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Chemical Inducers in Priming the Induction of Defense Enzymes and Phenols in Banana and Resistance to Soft Rot Disease Caused by *Pectobacterium carotovorum* subsp. *carotovorum*

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Chemical inducers facilitate broad-spectrum of induced resistance against plant pathogens and thus reduced the toxicity of the chemicals used in excess by the farmers for the management of plant diseases. Banana soft rot incited by *Pectobacterium carotovorum* subsp. *Carotovorum* (*Pcc*) cause huge economical loss to the farming if infected at an early stage. In the present study, chemical inducers *viz.*, salicylic acid, potassium silicate, potassium sulphate, fosetyl aluminium, humic acid @ 1000 ppm and COC 3g/lit + streptocycline 300ppm (check) were tested against *Pcc*under glass house conditions. The results revealed that salicylic acid @ 1000 ppm effectively reduced the soft rot disease incidence (58.33 % disease reduction over inoculated control) and it was followed by potassium silicate (50 %) and fosetyl aluminium (45.83 %). Besides, chemical inducers induced defense enzymes *viz*., PO, PPO, PAL, SOD and phenols on 5thday after inoculation in addition to plant growth promotion. Thus, it is concluded that resistance is induced in banana by chemical inducers and play a major role in the management of banana soft rot disease.

Introduction

Banana (Tomlinson *et al.,* 1987) is an important fruit crop which serves as a good source of food for millions of people all around the world. Internationally, it is the fifth largest agricultural commodity after cereals, sugar, coffee and cocoa and are rich in vitamins, minerals, carbohydrates, flavanoids and phenols(Winston and Beck, 1999). Tissue culture technique is widely employed for its propagation throughout the world, where India leads highest production in the world (Singh *et al.,* 2011). Banana is affected by many fungal, bacterial and viral diseases, among which soft rot/rhizome rot caused by *Pectobacterium carotovorum* subsp. *Carotovorum* (*Pcc*) is one of the serious disease. The pathogen is an important threat in India, mostly to the young micropropagated plants and cause a yield loss upto 77-80% (Loganathan *et al.,* 2019, Rajamanickam *et al.,* 2018).

Plant generally develops unique defense mechanism against biotic stresses as Induced Systemic resistance (ISR) and / Systemic Acquired Resistance (SAR) (Van Wees *et al.*, 2008). In this study, chemical exploiters and otherwise known as chemical inducers act as an alternative promising tool to enhance the primary endogenous immunity of plants to combat pathogenic invasions rather than kill the pathogens directly by activating various defense biosynthetic pathways (Zhou and Wang, 2018, Thakur and Sohal, 2013). Chemical inducers or exploiters such as salicylic acid, potassium silicate and fosetyl aluminium helps in minimal inhibition of bacterial pathogen and enhance defense mechanism in plants (Sampath kumar *et al.,* 2018). Similarly, exogenous application of salicylic acid has found to inhibit various bacterial pathogens *viz., Dickeya solani* (Czajkowski *et al.,* 2015), *Pectobacterium carotovorum* and *Pseudomonas syringe* (Lagonenko *et al.,* 2013). Potassium silicate helps to promote plant growth activity in banana (El-mehrat *et al.*, 2017) and spraying of fosetyl aluminium helps in inhibition of *Erwinia* in fruit crops (Petre *et al.,* 2015).

Materials and Methods

Isolation of pathogen

Soft rot infected banana samples were washed properly in tap water and allowed to dry for few minutes. The infected sample cut in to small rhizome bits (half infected and half healthy) using sterile knife and sterilized using 1% sodium hypochlorite for 30 sec followed by washing three times in sterile water. The sterilized bits were kept in a cavity slide and a drop of sterile water was poured and left free for 5 minutes to squeeze out the bacterial ooze. A loop full of bacterial ooze was taken and streaked on the Nutrient Agar (NA) medium and allowed for incubation at 28ºC for 48hrs. Based on the morphological character, individual colony on the NA medium was taken and streaked in Crystal Violet Pectate (CVP) medium (a specific media for bacterial soft rot pathogens).The pathogen was further confirmed using cavity formation in the CVP medium (Cuppels and Kelman, 1974), positive activity to plant cell wall degrading enzymes, gram's staining, carrot soft rot test and KOH test. Isolates were maintained in 4ºC on NA medium and stored at 80% glycerol stock at -80ºC for long time storage and for future use.

