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Vitamin B_1 Induced Resistance in *Solanum lycopersicum* Linn against Early Blight Disease

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Abstract

In contemporary times, elicitors have emerged as a viable solution for managing plant diseases due to their cost-effectiveness and widespread accessibility. The utilization of vitamins as elicitors presents a pioneering strategy in combating plant diseases, characterized by its eco-friendly nature. In the current study, vitamin B_1 (thiamine) was used as an elicitor to investigate its effectiveness against early blight (EB) disease in tomato plants (PKM 1 Variety). Thiamine exhibited notable resistance against EB disease when applied at a concentration of 50 mM, showcasing its potential efficacy. Induced defense response that includes the accumulation of free radicals (H_2O_2 and superoxide anion) and antioxidant enzymes (superoxide dismutase, peroxidases, catalase and polyphenol oxidase) was observed in thiamine treated tomato plants. Accumulation of H_2O_2 (DAB staining) and superoxide anion (NBT staining) proved to show the well- established first line of defense against *Alternaria solani* in elicited tomato plants. The study demonstrated that the augmentation of defense enzymes resulted in a concomitant decrease in disease symptoms in tomato plants treated with thiamine, as evidenced by disease scale ratings.

***Key words*:** *Solanum lycopersicum*, Vitamin B_1, Induced resistance, Early blight

Solanum lycopersicum Linn. (Family: Solanaceae) commonly known as Tomato is a best studied and widely cultivated dicotyledonous plant that has been used as a model species for research [1-4]. Moreover, it is the world's second most consumed vegetable after potato [5-7]. Tomato plants are affected by various diseases, among which early blight disease causes 79% of annual economic yield loss [8-9]. EB disease is one among the most destructive diseases affecting tomato, decreasing its productivity by 80% [10-11]. The fungal pathogen, *Alternaria solani* (Ellis & Martin) Sorauer causes the most destructive diseases like collar rot on the basal stem of seedlings, early blight on foliage, lesions on stems of adult plant and rot on fruits of tomato [12-13].

Early blight disease management of tomato is often done by the use of fungicide, cultural practices like crop rotation, burning of infected crops and growing resistant cultivars [14]. However, it is difficult to manage EB disease as the pathogen isolates exhibit well-established variable levels of pathogenicity and prolonged active disease cycles [15]. Induced systemic resistance (ISR) is a mechanism wherein plants are induced or stimulated to express enhanced resistance upon entry of pathogen [16-18]. ISR is successful in opposition to broad spectrum of diseases [19]. It is phenotypically correlated with well- known Systemic acquired resistance (SAR) activated after the first line of infection by an antipathetic necrotizing pathogen [20]. Accumulation of ROS in plants is a sign of stress that causes oxidative damage, inducing the production of superoxide anion and hydrogen peroxide [21]. Plant cells respond to stress (biotic and abiotic) by intensifying the ROS detoxification pathways [22-23]. ROS are detoxified by antioxidant enzymes manufactured in plants during stress [24]. Such antioxidant enzymes include catalase (CAT), Guaiacol peroxidase (GPX), Superoxide dismutase (SOD), ascorbate glutathione cycle enzymes (AsS-GSH) such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX) and Glutathione reductase (GR) [25-26].

Elicitors play one of the major roles in induced systemic resistance in plant [27] and are proved to be successful in plant systems like grapevine [28] and muskmelon [29]. Several studies have revealed that vitamins, such as riboflavin (Vitamin B_2) [30] (Dong and Beer 2000), menadione sodium bisulphite (Vitamin K_3) [31] and thiamine (Vitamin B_1) [32-33] can trigger defense response against disease in plants, flowers or fruits [34-35]. Thiamine is well- known for its fundamental part as a coenzyme in ubiquitous metabolic pathways such as glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway [36]. Some earlier studies reveal that thiamine can trigger PR-1 gene expression in tobacco and induce resistance to Tobacco mosaic virus (TMV) in a Salicylic acid (SA) dependent manner [37]. The present investigation is first of its kind to document the effect of Thiamine (Vitamin B_1) as an elicitor against EB disease and the enhancement of defense mechanism in susceptible tomato plants.

MATERIALS AND METHODS

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Plant material

Solanum lycopersicum (Tomato) seeds susceptible to EB disease (Periyakulam-1) (PKM1) were obtained from Tamil Nadu Agricultural University (TNAU) Coimbatore, Tamil Nadu India. The tomato plants were grown in the pots in a soil mixture ratio of garden soil, vermiculite and compost (1:1:1) and regularly watered. These plants were used for elicitor treatment and pathogen infection after 25 days (Fig 1).

Fig 1 *Solanum lycopersicum* pre treated with different concentrations of thiamine and infecte with Alternaria solani
A) Control; B) Thiamine (25 mM) pre treated; C) Thiamine (50 mM) pre treated; D) Thiamine (75 mM) pre treated; E) *A. Solani*; F) Thiamine (25 mM) pre treated infected with *A. Solani*; G) Thiamine (50 mM) pre treated infected with *A. Solani*; H) Thiamine (75 mM) pre treated infected with *A. Solani*

Fungal culture

The fungal pathogen *Alternaria solani* (Ellis and Martin) Sorauer (Accession No. 7114) was obtained from Marina labs, Chennai and was grown on potato dextrose Agar media for 4 days. Subsequently, fungal mycelial blocks were cut from the media and transferred onto the surface of corn meal agar media. The culture was then incubated in dark at 25 °C for 7 days.

Elicitor treatment

Thiamine hydrochloride (SRL chemicals) was used as an elicitor in this study. Different concentrations (25mM, 50mM and 75mM) of thiamine were prepared with glass distilled water and used as test solution in the form of foliar spray. Twenty-five days old tomato plants of PKM 1 variety with fully expanded leaves were sprayed until runoff with each elicitor concentration. Tomato plants sprayed with glass distilled water were maintained as control.

