Research Article

Induction of Defense Enzymes in Cotton treated with Chemical Inducers in relation to Resistance against *Xanthomonas citri* pv. *malvacearum*

A. Sampath Kumar[1]*, K. Eraivan Arutkani Aiyanathan[2], S. Nakkeeran[1] and S. Manickam[3]

[1]Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore - 641003, India
[2]Department of Plant Pathology, Agricultural College and Research Institute, TNAU, Madurai- 625104
[3]ICAR-Central Institute for Cotton Research Regional Station, Maruthamalai Road, Coimbatore – 641003, India

Abstract

Chemical inducers such as potassium sulphate (1000 ppm), potassium silicate (1000 ppm), salicylic acid (1000 ppm), fosetyl aluminium (1000 ppm) and humic acid (1000 ppm) along with streptocyline (250 ppm) and copper oxychloride (2000 ppm) as standard chemical control (check) were tested for its effectiveness and induction of defense related enzymes against cotton bacterial blight caused by *Xanthomonas citri* pv. *malvacearum*. Salicylic acid (1000 ppm) and potassium silicate (1000 ppm) effectively reduced the disease and recorded with 38.0 and 37.9 per cent reduction in disease over control, respectively. The defense enzymes namely peroxidise (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and superoxide dismutase (SOD) were assayed for the above treatments in cotton seedlings and then challenge inoculated with *Xanthomonas citri* pv. *malvacearum*. PO, PPO and PAL activities were recorded maximum at 72 h after pathogen inoculation, where as SOD recorded maximum at 24 h after pathogen inoculation. Among the various chemical inducers, salicylic acid (1000 ppm) and potassium silicate (1000 ppm) found to show maximum PO, PPO, PAL and SOD activities.

Keywords: Defense enzymes, chemical inducers, Cotton, *Xanthomonas citri* pv. *malvacearum*

***Correspondence**
Author: Sampath Kumar
Email: sampath000@gmail.com

Introduction

Cotton (*Gossypium* spp.) is recognised as "White gold" and the world's leading natural textile fibre. Cotton is cultivated in eleven states of India. The crop is affected by several diseases and bacterial blight caused by *Xanthomonas citri* pv. *malvacearum* is the major disease occurring in entire cotton growing regions of India. Thirty per cent yield losses are common in different states of India by various races of *X. citri* pv. *malvacearum* [1]. Many synthetic chemicals have been used to induce resistance in plants through various chemical pathways [2] and [3]. Acibenzolar-S-methyl (ASM) was the first chemical inducer successfully commercialised and found to show varied levels of resistance to many pathogens on several plant hosts [4]. Likewise, the salicylic acid treatment in tomato plants challenged with wilt pathogen *Ralstonia solanacearum* recorded significantly increased peroxidase activity and lignin synthesis [5]. The salicylic acid treated citrus leaves showed higher level of phenyl ammonia lyase (PAL) activity and effectively reduced canker disease caused by *Xanthomonas citri* pv. *citri* [6]. The application of salicylic acid suppressed the bacterial leaf blight incidence in rice caused by *Xanthomonas oryzae* pv. *oryzae* [7]. With this background, the present experiment was carried out to test the various chemical inducers for its effectiveness and induction of defense related enzymes in cotton against bacterial blight.

Materials and methods

Efficacy of chemical inducers on bacterial blight incidence

The five different chemical inducers namely potassium sulphate, potassium silicate, salicylic acid, fosetyl aluminium and humic acid were tested at 1000 ppm concentration on cotton seedlings (LRA5166) for its efficacy against bacterial blight of cotton. The seedlings were raised in protrays using coco pith as growing medium. The treatments were given as seed treatment by soaking of seeds for 6 h before sowing and foliar spray at 20 days after sowing. The streptocyline (250 ppm) and copper oxychloride (2000 ppm) served as standard chemical control and applied as slurry seed treatment and foliar spray after sowing. Inoculated and uninoculated controls were also maintained. The virulent isolate of *X. citri* pv. *malvacearum* was spray inoculated (2 X 10^6 CFU/ml) one day after foliar spray of treatments.

The inoculated seedlings were incubated in plant growth chamber at 28º C, 90% RH and 3000 LUX light intensity during day time and 22º C, 90% RH and absence of light during night time (Labtech - LGC 5101, Daihan Labtech India Pvt. Ltd) in the Department of Plant Pathology. Plants were examined for the appearance of disease and the per cent disease index (PDI) was calculated at 20 and 30 days after inoculation of pathogen and the mean PDI was worked out. The per cent disease reduction over control was also calculated. Three replications were maintained.

