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Riboflavin and Humic acid Induced Resistance in Tomato Against *Tobacco mosaic virus* (TMV)

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ABSTRACT

Riboflavin (R) as an antioxidant and Humic acid (HA) as a phenolic compound both can act as activators of defense mechanisms against different plant pathogens but no clear answer concerning to viral diseases. Exogenous application of R at (0, 0.5 and 1.0 mM) and HA at (0, 1 and 2 g/L) on tomato plants were conducted to induce systemic resistance against *Tobacco mosaic virus* (TMV). All possible combination of both inducers was also investigated. Spraying riboflavin (R2) at 1.0 mM individually and HA1+ R2 at (1 g / L & 1.0 mM), HA2+ R2 at (2 g / L &1.0 mM) in combination treatments reduced the symptoms severity. Moreover, the effect on vegetative growth parameters were clearly decreased in the similar treatments compared to the other treatments. These results were serologically confirmed by ELISA test. The activity of peroxidases enzyme and the total of phenolic compounds were significantly increased in inoculated leaf tissues treated with R2, HA1+ R1 and HA1+ R2. There were increases in reactive oxygen species (ROS) as indicated by elevating the histochemical localization of H_2O_2 using the 3, 3-diaminobenzidine (DAB) - staining technique in the inoculated leaf tissues treated with R2 followed by HA1+ R2 and HA1+ R1. These findings revealed that, foliar spraying with riboflavin as a single treatment or in combination with humic acid can induce plant defense against TMV.

Key words: Tomato plant, TMV, Symptoms, ELISA, POD, ROS, phenolic compounds

Introduction

Induced resistance (IR) is a phenomenon by which the plant can utilize own defense mechanism using biotic or abiotic inducing agents. These inducers improve the plant's system through activating the different defense responses to increase the level of resistance to pathogens. Moreover, vitamins act as a potential organic compounds which used as elicitors of IR in plants (Reignault and Walters, 2007).

Riboflavin, known as vitamin B2, It is a nutritional supplement that is usually used to increase resistance to infection in human and animals (Sundravel *et al*., 2003) by interfering in antioxidation , peroxidation (Perumal *et al*., 2005; Nazarul *et al*., 2006), or motivation of several defense mechanisms (Verdrengh and Tarkowski, 2005; Zhang *et al*., 2009). Riboflavin can generate reactive oxygen species (ROS) such as superoxide (O_2), hydroxyl radical (OH) and hydrogen peroxide H_2O_2 at the cellular level in plants and these play a critical role in activating resistance mechanisms during plant-pathogen interactions (Tzeng *et al*., 1996 ; Low and Merida, 1996) environmental conditions and defense activators (Van acker *et al.,* 2000). The role of ROS in plant defense mechanisms has been suggested for cell wall strengthening by oxidative cross-linking of cell walls (Brisson *et al*., 1994) lipid peroxidation, and induction of hypersensitive reaction as a signaling molecular (Morkunas and Gemerek, 2007; Thabet *et al*., 2012) as well as, stimulation the defense-related genes as a secondary signaling factor Lamb and Dixon, (1997).

Humic acid (HA) is a compound, constructed on potassium-humates, which can be utilized successfully in many parts of plant production as a plant growth promoting or soil conditioner for enhancing natural resistance against plant diseases (Scheuerell and Mahaffee, 2006). HA inducement plant growth through increasing cell division and optimizing uptake of nutrients (Chen *et al*., 2004). Moreover, humic acid trigger the soil microorganisms (Qualls, 2004). The most commercial humic acid is almost applied directly to the soil and/or as a foliar application to the plants. Several reports

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indicated that, HA directly or indirectly play as indicator for induced systemic resistance (Abd-El-Kareem, 2007; El-Mohamedy and Ahmed, 2009). Bio-organic fertilizer has been stated to be essential for reducing the chemical fertilizers application as a result decreasing the environmental pollution and the production cost (Mac Carthy *et al.*, 1999).

The main targets of the current work were proposed to study the effect of foliar applications with riboflavin and humic acid on the induction of systemic resistance against TMV infection in tomato plants.