Pathogen inoculation

After 2 days of elicitor treatment, the leaves of elicitor treated and control plants were inoculated with the pathogen *A. solani*. The leaves were wounded (one wound per leaf) by making punctures with the help of a sterile needle and a mycelial block (5mm diameter) of *A. solani* from the actively growing PDA culture was placed on each wound. Leaf samples for enzyme assay were collected at every 12 hours interval up to 48 hours.

Estimation of protein

Protein quantification was done by the dye binding method of Bradford [38] using Bovine Serum Albumin (BSA) as a standard. A mixture of sodium phosphate buffer, distilled water and Bradford reagent was used as blank. The protein content in the extract of tomato leaves pretreated with thiamine was read at 595 nm in spectrophotometer (Shimadzu LMSP-V325, India). The protein content of the samples was calculated using a standard graph constructed with BSA.

Histochemical staining of superoxide anion and hydrogen peroxide

The level of superoxide anion radicals was visually analyzed using nitro blue tetrazolium chloride (NBT) staining solution as described by Kumar *et al.* [39]. The superoxide anion generated was stained blue. Histochemical staining of H_2O_2 in the leaf samples was done using 3, 30-diaminobenzidine (DAB) staining following the method of Daudi and Brien [40]. The H_2O_2 generated region was stained brown.

Determination of antioxidant enzymes

Catalase (CAT) assay

Catalase activity was assayed quantitatively following the method described by Volk and Feierabend [41]. The increase in absorbance was read at 240 nm (Shimadzu LMSP-V325, India). The enzyme activity was determined using the molar extinction co-efficient 36 $M^{-1}cm^{-1}$. The catalase activity was expressed in min mg^{-1} protein.

Guaiacol peroxidase (GPX) assay

Guaiacol peroxidase activity was assayed quantitatively following the method described by Volk and Feierabend [41]. The increase in absorbance was read at 470 nm (Shimadzu LMSP-V325, India). GPX activity was expressed in $\Delta 470$ mg^{-1} protein.

NATIVE-PAGE was performed in polyacrylamide slab (7% (w/v)) separating and (5% (w/v)) stacking gel according to Davis system [42] for qualitative analysis. The staining method for guaiacol peroxidase was followed according to Ulmer *et al*. [43]. NBT staining of treated and untreated tomato leaves revealed the presence of superoxide anion which is the first molecule of defense response at lower concentration. At 24 and 36 h after treatment with thiamine at a concentration of 50 mM, maximum accumulation of superoxide anion was evident. The results obtained are similar to the study made by Ge *et al*. [29] in Musk melon fruits treated with thiamine as an elicitor.

Ascorbate peroxidase (APX) assay

Ascorbate peroxidase activity was assayed quantitatively following the method of Volk and Feierabend [41]. The APX activity was monitored spectrophotometrically at 290 nm (Shimadzu LMSP-V325, India).

Superoxide dismutase (SOD) assay

Superoxide dismutase activity was assayed quantitatively following the method described by Beauchump and Fridovich [44]. The SOD activity was determined by its reduction of Nitroblue tetrazolium chloride (NBT). The assay was determined spectrophotometrically at 560 nm (Shimadzu LMSP-V325, India). The detection of isoforms by extraction of SOD from the leaves and separation on 7% NATIVE- PAGE gel under non-reducing condition following the method of Davis [42] at 4 °C.

Polyphenol oxidase (PPO) assay

PPOs catalyze the O_2 dependent oxidation of phenols to quinine, reactive molecules that induce cell death and remain barriers to secondary infection [45]. The PPO assay was done by following the method of Brueske [46]. The absorbance was read at 410 nm at 50 C (Shimadzu LMSP-V325, India). The

blank reading contains the above except the enzyme extract. For the detection of isoforms, PPO was extracted from the leaves and separated on a 7% native PAGE gel under non-reducing condition following the method of Davis [42] at 4 °C.

Estimation of photosynthetic pigments

The photosynthetic pigments chlorophyll a, chlorophyll b and total chlorophyll were quantified in the thiamine treated tomato plants at various concentrations following the method of Arnon [47].

$$\text{Chlorophyll a} = \frac{12.7\,(A_{663}) - 2.69\,(A_{645})}{a \times 1000 \times W}$$

$$\text{Chlorophyll b} = \frac{22.9\,(A_{645}) - 4.68\,(A_{663})}{a \times 1000 \times W}$$

$$\text{Total chlorophyll} = \frac{20.2\,(A_{645}) + 8.02\,(A_{663})}{a \times 1000 \times W}$$

Where;
A_{663} is the absorption maxima for Chlorophyll a
A_{645} is the absorption maxima for Chlorophyll b

Direct inhibition assay

Agar well diffusion assay was done by following the method of Perez *et al*. [48] to check the direct inhibition of thiamine against *A. solani*.

Early blight disease assessment

Disease assessment in infected tomato plants from the different thiamine treatment was recorded by giving a severity rating of 0-5 following the method of Pandey *et al*. [49].

Description of disease rating scale (0-5), for early blight in tomato is given as:

0- Free from infection; **1-** <10% surface area covering leaf, stem and fruit infected by early blight; **2-** 11-25% foliage of plant covered with a few isolated spots; **3-** Many spots coalesced on the leaves, covering 26-50% surface area of plant; **4-** 51-75% area of plants infected, fruits also infected at peduncle end defoliation and blightening started. Sunken lesions with prominent concentric ring on stem, petioles and fruits; **5-** <75% area of plant part blighted, severe lesion on stem and fruit rotting on peduncle end.

Statistical analysis

All the experiments were conducted in triplicates and the data obtained were subjected to Analysis of Variance (ANOVA) by two-way method using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Protein quantification

The highest protein content was observed in 75 mM thiamine pre-treated leaves compared to untreated leaves and other treatments. Estimation of protein content in thiamine treated and untreated plants showed that the total protein content was maximum in plants treated with thiamine at 50 mM concentration and inoculated with *A. solani* (Fig 2). The increase in the protein content may be due to the induction of antioxidant enzymes in the plant system. In general, during plant pathogen interaction, there is a modification of protein structures to activate defense responsible genes [50].

