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Vitamin B Mediated Priming of Disease Resistance and Defense Responses to Tobacco Mosaic Virus in *Capsicum annuum* L. Plants

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Abstract

Thiamin (B1) and riboflavin (B2) can act as activators and priming factors of defense mechanisms for Tobacco Mosaic Virus (TMV) infection in *Capsicum annuum* plants. Effect of exogenous application of each vitamin on *C. annuum* leaves was demonstrated to induce defense responses and systemic resistance against TMV in the untreated parts of the plant. A range of concentrations was used of both vitamins. 70% of inhibition of TMV for thiamin and 64.1% for riboflavin were achieved, when applied just before virus inoculation. The induction of disease resistance and reduction of virus infectivity in *C. annuum* leaves were determined by indirect ELISA and local lesion host plant assay. The synergetic effect of both vitamins on TMV disease reduction was studied. To investigate the defensive enzymes responsible for the induction of resistance, the levels of Phenylalanine ammonia-lyase (PAL), Polyphenol Oxidase (PPO), and Peroxidase (POD) were examined by specific enzyme assay for each one, and the accumulation of the enzymes was detected 0 to 20 days after treating with the vitamins. Also, the up-regulation and expression of the defense genes POD, PPO, PAL, and some of the pathogenesis related proteins, PR4, PR9, and PR10 were studied by reverse transcriptase polymerase chain reaction (RT-PCR). Application of vitamins B1, and B2 significantly increased the activities of some of the pathogenesis related enzymes, and genes. The possible correlation between timing of application of elicitors and expression of defensive genes was also studied.

Introduction

TMV is one of the most common causes of plant virus diseases and causes a serious loss of crop worldwide. It was known that TMV can infect more than 150 types of plants including vegetables, flowers, and weeds. Due to the high genetic variation of TMV, conventional chemical treatments have no effect on protecting the plant from viral infection.

Plants are under constant threat of microbial pathogen attack, and they also posses many defense mechanisms. These defense mechanisms can be non-inducible like cell walls, cuticles, physical and chemical barriers that limit access of microbes to plant cells [1], and can be inducible, which can further be biotic and/or abiotic. In addition to these natural defense mechanisms, there are elicitor compounds that can artificially induce signals in the plant [2]. The plant's innate immune system recognizes these signals and responds to them and starts to prime/induce the defense responses [3].

Elicitor compounds can be biotic that are biological in origin, or derived from either the plant or the microbe [4]. Elicitor compounds can also be synthetically generated like oligo saccharides, glycosides, amides, carboxylic acids, and aromatic compounds [5-7] and vitamin [6,8,9].

There has been also a strong trend to protect crops using natural substances like vitamin B1, B2 and K3, as inducers of systemic acquired resistance (SAR) [10,11] as they are very cost effective and safe for the environment [12]. Treatment of plant surfaces with elicitor alone or with the infecting virus activates defense mechanism through multiple signaling pathways [13].

When plants are treated with elicitors, priming happens and consequently, multiple defense signaling pathways evolve to cope with adverse environmental conditions and invading pathogen [14], and starting to induct defense mechanism in elicitor-treated plants [15,16).

Phenylalanine ammonia-lyase (PAL) plays an important role in plant defense; as a key enzyme in phenylepropanoid pathway it is involved in the biosynthesis of salicylic acid (SA), an essential signal involved in plant systemic resistance [17]. PAL catalyses biotransformation of L. Phenylalanine to trans-cinnamic acid and ammonia plays an important role in plant defense through biosynthesis of salicylic acid [18], essential signal pathways in plant resistance [19]. Peroxidase (POD), on the other hand, is an oxido-reductive enzyme that participates in the cell wall polysaccharide processes such as oxidation of phenols, and lignification of host plant cells during the defense reaction against pathogenic agents [20]. Resistance is associated with the induction of peroxidase [21] in host tissues.

Systemic acquired resistance is often characterized by localized necrosis, expression of pathogenesis related (PR) protein genes. Plants produce a number of compounds and proteins in response to pathogen infection, these compounds and proteins are believed to have a high importance in protecting them from the deleterious effects of the pathogen. These include pathogenesis-related proteins such as PR-1, PR-2, and PR-5 have been implicated with elicitor treatments just like SA [22].

The PR proteins have been classified into 14 families based on the amino acid sequences, serological relationship and/or enzymatic or biological activity. Many PR proteins exhibit direct antifungal activity against a wide range of fungal pathogens [23], or viral pathogen [24]. There were some other defense related pathogenesis related proteins discovered in pepper and other plants, like PR4 and PR9 [6], which can be induced and accumulated in response to stress or an invading

pathogen [25]. PR10 family, one of the pathogenesis-related groups, it has been reportedly stated that they posses distinct functions in developmental processes, secondary metabolism, and antimicrobial activity [26].

One of the commonly recognized vitamins is riboflavin [27], and thiamin [28] which can be produced by plants and microbes and acts as a coenzyme in many physiological processes in animals, plants, and microbes [29].

Purpose of this research is to find organic or natural substances that can fight viruses and protect crops without compromising the health of the human consumer [30,31]. Thus, it is urgent to study new bio-control agents for plant diseases. Present study focuses on the effect of thiamin and riboflavin in the induction of disease resistance in the TMV infected *C. annuum* plants as well as an investigation of the various enzyme activity after treatment with the inducers and the expression patterns of five defense related genes of (POD, PPO, PAL, PR4, PR9, and PR10) were studied.

Materials and Methods

Virus isolates and sources

Tobacco mosaic virus (TMV) was maintained in an aqueous solution (20 mg/ml) at 4°C. A diluted solution of 1 µg/ml was used to inoculate leaves of *C. annuum* by rubbing the leaf lamina with a finger dipped in inoculum mixed with carborandum as an abrasive material. *C. annuum* plants were inoculated with TMV. The inoculated plants displayed symptoms of leaf mosaic, leaf curling and stunted growth. Chenopodium amranticolor was used as a local lesion host showing local lesions symptoms when inoculated with TMV.

Maintenance of the host plants

Pepper seeds (*C. annuum L.*), and *Chenopodium amaranticolor* L. seeds were surface-sterilized using 5% sodium hypochlorite for 10 min, and rinsed five times with sterile distilled water. The seeds were then placed on pots containing sand and soil in 1:2 ratios, and grown in a growth chamber at 25°C under a 16 h/8 h light/ dark photo cycle. Germinated pepper seedlings were maintained in insect-proof screen house conditions.

Preparation of inducers

Different concentrations of thiamin and riboflavin (Sigma-Aldrich Chemical Co., St. Louis, MO) were prepared as follows, (0, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, and 15.0 mM). Plants were sprayed with water or different concentrations of thiamin and riboflavin respectively.

Seedling treatment

Thiamin B1 and riboflavin B2 were prepared as indicated above. Each vitamin was sprayed onto twenty-day old pepper seedlings and after 24h they were challenge-inoculated with TMV.

Effect of thiamin and riboflavin treatment on disease development in *C. annuum* and TMV infection

C. annuum, seeds were sown and maintained as described above. The obtained seedlings (20-day-old) were treated by spraying with thiamin and riboflavin independently, and after 24 h of treatment, the treated *C. annuum* was inoculated with TMV. After 3 weeks, the leaf samples (3^{rd}) were harvested and the virus concentration was quantified by using ELISA and local lesion host.

Quantification of virus concentration by using Enzyme linked immunosorbent assay (ELISA): The leaves harvested from untreated or treated plants showing viral symptoms were crushed. The homogenate was centrifuged at 10,000 rpm for 5 mins at 4°C. The supernatant extracted from the *C. annuum* was subjected to indirect-ELISA against anti-TMV respectively according to [32]. The virus concentration in the inducer-treated and untreated inoculated or non-inoculated as well as the control host plants were assayed.

Quantification of the virus concentration by using local lesion host: Virus concentration in infected *C. annuum* have been quantified by extraction of infected leaves treated and un-treated with the inducers. The leaf homogenate was then used as a source of TMV and used in the inoculation of *C. amaranticolor* with abrasive. Evaluation of virus infection and disease resistance was assayed at the local lesion host (*C. amarantocolor*) after 7 days from inoculation. The number of lesions per leaf was counted.

% Inhibition = (number of local lesions of sample / number of local lesions of control) * 100

Time course effect of thiamin and riboflavin treatment on the induction of resistance

The primary leaves of seedlings were sprayed by 1ml of the vitamin solution at 4 mM of thiamin and 2 mM of riboflavin. After 24 h of treatment, the treated *C. annuum* seedlings were inoculated with TMV. After inoculation, the leaf samples (4^{th}) harvested at different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, and 20 days for enzymes, and 12 h, 1, 3, and 5 days for genes expression). The harvested leaf material was frozen in liquid nitrogen and stored at -80ºC. The different defense related enzymes and genes expressed due to the treatment of thiamin and riboflavin were determined.

Enzymatic assays

Estimation of peroxidases (POD assay): For estimation of peroxidases, one gram of inducer treated/un-treated TMV infected/un-infected leaves, sample was suspended in 2 ml of cold freshly prepared 10% polyvinylpyrrolidone in 0.5 M tris HCl buffer (p^H 7.2) and the ratio was kept 2:1 (v/w) for buffer and material. The extract was centrifuged at 14000 rpm for 20 minutes at 4°C, and the resulted supernatant was used for enzyme assay.

The reaction mixture was prepared as previously described by Malik & Singh [33]. The mixture contained, 0.5 ml phosphate buffer p^H 7; 0.2 ml enzyme source; 0.3 ml of 0.05 M pyrogallol; 0.1 ml of 1%(v/v) H_2O_2. The total mixture volume was raised to 3 ml using distilled water. The reaction mixture was incubated at 30°C for 5 min. Then the reaction stopped by adding 0.5 ml of 5 % (v/v) H_2O_2. One unit of peroxidase activity was expressed as the changes in absorbance at 425 nm and expressed as unit's min^{-1} g^{-1} fresh wt.