Screening of chemical inducers against *Pectobacterium carotovorum* subsp. *Carotovorum* under greenhouse condition

Five different chemical inducers reported previously for as resistance inducers were tested @ 1000 ppm concentration on one month old tissue culture plant *cv.* Grande naine (AAA) for its efficacy against *Pectobacterium carotovorum* subsp. *carotovorum.* The experiment consisted of eight treatments replicated thrice with five plants per replication. Pot mixture was comprised of soil, sand and compost (2:1:1; v/v) and the soil mixture was pre-sterilized for 30 min in an autoclave at 121 ºC. The treatment was given by each chemical inducer by soil application @ 10 ml / pit followed by soil drenching @ 10 ml / pit @ 30^{th}, $45^{th}$and 90^{th} days after planting (DAP). Copper oxychloride (COC) @ 3g/lit + streptocycline 300ppm on 30^{th}, 45^{th} and 90^{th} DAP served as standard chemical check. An inoculated and un-inoculated control plants were also maintained. The virulent isolate of

Pectobacterium carotovorum subsp. *Carotovorum* (BV1) was inoculated by soil drenching @ 10^8 cfu / ml bacterial suspension and the respective treatments were examined for the disease symptom expression at 30th, 45th and 90th day and calculated the per cent disease incidence (PDI) and plant growth characters *viz*., pseudostem length, pseudostem girth, root length, number of leaves and leaf area were examined at 120 days after the complete experiment.

Assay of defense related- enzymes and total phenols

The chemical inducers treated plants challenged with the soft rot pathogen; *Pectobacterium carotovorum* subsp. *carotovorum*was uprooted carefully without any damage to root tissue at 0,3,5,7, 9 day intervals. Two samples from each replicate in all treatments were taken and kept separately in a sterilized zip-lock cover in ice cool box. The fresh root sample was washed clearly with sterile water and homogenized well with liquid nitrogen in a pre-chilled mortar and pestle and kept in -80 ºC for the estimation of enzymes and phenols (Anita and Samiyappan, 2012).

Assay of peroxidase (PO)

One gram root sample kept in -80 °C was homogenized in 2 ml of 0.1M phosphate buffer (pH -7.0) at 4 °C. The homogenate was centrifuged at 10,000 rpm @ 4 °C for 30 min and the supernatant used for the enzyme activity. The reaction mixture consisted of 2.0 ml of mixture containing 1.0 ml of 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.5 ml of 0.1 M hydrogen peroxide and 0.5 ml enzyme extract and kept in room temperature (28 ±2 $^{\circ}$C) for 3 minutes. The changes in absorbance at 420 nm was recorded at 30 sec intervals for 3 min and the enzyme activity was expressed as changes in the absorbance $min^{-1}g^{-1}$ root tissue (Zieslin and Ben-Zaken, 1993).

Assay of polyphenol oxidase (PPO)

One gram root sample kept in -80 °C was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 10,000 rpm for 3 min at 4 °C and the supernatant served as enzyme source (Mayer *et al.*, 1965). The reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. The reaction was initiated by the addition of 200 µl of 0.01 M catechol and the activity was measured calorimetrically and expressed as change in absorbance at 495 nm min^{-1}g-1 of root tissue (Anita and Samiyappan, 2012).

Assay of phenylalanine ammonia lyase (PAL)

One gram root sample kept in -80 °C was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer (pH 7.0), containing 1.4 mM of 2-mercaptoethanol and 50 mg of insoluble polyvinyl pyrrolidone (PVP). The enzyme extract was filtered through muslin cheese cloth and the filtrate was centrifuged at 10,000 rpm for 30 min at 4 ºC and the supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *etal*. (1984). Sample containing 400 µl 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 ºC. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 m^{-1} (Dickerson *et al.*, 1984). Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} root tissue(Anita and Samiyappan, 2012).

Assay of superoxide dismutase (SOD)

One gram root sample kept in -80 °C was homogenized in 2 ml of 0.2 M citrate phosphate buffer (pH 6.5) at 4 ºC. The homogenate was centrifuged @10,000 rpm at 4ºC for 30min. The supernatant served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT (Giannospolitis and Ries, 1977). The reaction mixture (3ml) consists of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 0.5ml of the enzyme extract and 2 µM riboflavin, the reaction mixture was shaken and placed under a 40-W fluorescent lamp at 25 ºC. The change in absorbance at 560 nm was measured at 30sec intervals for 3 min. The SOD activity was expressed as changes in the absorbance as units g^{-1}tissue (El-Moshaty *et al.,* 1993).