In the enzyme staining activity of guaiacol peroxidase, superoxide dismutase and polyphenol oxidase prominent bands were observed in all the thiamine pretreated tomato plants (25 mM, 50 mM and 75 mM concentration). The results of the enzyme staining in Native-PAGE correlated with the observations made in quantitative assays.

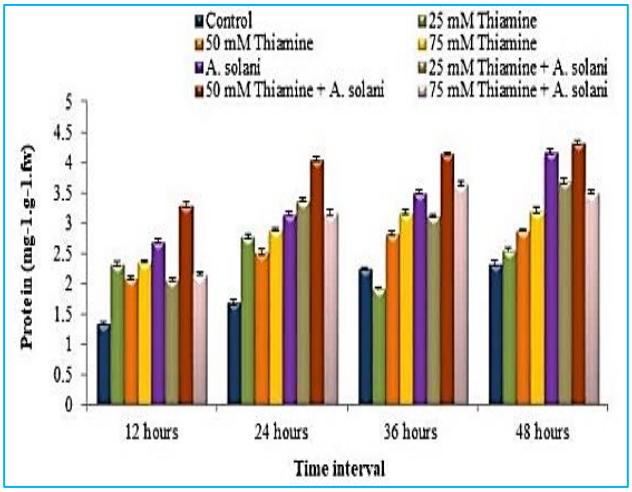

Fig 2 Protein content in leaves of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

ROS - Histochemical staining of hydrogen peroxide (DAB staining)

Accumulation of hydrogen peroxide was macroscopically detected in the leaves of tomato pretreated with thiamine and/or infected with pathogen (Fig 3). Thiamine pretreatment at concentration 25 mM, 50 mM and 75 mM showed accumulation of hydrogen peroxide at a time interval of 24 h, 36 h and 48 h. Higher accumulation of hydrogen peroxide was evident in 50 mM pre-treated leaves at 36 h, whereas at 24 h the hydrogen peroxide accumulation was maximum at 75 mM thiamine pre-treated leaves. Notably, thiamine (50 mM) pre-treated leaves followed by pathogen infection were recorded to have high accumulation of hydrogen peroxide at all-time points.

ROS - Histochemical staining of superoxide anion (NBT staining)

Histochemical analysis was carried out in leaves of experimental tomato plants. Thiamine pre-treated leaves at 50 mM and 75 mM concentration showed rapid accumulation of superoxide anion at 24 h, 36 h and 48 h whereas, pathogen alone infected tomato leaves showed delayed accumulation of superoxide anion at 36 h and 48 h. In thiamine (25 mM, 50 mM and 75 mM) pretreated followed by pathogen infected leaves, there was a rapid accumulation of superoxide anion at 24 h, 36 h and 48 h. Thiamine (25 mM and 75 mM) treatment followed by pathogen infection recorded maximum accumulation of superoxide anion only at 36 h. Thiamine pre-treated leaves followed by pathogen infection showed marked activity at 36 h (Fig 4).

ROS is the first and foremost response to biotic stress [51]. It includes H_2O_2, superoxide anion and singlet oxygen. ROS serves as an important signaling molecule during defense mechanism though ROS leads to cell death, in lower concentrations acts as a regulator for plant defense response [21]. Onset of pathogenic infection on the host plants results in the increased production of Reactive Oxygen Species which causes programmed cell death and also confers systemic resistance. Induced Systemic Response is activated in the host plant only after the proper stimulus is initiated by the pathogen and pest entry Thakur *et al*. [52].

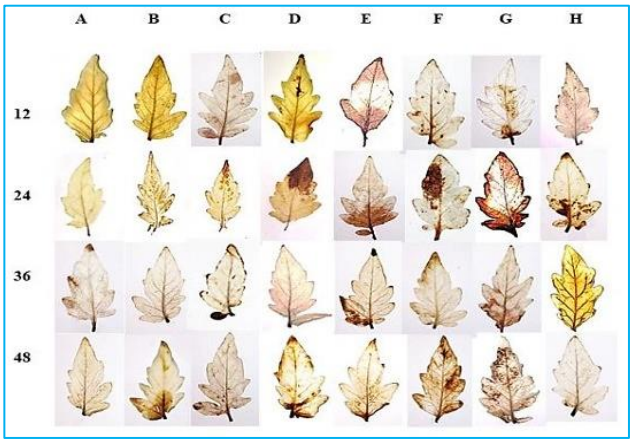

Fig 3 DAB staining of hydrogen peroxide in tomato leaves pre-texted with thiamine and infected with *A. solani*
A) Control; B) Thiamine (25 mM) pre treated; C) Thiamine (50 mM) pre treated; D) Thiamine (75 mM) pre treated; E) *A. Solani*; F) Thiamine (25 mM) pre treated infected with *A. Solani*; G) Thiamine (50 mM) pre treated infected with *A. Solani*; H) Thiamine (75 mM) pre treated infected with *A. Solani*

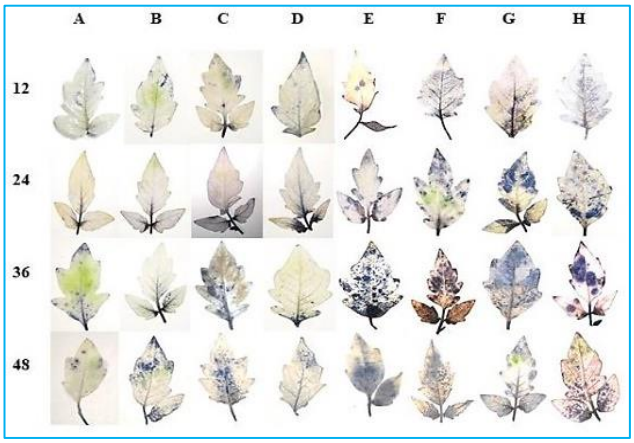

Fig 3 NBT staining of superoxide anion in tomato leaves pre-texted with thiamine and infected with *A. solani*
A) Control; B) Thiamine (25 mM) pre treated; C) Thiamine (50 mM) pre treated; D) Thiamine (75 mM) pre treated; E) *A. Solani*; F) Thiamine (25 mM) pre treated infected with *A. Solani*; G) Thiamine (50 mM) pre treated infected with *A. Solani*; H) Thiamine (75 mM) pre treated infected with *A. Solani*

