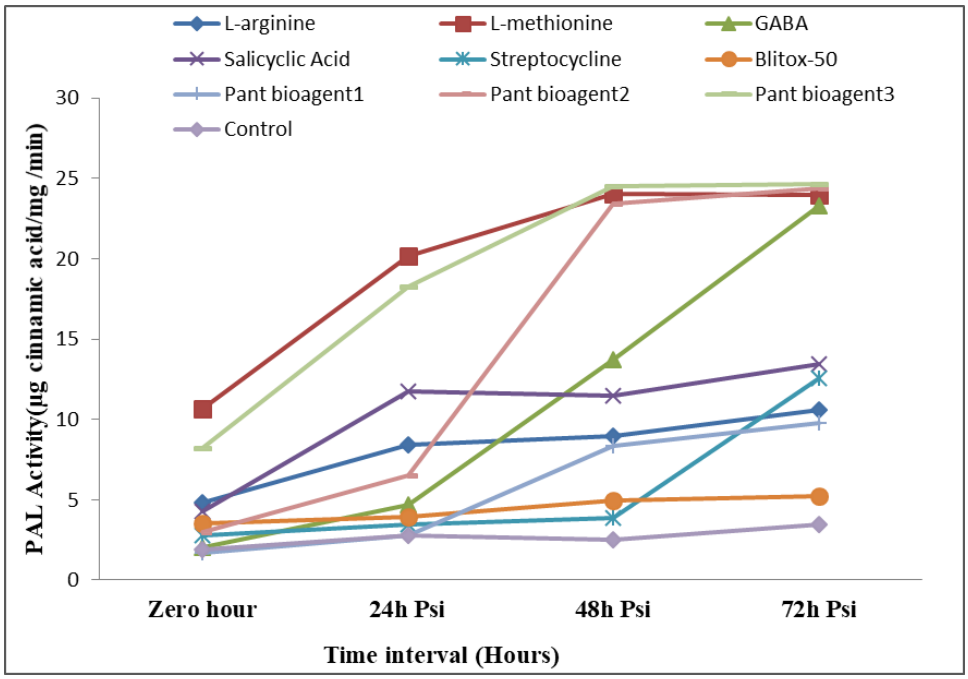

Fig 1: Phenylalanine ammonia lyase (PAL) activity in *Xanthomonas axonopodis* pv. *citri* isolate

3.2. Catalase (CAT) activity

In glasshouse study, an increase in catalase activity in plants was observed in all the treatments including control at 24 hour after pathogen inoculation. The results showed that catalase activity in plants pretreated with L-methionine, GABA, Streptocycline, blitox-50, Pant bioagent 1, Pantbioagent 2 and Pant bioagent 3 was significantly increased at 24 hour Post inoculation (24h Psi) over catalase activity before inoculation of the pathogen, then it followed a decreasing trend upto 72h Psi. While catalase activity pretreated with L-arginine and Salicylic acid was significantly increased upto 48hPsi and then decreased at 72 h Psi. It seems that, in these conditions, the antioxidant activity of plant catalase was active and scavanging the reactive oxygen species. Catalase has been reported as a free radical scavenger and as a suppressor mechanism in a number of host- pathogen/ elicitor interactions (Scandalios, 1993). Significantly maximum catalase activity (ΔA_{415}/mg /min) at 24h Psi was observed in Pant bioagent 3 (0.022) followed by Pant bioagent 2 (0.014), L-arginine (0.0132), L-methionine (0.0129) and Pant bioagent 1 (0.0126) while minimum activity was recorded in GABA (0.0067), followed by blitox-50 (0.0092), Salicylic acid (0.0101) and control (0.011) (Fig.2). The enzyme activity treated with GABA, blitox-50, Salicylic acid compared to control was not significant. The present findings are in accordance with the report of Hasabi *et al.*, 2014 [11] who observed that catalase activity, in cirus plants treated with Amino acids compared to the control, was not significant.