Estimation of total phenols

One gram root sample kept in -80 °C was homogenized in 10 ml of80% methanol 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-Cicalteau reagent (1 N) and the solution was kept at 25 ºC. After 3 min, 1 ml of saturated solution of sodium carbonate and one ml of distilled water was added and the reaction mixture was incubated for 1h at 25 °C. The absorbance of the developed blue colour was measured using a spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol mg^{-1} protein (Zieslin and Ben-Zaken, 1993).

Statistical analysis

The pot culture experiment was conducted by following Completely Randomized Design (CRD) and the statistical analysis was done by following SPSS method (Statistical Package for Social Sciences)and AgRes(Gomez and Gomez, 1984).

Results and Discussion

Effect of chemical inducers against soft rot disease

In the glass house conditions, soil drenching of chemical inducers *viz*., salicylic acid (SA), potassium sulphate (PS), potassium silicate (PSI), fosetyl aluminium (FA) and humic acid (HA) @ 1000 ppm were tested against soft rot disease. Among them, salicylic acid was effective against banana soft rot disease (*Pectobacterium carotovorum* subsp. *carotovorum*),which recorded a minimum disease incidence of 33.54 % followed by potassium silicate (40.06 %) and fosetyl aluminium (43.34 %) which were on-par to each other (Fig 1; Table1). Salicylic acid treatment @ 1000 ppm was also found to show the highest growth promotion *viz.*,increased plant height (29 cm), girth (9 cm), root length (3.47cm) and number of leaves (8.2), followed by potassium silicate treatment. Growth promotion activity was more in plants treated with chemical inducers and also in chemical check *i.e.* COC @ 3g/lit + streptocycline 300ppm, whereas very low growth attributes were observed in the control plants (Table 1).

Induction of defense enzymes and total phenols in banana against soft rot disease using chemical inducers

Banana plants treated with chemical inducers were challenge inoculated with *Pcc.* The highest activity of defense enzymes and phenols were observed in banana roots after 5^{th} day after inoculation of the pathogen. The phenols (Fig 2e) and defense enzyme *viz*., peroxidase (Fig 2a), polyphenol oxidase (Fig

2b), phenylalanine ammonia lyase (Fig. 2c) and superoxide dismutase (Fig 2d) were increased after 24 hours of treatment and the activity increased rapidly upto 96 hours and thereafter declined in the next 48 hours (Fig 2). Among the five inducers, salicylic acid recorded higher PO, PPO, PAL, SOD and phenols activity against *Pcc*, which was followed by potassium silicate, fosetyl aluminium and potassium sulphate which were almost on-par with each other in the production of PO, PPO and PAL activity but SOD and phenol activity showed variation between each chemical inducers (Table 2).

Table.1 Effect of chemical inducers on plant growth promoting activity and soft rot incidence in banana cv. Grand naine under glass house conditions

Sl. NO	Treatments	Percent disease incidence **	Percent disease reduction over inoculated control	Plant height (cm) ***	Plant grith (cm) ***	Number of leaves ***	Leaf area (cm^2) ***	Root length (cm) ***
1.	Salicylic Acid – 1000ppm	33.54 (35.38)	58.33	29.74 (5.45)	8.93 (2.98)	8.2 (2.86)	2346.05	31.47 (5.60)
2.	Potassium Sulphate – 1000ppm	50.00 (45.00)	37.50	24.38 (4.93)	8.31 (2.87)	7.8 (2.78)	1812.09	23.63 (4.85)
3.	Potassium Silicate – 1000ppm	40.06 (39.26)	50.00	27.85 (5.27)	8.63 (2.93)	8.0 (2.82)	2097.15	29.72 (5.44)
4.	Fosetyl Aluminium – 1000ppm	43.34 (41.16)	45.83	26.35 (5.12)	8.61 (2.92)	8.0 (2.82)	2072.57	27.23 (5.21)
5.	Humic Acid - 1000ppm	56.66 (49.02)	29.18	22.96 (4.76)	8.04 (2.83)	7.7 (2.77)	1759.29	25.39 (5.03)
6.	COC 3g/lit + Streptocycline 300ppm (Check)	23.34 (28.89)	70.83	30.27 (5.49)	9.26 (3.3)	8.4 (2.89)	2512.34	32.11 (5.66)
7.	Inoculated control	80.00 (62.45)	-	15.64 (3.95)	6.0 (2.44)	6.0 (2.44)	635.71	8.22 (2.86)
8.	Un-inoculated control	0.00 (0.58)	-	24.81 (4.97)	8.0 (2.82)	7.8 (2.78)	1834.68	20.78 (4.55)
	SEd **CD = (0.05)**	0.72 1.53		0.05 0.10	0.03 0.06	0.02 0.04	0.49 1.05	0.03 0.06