Materials and Methods

Foliar application with riboflavin and humic acid under greenhouse conditions:

Healthy tomato seeds (cv. Stren B) were sown under greenhouse conditions. Seedlings at 3-4 leaf stage were transplanted to 20 cm diameter plastic pots filled with sterilized mixture of soil and sand (1:1 w/w) which were kept in insect proof cages. Tomato plants at four-leaf stage were foliar-sprayed with water as a control, riboflavin at (0.5, 1.0 mM), humic acid in a solid form as potassium humate 85% at rate of (1, 2 gram / liter) containing 0.01% Tween-20 as surfactant and combination of both inducers as follow in Table (1).

In case of combination with both inducers after 2 days from the first application of humic acid at (1, 2 g / L), the plant treated with riboflavin at (0.5, 1.0 mM), this procedure could help plants to assimilate the both examined compounds in their tissues. Furthermore, to avoid the washing effect of both compounds to each other which may occur when applied the two compounds simultaneously, tween 20 at 0.05 ml / L^{-1} was used as a wetting agent.

All treatment application were conduct under two different conditions, first without inoculation with TMV as control and the second inoculated with TMV. Three replicates of 4 tomato plants (6 - week old) were maintained for each treatment. All the pots were arranged in a completely randomized block design (22 - 28 ^{o}C), 70 - 80% relative humidity and 12 h photoperiod and watered as needed.

Table 1: Foliar application treatments under greenhouse conditions.

Treatment	Concentration
Humic acid (HA1)	1 g / L
Humic acid (HA2)	2 g / L
Riboflavin (R1)	0.5 mM
Riboflavin (R2)	1.0 mM
Humic acid + Riboflavin (HA1+R1)	1 g / L + 0.5 mM
Humic acid + Riboflavin (HA2+R2)	2 g / L + 1.0 mM
Humic acid + Riboflavin (HA1+R2)	1 g / L + 1.0 mM
Humic acid + Riboflavin (HA2+R1)	2 g / L + 0.5 mM
(Control)	distilled water

Inoculation procedures:

TMV kindly provided by Plant Virus Lab., Department of Plant Pathology, Faculty of Agriculture, Ain Shams University. Virus was propagated on *Nicotiana tabacum*.

The plants were mechanically inoculated with TMV using phosphate buffer solution (Garcia-cano *et al*., 2006). On the other hand, Leaves of control plants were dusted with carborandum and inoculated with buffer solution. All plants were kept under greenhouse conditions. Symptoms were recorded 14, 21 and 30 days after inoculation.

Plant growth parameters of tomato plants:

The plant length (cm) and number of branches /plant were measured after 30 days post inoculation in ten tomato plants from each treatment.

Relative concentration of TMV in infected plants:

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) Clark and Adams, (1977) was used to determine TMV levels in sap of 0.2 g young leaves of infected tomato plants at 7, 14 and 30 days post inoculation. Absorbance values were determined using ELISA reader (BIE & BERNTSEN A.S) at 405 nm [1 hr. after addition of the substrate].

Extraction and assay of peroxidases (POD):

The extraction process was essentially based on the methods described by Biles and Martyn, (1993) as follow: 1 g of fresh leaf tissue (7 and 30 days post inoculation) was taken, then frozen by liquid nitrogen and ground in 2 ml sodium phosphate buffer (pH 6.5, 0.1 M) using mortar and pestle. Samples were transferred to eppendorf tubes, and then centrifuged for 20 min at 12000 rpm at 4°C. The peroxidase activity was directly determined according to Liu *et al*., (2010). The reaction mixture consisted of 2.9 ml of a 100 mM sodium phosphate buffer (pH 6.0) containing 0.25 % (v/v) guaiacol (2- methoxy – phenol) and 100 mM H_2O_2. The reaction was started by adding 100 µl of the crude enzyme extract. The change in absorbance was recorded for 3 min at 470 nm. Enzyme activity was expressed as increase in absorbance min^{-1} g^{-1} fresh weight by using spectrophotometer (Unico-2100).

Determination of total phenolic compounds:

Total phenolic compounds were extracted with ethanol from fresh leaf samples (30 days post inoculation) according to the method described by Swain and Hillis, (1955). Determination of the total phenols was carried out according to Vlase *et al*. (2014). In this method, 1 ml of the above extracted ethanol was mixed with 0.25 ml of concentrated HCL boiled in water bath for 10 min, then cooled. The mixture was mixed with 1 ml of Folin -Denis reagent and 6 ml of 20 % sodium carbonate. The mixture was left for 30 minutes and diluted to 10 ml with distilled water. The optical density of the blue color developed was measured at 725 nm by using spectrophotometer (Unico-2100). Total phenols were quantified using a standard curve of catechol.