Catalase activity (CAT)

The catalase activity (CAT) activity was observed to be maximum in thiamine (75 mM) pre-treated leaves and untreated control at 12 h and 24 h respectively, whereas in 36 h and 48 h, the catalase activity (CAT) activity was maximum in thiamine (50 mM) pre-treated leaves. The catalase activity of only pathogen inoculated leaves was recorded to be high at 12 h with a drastic declination of CAT activity 24 h, 36 h and 48 h. The catalase activity in the tomato leaves pre-treated with thiamine (50 mM) followed by pathogen infection was observed to be significantly maximum at 12 h, 24 h, 36 h and 48 h compared to other treatments (Fig 5).

Ascorbate peroxidase activity (APX)

At 12 h and 48 h, APX activity was observed to be significantly high in thiamine (25 mM) pre-treated leaves compared to untreated control and other treatments. The APX activity was observed to be maximum in thiamine (50 mM) and thiamine (75 mM) pre-treated leaves at 24 h and 36 h respectively. There was a significant increase in the APX activity in tomato leaves pre-treated with thiamine (75 mM) followed by pathogen infection at 12 h and 24 h compared to other treatments. In contrast, at 36 h and 48 h the APX activity was recorded to be maximum in thiamine (50 mM) pretreated leaves followed by pathogen infection. Overall, tomato leaves treated with thiamine (25 mM, 50 mM and 75 mM) followed by pathogen infection showed increase in APX activity (Fig 6).

Ascorbate peroxidase (APX) is involved in scavenging hydrogen peroxide to eliminate the damage in host tissues [33]. In the current study, APX was observed to be 3.74 Δ290 $min^{-1}mg^{-1}$ at 48 h in 50 mM concentration of thiamine treated tomato plants. APX activity was induced in tomato leaves significantly by thiamine treatment.

Antioxidant enzymes are molecules that scavenge reactive oxygen species to reduce oxidative stress in infected plants. They include catalase, ascorbate peroxidase, peroxidase, superoxide dismutase [53]. Thiamine treated *Solanum lycopersicum* Linn. var PKM-1 plants were further analyzed for antioxidant enzymes.

Catalase activity mediating the conversion of H_2O_2 into water and oxygen plays an important role in reducing oxidative damage [54]. In the current study, activity of catalase was significant at $p<0.001$ in all the time intervals (24 h, 36 h and 48 h) at 50 mM concentration.

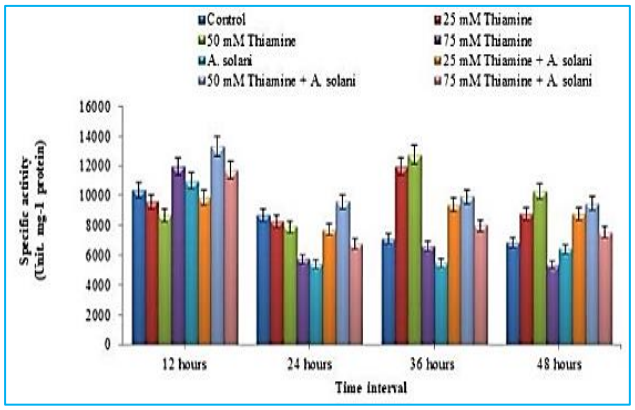

Fig 5 Catalase activity in leaves of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

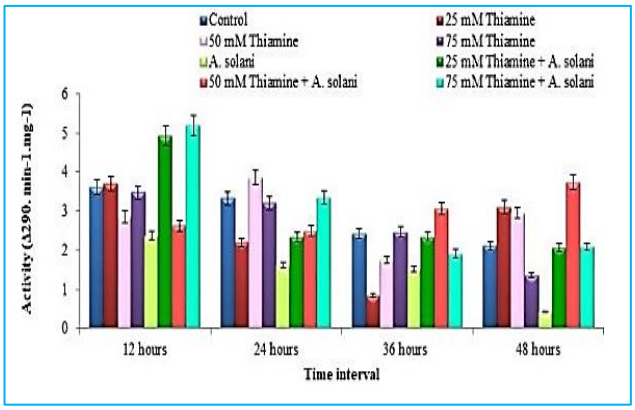

Fig 6 Ascorbate peroxide activity in leaves of *S. lycopersicam* pre-treated with thiamine and infected with *A. solani*

Guaiacol peroxidase activity (GPX)

There was a significant increase in the GPX activity in tomato leaves pre-treated with thiamine (25 mM) at 12 h compared to the untreated control. At 36 h and 48 h, the GPX activity was significantly maximum in thiamine (50 mM) and thiamine (75 mM) pretreated leaves respectively. The GPX activity was maximum in thiamine (75 mM) pre-treated leaves followed by pathogen infection at 12 h. In contrast, GPX activity was maximum in thiamine (50 mM) pre-treated leaves inoculated with pathogen at 24 h and 48 h. At 36 h, GPX activity

was observed to be maximum in thiamine (25 mM) pre-treated leaves followed by pathogen infection (Fig 7).