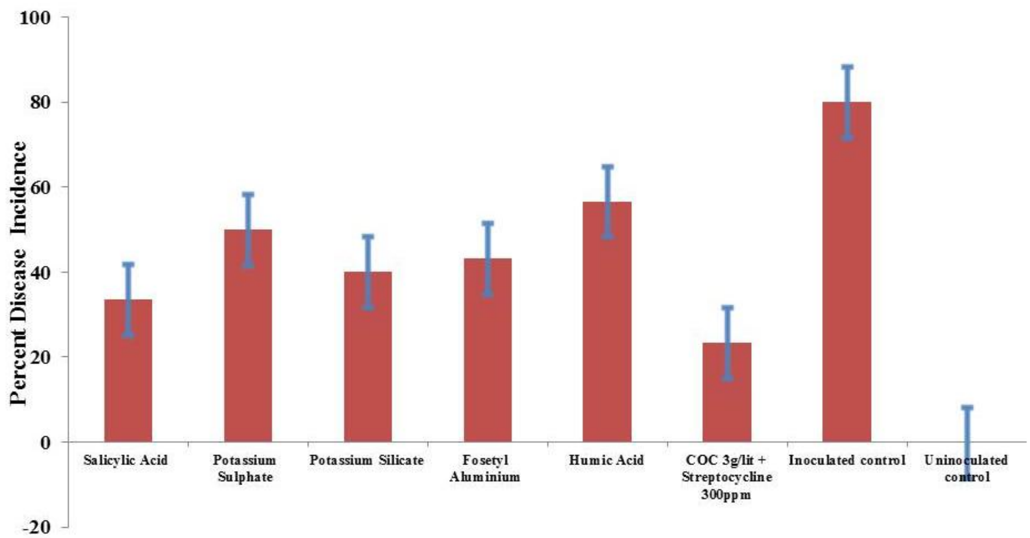
Values are mean of three replications
Values in parentheses are arc sine** and square root *** transformed values
Means followed by a common letter are not significantly different at 5% levels by LSD

Table.2 Defense enzymes activity in banana treated with chemical inducers against soft rot disease caused by *Pectobacterium carotovorum* subsp. *Carotovorum*

SL.NO	Treatments	PO	PPO	PAL	SOD	Total phenols
1.	Salicylic Acid – 1000ppm	2.65^{a}	1.36^{a}	2952.55^{a}	23.54^{a}	820.41^{a}
2.	Potassium Sulphate – 1000ppm	2.01^{ab}	1.10^{ab}	2460.45^{c}	16.53^{b}	707.62^{b}
3.	Potassium Silicate – 1000ppm	2.24^{ab}	1.24^{ab}	2728.47^{b}	16.77^{b}	794.84^{a}
4.	Fosetyl Aluminium – 1000ppm	2.12^{ab}	1.13^{ab}	2805.95^{b}	12.10^{bc}	725.69^{b}
5.	Humic Acid- 1000ppm	1.57^{bc}	0.89_{bc}	2237.61^{d}	10.60^{bcd}	650.13^{c}
6.	COC 3g/lit + Streptocycline 300ppm	1.77^{bc}	0.92^{bc}	1964.94^{e}	10.54^{bcd}	619.58^{d}
7.	Inoculated control	1.06^{cd}	0.59^{cd}	1656.14^{f}	6.13^{cd}	454.21^{e}
8.	Uninoculated control	0.75^{d}	0.41^{d}	1491.27^{g}	4.63^{d}	340.30^{f}
	SEd **CD (P = 0.05)**	0.03 0.07	0.01 0.03	0.39 0.84	0.03 0.06	0.28 0.61