Assay of defense enzymes

To assay the various enzymes (PO, PPO, PAL and SOD), the leaf samples were collected separately at 0, 24, 72, 120 and 168 h after challenge inoculation of pathogen.

Peroxidise (PO)

The peroxidase enzyme activity was determined by measuring the increase in absorbance at 470 nm due to oxidation of guaiacol to tetraguaiacol [8]. One gram of fresh cotton leaf sample was ground in one ml of 0.1 M sodium phosphate buffer pH 7.0 in a pre chilled pestle and mortar. The homogenate was centrifuged at 12,000 rpm at 4 °C for 15 min. The supernatant was used as enzyme source. The reaction mixture consisted of 2.5 ml of a mixture containing 1.5 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. Enzyme extract (0.1ml) was added to initiate the reaction and change in absorbance of reaction was measured calorimetrically at 470 nm at 30 sec intervals for 3 minutes at room temperature (28 ±2^{o}C) Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. The boiled enzyme was used as blank. Activity was expressed as the increase in absorbance at 470 nm $min^{-1}g^{-1}$ of fresh tissue of leaf sample. Each treatment consisted of three replicates (leaves) and two spectrophotometric readings per replicate using ELICO Double beam SL210 UV VIS spectrophotometer.

Polyphenol oxidase (PPO)

Polyphenol oxidase activity was determined by monitoring the increase in absorbance at 490 nm due to oxidation of catechol [9]. One gram of cotton leaf sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4° C. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant served as enzyme source to assess the polyphenol oxidase activity. The reaction mixture consisted of 1.5 ml of 0.1 M 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 490 nm $min^{-1}g^{-1}$ of fresh leaf tissue.

Phenylalanine ammonia lyase (PAL)

One gram of cotton leaf sample 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 polyvinylpyrrolidone (PVP). The enzyme extract was filtered through muslin cheese cloth and the filtrate was centrifuged at 12,000 rpm for 15 min at 4° C. The resulting supernatant was used as the enzyme source. The PAL activity was determined by the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. 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 30min at 30°C. The amount of trans-cinnamic acid synthesized was calculated by using its extinction coefficient of 9630 $M^{-1}cm^{-1}$ [10]. The enzyme activity was expressed in fresh weight basis as nmol trans-cinnamic acid $min^{-1}g^{-1}$ of leaf sample.

Superoxide dismutase (SOD)

The enzyme extract was prepared by homogenizing 1g leaf tissue of cotton in two ml of 0.2 M citrate phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 12,000 rpm at 4°C for 30min. The supernatant served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT [11]. The assay mixture (3ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 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 in parallel to the sample tubes for blank. Each extract was subtracted from the blank and mathematical difference was then divided by blank and multiplied by 100 to obtain the percentage inhibition of NBT photo-reduction. The SOD activity was expressed in SOD units g^{-1} tissue (50% NBT inhibition = 1 unit) [12].

Results and Discussion

Among the five different chemical inducers, salicylic acid (1000 ppm) and potassium silicate (1000 ppm) effectively reduced the disease incidence and recorded with 38.0 and 37.9 per cent reduction in disease over control (**Figure 1**). These two treatments were statistically on par with each other. Assay of PO activity in cotton seedlings treated with chemical inducers and inoculated with bacterial blight pathogen showed significant differences between the treatments. Among the treatments, the maximum peroxidise activity of 3.93 changes in absorbance/min/g of leaf tissue was observed in salicylic acid (1000 ppm) followed by potassium silicate (1000 ppm) at 72 h after challenge inoculation of the pathogen (**Figure 2**).

