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EFFECT OF BION AND SALICYLIC ACID ON PEROXIDASE ACTIVITY AND TOTAL PHENOLICS IN TOMATO AGAINST *Alternaria solani*

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Early blight disease caused by *Alternaria solani* is the most damaging disease of tomato. This disease causes severe yield losses in tomato. This study was planned to find out the efficacy of Bion and salicylic acid against *A. solani* under greenhouse conditions. The role of resistance inducers in triggering biochemical activities required for resistance induction like peroxidase and total phenolics during 2015-16 were also studied. Test cultivar (Prescot) was treated with Bion (125 mgL^{-1}) and salicylic acid (2 mM) through foliar and seedling root dipping methods to assess the reduction in disease severity and alterations in biochemicals (Peroxidase and total phenolics). Biochemical changes were analyzed from 0 to 10 days (0, 2, 4, 6, 8, 10). Maximum reduction in disease severity was observed in Bion treated plants during 2015-16 (54.47 and 53.56%, respectively) while in Salicylic acid treated plants (38.84%, 38.01%) reduction was observed in both years. Maximum biochemical activity was observed after 10 days of Bion and Salicylic acid application. Foliar application of resistance inducers enhanced the biochemical activity as compared to seedling root dipping method. Significant increase in total phenolics (67.96 and 84.26%) and POD activity (11.35 and 8.69%) was observed where Bion was applied on pathogen inoculated plants during both years respectively. Results confirmed that foliar application of Bion could protect the tomato plant from *Alternaria solani* directly by strengthening the defense system.
Keywords: Biochemicals, foliar spray, seedling root dipping, induced resistance

INTRODUCTION

Alternaria solani is the most destructive pathogen of tomato (*Solanum lycopersicum* L.) in the world (Khan, 2002), causing early blight disease and contributing to a heavy reduction in fruit yield (Abbasi *et al.*, 2002). The *Alternaria* fungus can damage all plant parts including leaves, stem, twigs and fruits, ultimately affects the plant growth process (Abada *et al.*, 2008). Fungicides are major source to control this disease. Nevertheless, the world is moving towards ecofriendly and less hazardous ways of disease management. Systemic acquired resistance (SAR) is a phenomenon useful against a wide spectrum of infective factors, including viruses, and it is played along with assembly of pathogenesis-related proteins and facilitated through a salicylic acid (SA) based pathway (Hammerschmidt, 1999). SAR initiation starts from a few hours to few days and strengthens the plant for a long time during its development process. A systemic signal is necessary in fast and efficient response of tissues to the infection caused by some pathogen. This process is usually stated as "conditioning" (Métraux *et al.*, 2002). Consequently, SAR protects the plants against several diseases through various defense related enzyme production and it can replace the typical fungicidal control of diseases, which are harmful to the environment. Benzothiadiazole with the trade name Bion is an ecofriendly and non-toxic resistance inducer effective against many pathogens, including viruses (Anfoka, 2000; Baysal *et al.*, 2005). Bion is less toxic, quickly decomposable in plant tissue and human and nature friendly, thus can be applied in the field or greenhouse (Soylu *et al.*, 2003: Cao *et al.*, 2011). Oostendrop *et al.* (2001) reported that the Bion is the best abiotic inducer currently known. Salicylic acid (SA) is another key factor in triggering both local and systemic acquired resistance (Pieterse and Loon, 1999). SA application to the plants triggers the resistance induction enzymes and pathogenesis-related proteins that help to activate the resistance genes (Yao and Tian, 2005; Fernandes *et al.*, 2006; Chen *et al.*, 2006). When cucumber leaves were inoculated with some pathogenic microbes capable of SAR induction, amount of SA significantly increased in hypocotyl and roots of the cucumber plant (Kubota and Nishi, 2006). SA found effective in activating SAR in sweet cherry fruit against *Penicillium expansum* (Xu and Tian, 2008).
Biochemical changes involved in initiating SAR by resistance inducers can act as marker of induced resistance (Hashem and Abo-Elyousr, 2011). These include increase of phytoalexins, cross linkages of cell wall with lignin (Thangavelu *et al.*, 2003), and increase in certain defense related enzyme (He *et al.*, 2002). The enzyme peroxidase (POD) has been involved in the program cell death, the formation of papillae, and the polymerization of lignin from monomeric lignols (Shah *et al.*, 2004). Comparison of foliar application and seedling root