Values are mean of three replications
Means followed by a common letter are not significantly different at 5% levels by LSD

Figure.1 Screening of chemical inducers against banana soft rot disease caused by *Pectobacterium carotovorum* subsp. *Carotovorum*



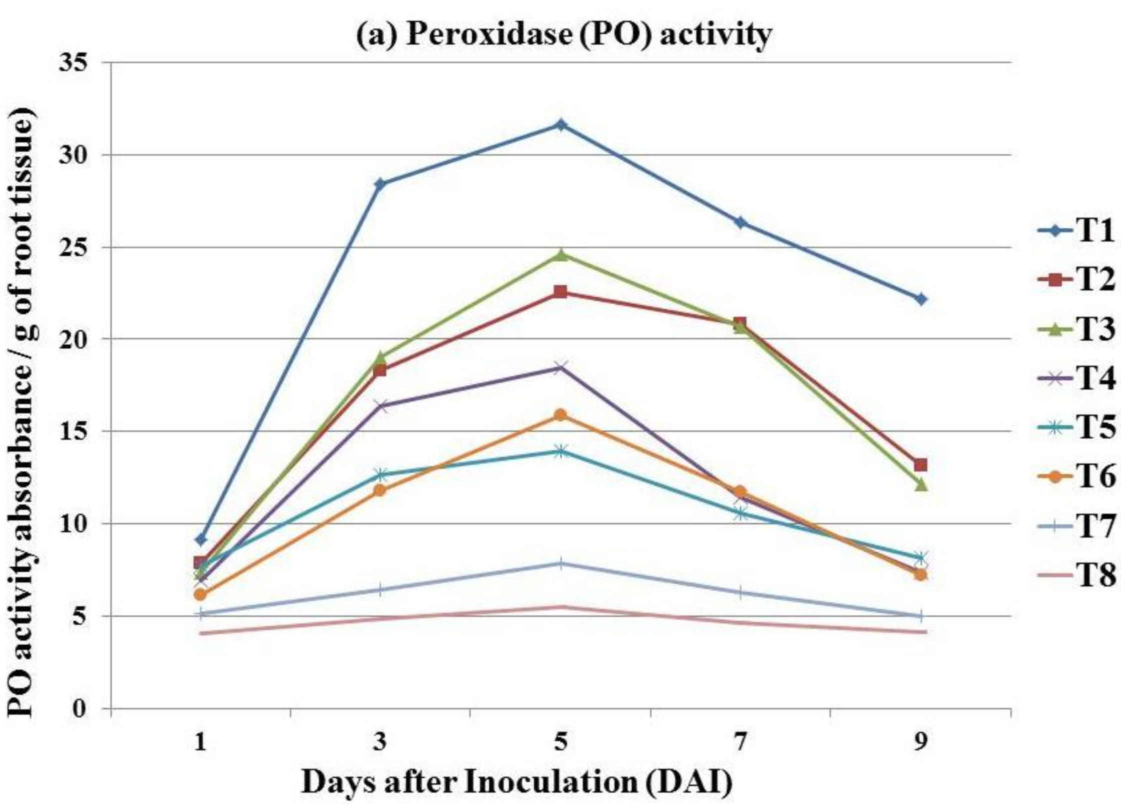
Error bars indicate standard deviation obtained from three replicates per treatment

Figure.2 Expression of defense enzymes in banana cv. Grand naine treated with chemical inducers