Polyphenoloxidase activity: The activity of PPO was determined by adding 50 µl of the crude extract of 1 g of treated sample to 3 ml of a solution containing 100 mM potassium phosphate buffer at p^H 6.5 and 25 mM pyrocatechol. The increase of absorbance at 410 nm, for 10 min at 30°C, was measured. One PPO unit was expressed as the variation of absorbance at 410 nm per mill igram of soluble protein per minute, and expressed as unit's min^{-1} g^{-1} fresh wt [34].

PAL activity assay: Samples were homogenized by grinding with liquid nitrogen in a mortar and then extracted at 4ºC with 0.1 M sodium acetate buffer (p^H 5.0; 1:1 w/v) containing various protease inhibitors. Homogenates were centrifuged at 10 000 rpm for 30 min. at 4ºC and the supernatant was used as the enzyme source.

PAL activity was assayed by the method suggested by Sadasivam and Manikam [35] with slight modifications. The leaf tissue (1:10 w/v) was macerated with borate buffer containing 25 µL β-mercaptoethanol, 50 µL PMSF, 50 µL cystein-HCl, and 50 µL ascorbic acid using a chilled mortar and pestle. The homogenate was clarified by centrifugation at 12000 rpm for 20 min. at 4ºC. Supernatant was used as enzyme source. The assay was initiated by the addition of 1 ml L-phenyl alanine solution to the mixture containing 0.5 ml borate buffer, 0.2 ml of enzyme solution, and 1.3 ml of distilled water. The mixture was incubated for 1 h at 32ºC and the reaction stopped by adding 0.5 ml of trichloroaceticacid in the reaction system. A control was run by adding phenylalanine solution following the addition of trichloroacetic acid. Absorbance was measured at 290 nm and the enzyme activity expressed as micromole trans-cinnamic acid formed per mg protein per min.

Enzyme assay calculation: Samples of 1.0 g of the leaves were extracted for each enzyme system. The percentage of increase over control (fold increase) of each enzyme in infected and non-infected plants was calculated using the formula:

((T-C)/C)×100

Where: C=enzyme level in plant treated with only water to act as a control,

T=enzyme level in virus, or vitamin treated infected or non-infected plants.

Reverse transcription (RT)-PCR analysis of the expression of *C. annuum* defense-related genes

RT-PCR analysis was conducted to determine the expression of selected defense related genes in thiamin and riboflavin treated *C. annuum* plants. Each *C. annuum* plant at the 20 days leaf stage was sprayed with 2 mM of riboflavin and 4 mM of thiamin individually and after 24 hours challenged with TMV inoculation. Total RNA was extracted, using Trizol reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer instructions, from treated *C. annuum* leaves after 0, 12hours, 1, 3, and 5 days of treatment. The quality of extracted RNA was tested by using the A260/280 ratio. The transcription levels of genes were detected by RT-PCR using a PrimeScript First Strand Complementary DNA (cDNA) Synthesis kit (TaKaRa, Japan). qRT-PCR analysis was performed using combinations of gene-specific primers for each cDNA. qRT-PCR was performed with an iCycler iQ Multicolor PCR Detection System (Bio-Rad, Hercules, CA, USA). qRT-PCR was carried out with cDNA in triplicate in 96-well plates using SYBR premix Ex Taq II (TaKaRa, Japan). Each reaction (20 µL) consisted of 10µL SYBR premix ExTaq II, 2 µL diluted cDNA, and 0.4 µM forward and reverse primers. qRT-PCR cycling conditions were as follows: 95ºC for 1min and 45 cycles of 95ºC for 15s, 52ºC for 20s, and 72ºC for 30s. RT-PCR products were loaded on 1.0% agarose gel and the relative quantity of each band was estimated by a gel pro analyzer (GeneGenius). Five genes involved in different plant defense pathways were selected: *PAL,* POD, PR4, PR9, and PR10. The *actin* gene was used as an internal control. Gene-specific primers of these genes are shown in Table 1.

Gene	Primer Forward	Primer Reverse	Source
POD	5`-5AAGGTATTAGGGCTCAGGGGA-3′	5`-CGAACCTATGTAAAAAGAGTGTAA-3′	[36]
PAL	5′-GGTTTTGGTGCAACATCACATAGGAG-3′	5′-ATTGTCAAAGTTCTCTTAGCTACTTGGC-3′	[37]
PR4	5′-AACTGGGATTTGAGAACT-GCCAGC-3′	5′-ATCCAAGGTACATATAGAGCTTCC- 3′	[6]
PR9	5′-GACTAGTTTCAAGAGCATCA-3′	5′-AATTGTATAGCCTGTAGCTG-3′	[38]
PR10	5′-ATGTTGAAGGTGATGGTGGTGCTG-3′	5′ -TCCCTTAGAAGAACTGATACAACC-3′	[26]
CaActin (Control)	5′-CACTGAAGCACCCTTGAACCC-3′	5′-GAGACAACACCGCCTGAATAGC-3′	[39]

Table 1: Primers used in Real time RT-PCR for genes POD, PAL, PR4, PR9, and PR10 with CaActin as a control.

Results

Effect of thiamin and riboflavin treatment on disease development in *C. annuum* TMV infection by using ELISA

Thiamin and riboflavin treatments on *C. annuum* seedlings showed significant reduction in the viral concentration in comparison with control seedlings as evident from the results of indirect ELISA carried out by using the antibodies of TMV. Virus concentration started to decrease; indicating the start of resistance, at 1mM of riboflavin treated plants and showed a considerable decrease in the virus concentration at 2 mM. On the other hand, thiamin treated *C. annuum* plants started to show decrease in TMV concentration at 2 mM of the vitamin dose, and showed a considerable decrease in the virus at 4 mM (Figure 1).

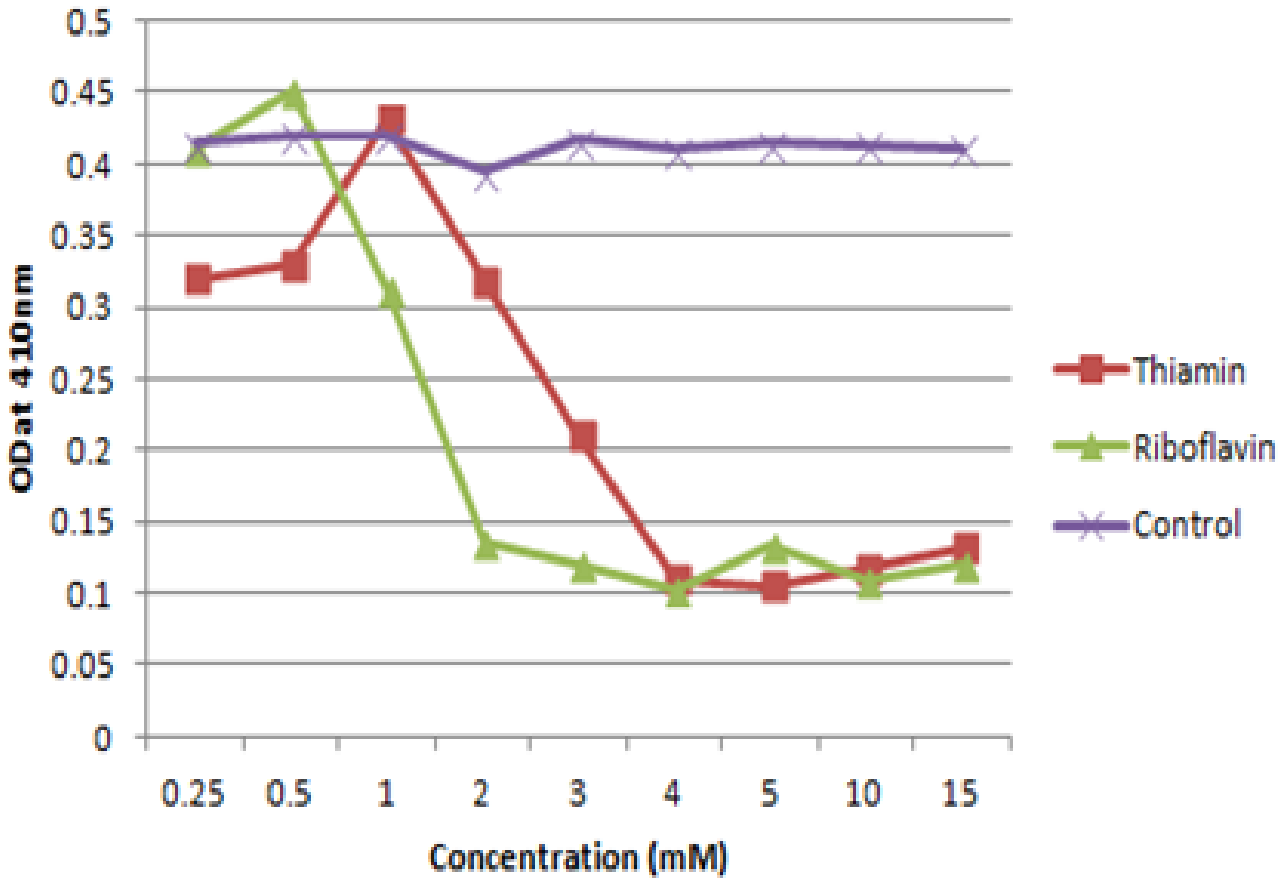

Figure 1: Effect of different concentrations of thiamin and riboflavin on reduction in virus concentration in *C. annuum* treated seedlings after 20 days (Indirect ELISA absorbance at 410nm). Each value in the results shown is the mean of three replicates for each treatment.

Effectiveness of thiamin and riboflavin treatment on disease development and TMV infection in *C. annuum* by using local lesion host (*C. amaranticolor*)

The other part of leaf homogenate from different treated/untreated *C. annuum* plants was used in the inoculation of *C. amaranticolor* with the abrasive as a source of TMV. After 7 days, local lesions will be counted and used in the quantification of virus infection on *C. annuum*. A remarkable reduction in infection with TMV was observed in thiamin and riboflavin treated *C. annuum* plants, while SDW treated plants (control) exhibited heavy infection (Figure 2).