dipping method has not been studied yet. The aims of this study were to investigate the effect of Bion and SA on induction defense related enzyme (POD) and total phenolic contents against early blight and to evaluate their efficacy through foliar and seedling root dipping method of application under greenhouse conditions.

MATERIALS AND METHODS

***Plant growth conditions*:** Greenhouse grown tomato seedlings were used in this study. Tomato plants of test cultivar (Prescot) were sown in trays at greenhouse. Conditions maintained at 25 ± 2°C during the day time and 20 ± 2°C at night. 8 weeks old seedlings were transferred to 30 cm diameter pots filled containing sandy loam soil mixed with peat moss (1:1).

***Pathogen and treatment application*:** Pure culture of *A. solani* was dissolved in distilled water and applied to tomato seedlings at the rate of 5×10^4 conidia/ml with the help of atomizer till run off. Bion (Syngenta Crop Protection, Inc., Greensboro, NC) and salicylic acid (Spectrum Chemical Mfg. Corp. California USA) were dissolved in distilled water to give 125 mgL^{-1} and 2 mM, respectively, and applied to whole plants at the rate of 50 ml for per plant two days prior to inoculation. For seedling root dipping, before transplanting, roots of seedlings were dipped in Bion and salicylic acid solutions for one hour and transferred to pots. After treatments application, plants were kept in a greenhouse as mentioned earlier. Healthy (water treated) and infected controls (*A. solani* inoculated only) were maintained. This study was carried out under Complete Randomized Design (CRD) and repeated twice under greenhouse conditions in 2015 and 2016. There were four pots in each replication, and total number of replications were three. Six treatment groups were made (Healthy control, control+*A. solani* (diseased control), Bion, Bion+ *A. solani*, salicylic acid and salicylic acid+*A. solani*). The disease severity was recorded according to the rating scale of 0 to 9 (Mayee and Datar, 1986).

***Sample preparation for enzyme extraction*:** Two samples of leaf tissues from each treatment group (1g fresh weight) were collected at 0, 2, 4, 6, 8, and 10 days after treatment application, dipped in liquid nitrogen for enzyme extraction. The frozen samples were placed (1:5 w/v) in an ice-cold mortar using 50 mM potassium phosphate buffer (pH 7.0) containing 1 M NaCl, 1% polyvinylpyrrolidone, 1 mM EDTA and 10 mM β-mercaptoethanol for homogenizing. The homogenized samples were centrifuged at 17,000 g for 20 min at 4°C. The crude enzyme extract was obtained and divided into 1.5 ml portions. Protein concentrations were determined according to the protocol given by Bradford (1976). The extract was then used to determine the activities of peroxidase.

***Peroxidase (POD) activity*:** Peroxidase activity was estimated by following the protocol given by Urbanek *et al.* (1991).

***Estimation of total phenol content*:** Phenol contents were calculated by following the protocol given by Malick and Singh (1980).

***Statistical analysis*:** The data was analysis was done through computer software Statstix 8.1 using Fisher's analysis of variance technique. The means of treatments were compared by least significance difference (LSD) test at 5% probability level (Steel *et al*., 1997). The main factors are presented in tables and only significant interactions are presented in graphs.

RESULTS

***Bion and salicylic effect on disease severity (%)*:** Bion and salicylic acid applied through various application methods significantly ($p \leq 0.05$) reduce the disease severity in tomato plants. Maximum decrease in disease severity was noticed at 5th day when Bion was applied through foliar application followed by salicylic acid and water treated control. A Similar but decreasing trend was observed in 2nd year of study (Table 1).