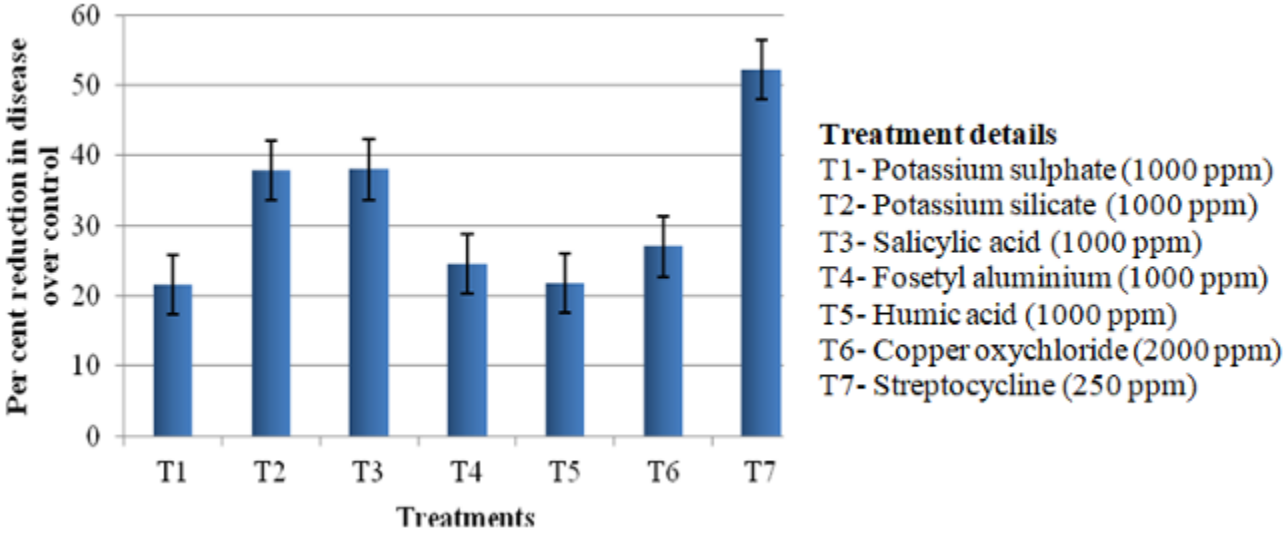
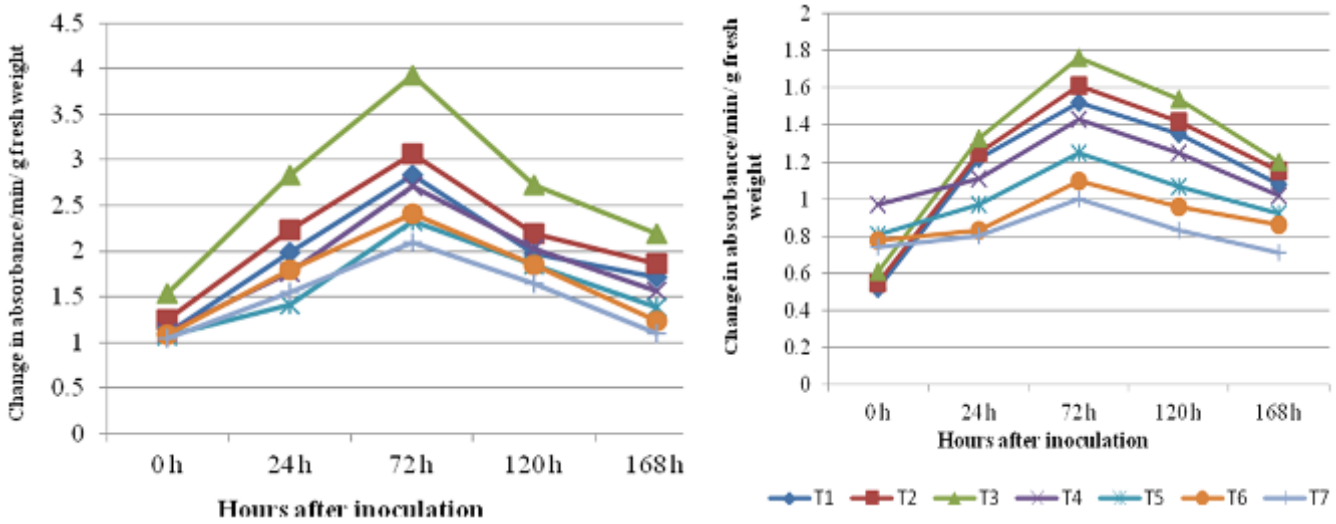


Figure 1 Efficacy of chemical inducers on cotton bacterial blight (*X. citri* pv. *malvacearum*) incidence