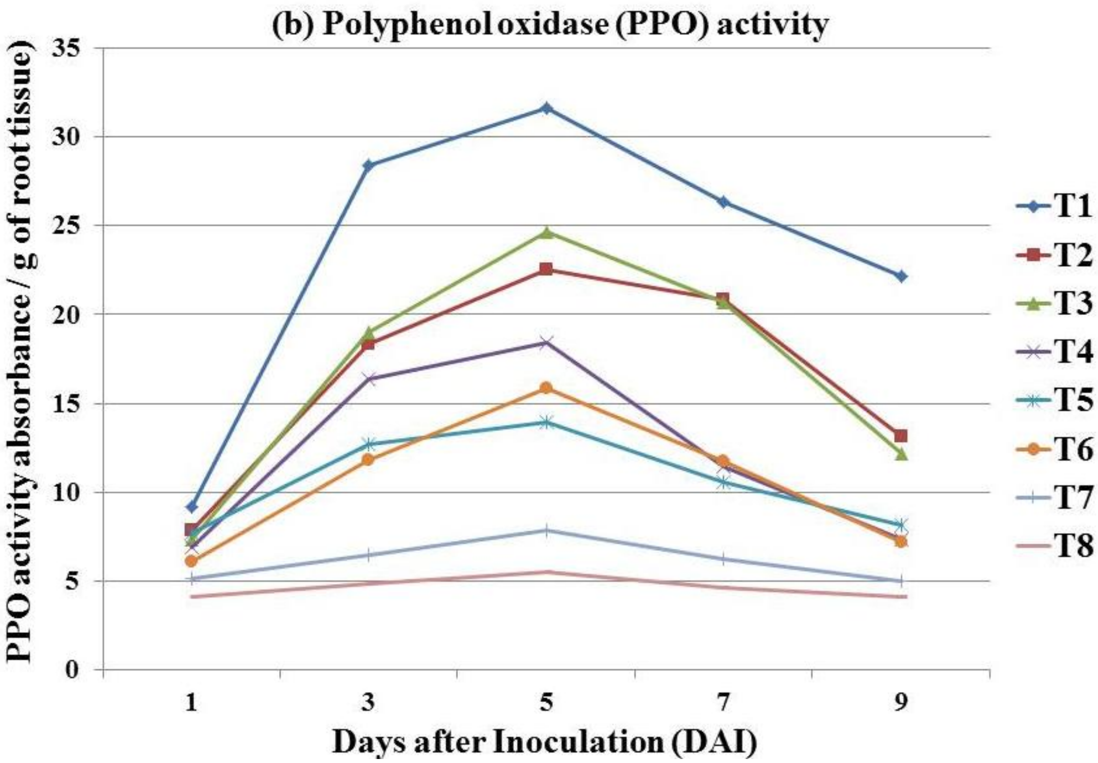
Treatment details

- T1 Salicylic Acid - 1000ppm
- T2 Potassium Sulphate - 1000ppm
- T3 Potassium Silicate - 1000ppm
- T4 Fosetyl Aluminium - 1000ppm
- T5 Humic Acid - 1000ppm
- T6 COC 3g/lit + Streptocycline - 300ppm
- T7 Inoculated control
- T8 Uninoculated control