Histochemical localization of H_2O_2:

Detection of H_2O_2 was carried out using the 3, 3-diaminobenzidine - staining method as described by Thordal-Christenssen *et al*. (1997). The inoculated tomato leaves (7, 30 days post inoculation) were cut from the base of the stem and the cut ends were immersed for 8 h under light at 25°C in solution containing 1mg / ml^{-1} diaminobenzidine (DAB) dissolved in acidified HCl (pH 3.8), to allow DAB uptake and reaction with H_2O_2. Leaves were fixed and decolorized in boiling 95% ethanol for 10 min before being cleared in saturated chloral hydrate. Subsequently, leaves were stored in solution (50% glycerol).

Statistical analysis:

The experimental data were subjected to analysis of variance (two-way ANOVA) procedures using the statistical software package procedures SAS Institute Inc, (1988). Duncan's test ($P \leq 0.05$) was used to determine significant differences among treatments means.

Results and Discussion

Effect of inducers on disease development.

Several natural and synthetic chemical agents have been described as activators of defense - related processes when applied to plants (Yamaguchi, 1998). We investigated the ability of exogenously administered tomato plants which sprayed with riboflavin and humic acid to induce resistance against *Tobacco mosaic virus* (TMV)

Tomato plants grown under greenhouse conditions were inoculated with TMV after spraying with riboflavin, humic acid or different mix combinations, Inoculated plants were kept for investigating the development of external symptoms. Data were recorded 14, 21 and 30 days post inoculation. All inoculations were carried out mechanically using buffer solution as mentioned before.

Tomato plants showed different responses due to treatments (Table, 2). Regarding to the effect of time on symptoms development, generally expression of symptoms was appeared later on tomato plants in single treatment with R2 than the other single treatments, also in combination treatments HA1 + R1, HA2 + R2 and HA1 + R2 symptoms were appeared later than the other combination treatments.

Generally, after 14 dpi tomato plants treated with both concentrations of humic acid showed mosaic. On the other hand all treatments were showed mild mosaic symptoms. After 21 dpi, all the treatments showed mosaic symptoms except R2, HA1 + R1, HA2 + R2 and HA1 + R2 were showed mild mosaic. After 30 dpi, all the treatments showed severe mosaic except HA1 + R2 and HA2 + R2 symptoms progress still slow and showed mosaic. Instead, R2 showed mosaic and yellowing. Generally, all treatments with R2 combined with both concentration of HA were showed low symptoms expression. In contrast, spraying plants with HA1 showed, severe mosaic, stunting and leaf malformation. HA2 and R1 showed severe mosaic and leaf malformation. HA1 + R1 and HA2 + R1 showed sever mosaic and stunting. Control plants (untreated) inoculated with TMV showed severe mosaic, stunting, leaf malformation and yellowing.

The growth performances were more vigor due to treatment with R2, HA1 + R2, HA2 + R2 and HA1 + R1 as compared to the other treatments. These results in harmony with Packer *et al*., (1996) and Zubay, (1998) they reported that, riboflavin play important role in antioxidation and peroxidation. Similarly, these results may be attributed to the positive effect of riboflavin that described by Dong *et al*., (1995) and Dong and Beer, (2000) which they indicated that, foliar application with riboflavin efficiently controlled several diseases of tobacco plant.

Table 2: External symptoms on tomato plants after 14, 21 and 30 days post inoculation with TMV in different treatments combinations.

Treatments \ Symptoms*		14 days		21 days		30 days				
		Mosaic	M.M	Mosaic	M.M	Mosaic	S.M	Stunting	Mal.	Y.
Single	HA1	+	-	+	-	-	+	+	+	-
	HA2	+	-	+	-	-	+	-	+	-
	R1	-	+	+	-	-	+	-	+	-
	R2	-	+	-	+	+	-	-	-	+
Double (combination)	HA1+R1	-	+	-	+	-	+	+	-	-
	HA2+R2	-	+	-	+	+	-	-	-	-
	HA1+R2	-	+	-	+	+	-	-	-	-
	HA2+R1	-	+	+	-	-	+	+	-	-
Control inoculated		+	-	+	-	-	+	+	+	+
Control (uninoculated)		-	-	-	-	-	-	-	-	-

**: M.M= mild mosaic, S.M= sever mosaic, Mal. = malformation, Y= yellowing, + = symptoms, - = no symptoms*

Effect of virus infection on plant length and number of branches.