Table 1. Impact of Bion and Salicylic acid applied through various methods on disease severity (%) at different days

Factors	**Disease Severity (%)**	
Days (D)	**2015**	**2016**
5	22.53 c	21.46 c
10	30.97 b	29.34 b
15	35.24 a	35.04 a
Method (M)		
Foliar Application	28.26 b	27.72 b
Seedling root dipping	30.90 a	29.72 a
Treatments (T)		
Control	42.94 a	41.69 a
Bion	19.55 c	18.12 c
Salicylic acid	26.26 b	25.84 b
LSD ($p \leq 0.05$)		
D	1.09	1.15
M	0.91	0.96
T	1.11	1.17
D × M	**	NS
D × T	**	*
M × T	**	**
D × M × T	NS	**

Any two means sharing same letters are not significant at $p \leq 0.05$. n = 3 ** = Highly Significant at $p \leq 0.01$; * significant at $p \leq 0.05$; NS = Non-significant

Regarding the interactive effect of chemicals × days, minimum disease severity was noticed at day 5, while maximum disease severity was observed at day 15. Bion reduced the disease severity followed by salicylic acid at respective days after application in 2015-16 (Fig. 1). Regarding the interactive effect of days × method, significant reduction in disease severity was observed when resistance inducers applied through foliar application at 5, 10 and 15 days respectively during 1st year of study (Fig. 2). Interactive effect of chemicals × application methods showed maximum decrease in disease severity in case of Bion applied through foliar application followed by salicylic acid as compared to control followed by seedling root dipping method in both years of study (Fig. 3). Interactive effect of chemicals × application methods × days showed that maximum reduction in disease severity was observed when Bion was applied through foliar application on 5th day followed by salicylic acid over control as compared to seedling root dipping method in 2016 (Fig. 4).

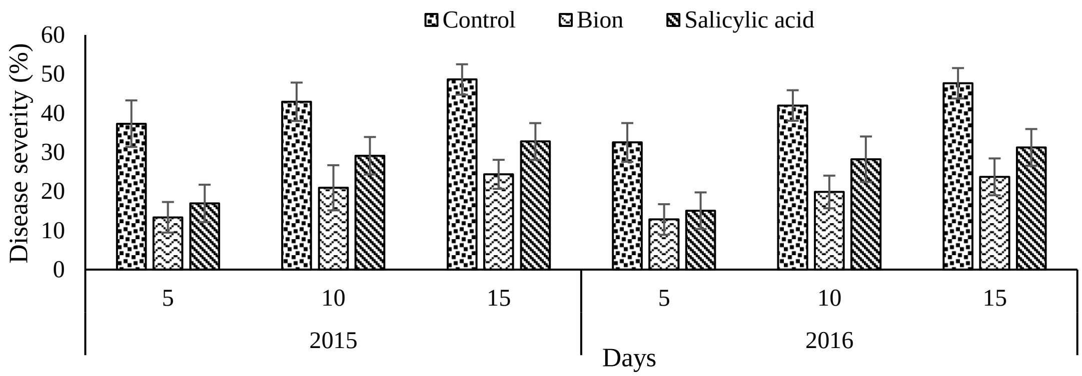

Figure 1. Interactive effect of chemicals × days on disease severity as influenced by the different resistance inducers applied through various methods.