The disease severity in the treated plants with thiamin and riboflavin was significantly lower than that of the control. The dose effect of riboflavin and thiamin showed that the concentration of 2 and 4 mM respectively, were most effective and sufficient for induction of resistance to TMV (64.1 and 70.0% protection). Concentrations higher than 2 and 4 mM in the case of riboflavin and thiamin respectively did not show any increase in resistance. However, less than 2 and 4 mM concentration was less effective, suggesting that there is a threshold concentration for these vitamins to start inducing the viral resistance in the plant. After this threshold is reached, the concentration is not an issue any more. These threshold doses of the vitamins will be called the effective dose.

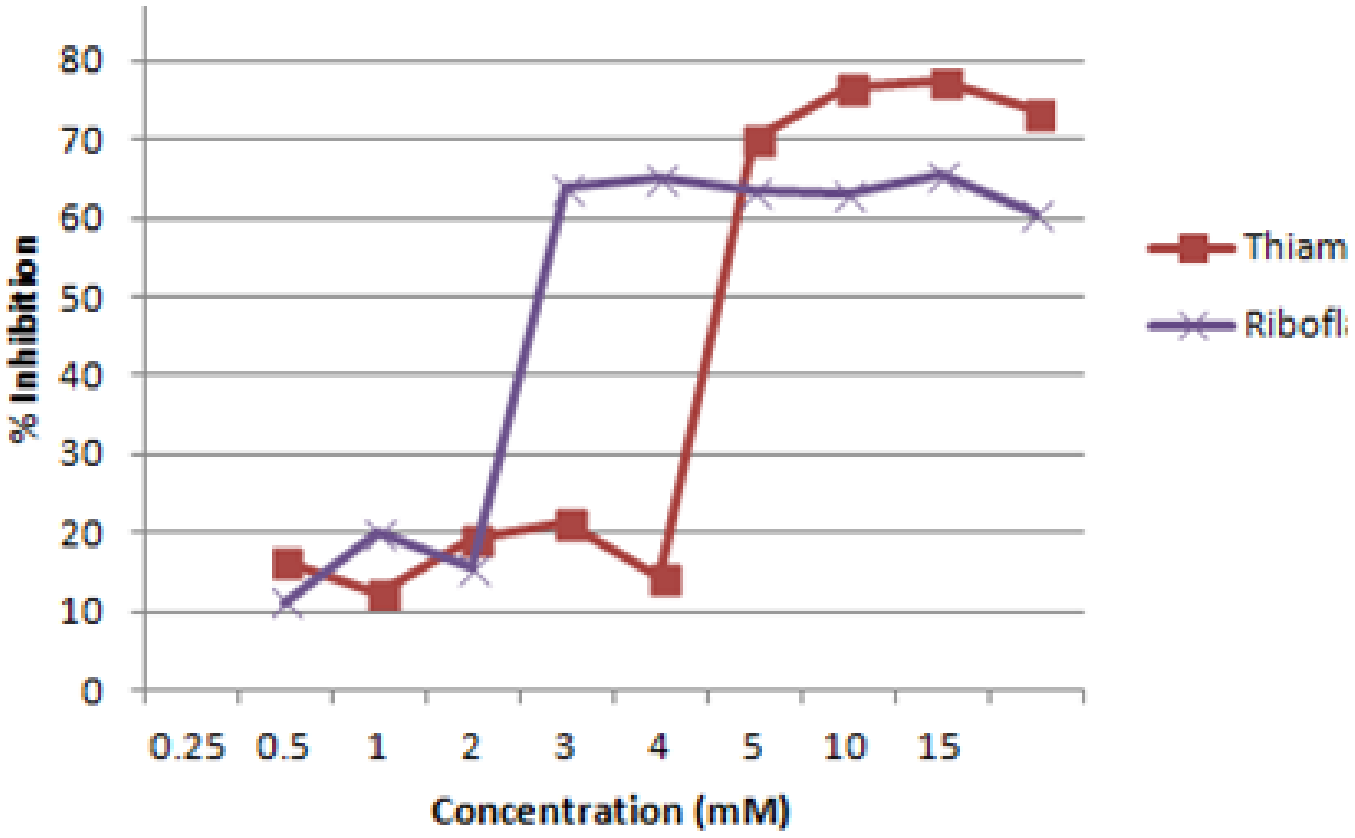

Figure 2: Effect of different concentrations of thiamin and riboflavin on percent inhibition of *C. annuum* plants challenge inoculated with TMV (as indexed by *C. amaranticolor* test). Each value in the results shown is the mean of three replicates for each treatment.

Synergetic Effect of thiamin and riboflavin on disease severity in *C.annuum* plants

Equal amounts of thiamin, at effective concentration 4 mM, and riboflavin, at effective concentration 2 mM, were mixed and sprayed to *C. annuum*. The leaves were taken and ground, their homogenate was tested using ELISA. On the other hand thiamin, riboflavin, and TMV only treated plants were tested using ELISA. Results in Figure 3 below showed that adding riboflavin to the TMV treated plants reduced the TMV concentration in the plant leaves by 65%, on the 5th day of treatment. Adding thiamin showed more reduction in virus concentration. Adding both riboflavin, and thiamin to TMV treated plant leaves, on the other hand reduced the virus concentration on the leaves by around 83%. Virus concentration increased slightly on the 10th day for all treatments, indicating a reduction in virus resistance, and kept fluctuating afterwards with no major increase or decrease till the 20th day for all treatments (Figure 3).

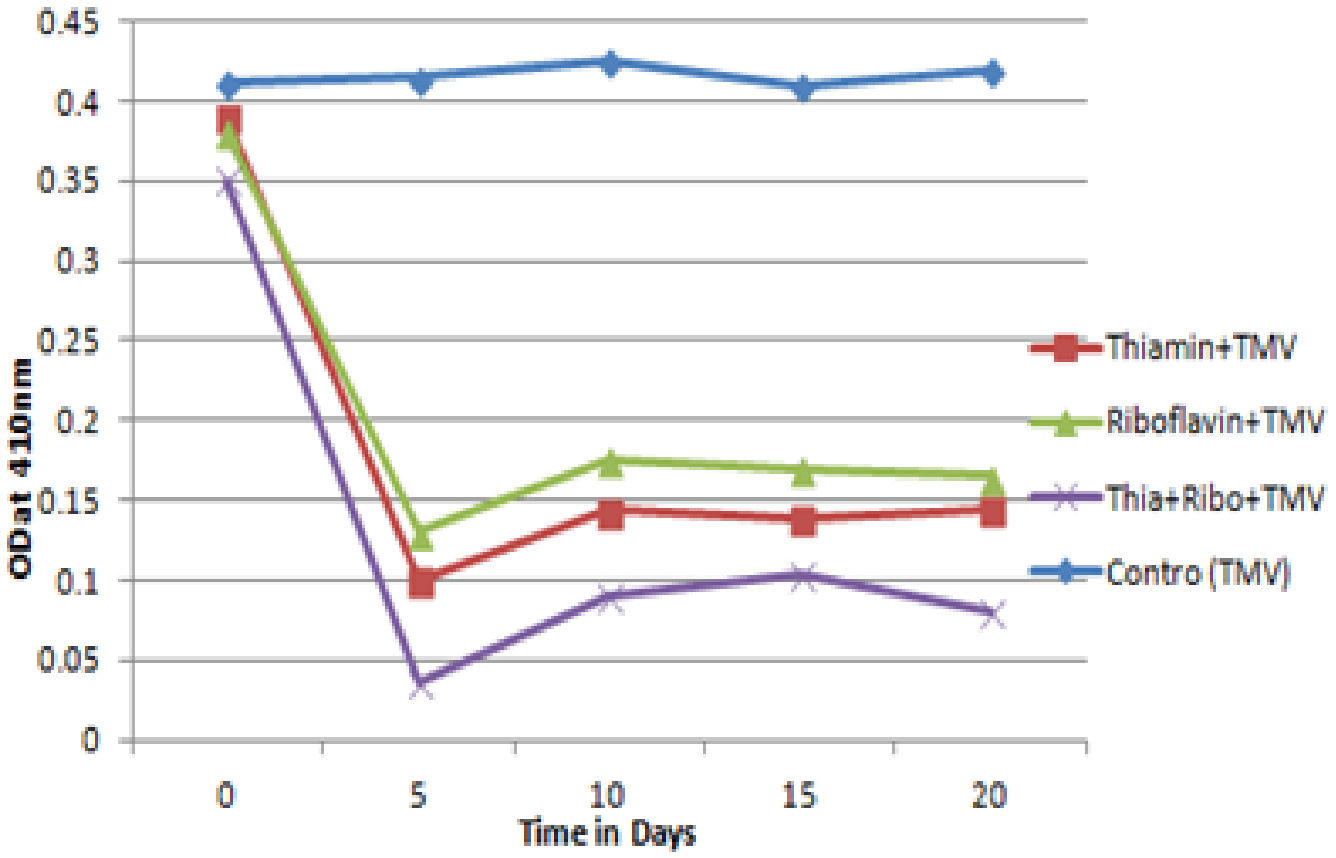

Figure 3: Time course effect of thiamin, riboflavin, and the combination of both on reduction of virus concentration in *C. annuum* treated seedlings after 20 days (Indirect ELISA absorbance at 410 nm) each value in the results shown is the mean of three replicates for each treatment.

Effect of thiamin and riboflavin on PAL, POD, and PPO enzyme activity

C. annuum plant, the activities of PAL, POD, and PPO enzymes were analyzed, as they are the key factors in the induced resistance. The data presented in Figure 4 below shows six curves representing all the different treatment as indicated below. As, shown in Figures 4a-4c below, the plants treated with only water show low levels of the PAL and POD enzymes, and a slightly higher level of the PPO enzyme. A slight upraise in the enzyme levels happens from 3 to 5 days after treatment with TMV in case of PAL and POD. Plants treated with TMV only showed also a slight upraise in the PPO levels, and it was higher than those of PAL and POD.