Data in Table, (3) indicated that, thirty days post inoculation, plant length and number of branches were increased significantly ($P \leq 0.05$) in non-inoculated plants than the inoculated. As well, foliar application of R2 as single treatment, HA1 + R2 followed by HA2 + R2 and HA1 + R1 as a double treatment increased significantly ($P \leq 0.05$) both of growth parameters on inoculated and non-inoculated plants. Generally, the foliar application with HA1 + R2 was the most significantly treatment on both plant length and number of branches in inoculated and non-inoculated plants.

The enhancing effect of riboflavin at 1.0 mM, HA1 + R2 and HA2 + R2 as a double treatment on plant growth might be attributed to vitamin B2 (riboflavin) which produced by plants and microbes that acts as a coenzyme in many physiological reactions in plants ((Wolinsky and Driskell, 1997; Gastaldi *et al.,* 1999).). Similar trend of results was also detected by Neelamathi *et al*., (2005) they reported that, riboflavin induced high significant variation in induction of shoots in different concentration. It used as antioxidant and has an auxin action that improved vegetative growth parameters.

As well, the role of humic acid contains many elements which improves fertility and increase the availability of nutrients then consequently increase plant growth and yield. These results in parallel with Magdi *et al*., (2011) they confirmed that, the combination of chemical fertilizer and application of humic substances improves growth and yield of cowpea plants. Similar trend of results were also stated by Maral Moraditochaee, (2012) which found that, foliar spraying with humic acid and nitrogen make significant increase in yield of peanut, and foliar spraying with 40 mg / L humic acid was superior. Another study was in agreements with our data obtained by Gad El-hak *et al*., (2012) which they revealed that, foliar application of pea plants with salicylic acid at concentration of 200 ppm and humic acid at rate of 1g / L produce high quality fresh pods and seed yields under sids area and similar growing conditions.

Table 3: Effect of foliar application with riboflavin and humic acid on some growth parameters of tomato plants 30 days post inoculation with *Tobacco mosaic virus* (TMV).

Treatments	Plant length (cm)			Number of branches		
	Inoculated TMV	Non Inoculated	Mean	Inoculated TMV	Non Inoculated	Mean
Control	24.66 m	39.73 e	32.20 F	7.66 k	12.33 f	10.00 E
HA 1	27.26 l	39.76 e	33.51 E	8.66 j	12.66 f	10.66 D
HA2	28.86 k	40.23 e	34.55 D	9.66 hi	13.00 f	11.33 C
R1	33.3 h	41.56 d	37.43 C	9. I0 j	14.33 e	11.66 C
R2	35.26 fg	42.70 c	38.98 B	10.00 h	15.33 d	12.66 B
HA1 R1	31.36 i	44.13 b	37.75 C	11.00 g	16.33 bc	13.66 A
HA2 R2	34.7 g	44.23 b	39.46 B	10.00 h	17.00 ab	13.50 A
HA1 R2	36.03 f	45.13 a	40.58 A	9 .I0 J	17.33 a	13.16 A
HA2 R1	30.13 j	44.16 b	37.15 C	9 .I0 J	16.00 cd	12.50 B
Mean	31.28 B’	42.40 A’		9.33 B’	14.92 A’	

**: Ten plants were used in each trial - Means having the same letters (s) are not significantly different. Duncan's multiple rang test at (P≤0.05).*

Relative concentration of TMV in infected tomato plants:

DAS-ELISA was used to determine the relative concentration of TMV in infected tomato plants. Absorbance values (at 405 nm) of the infectious sap containing TMV were reduced on 7, 14 and 30 days post inoculations (Table, 4). Relative concentration of TMV after 7 dpi was decreased significantly (P≤ 0.05) in plants treated with R2 compared with the other treatment. Conversely, after 14 and 30 dpi the lowest values were significantly (P≤ 0.05) resulted in plants treated with HA1+R2 followed by HA2+ R2 compared with the other treatments. These results were in parallel with those obtained by Aly Torky, (2016) which cleared that, application of vitamins Thiamin (B1) and riboflavin (B2) activated the defense mechanisms against *Tobacco mosaic virus* (TMV). The synergetic effect of both vitamins reduced the TMV infection in *Capsicum annuum* plants. In addition to that, these results confirmed by indirect ELISA and local lesion host plant assay. In general, the obtained data clearly showed that, foliar application of riboflavin as a single treatment especially at concentration (1.0 mM) and in combination with humic acid at (1 gm / L) induce resistance against TMV in tomato plants.

Induced resistance was announce to be activated by exogenous application of riboflavin (Ahn *et al*., 2005; Saikia *et al*., 2006). The efficiency of riboflavin and humic acid were depended on many features like concentration, time of application, besides the plant stage when the inducers were applied.

The obtained data clearly showed that, riboflavin as a single treatment or in combination with humic acid induced resistance against TMV in tomato plants. Data in Table (4) showed that, there is a close relationship between low symptoms expression and a low relative concentration of TMV in infected tomato plants. The reduction due to altering in the activity of phenolic compounds, related enzymes including peroxidase (PO) and generation of ROS in plants. These results run in the same trend with those of Upreti *et al*. (1991) and Taheri and Höfte, (2006) which they confirmed that, riboflavin had an important role as an antioxidant which induces several defense mechanisms finally plant resistance increase.

Table 4: Effect of different treatments on relative concentration of TMV infection in tomato leaves after 7, 14 and 30 days post inoculations as determined by ELISA reader at 405 nm.

Treatments		Time	
	7 dpi	14 dpi	30 dpi
HA1	1.3417 de	2.9917 b	3.5217 b
HA2	2.5053 ab	2.6200 c	3.4770 ab
R1	2.0547 bc	2.5293 cd	3.1747 bc
R2	1.1353 e	2.2397 e	3.0517 bcd
HA1 + R1	2.1090 bc	1.8837 f	2.7187 cde
HA2 + R2	1.7607 cd	2.1807 e	2.3633 de
HA1 + R2	1.5707 de	1.7787 f	2.1287 e
HA2 + R1	2.3917 ab	2.3263 de	2.5583 cde
Control (inoculated)	2.6343 a	3.3950 a	4.1593 a
Control (non-inoculated)	0.4980 f	0.5180 g	0.5137 f

Absorbance values were determined with ELISA reader at 405 nm. Means having the same letters (s) are not significantly different. Duncan's multiple rang test at (P≤0.05).

In the current study, the simultaneous exposure with riboflavin and humic acid on infected tomato plants exhibited a synergistic effect on decrease symptoms expression and relative concentration of TMV, phenolic compounds and their related enzymes (Table 2, 4, 5 & Fig. 1). These inducers could play a protective role against TMV infection. Also these results in harmony with Dong and Beer, (2000) they reported that, treated *Arabidopsis thaliana* with riboflavin established systemic resistance to *Peronospora parasitica* and *Pseudomonas syringae pv. tomato*, on the other hand, tobacco plants developed systemic resistance to *Tobacco mosaic virus* (TMV) and *Alternaria alternate.* These results occurred due to founding of Ryals *et al*. (1996) and Delaney, (1997) which they existing evidence that, riboflavin acts as a signaling pathway leading to systemic resistance and activating PR-genes in *A. thaliana* and tobacco.

Effect of different inducers on peroxidase (POD) activity:

Peroxidase (POD) is one of the most important enzymes in plants, mainly due to involvement in so many molecular, physiological and morphological events in the plant life cycle. This enzyme localizes in different sites within plant cell (Hiraga *et al*., 2001; Delannoy *et al*., 2006). It is well known to catalyze the oxidation of a wide variety of substrates, using H_2O_2 such as a phenol which plays a considerable role in lignin synthesis (Goldberg *et al*., 1987)