Guaiacol peroxidase activity (GPX) was observed to be significantly elevated (6.07 Δ470 $min^{-1}mg^{-1}$) at 12 h in 75 mM concentration and at 24 and 48 h in 50 mM concentration when compared to control plants. Decreased level of GPX indicates that thiamine might be involved in the formation of lignin to control the entry and movement of fungal pathogens in the system thereby reducing the disease severity [55].

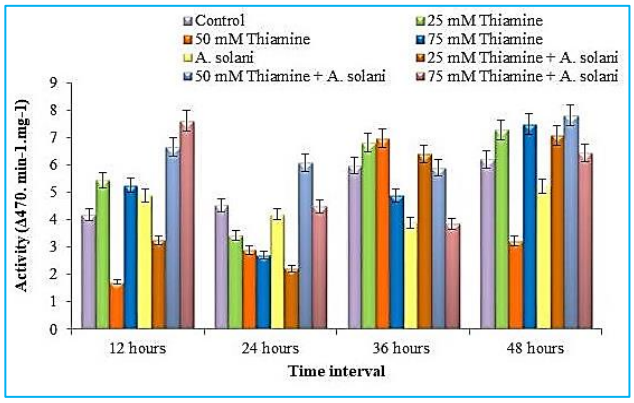

Fig 7 Guaiacol peroxide activity in leaves of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

Enzyme staining activity of guaiacol peroxidase

A rapid increase in the generation of guaiacol peroxidase was evident in thiamine pre-treated leaves at 24 h, 36 h and 48 h (Fig 8). The increase in generation of GPX activity was observed in thiamine pre-treated leaves (25 mM, 50 mM and 75 mM) followed by the pathogen infection whereas only pathogen infected tomato leaves showed a delayed increase in GPX level at 12 h, 24 h and 36 h. Very prominent and distinct GPX enzyme bands were observed in thiamine pre-treated leaves followed by pathogen infection in all time points but only pathogen infected leaves showed less activity.

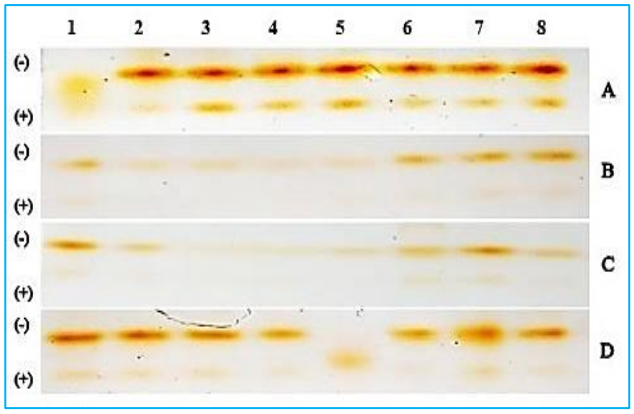

Fig 8 Enzyme activity staining of guaiacol peroxidase of leaves *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

A- 12 hours; B- 24 hours, C- 36 hours, D- 48 hours

Lane 1- Control; Lane 2- Thiamine (25 mM); Lane 3- Thiamine (50 mM); Lane 4- Thiamine (75 mM); Lane 5- *A. Solani*; Lane 6- Thiamine (25 mM) + *A. Solani*; Lane 7- Thiamine (50 mM) + *A. Solani*; Lane 8- Thiamine (75 mM) + *A. Solani*

Superoxide dismutase activity (SOD)

SOD activity was significantly maximum in tomato leaves pre-treated with thiamine (25 mM) at 12 h, whereas in 24 h, SOD activity was maximum in untreated control. In 36 h and 48 h, the SOD activity was maximum in thiamine (75 mM) and thiamine (50 mM) pre-treated leaves respectively. The SOD activity was maximum in thiamine (25 mM) pre-treated leaves followed by pathogen infection at 24 h. In contrast, SOD activity was maximum in thiamine (50 mM) pre-treated leaves inoculated with pathogen at 12 h, 36 h and 48 h. Tomato leaves pre-treated with thiamine (25 mM, 50 mM and 75 mM) followed by pathogen infection showed increase in SOD activity (Fig 9).

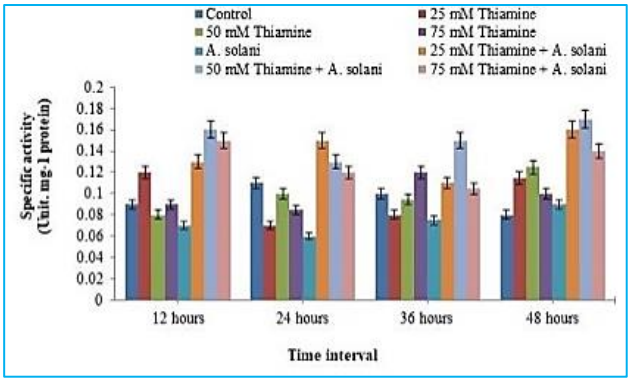

Fig 9 Superoxide dismutase activity in leaves of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

Enzyme staining activity of superoxide dismutase

A rapid increase in the generation of SOD was evident in thiamine pre-treated leaves at 24 h, 36 h and 48 h (Fig 10). An increase in the generation of SOD was observed in thiamine pre-treated leaves (25 mM, 50 mM and 75 mM) followed by the pathogen infection whereas only pathogen infected tomato leaves showed rapid increase in SOD level at 12 h and 24 h whereas, at 36 h and 48 h, it showed less quality of SOD activity. Thiamine (50 mM and 75 mM) pre-treated leaves followed by pathogen infection was recorded to show distinct bands at 24 h, 36 h and 48 h but at 12 h SOD activity was less.