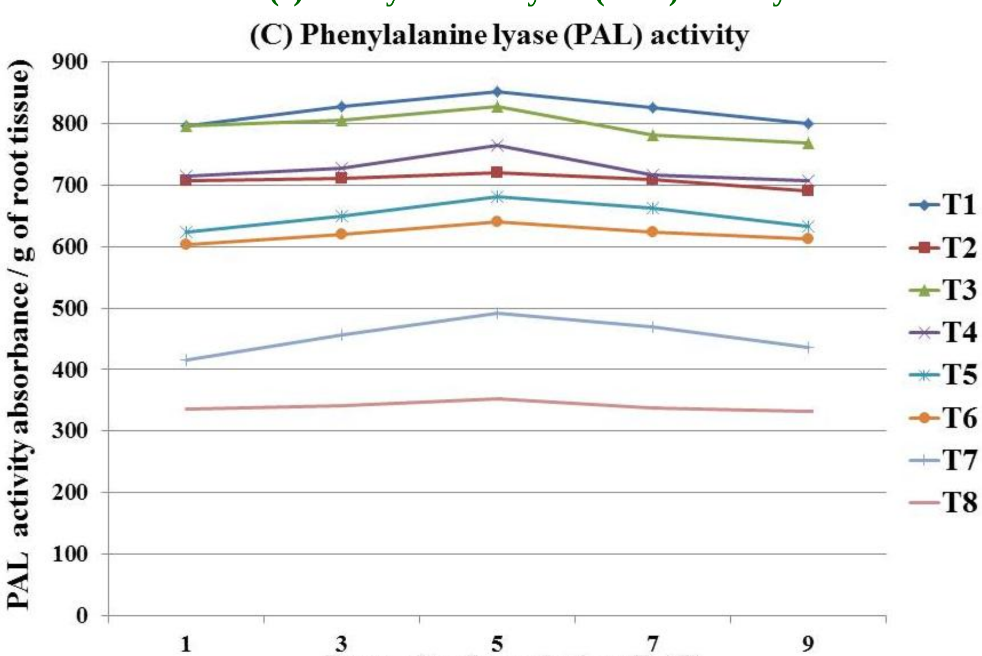
(a) Peroxidase (PO) activity



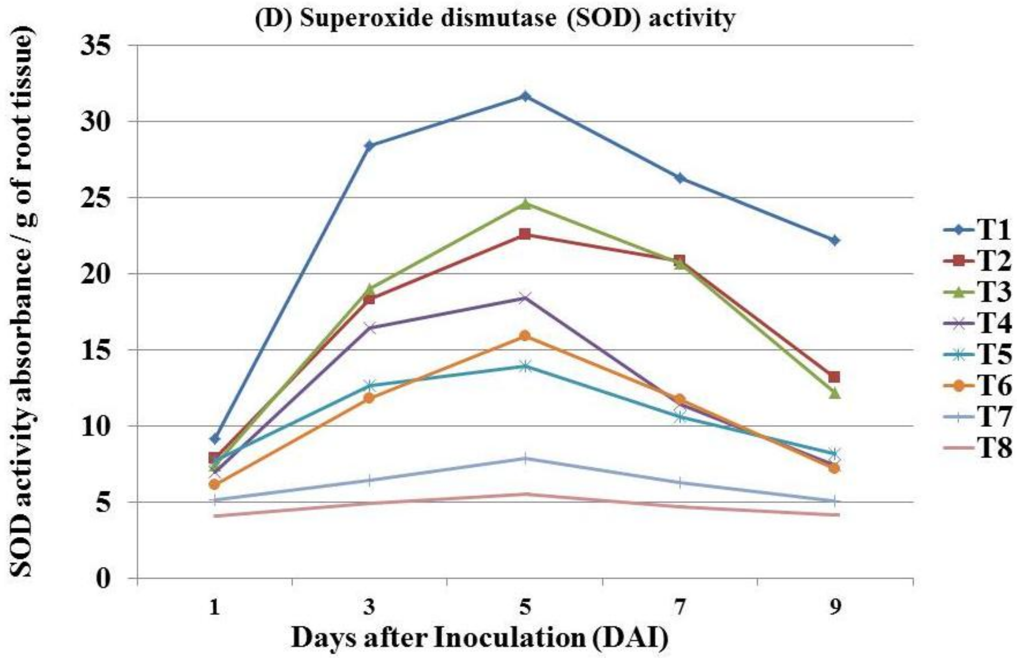
(b) Polyphenol oxidase (PPO) activity



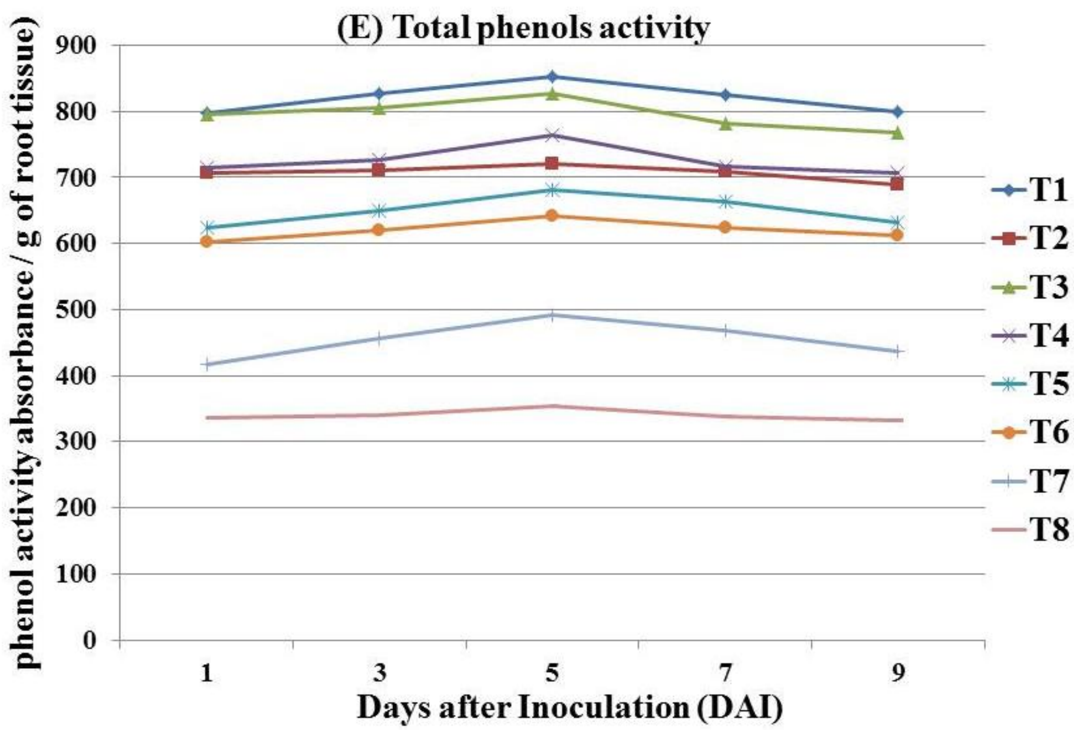
(c) Phenylalanine lyase (PAL) activity



(d) Superoxide dismutase (SOD) activity



(e) Total phenols activity



The study conducted to study the efficacy of chemical inducers *viz.*, salicylic acid, potassium sulphate, potassium silicate, fosetyl aluminium and humic acid evinced that these chemicals act as an elicitor to induce resistance against *Pcc* by enhancing defense related enzymes and phenols in banana. *Pcc* is the major problem in young banana tissue culture plantlets and the initial application of chemical inducers may favor basal resistance and only limited research work has been carried out to manage bacterial rhizome rot or soft of banana (Rajamanickam *et al.,* 2018; Loganathan *et al*., 2019). This is the first study in banana under controlled conditions, where chemical inducers were used to induce defense against *Pectobacterium carotovorum* subsp. *carotovorum*. Ntushelo (2017) reported the inhibitory activity of salicylic acid @ 1200 ppm against *Pectobacterium carotovorum* subsp. *carotovorum* in tobacco and the reduction was found to be 35 per cent but in lower concentration @ 500 ppm, promotion in bacterial growth was observed. In the present study also the pathogen was suppressed upto 33.5 per cent by treating with salicylic acid. Similarly, Thakur and Sohal (2013) reported that salicylic acid reduced the *Ralstonia solanacearum* priming the immune system and depressed the expression of pathogen virulence factor by activating multiple defense actions*viz.,* hypersensitive response, reactive oxygen species (ROS), defense genes expression PR1 and systemic acquired resistance.