The activity of proxidase (POD) was determined at 7 &30 days post inoculation (dpi) with TMV virus. Enzyme activity was detected for treated plants, either inoculated or non-inoculated. All treatments increased POD activity at inoculated and non-inoculated plants compared to control. At 7 & 30 dpi inoculated leaves showed higher (POD) activity as compared to the non-inoculated (Fig.1). At 7dpi the highest increase was detected in the treated leaves (inoculated and non-inoculated) with R2, followed by HA1+R1 and HA1+R2. However, at 30 dpi the highest increase was detected in the leaves treated with HA1+R1 followed by HA1+R2 and R2 (Fig.1). In general, the increase of POD activity was markedly observed at the 30 dpi than that at 7dpi. The abovementioned trend was clearly observed for samples obtained from treated plants, either inoculated or non-inoculated. These results come in agreement with that found by Aly Torky, (2016) which revealed that, treated *C. annuum* plants with thiamin and riboflavin showed significant increase in the activity of peroxidase, phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO).

Effect of different inducers on total phenolic compounds:

The changes in cellular responses due to infections has been reported to be potentiated by phenolic compounds, including alterations in ion transport across the plasma membrane, synthesis of antimicrobial secondary metabolites like phytoalexins, cell wall phenolics and lignin-like polymers, and motivation of various defense genes (Conrath, 2009; Boubakri, (2013).

Phenolic compounds were determined in inoculated or non-inoculated tomato leaves at 30 days post inoculation (Table 5). The total phenols were increased due to applying different inducers as compared to the control plants (inoculated or non-inoculated). Phenolic compounds generally were increased significantly ($P \leq 0.05$) in inoculated than non-inoculated tomato plants. The maximum significantly increase were obtained due to spraying with riboflavin at 1.0 mM as a single treatments and HA1+R2 followed by HA1+R1 as a double treatments of inoculated or non-inoculated plants.

The increase level of enzymatic activity in treated tomato plants indicate that, induced resistance was related to the increase in POD and phenolic compounds. Similar trend of results described by Song *et al*., (2013) and Paz Aranega-Bou, (2014). Furthermore, induced resistance in a number of plant-pathogen interactions has been associated with accumulation of phenolic compounds. On the other hand, PO enzyme acting as a catalyst for the polymerization of phenolic compounds to form lignin and suberin in the cell wall, which can turn as mechanical barriers to block the spread of the pathogen in the plant cell (Faize *et al*., (2012).

Table 5: Total phenolic compounds (µg / g fresh weight) of inoculated or non-inoculated tomato plants, as determined at 30 days post inoculation with TMV.

Treatment	Total phenolic compounds (µg/g fresh weight)		
	Inoculated TMV	Non-Inoculated	Mean
Control	259.34 dc	142.856 d	201.10 C
HA 1	366.3 bcd	241.76 cd	304.03 BC
HA2	318.68 bcd	234.433 cd	276.56 C
R1	311.356 bcd	245.423 cd	278.39 C
R2	747.253 a	509.156 abc	628.21 A
HA1 R1	512.82 abc	424.906 bcd	468.86 AB
HA2 R2	369.963 bcd	344.32 bcd	357.14 BC
HA1 R2	582.42 ab	366.303 bcd	474.36 AB
HA2 R1	377.29 bcd	318.683 bcd	347.99 BC
Mean	427.39 A’	314.20 B’	

HA1: *Humic acid (1g/L),* ***HA2***: *Humic acid (2g/L),* ***R1***: *Riboflavin (0.5mM),* ***R2***: *Riboflavin (1mM),* ***HA1+R1***: *Humic acid (1g/L) & Riboflavin (0.5mM),* ***HA2+R2***: *Humic acid (2g/L) & Riboflavin (1mM),* ***HA1+R2***: *Humic acid (1g/L) & Riboflavin (1mM),****HA2+R1***: *Humic acid (2g/L) & Riboflavin (0.5mM). Means having the same letters (s) are not significantly different. Duncan's multiple rang test at ($P \leq 0.05$).*

Detection of Reactive Oxygen Species (ROS)

In the primary stages of the plant - pathogen interactions, H_2O_2 formation has been strongly linked to the establishment of riboflavin-IR in various plant-pathosystems (Liu *et al*., 2010). ROS / HR can be detected by measuring hydrogen peroxide (H_2O_2) in living tissues by 3, 3-diaminobenzidine (DAB), which producing a brown stain (Thordal *et al*., 1997).