Treating the plants with thiamin or riboflavin alone, on the other hand, increased the levels of the enzymes slightly from the 1st day, showing a peak around the 3rd day for PAL, POD, and PPO. This activity started to decrease slightly afterwards, indicating that the vitamin itself has the ability to induct the enzyme activity even when the plant is not in the attack/defense state. As for the case of treating the TMV inoculated plants with the vitamins, Figure 4a shows that the PAL level for Thiamin and TMV treated plants reached a peak in the third day. Afterwards the level of PAL activity showed very slight variance around the peak, and it persisted till the 20th day of treatment. As for the activity of the POD enzyme for thiamin treated TMV inoculated plants, it started with low levels and increased from the 1st day to reach a peak at 3rd day after treatment. This peak showed some decrease for three days and persisted till the 15th day of treatment and then started to decrease. Figure 4c showed a low start in the PPO enzyme level for thiamin treated TMV inoculated plants which started to incline until it reached a peak on the 3rd day of treatment. Thiamin TMV treated plant curve remained steady and persisted the enzyme level till the 20th day of treatment and started to decline.

As for the riboflavin TMV treated plants, PAL activity showed a peak on the 4th day of treatment, this peak had a decrease on the next two days and then started to be steady till the 20th day after treatment, Figures 2b on the other hand, is showing that the riboflavin treated TMV inoculated plants showed a peak in their POD level activity at the 5th day, which persisted till the 20th day of treatment. Finally, for the riboflavin TMV treated plants, it started with a higher PPO enzyme level relatively, and reached its peak on the 4th day. PPO enzyme level kept oscillating around the peak afterwards till reaching the 20th day of treatment with an enzyme level very close to the peak value.

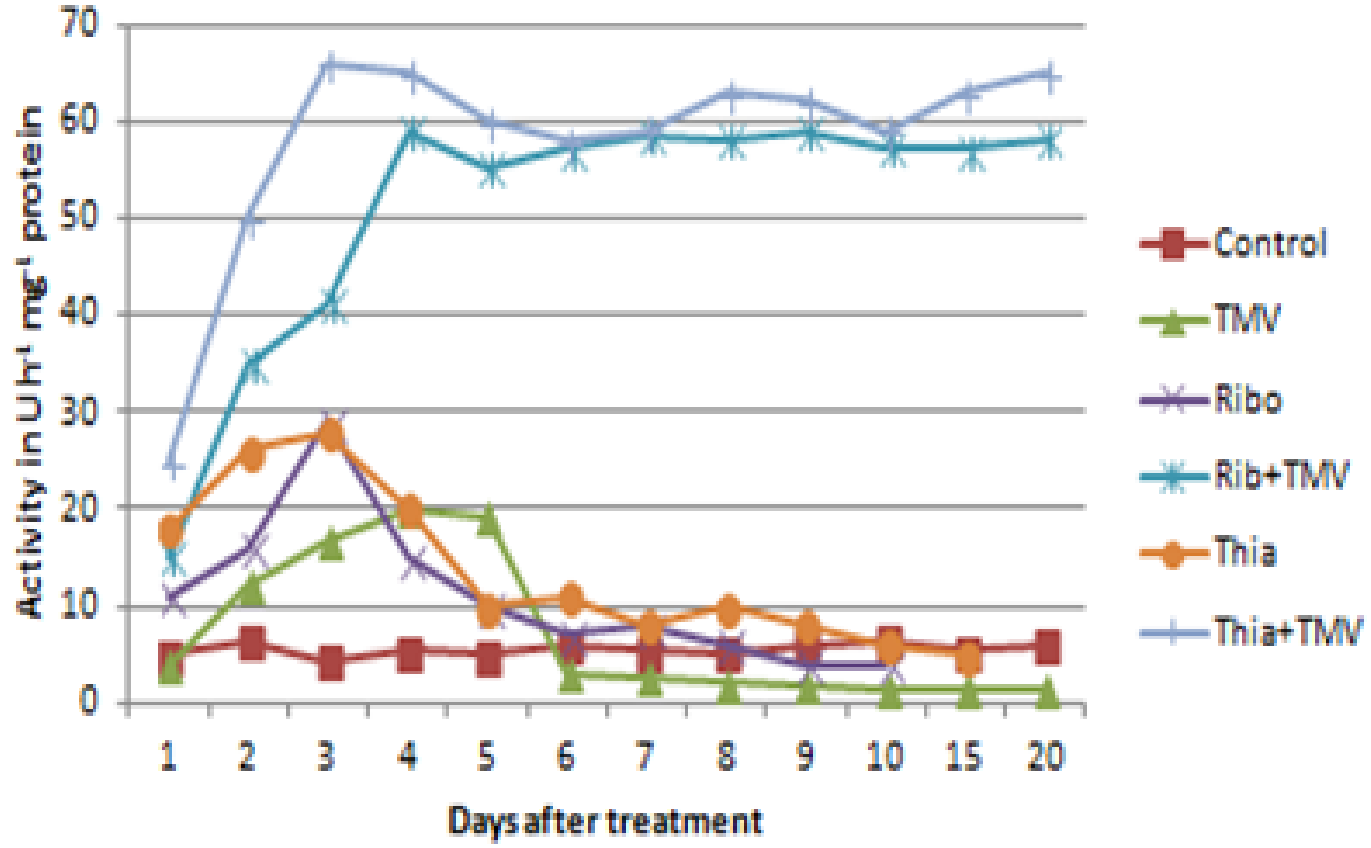

Figure 4a: Effect of time on enzyme activity of riboflavin and thiamin treated and non-treated TMV infected *C. annuum* plants, PAL, Curves are described as follows: for each enzyme activity, one for the plant treated with water only, one for TMV only treatment to take into consideration other factors or resistance inducers TMV may induct in the plant, one for each vitamin treatment alone to investigate its effect without the virus on the plant, and finally one for each vitamin with the virus. Hence, the curve measuring the enzymatic activity of the plant treated with water only should serve as a control for the two curves measuring the enzymatic activity for the plants treated with the vitamin alone, and the curve measuring the enzymatic activity of the plants inoculated with the virus alone, should serve as a control for the two curves measuring the enzyme levels for the plants treated with the vitamin and inoculated with the virus.

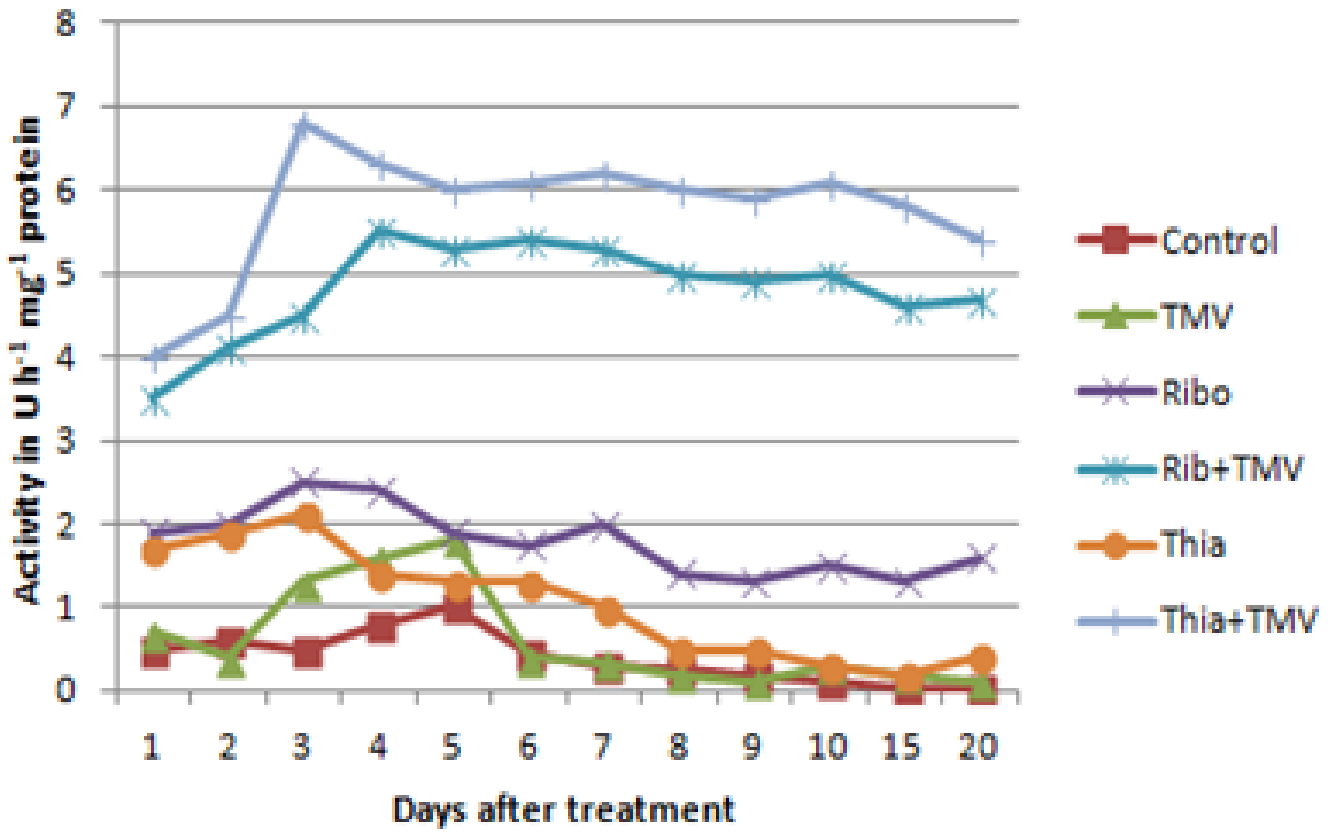

Figure 4b: Effect of time on enzyme activity of riboflavin and thiamin treated and non-treated TMV infected *C. annuum* plants, POD, Curves are described as follows: for each enzyme activity, one for the plant treated with water only, one for TMV only treatment to take into consideration other factors or resistance inducers TMV may induct in the plant, one for each vitamin treatment alone to investigate its effect without the virus on the plant, and finally one for each vitamin with the virus. Hence, the curve measuring the enzymatic activity of the plant treated with water only should serve as a control for the two curves measuring the enzymatic activity for the plants treated with the vitamin alone, and the curve measuring the enzymatic activity of the plants inoculated with the virus alone, should serve as a control for the two curves measuring the enzyme levels for the plants treated with the vitamin and inoculated with the virus.