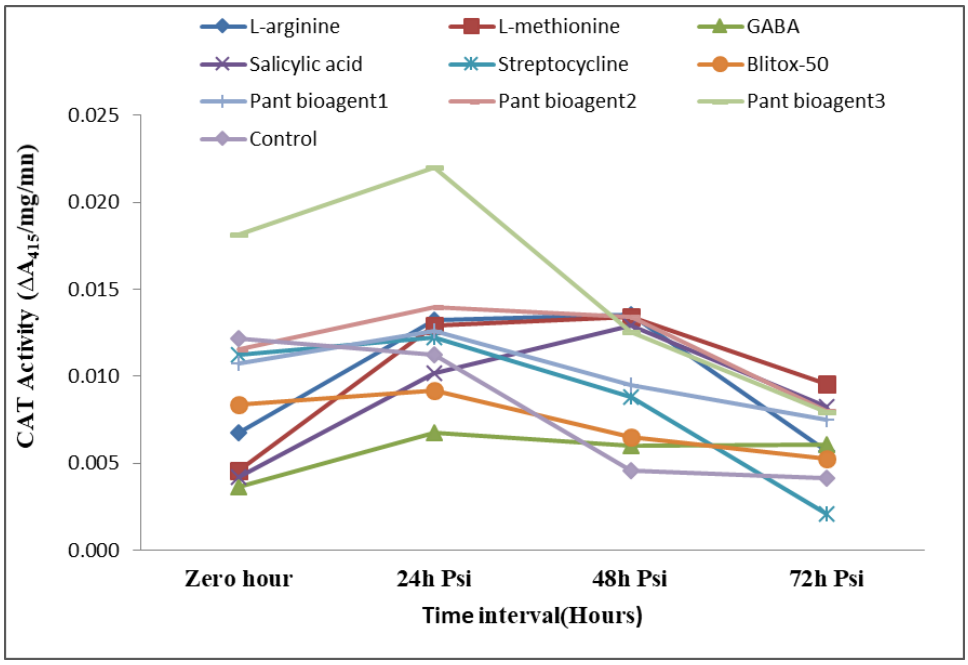

Fig 2: Catalase (CAT) activity in *Xanthomonas axonopodis* pv.*citri* isolate

3.3. Peroxidase (PO) activity

The result indicated that with time there is increase in Peroxidase activity in citrus plants by all the treatments including check after pathogen inoculation. Before inoculation treatments Pant bioagent 3 (0.0133), Pant bioagent 2 (0.0126) and L-methionine (0.0110) showed the higher level of the PO activity (ΔA_{470}/mg /min) as compared to check (0.0048). While at 72h Psi maximum PO activity (ΔA_{470}/mg /min) was observed in Pant bioagent 3(0.0191) followed by Pant bioagent 2 (0.0172), Pant bioagent 1 (0.0166) and L-methionine (0.0163). While minimum PO activity was shown by L-arginine (0.0116) and Control (0.0125) (Fig. 3)

Peroxidase controls the availability of H_2O_2 in the cell wall, which is a prerequisite for the cross-linking of phenolic groups in response to various external stresses, such as wounds, pathogen interactions and environmental constraints, through the formation of a physical barrier of lignin or suberin. Thus it protects plant from stress conditions. High concentrations of phenolic compounds around wounds or pathogen-infected areas can restrict or weaken the pathogen growth (Reimers and Leach 1991) [13]. Ballester *et al*. (2010) [1] found that soluble peroxidase contributes to the beneficial effect of pathogen infection treatment by reducing disease incidence. Thus, the increase in peroxidase is one of the markers of induced resistance. The present findings are in accordance with the report of Hasabi *et al.*, 2014 [11], who reported that peroxidase activity showed pretreatment of plants with amino acid methionine increased the activity of this enzyme at 48 and 72 hours post inoculation in citrus plant.

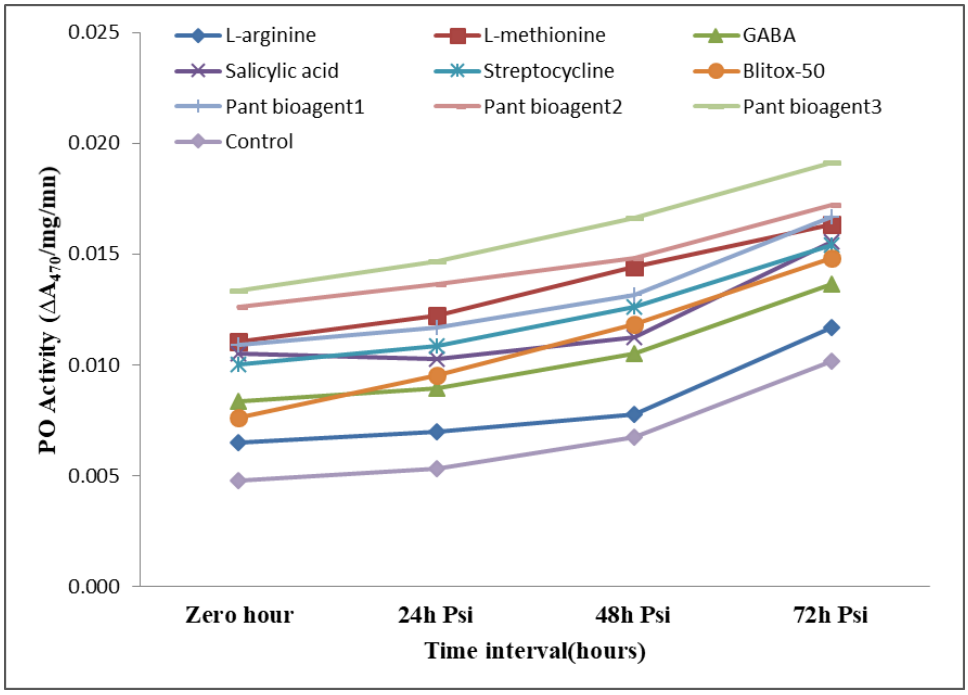

Fig 3: Peroxidase (PO) activity in *Xanthomonas axonopodis* pv.*citri* isolate

4. Conclusion

The results from the study indicated that pretreatment of citrus plants with Pant bioagent 2, Pant bioagent 3, L-methionine, GABA and Salicylic acid induced the levels of plant defense and thus induced disease resistance against *X. axonopodis* pv.*citri*. These compounds can be used as effective alternative compounds to copper bactericides for the management of citrus canker. Since the use of copper bactericide for long term induces copper resistance in Xanthomonands population. Also the excessive use of copper bactericides led to accumulation of copper in citrus soils that has potential phytotoxic and adverse environmental effects. So their usage should be minimized and safer strategies as biological compounds application should be more publisized in future.

5. References

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