The accumulation of hydrogen peroxide (H_2O_2) were analyzed in TMV inoculated tomato leaves at (7 & 30 d post inoculation), leaves showed a brownish color was observed at the base of the leaf and directly appearing in primary and secondary veins from leaves (Fig. 2). In contrast, the generation of H_2O_2 was not observed in the control plant that was only sprayed with water. Production of reactive oxygen species (ROS) is one of the earliest responses in plant tissues due to the infections (Wojtaszek, 1997), which prevents further spread of different pathogens (Gadjev *et al*., 2008).

The intensity of the stain increased with the development of the infection and was much more pronounced in leaves treated with R2 followed by HA1+R2 and HA1+R1 as compared to control plants. Similar trend of results was also detected by Moshe *et al*., (2012), their data revealed that, riboflavin-IR is tightly associated with preparing of the host tissue for enhance activation of a multifaceted cellular defense response, including highly localized accumulation of H_2O_2 in the epidermis.

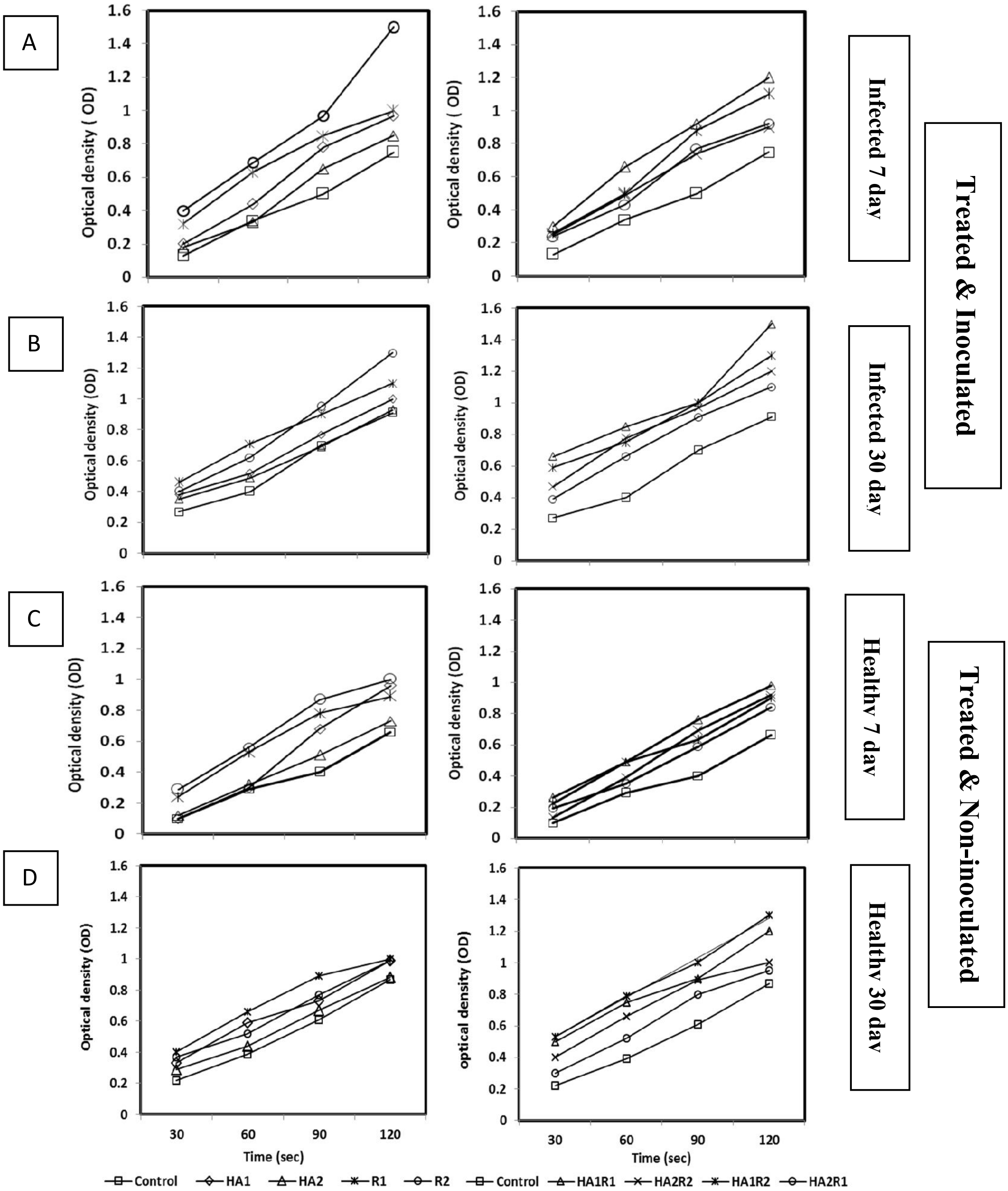

Fig. 1: Effect of spraying different inducers on peroxidase activity of tomato plants. (A); (B) Samples were taken of treated and inoculated 7 & 30 dpi (C); (D) treated and non-inoculated 7 & 30 dpi. TMV virus was used for artificial infection. **HA1**: Humic acid (1g/L), **HA2**: Humic acid (2g/L), **R1**: Riboflavin (0.5mM), **R2**: Riboflavin (1mM), **HA1+R1**: Humic acid (1g/L) & Riboflavin (0.5mM), **HA2+R2**: Humic acid (2g/L) & Riboflavin (1mM), **HA1+R2**: Humic acid (1g/L) & Riboflavin (1mM), **HA2+R1**: Humic acid (2g/L) & Riboflavin (0.5mM).

Treatment	Treated & Non infected	Treated & Infected 7 dpi	Treated & Infected 30 dpi
Control (Water)			
R2			
HA1+R2			
HA1+R1			

Fig 2: Accumulation of H_2O_2 in tomato leaves inoculated with TMV at 7 & 30 days post inoculation. Localization of H2O2 was marked as a reddish brown staining. **R2**: Riboflavin (1mM), **HA1+R2:** Humic acid (1g/L) & Riboflavin (1mM), **HA1+R1**: Humic acid (1g/L) & Riboflavin (0.5mM).