Sampath Kumar *et al.,* (2018) reported that cotton bacterial blight caused by *Xanthomonas citri*pv. *malvacearum* was inhibited by salicylic acid and potassium silicate. Petre *et al.,* (2015) assessed the efficacy of fosetyl aluminium in inducing defenses and reported reduction in host susceptibility and disease incidence by *Erwinia amylovora* and *Pseudomonas syringae*. Vicente and Plasencia (2011) reported the role of salicylic acid in biotic and abiotic stress conditions and its molecular mode of action in physiological process that supports plant growth and development in many crops *viz.,* soybean, wheat, maize *etc*. El-mehrat *et al.,* (2017) reported the efficacy of potassium silicate along with compost application in banana cv. Grand Naine and recorded higher plant growth promotions, yield and fruit quality when compared to control plant. Similarly, potassium silicate as combined application by dipping and soil drenching of onion bulbs helps to suppress the onion white rot disease, soil bacterial invasions and also helps to promote p lant fresh weight (El-Sheery, 2017).

Ngadze *et al.,* (2012) analyzed the resistance mechanism in potato against *Pcc* and reported that polyphenol oxidase (PPO), peroxidase, phenylalanine ammonia lyase (PAL), chlorogenic acid, and total soluble phenols imparted resistance to soft rot pathogens (Waleron *et al*., 2002). The application chemical inducers in rice helps to increase the defense enzymes such as ascorbate peroxidase, dehydroascorbate reductase, superoxide dismutase and trigger soil PGPR activity (Liu *et al.,* 2013). Thus, it is concluded that salicylic acid treatment @ 1000 ppm promoted the primary endogenous immunity against *Pcc* through defense enzymes such as PO, PPO, PAL, SOD and phenols and also induced plant growth promotion at the initial planting stage of banana. This can be exploited for the management of banana soft rot.

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