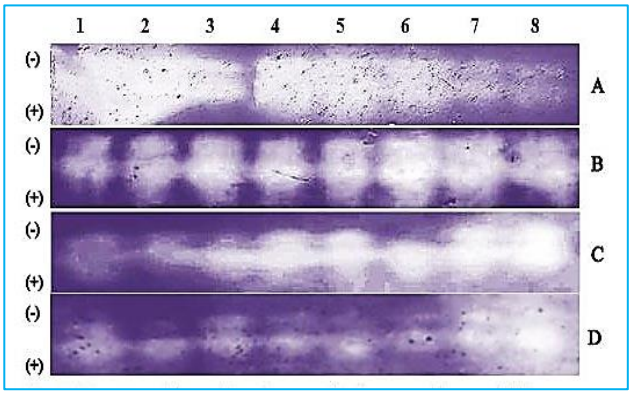

Fig 10 Enzyme activity staining of superoxide dismutase of leaves *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

A- 12 hours; B- 24 hours, C- 36 hours, D- 48 hours

Lane 1- Control; Lane 2- Thiamine (25 mM); Lane 3- Thiamine (50 mM); Lane 4- Thiamine (75 mM); Lane 5- *A. Solani*; Lane 6- Thiamine (25 mM) + *A. Solani*; Lane 7- Thiamine (50 mM) + *A. Solani*; Lane 8- Thiamine (75 mM) + *A. Solani*

Polyphenol oxidase activity (PPO)

PPO activity was recorded to be significantly high in tomato leaves pre-treated with thiamine (50 mM) and thiamine (75 mM) at 24 h and 36 h respectively. At 12 h and 48 h, the PPO activity was high in tomato leaves pre-treated with thiamine (25 mM) compared to the untreated control. The PPO activity was high in thiamine (75 mM) pre-treated leaves

followed by pathogen infection at 12 h and 24 h. In contrast, PPO activity was maximum in thiamine (50 mM) pre-treated leaves inoculated with pathogen at 36 h and 48 h. The recorded results reveal that the PPO activity was observed to be maximal in thiamine pre-treated leaves (25 mM, 50 mM and 75 mM) and thiamine pre-treated leaves (25 mM, 50 mM and 75 mM) followed by pathogen infection (Fig 11).

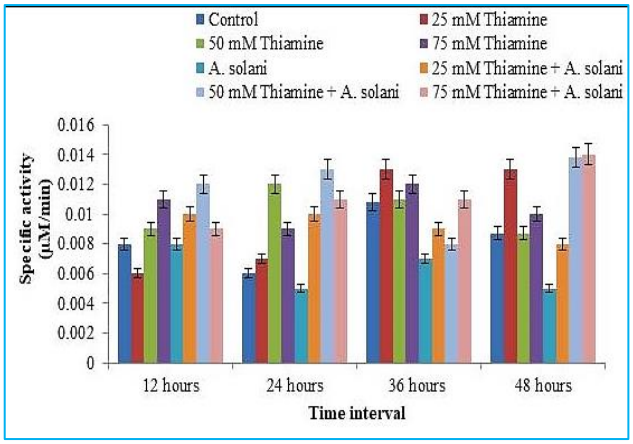

Fig 11 Polyphenol oxidase activity in leaves of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

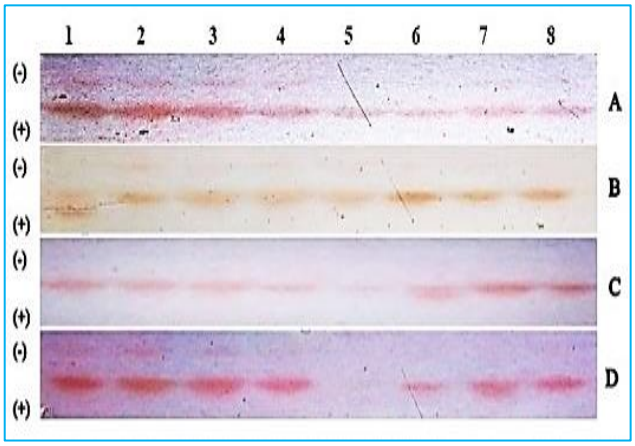

Fig 12 Enzyme activity staining of polyphenol oxidase of leaves *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

A- 12 hours; B- 24 hours, C- 36 hours, D- 48 hours

Lane 1- Control; Lane 2- Thiamine (25 mM); Lane 3- Thiamine (50 mM); Lane 4- Thiamine (75 mM); Lane 5- *A. Solani*; Lane 6- Thiamine (25 mM) + *A. Solani*; Lane 7- Thiamine (50 mM) + *A. Solani*; Lane 8- Thiamine (75 mM) + *A. Solani*

Superoxide dismutase is a significant antioxidant enzyme that converts the anion into hydrogen peroxide and oxygen thereby reducing the stress induced by free radicals. In the present study maximum elevation of the enzyme activity was observed in 50 mM thiamine treated tomato plants. This was found to be significantly more when compared to the other treatments and untreated control. Similar observations were demonstrated in *Mentha piperita* treated with plant-growth promoting rhizobacteria [56].

Enzyme staining activity of polyphenol oxidase

There was a rapid generation of PPO in thiamine pre-treated leaves and untreated control at all time points (Fig 12). An increase in the generation of PPO was observed in thiamine pre-treated leaves (25 mM, 50 mM and 75 mM) followed by the pathogen infection whereas only pathogen infected tomato leaves showed good activity at 24 h. At 48 h, PPO bands were found as a trace in only pathogen infected leaves. PPO activity was evident in all the treatments at all time points compared to untreated control and pathogen infected leaves.

The increase in polyphenol oxidase will decrease the disease severity in infected plants. Also, the oxidative reaction catalyzed by PPO results in fungitoxic polymerization of quinones that induces an incompatible environment for fungal growth [55]. The results indicated that PPO activity was greatly enhanced in all the three concentrations of thiamine treated tomato plants. However, there was a delayed but higher induction of PPO in 75 mM thiamine treated tomato plants when compared to others.

Photosynthetic pigments

Chlorophyll a

In the present study, high amount of chlorophyll a content was recorded in thiamine (50 mM) pre-treated leaves at 24 h compared to other treatments and untreated control (Fig 13). There was an increase in chlorophyll a content in thiamine (25 mM) pre-treated leaves followed by pathogen infection at 36 h. Chlorophyll a content was recorded to be minimum in leaves infected with pathogen at 48 h.