Generally, we try to mix of riboflavin and humic acid at different concentration to study the synergetic effect of both compounds on the induction of resistance against TMV infection. Our results showed that, combining of both compounds provided a higher induction of virus resistance. These effects may be due to that inducers displayed a resistance mechanism and promoting an enzymatic defense system involving POD, H_2O_2 and phenolic compounds. Saikia *et al*., (2006) informed that, the activation of POD, PAL and PPO lead to formation of antiseptic secondary products. Furthermore, riboflavin is found to be involved in anti-oxidation and peroxidation processes which, affect in the production of ROS in oxidative burst (Boubakri, 2013; Ashoori and Saedisomeolia, 2014). Some studies observed that, foliar application of riboflavin successfully controlled several diseases of tobacco plants (Dong and Beer, 2000). Riboflavin is a cofactor of enzyme flavoproteins, which catalyze lipid peroxidation that serve as a signaling network in plant immune responses (Baker *et al*., 1997; Sierra and Vidal-Valverde, 1999). Additionally, application of Humic acid (HA) consistently enhanced the concentration of some antioxidants such as, β-carotene, superoxide dismutases, and ascorbic acid. These antioxidants may play an important role in the regulation of plant development and chilling of disease resistance (Dmitrier *et al*., 2003). HA is considered to increase the permeability of plant membranes and enhance the uptake of soil nitrogen and nutrients such as potassium, calcium, magnesium and phosphorus, which making these elements more mobile and available to plant root system (Piccolo *et al*., 1992).

Conclusion

The results of this study suggest that, the treatments of Riboflavin (R2) and /or in combination with Humic acid (HA) can act as activators of defense mechanisms in tomato plants against TMV infection. It appears that treatments have many significant effects on the plant growth and reduction of symptoms severity through modifying some biochemical metabolites in tomato plants. Generally, the treatment of R2 at 1.0 mM individually and HA1+ R2 at (1 g / L & 1.0 mM), HA2+ R2 at (2 g / L &1.0 mM) in combination treatments gave the highest results in this respect. Also, it was obvious that, there was an increase in the resistance to TMV through significant correlations were found between increase the activity of peroxidases enzyme, the total of phenolic compounds and reduction of TMV concentration in tomato leaves that treated with R2 and/or HA. These findings supposed that, foliar spraying with riboflavin as a single treatment or in combination with humic acid can induce plant defense against TMV.

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