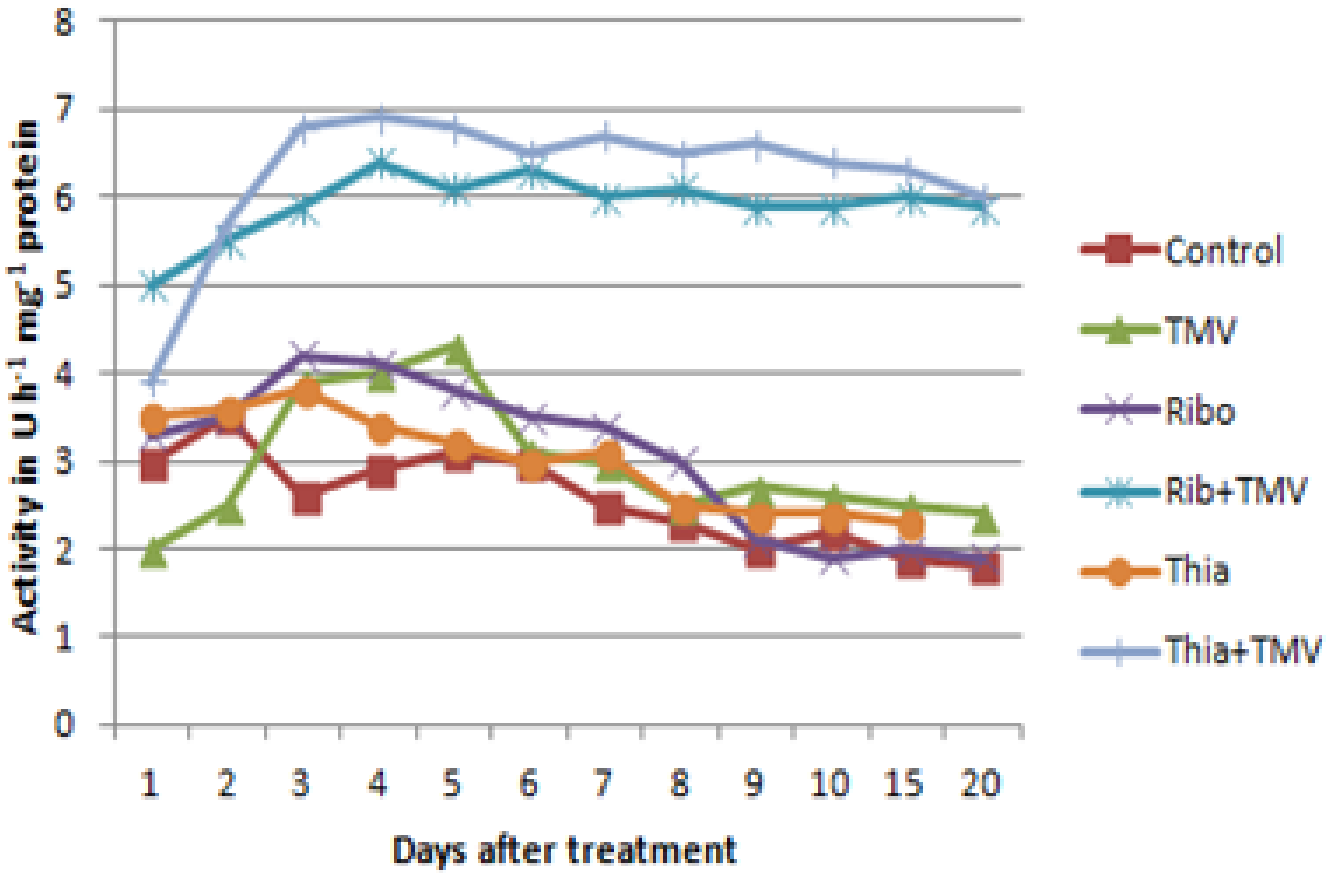

Figure 4c: Effect of time on enzyme activity of riboflavin and thiamin treated and non-treated TMV infected *C. annuum* plants, PPO Curves are described as follows: for each enzyme activity, one for the plant treated with water only, one for TMV only treatment to take into consideration other factors or resistance inducers TMV may induct in the plant, one for each vitamin treatment alone to investigate its effect without the virus on the plant, and finally one for each vitamin with the virus. Hence, the curve measuring the enzymatic activity of the plant treated with water only should serve as a control for the two curves measuring the enzymatic activity for the plants treated with the vitamin alone, and the curve measuring the enzymatic activity of the plants inoculated with the virus alone, should serve as a control for the two curves measuring the enzyme levels for the plants treated with the vitamin and inoculated with the virus.

Effect of thiamin and riboflavin on the expression of genes in treated *C. annuum* plants infected with TMV

Now to gain more information about the genes that can be responsible for the defense and the release of the defense enzymes shown on the previous experiment, gene expression of selected defense related genes was conducted using RT-PCR. Five common genes were examined for their expression in riboflavin and thiamin treated TMV infected *C. annuum* plants with reference to actin gene, those genes are PAL, and POD which already showed good enzymatic activity in the previous experiment, besides three more genes PR4, PR9, and PR10. Time course used in the experiment is after 12 hours of treatment to detect the early gene appearance, and then at 1, 3, and 5 days of treatment. According to the values estimated for each band by gel analyzer, the results were as follows, TMV infected plants showed PAL gene activity starting at the 1st day of treatment and persisted till the 3rd day and disappeared on the 5th, thiamin on the other hand showed an early detection of the PAL gene activity after 12 hours of treatment and persisted till the 3rd day as shown in Figure 5A.

An early high PAL gene expression for thiamin TMV treated plants after 12 hours of treatment which even increased at the 1st day of treatment, and gene expression kept almost the same till the 5th day of treatment. Riboflavin treated plants in Figure 5B, on the other hand showed some activity on the 3rd day of treatment which did not persist till the 5th day, Riboflavin TMV treated plants showed an early high PAL gene expression after 12 hours and 1st day, and underwent a slight decrease in the 3rd and 5th days but still with observable values. As for the POD, it showed a considerable activity with TMV treated plants on the 1st day of treatment till the 3rd day, and less activity with thiamin and riboflavin treated plants at the 1st day which again persisted till the 3rd day. A very high gene expression early at 12 hours of thiamin TMV treatment showed up in Figure 5A, which persisted till the 5th day of treatment. Riboflavin TMV treated plants, on the other hand did not show any POD gene expression value at 12 hours of treatment. Some POD gene activity started to show up after the 1st day of inoculation, and persisted till the 5th day. PR4 gene started at a very low activity at the 1st day of TMV treated plants, and did not show any activity afterwards, PR4 gene also showed a weak activity at 12 hours of

thiamin treatment and this activity disappeared after the 1st day of treatment, riboflavin treated plants on the other hand showed a weak activity at the 1st day of treatment and it got a lot weaker on the 3rd day, and vanished afterwards. Figure 5A shows that PR4 gene showed a weak activity at 12 hours of thiamin and TMV treated plants. PR4 gene reached a slightly higher expression at the 1st day of treatment, which then totally disappeared at the 3rd day. Riboflavin TMV treated plants showed also a low expression of PR4 gene activity after 12 hours of treatment, and got higher at the 1st day of treatment, and then vanished at the 3rd day of treatment. PR9 gene showed some activity at the 1st day of TMV treatment and showed no activity afterwards, a hardly recognizable activity at 12 hours and 1st day of thiamin treated, and a very weak activity on the 1st day of riboflavin treated plants. A very weak activity at PR9 gene expression shown in Figure 5A showed also a relatively lower gene expression at 12 hours of thiamin TMV treated plants, and got even lower at the 1st day and disappeared afterwards. Riboflavin TMV treated plants in Figure 5B, showed a very weak expression presenting a very low starting point of PR9 gene expression at 12 hours of treatment, which increased at the 1st day reaching a peak gene expression and then started to gradually fall till the 5th but still observable. Finally, gene PR10 shown in Figure 5A showed good activity for TMV treated plants showing late at the 3rd and 5th days of treatment, very low activity at the 1st and 3rd days of thiamin treated plants, and after 12 hours and 1st days of riboflavin treatment, a high starting gene expression for thiamin TMV treated plants at 12 hours of inoculation which kept increasing and reached a peak at the 3rd day, and then started to fall at the 5th but still existent. Riboflavin TMV treated plants on the other hand, showed a very weak PR10 gene expression till 12 hours, and started to be observed at the 1st day of inoculation, when observed it had the same magnitude and its values started to get higher as days post inoculation increase till the 6th day (Figure 6).

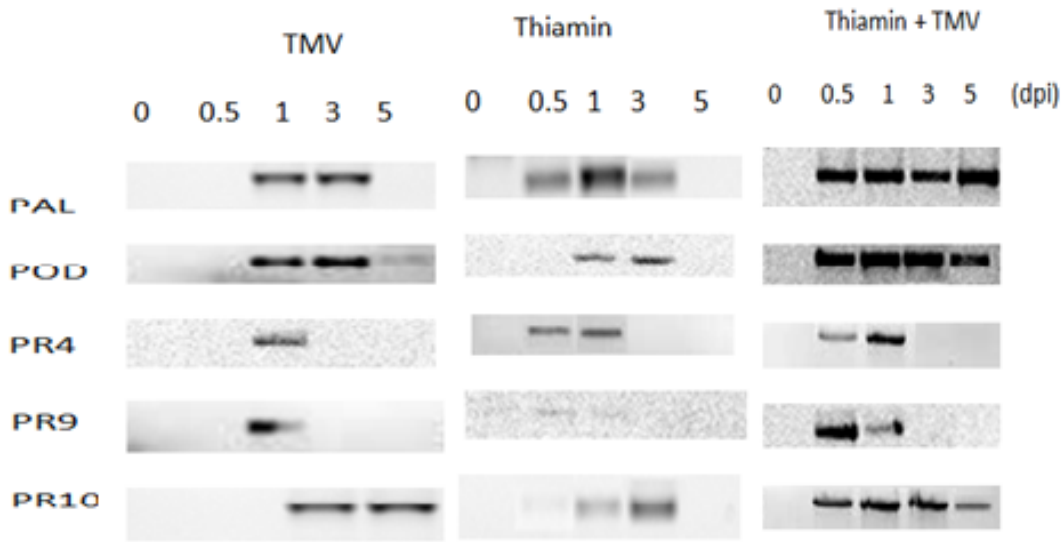

Figure 5A: Defense related gene expression of PAL, POD, PR4, PR9, and PR10 induced by TMV, thiamin and thiamin + TMV treated *C. annuum* plants.