Chlorophyll b

Chlorophyll b content was observed to be maximum in thiamine (50 mM) pre-treated leaves at 24 h compared to other treatments and untreated control (Fig 14). There was a significant increase in chlorophyll a content in thiamine (50 mM) pre-treated leaves followed by pathogen infection at 36 hours, whereas at 48 h maximum chlorophyll b content was recorded in thiamine pre-treated leaves. Chlorophyll b content was recorded to be minimal in leaves infected with pathogen at 48 hours.

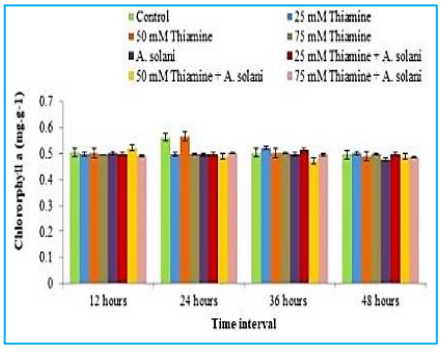

Fig 13 Chlorophyll a content of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

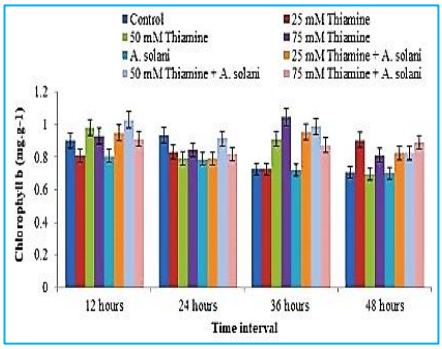

Fig 14 Chlorophyll b content of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

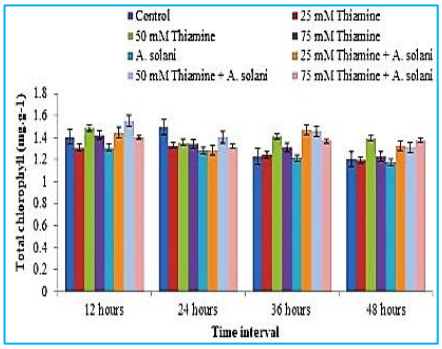

Fig 15 Total chlorophyll content of *Solanum lycopersicam* pre-treated with thiamine and infected with *Alternaria solani*

Total chlorophyll

In the present study, high amount of total chlorophyll content was recorded in 50 mM thiamine pretreated leaves at 12 h compared to other treatments and untreated control (Fig 15). There was a significant increase in total chlorophyll content in 50 mM thiamine pretreated leaves followed by pathogen infection at 12 h. In contrast, total chlorophyll content was recorded to be maximal in 75 mM thiamine pretreated leaves followed by pathogen infection at 48 h. Total chlorophyll content was recorded to be minimum in leaves infected with pathogen at 48 h. Chlorophyll degradation is associated with over accumulation of ROS and an increased susceptibility to necrotrophic fungal pathogens [57]. In the current study, estimation of total chlorophyll content is recorded to be maximum in 75 mM thiamine treated thiamine tomato plants infected with *Alternaria solani*. However, there was a lower content of the same in pathogen infected tomato plants untreated with thiamine. This may be due to the severe damages caused by singlet to the PS I and PS II of infected plants [58].

Direct inhibition assay (Agar well diffusion method)

Thiamine (25 mM, 50 mM and 75 mM) did not show direct inhibition against *Alternaria solani* in direct inhibition assay done by agar well diffusion method (Fig 16).

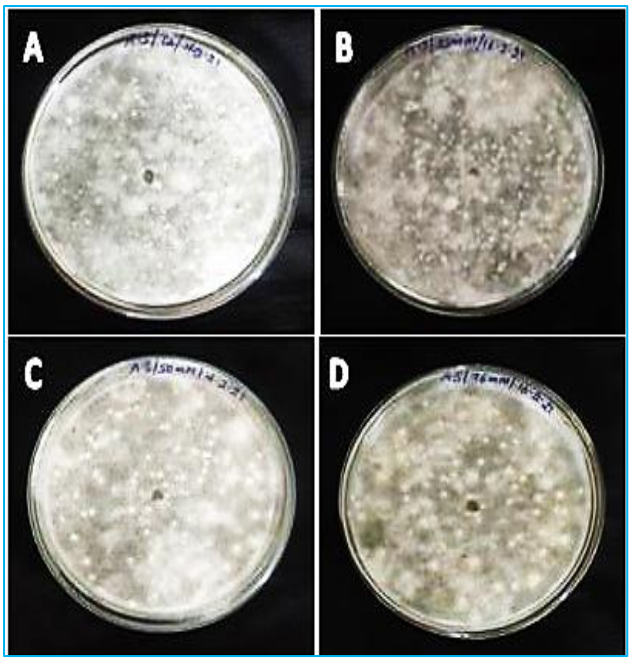

Fig 16 Direct inhibition assay
A) Control; B) 25mM thiamine; C) 50 mM thiamine; D) 75 mM thiamine

Table 1 Disease rating in thiamine treated and control plants

Treatment	Disease rating
Control	0
25 mM Thiamine	0
50 mM Thiamine	0
75 mM Thiamine	0
A. solani	4
25 mM Thiamine + *Alternaria solani*	1
50 mM Thiamine + *Alternaria solani*	0
75 mM Thiamine + *Alternaria solani*	1

Morphological studies for disease assessment

The untreated control plants, thiamine pre-treated (25 mM, 50 mM and 75 mM) plants and thiamine pre-treated (50 mM) plants followed by pathogen infection were rated with scale 0 because these plants were free of early blight symptoms. Thiamine pre-treated (25 mM and 75 mM) plants followed by pathogen infection were rated with scale 1 as they showed limited leaf spots. Only pathogen infected plants were rated with scale 4 as the 50 % to 60 % of leaf surface were covered with spots (Table 1, Fig 17).