(a) Peroxidase (PO) activity (b) Polyphenol oxidase (PPO) activity

Treatment details

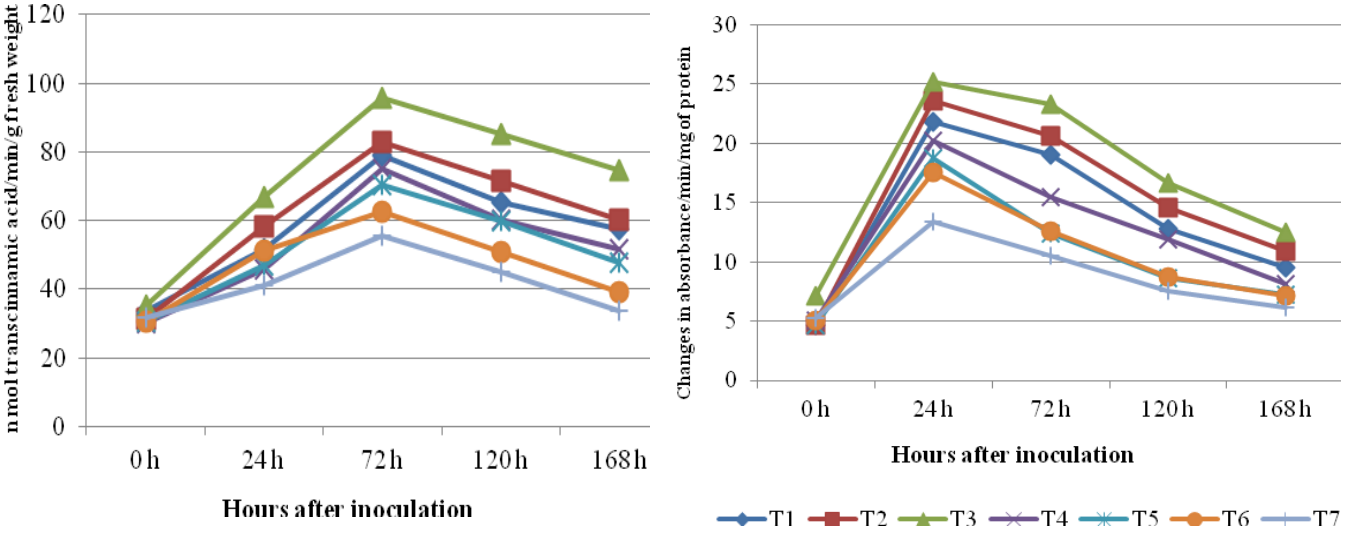
T1-	Potassium sulphate (1000 ppm)	T5-	Humic acid (1000 ppm)
T2-	Potassium silicate (1000 ppm)	T6-	Copper oxychloride (1000 ppm)
T3-	Salicylic acid (1000 ppm)	T7-	Streptocycline (250 ppm)
T4-	Fosetyl aluminium (1000 ppm)		

Figure 2 Induction of defense enzymes in cotton seedlings by chemical inducers (a) Peroxidise (PO) activity (b) Polyphenol oxidase (PPO) activity

The increased activity of PPO was observed in all the treatments. The maximum PPO activity was found in 72 h after various treatments and then followed the declining trend. Salicylic acid (1000 ppm) recorded the highest PPO activity of 1.76 changes in absorbance/min/ g of leaf tissue. Potassium silicate (1000 ppm) and potassium sulphate (1000 ppm) were the next best treatments (Figure 2). These three treatments were statistically on par with each other.

Gradual increase in phenylalanine ammonia lyase (PAL) was recorded in all the treatments up to 72 h after the challenge inoculation of pathogen and reduced after that period. Among the various treatments, salicylic acid (1000 ppm) recorded the maximum PAL activity (95.73 n mol transcinnamic acid/min/ g of fresh weight of leaf tissue), which was found to be more than double the time of pathogen inoculated control (**Figure 3**).

The SOD activity was appreciably increased in all the treatments and reached maximum at 24 h after the treatments and gradually decreased after that period. Increased SOD activity of 25.16 changes in absorbance/min/mg of protein was found in salicylic acid (1000 ppm) treatment, which was four times higher than the pathogen inoculated control. Potassium silicate (1000 ppm) and potassium sulphate (1000 ppm) were the next best treatments (Figure 3).