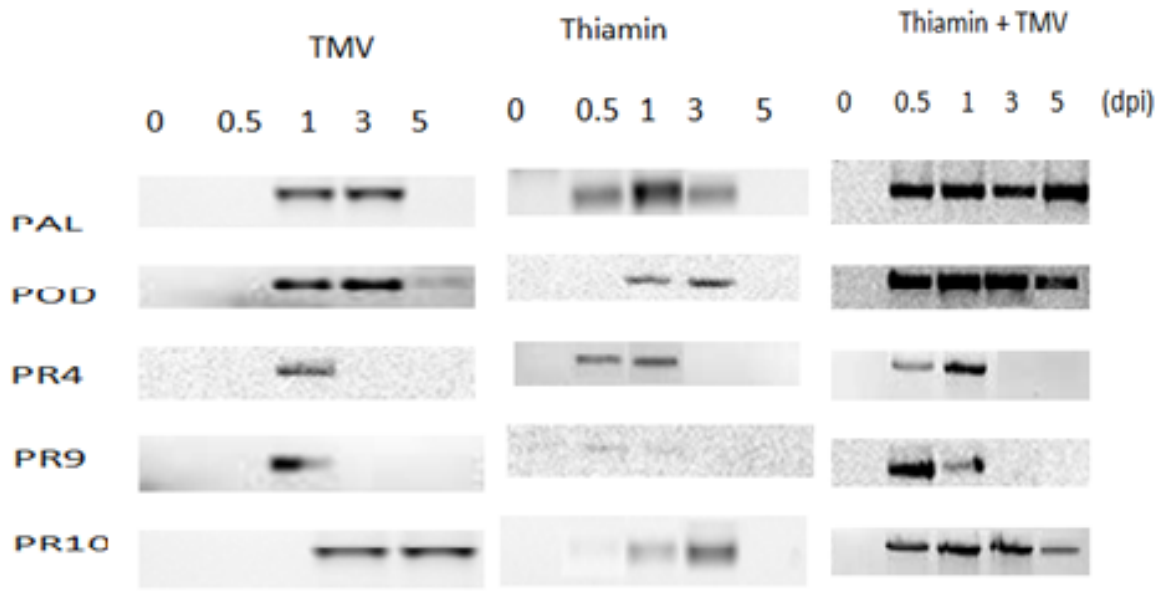

Figure 5B: Defense related gene expression of PAL, POD, PR4, PR9, and PR10 induced by TMV, riboflavin and riboflavin + TMV treated *C. annuum* plants.

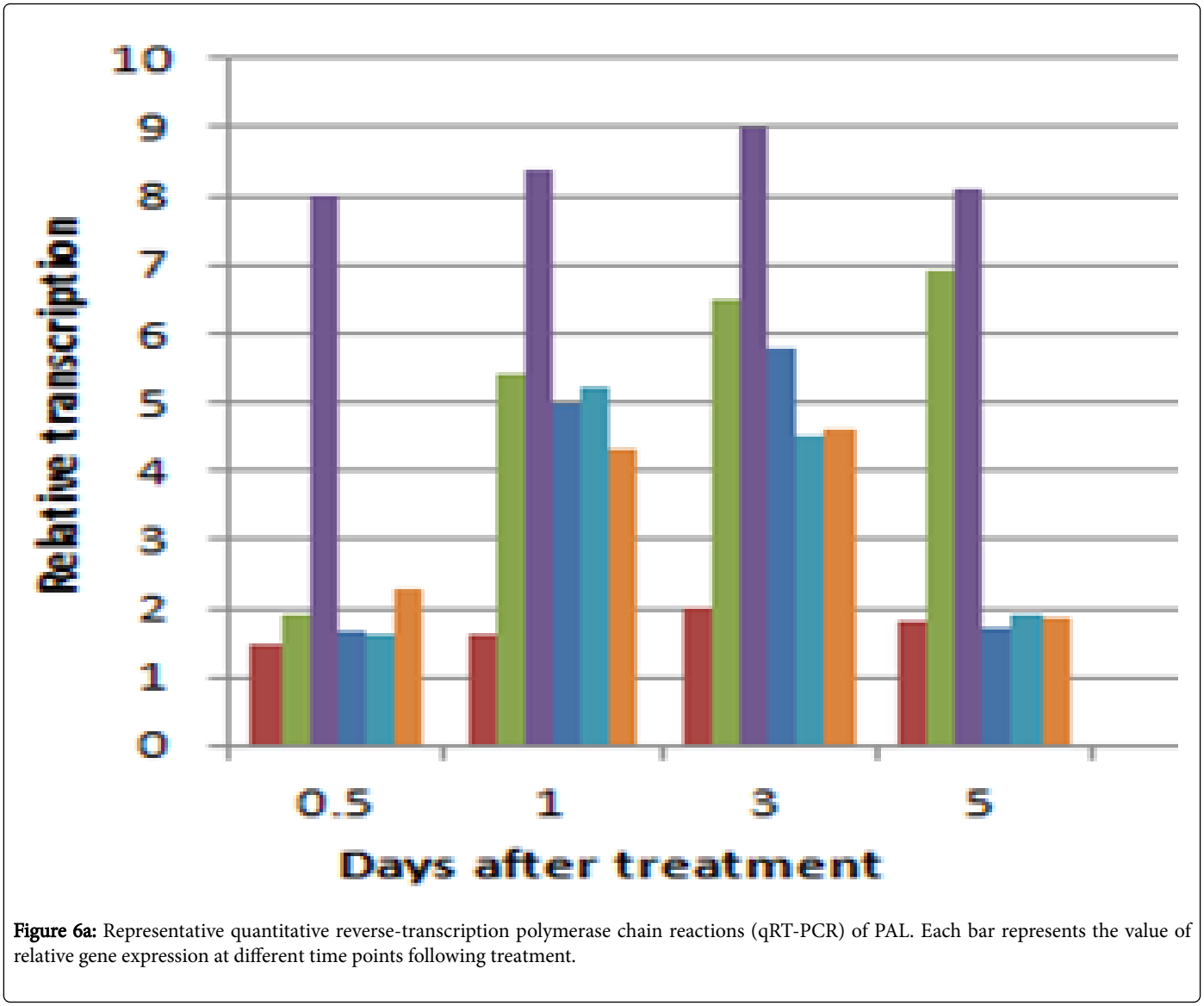

Figure 6a: Representative quantitative reverse-transcription polymerase chain reactions (qRT-PCR) of PAL. Each bar represents the value of relative gene expression at different time points following treatment.

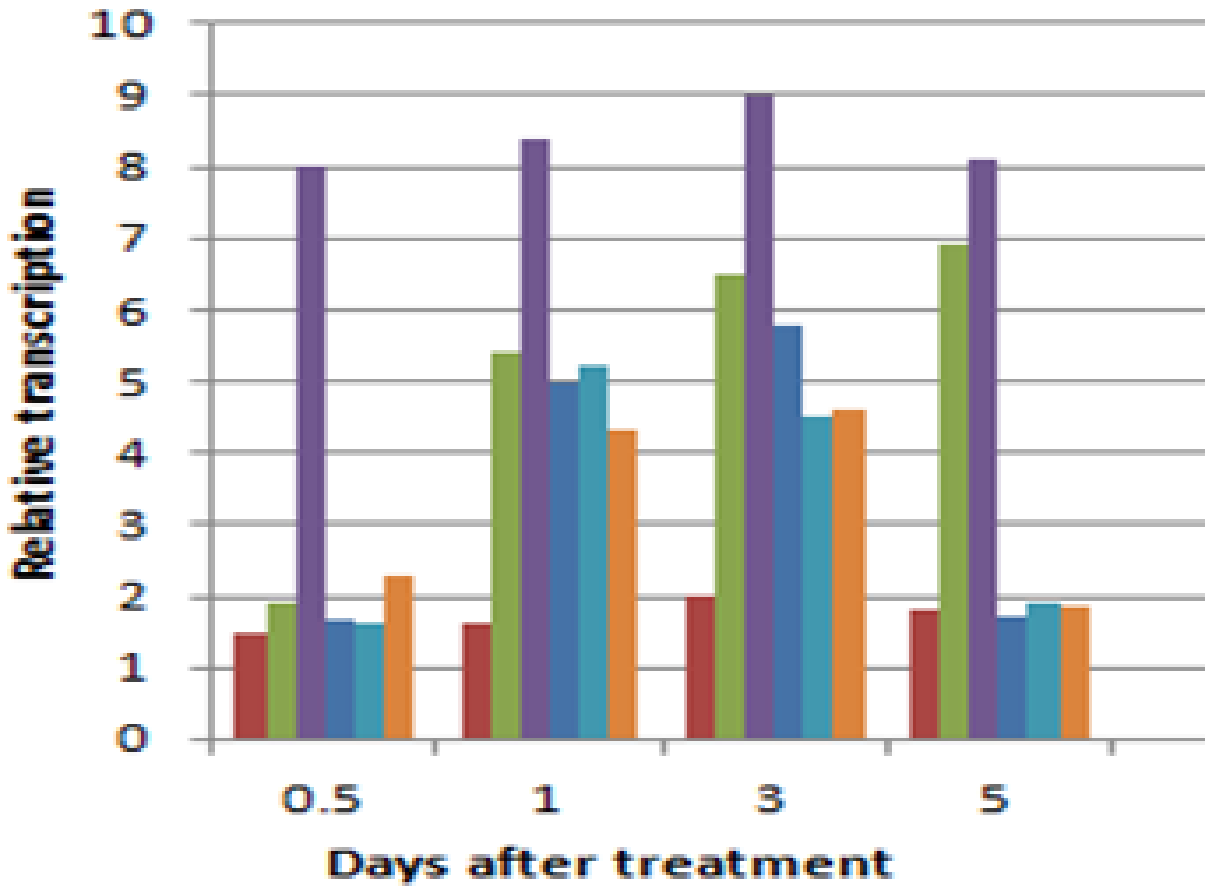

Figure 6b: Representative quantitative reverse-transcription polymerase chain reactions (qRT-PCR) of POD. Each bar represents the value of relative gene expression at different time points following treatment.