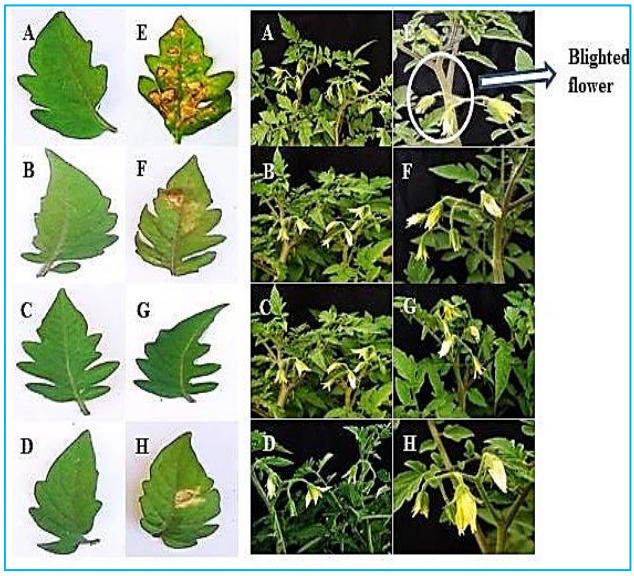

Fig 17 Manifestation of disease symptoms on tomato plants
A) Control; B) Thiamine (25 mM) pre treated; C) Thiamine (50 mM) pre treated; D) Thiamine (75 mM) pre treated; E) *A. Solani*; F) Thiamine (25 mM) pre treated infected with *A. Solani*; G) Thiamine (50 mM) pre treated infected with *A. Solani*; H) Thiamine (75 mM) pre treated infected with *A. Solani*

In the assays conducted for detecting antioxidant enzymes, tomato plants that received treatment with 50 mM concentration of thiamine showed better results than other treatments and untreated control. The present study did not reveal the direct effect of thiamine on *Alternaria* as observed in well diffusion assay although few research studies proved the inhibitory effect of thiamine on spore germination [28], [29]. Morphological assessment of early blight disease severity indicated that the thiamine pretreated tomato plants had a disease scale of 0-1 when compared to tomato plants infected with *Alternaria* of disease scale 3-4 [49]. Severe hypersensitive response to *Alternaria* was observed in control tomato plants whereas the leaves showed no or less symptom when treated with thiamine. Thus, the results observed demonstrates that thiamine could be efficiently used for eliciting resistance in tomato plants against the necrotrophic fungal pathogen *Alternaria* causing early blight disease. Further analysis of defense response pathways is needed to decipher the mode of disease resistance incurred by thiamine. However, thiamine could prove to be a promising alternative eco-friendly, cost-effective disease management strategy to the control of early blight disease of tomato. Modern methods of protecting plants with sustainable utilization of elicitors instead of chemicals and pesticides is the immediate urge to protect the environment and health of the individual with advanced efficacy [59]. In general, plants respond to stress of various ranges and defend actively during pathogen infection. These responses are critical and may reflect on coping up with necrotizing parasites or pathogen infection. Acquisition of defense responses can be triggered prior to infection by a stimulus of pathogen or chemical origin to overcome the stress resulting in the reduction of disease incidence [60]. In the present study, *Solanum lycopersicum* plants (PKM-1) susceptible variety for early blight triggered for

induction of defense responses by treatment with thiamine (Vitamin B_1), which plays a major role as enzymatic cofactor in universal metabolic pathway including glycolysis, pentose phosphate pathway and tricarboxylic acid pathway [36]. Thiamine was used as an elicitor molecule at a concentration of 25 mM, 50mM and 75 mM as foliar sprays to pre-treat 25 days old tomato plants for induction of resistance against *Alternaria solani*.

CONCLUSION

In conclusion, the study investigated the effects of thiamine pre-treatment on tomato plants, focusing on its impact on protein quantification, antioxidant enzyme activities, ROS accumulation, photosynthetic pigments, and disease resistance against Alternaria solani, the causative agent of early blight disease. The results demonstrated that thiamine pre-treatment, particularly at concentrations of 50 mM and 75 mM, significantly enhanced various defence mechanisms in tomato plants. Thiamine pre-treatment led to increased protein content, likely through the induction of antioxidant enzymes, which play crucial roles in defence against pathogen attack. Enzyme staining activities corroborated the quantitative assays, showing prominent bands in thiamine pre-treated plants. Furthermore, thiamine pre-treatment resulted in the accumulation of reactive oxygen species (ROS), particularly hydrogen peroxide and superoxide anion, which are vital signalling molecules in plant defence responses. The activities of antioxidant enzymes such as catalase, ascorbate peroxidase, guaiacol peroxidase, superoxide dismutase, and polyphenol oxidase were significantly elevated in thiamine pre-treated plants, indicating enhanced defence mechanisms against oxidative stress induced by pathogen infection. These enzymes play crucial roles in scavenging ROS and reducing oxidative damage in infected plants. Additionally, thiamine pre-treatment positively influenced photosynthetic pigment levels, particularly chlorophyll a, chlorophyll b, and total chlorophyll content, which are essential for photosynthesis and overall plant health. The study also evaluated the direct inhibitory effect of thiamine on *Alternaria* but did not observe significant inhibition in vitro. Morphological assessments revealed reduced disease severity in thiamine pre-treated plants compared to untreated controls, further indicating the potential of thiamine in enhancing plant resistance against early blight disease. Overall, the findings suggest that thiamine can effectively induce resistance mechanisms in tomato plants against *Alternaria*, making it a promising eco-friendly and cost-effective strategy for early blight disease management. Further studies are warranted to elucidate the underlying defence pathways activated by thiamine and to optimize its application for sustainable disease control in agriculture.

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