(a) Phenylalanine ammonia lyase (PAL) activity

(b) Superoxide dismutase (SOD) activity

Treatment details

T1- Potassium sulphate (1000 ppm)

T2- Potassium silicate (1000 ppm)

T3- Salicylic acid (1000 ppm)

T4- Fosetyl aluminium (1000 ppm)

T5- Humic acid (1000 ppm)

T6- Copper oxychloride (1000 ppm)

T7- Streptocycline (250 ppm)

Figure 3 Induction of defense enzymes in cotton seedlings by chemical inducers (a) Phenylalanine ammonia lyase (PAL) activity and (b) Superoxide dismutase (SOD) activity

In the present investigation, salicylic acid (1000 ppm) and potassium silicate (1000 ppm) were found to be superior in reducing the disease incidence. The reduced disease severity and number of lesions of bacterial leaf spot in tomato caused by *X. campestris* pv. *vesicatoria* by application of salicylic acid as foliar spray compared to seedling treatment [13]. The acibenzolar-S-methyl induced resistance in pepper plants against *Xanthomonas campestris* pv. *vesicatoria* by reducing the number and diameter of bacterial spots in growth chamber and open field conditions [14].

Plants induce well-organized and coordinated defense network in response to pathogen attack [15]. Induced resistance is an enhanced defense mechanism against broad spectrum of pathogens developed in plants when appropriately stimulated [16]; [17]. Peroxidase (PO), Polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL) and Superoxide dismutase (SOD) are reported to be the important defense-related enzymes. The use of chemical inducers for defense induction have been reported by earlier workers in various crops such as cotton against *X. ctiri*

pv. *malvacearum* [18] and [19], rice [20], lettuce [21], tomato [5], grapes [22] and citrus [6] against *Xanthomonas* spp.

Peroxidase is one of the key enzymes in the biosynthesis of lignin [23] and other resistance responses like suberization, cross-linking of cell wall proteins, generation of ROS and phytoalexin synthesis [24]. In the present study, seed soaking and foliar application of salicylic acid (1000 ppm) recorded maximum peroxidase activity at 72 h after challenge inoculation of pathogen. The results are in agreement with [18], they reported that salicylic acid (250 ppm) recorded induction of PO activity in cotton seedlings challenge inoculated with *X. citri* pv. *malvacearum* under pot culture conditions when compared to other treatments. The salicylic acid treated asparagus plants exhibited enhanced systemic resistance against pathogenic fungi *Fusarium oxysporum* f.sp. *asparagi* and increased the peroxidise activity as well as lignifications in the plants [25]. Polyphenol oxidase usually accumulates upon wounding and infection by pathogen in plants. In the present study, treatment with salicylic acid (1000 ppm) recorded the maximum polyphenol oxidase activity 72 h after challenge inoculation with *X. citri* pv. *malvacearum*. The induced PPO activity in cotton plants treated with endophytic *Bacillus* challenged with bacterial blight pathogen [26]. The highest activity of polyphenol oxidase was recorded in chick pea plants applied and treated with salicylic acid [27].

Early induction of PAL is important in plant disease resistance since it is the first enzyme in the phenylpropanoid pathway, which leads to the production of phenolic substances and structural barriers like callose deposition [28]. Salicylic acid (1000 ppm) recorded the maximum PAL activity when compared to untreated control in the present study. The same was reported in citrus against canker caused by *X. citri* pv. *citri* using salicylic acid, jasmonic acid, 3-indolacetonitrile, nicotinic acid and folic acid [6]. They recorded significantly low disease severity and higher level of phenylalanine ammonia lyase (PAL) activity in salicylic acid treated leaves. The increased SOD activity has also been observed in salicylic acid (1000 ppm) treated plants, which was four times higher than the pathogen inoculated control. The results are in line with the report of [29], they reported that exogenous application of salicylic acid in common bean increased the expression levels of superoxide dismutase (SOD), malate dehydrogenase (MDH) and phenylalanine ammonia-lyase (PAL) against the infection of bacterial blight pathogen *X. axonopodis* pv. *phaseoli*. The seed treatment and foliar sprays of salicylic acid reduced the disease severity in rice plant challenge inoculated with *X. oryzae* pv. *oryzae*. It also increased the levels of superoxide anion production and hypersensitive response [30].

Conclusion

Among five different chemical inducers tested on cotton against *X. citri* pv. *malvacearum*, salicylic acid (1000 ppm) was found to be the best for reducing the bacterial blight incidence in cotton by enhancing the activities of defense enzymes.

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