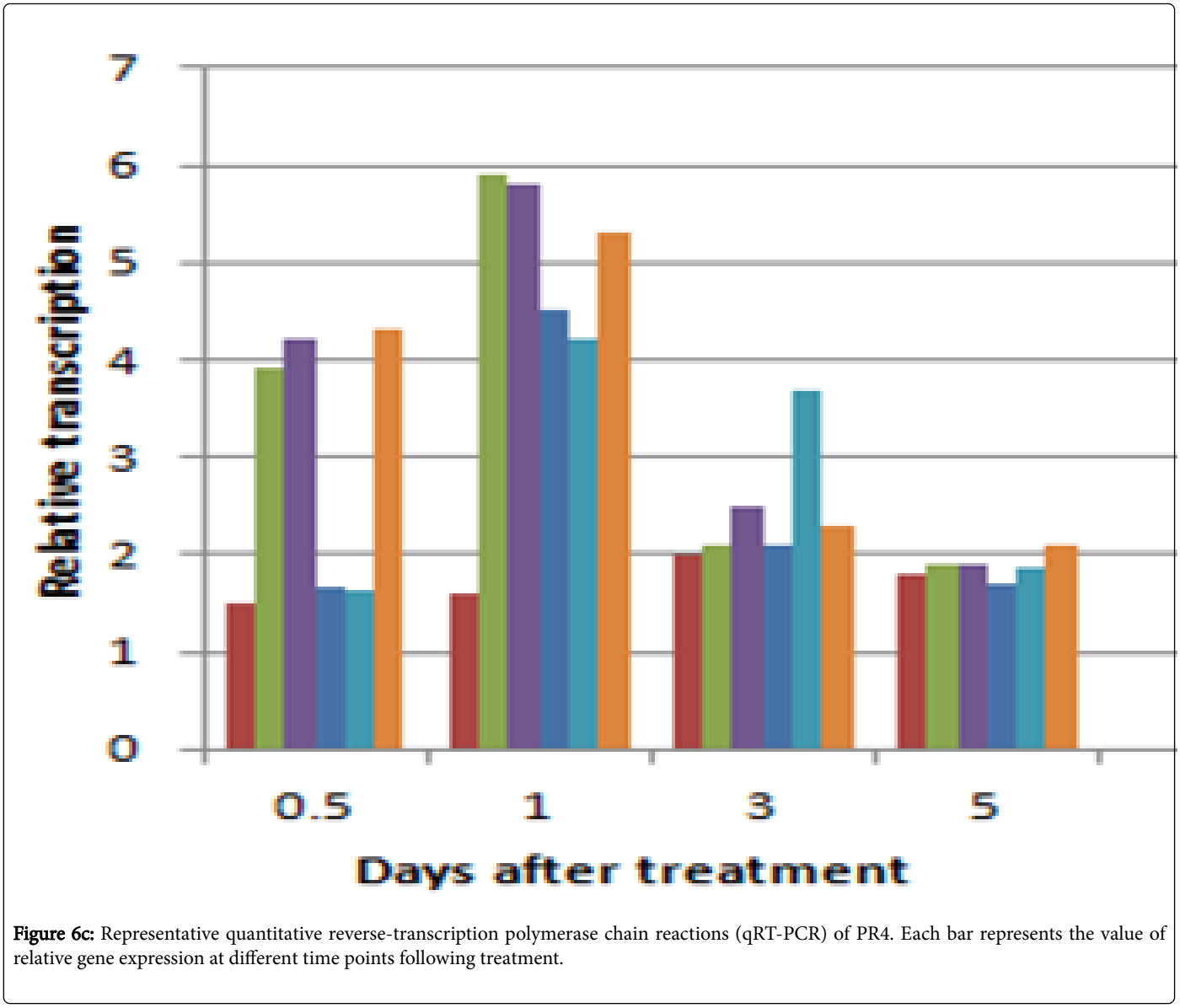

Figure 6c: Representative quantitative reverse-transcription polymerase chain reactions (qRT-PCR) of PR4. Each bar represents the value of relative gene expression at different time points following treatment.

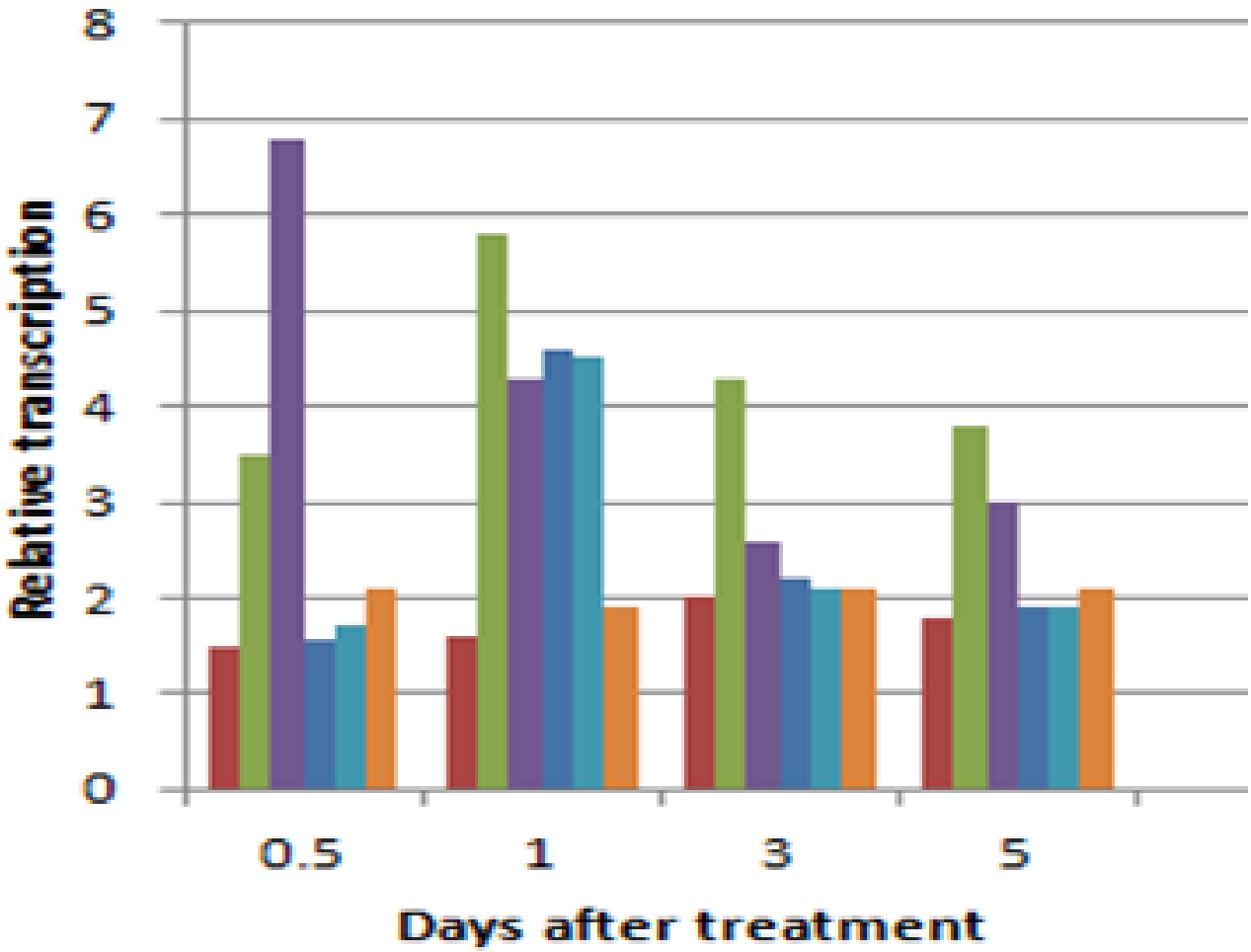

Figure 6d: Representative quantitative reverse-transcription polymerase chain reactions (qRT-PCR) of PR9. Each bar represents the value of relative gene expression at different time points following treatment.

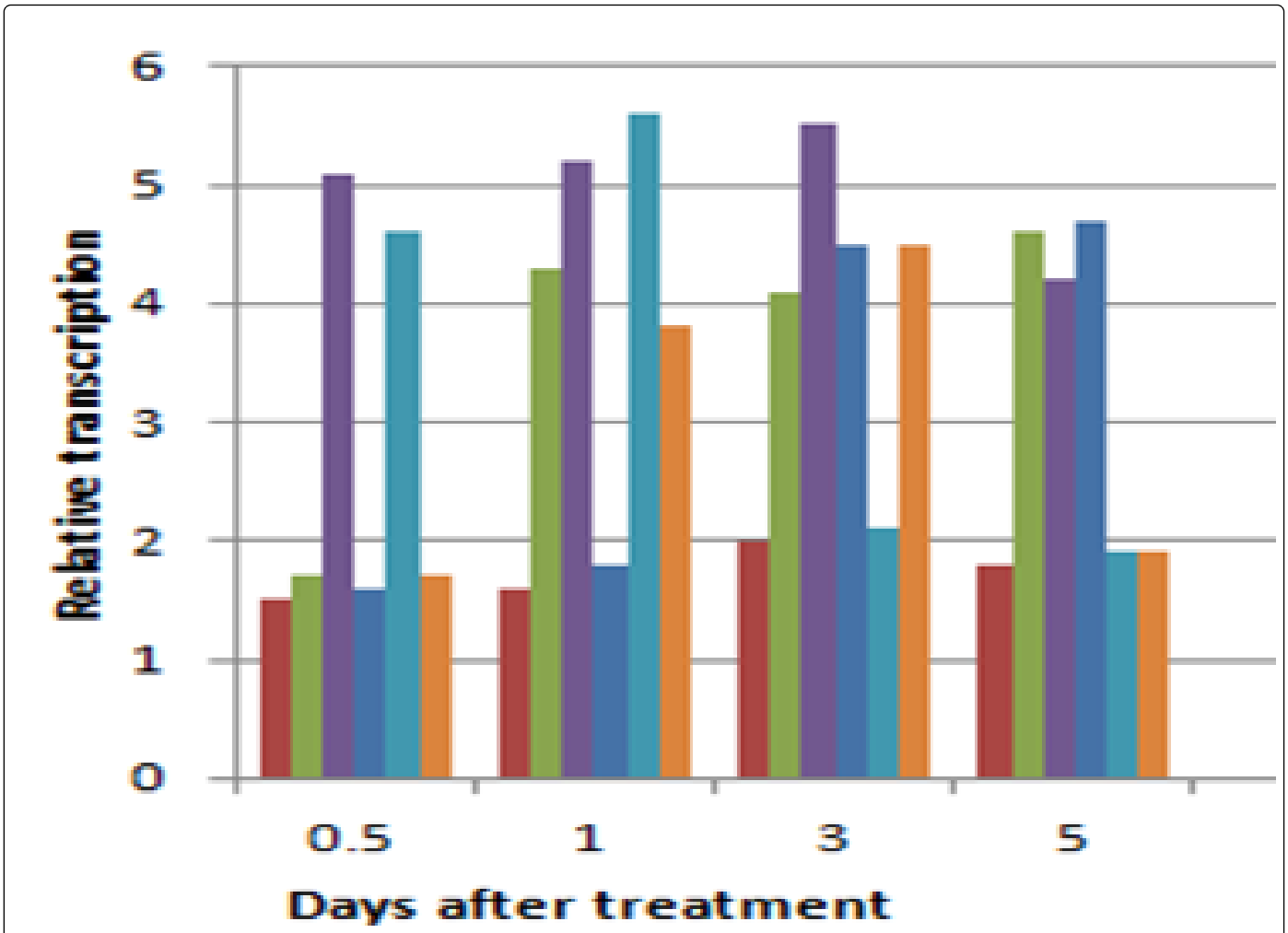

Figure 6e: Representative quantitative reverse-transcription polymerase chain reactions (qRT-PCR) of PR10. Each bar represents the value of relative gene expression at different time points following treatment.

Discussion

Thiamin and riboflavin are well known as safe, reliable and non-phytotoxic plant protection agents or resistance inducing elicitors, and were recently identified as novel disease-control compounds.

In this study, concentration, time of application, synergetic effect, induced enzyme levels, and relevant genes expressions were examined to understand how they affect the resistance of *capsicum annuum* plants to the TMV infection.

The treatment of *C. annuum*, with thiamin and riboflavin showed less viral concentration in comparison with controls as evident by the results of indirect ELISA and L.L. host plant tests. This confirms that thiamin and riboflavin induce resistance against TMV in both L.L. host and host plants, which reacts differentially after inoculation with TMV. Induced resistance was reported to be activated by exogenous application of thiamin and riboflavin [10,40]. The efficiency of riboflavin and thiamin was varied with various concentrations. The dose effect of 0.25 to 15 mM showed that 4 and 2 mM concentration of thiamin and riboflavin respectively, was sufficient for maximum induction of resistance; higher concentration did not increase the effect any further. The effect of elicitor depends on many factors like concentration, and time of application, as well as the plant stage when the elicitor was applied. A wide range of cellular responses due to priming has been reported to be potentiated by these compounds, including alterations in ion transport across the plasma membrane, synthesis and secretion of antimicrobial secondary metabolites(phytoalexins), cell wall phenolics and lignin-like polymers, and activation of various defense genes [41,42].

The % inhibition observed against TMV infection in *C. annuum* treated leaves with thiamin and riboflavin were 70% and 64% respectively. These results were obtained from the *C. amaranticolor* assay and they are in agreement with the results obtained from the ELISA absorbance values for thiamin and riboflavin at their effective doses. When studying the activity of resistance related enzymes like PAL, POD, and PPO, Plants treated with TMV only showed a higher level of enzymes than the control. This upraise in the enzymes levels shown in TMV treated plants may be explained by the fact that TMV as a pathogen may have inducted many resistance inducers in the plant which in turn raised the levels of the resistance enzymes for certain time, and then started to decline.

On the other hand, inoculated and non-inoculated plants with TMV showed a significant increase in the enzymatic activities and these components began to accumulate 2 days after treatment and reached maximum levels from 3 to 5 days after treatment, then the activities of these enzymes persisted around the peak value till 20 days after treatment and then started to decline. Thiamin treatment increased the activities of PO, PPO, and PAL in the inoculated and non-inoculated plants than the riboflavin treatment. Also, comparatively least disease severity was found at 3 days and 4 days after treatment with thiamin and riboflavin respectively. The level of enzymatic activity persisted for both thiamin and riboflavin treated plants till the 20th day of inoculation; this indicates that thiamin and riboflavin mediated induced resistance in *C. annuum* plants was related with the increase in PO, PPO, and PAL activities. These results come in agreement with those reported by [30,38].

Accumulation of phenolic compounds has been correlated with disease resistance in a number of plant-pathogen interactions. POD can be directly involved in defence mechanisms acting as a catalyst for the polymerization of phenolic compounds to form lignin and suberin in the cell wall, which can act as mechanical barriers to block the spread of the pathogen in the plant [43]. When plants are infected by the pathogen, complicated physiological and biochemical changes in the plant will occur to resist the invasion and the harm caused by the pathogen. After treatment with the inducers in TMV inoculated and non-inoculated plants, they displayed a resistance mechanism, promoting an enzymatic defense system, involving POD, PAL and PPO. Some studies have demonstrated that the POD, PAL and PPO are closely related to plant resistance [40,44]. The induction of POD, PAL and PPO activities lead to the formation of antiseptic secondary products. Reactive oxygen intermediates (ROIs), is another common term that refers to cell signaling and host resistance. Riboflavin, on the other hand is found to be involved in anti-oxidation and peroxidation [45] processes which affect the production of ROIs in oxidative burst and consequent HR [42].

Also, some studies observed that foliar application of riboflavin effectively controlled several diseases of tobacco [46]. While riboflavin is a cofactor of enzyme flavoproteins, some of which catalyze lipid perioxidation, main processes, in producing ROIs that serve as a signaling network in plant immune responses [47]. Glycosylated forms of riboflavin, which are considered unimportant in plant [48], may serve as a signal-storage compound. This function may be similar to that of glycosylated SA.

The role of thiamin in inducing resistance may be due to blocking of disease cycle, the direct inhibition of pathogen growth [49] and the induction of resistance to plant against pathogen infection [10]. Thiamin confers systemic acquired resistance (SAR) on susceptible plants through priming, leading to rapid counterattack against pathogen invasion triggered hydrogen peroxide accumulation, and callose induction [10].

When trying to mix equal amounts of thiamin and riboflavin with the virus to study the synergetic effect of the two vitamins on the induction of resistance against TMV infection, results showed that combined mix gave a higher induction of virus resistance on all the days tested after the treatment and this resistance to the virus persisted through the whole course of infection.

In the elicitor research field, the response of plant genes to elicitor treatment is of great interest and several gene expression studies have been conducted to gain greater knowledge of the diversity of genes induced after elicitor's treatment. Real-time PCR technology provides new opportunities to detect and study phytopathogenicity [50]. Because of its sensitivity, specificity and reproducibility, real-time PCR is suitable for identifying plant pathogens or for detecting minor changes in host resistance and susceptibility.

In this study, inducer treated/non-treated TMV inoculated and non-inoculated *C. annuum* were examined to evaluate the expression of genes involved in plant defense responses over time. The results showed that the highest gene expression level of PAL, POD, PR4, PR9, and PR10 happened at 1, 3, 1, 1, and 3 days after treatment correspondingly. Thus the PR genes expression took 1-3 days to reach its highest level to resistance.

It can be concluded that vitamin treatment activates plant signaling which induces series of intercellular events that in turn trigger transduction and hormonal pathways, which have their own role in defense mechanism by accumulation of molecular defense molecules [42].

Our study confirmed that priming is a sophisticated operating mechanism in elicitor induced disease resistance. Plant disease resistance is tightly correlated through an inter-related network signaling pathways for SA and JA. POD, PPO, and PAL are the key enzymes involved in the biosynthesis of the signal molecule [51-55].

This study demonstrated that both vitamins elicit systemic resistance indicated by the development of resistance by *C. annuum* aginst TMV after the inducer treatment indicating that both vitamins has potential for practical use in agriculture.

Thiamin and riboflavin have important role as priming agents, and have their effective therapeutic treatments in many physiological diseases for animal and humans. Thus, we can use external application of the vitamin in plants. Both enzymes have an anti-oxidant properties and other anti-oxidant induced disease resistance in plants [42,45].

Continuity of certain enzymes and genes through the whole time course of infection like PAL, POD, PR10, maybe the reason of disease resistance induction of *C. annuum* plants after vitamin B treatments.

Application of thiamin and riboflavin to *C. annuum* before challenge with the TMV triggered a set of plant defense reactions that resulted in the creation of an unsuitable environment for the virus infection, which protect the plant by different (physical and/or chemical) mechanisms.

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