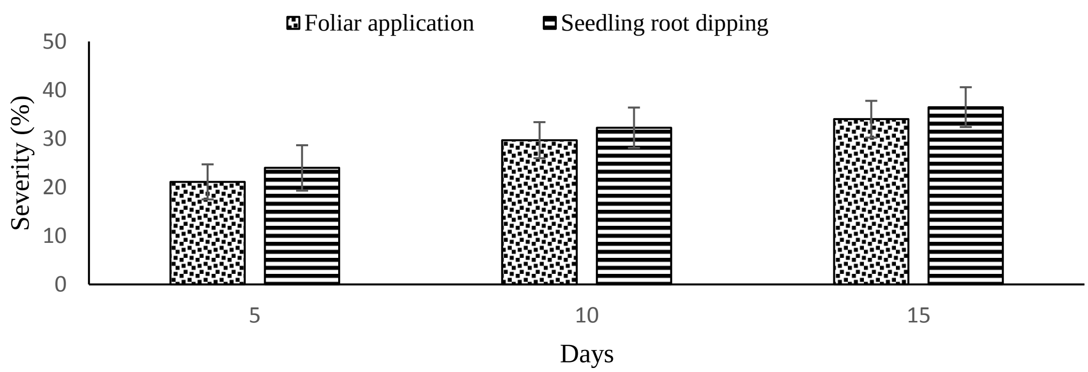

Figure 2. Interactive effect of days × application methods on disease severity as influenced by the different resistance inducers applied through various methods.

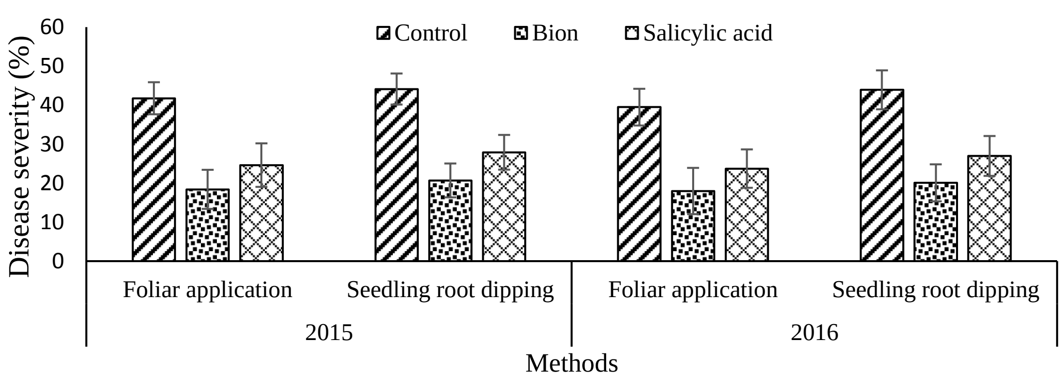

Figure 3. Interactive effect of chemicals × application methods on disease severity as influenced by the different resistance inducers applied through various methods.

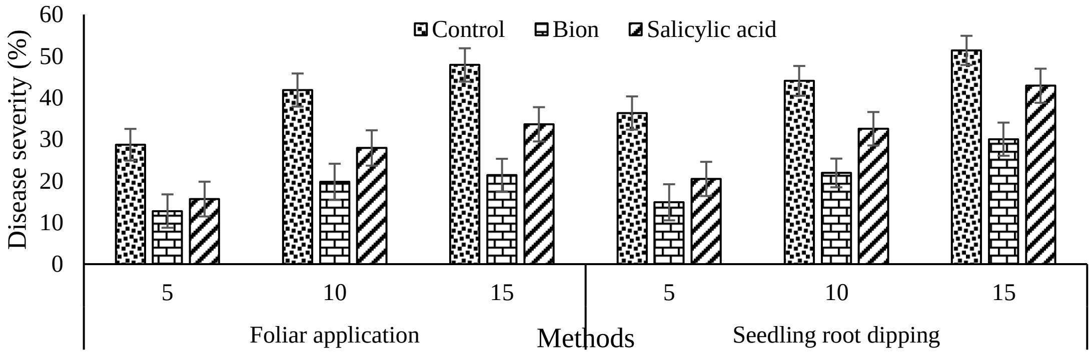

Figure 4. Interactive effect of chemicals × days × application methods on disease severity as influenced by the different resistance inducers applied through various method.

Table 2. Influence of resistance inducers applied through various methods on Peroxidase and Total phenolics at different days

Factors	**Peroxidase (POD)**		**Total phenolics**	
	Means		**Means**	
Days (D)	**2015**	**2016**	**2015**	**2016**
0	2.23 f	1.90 f	21.63 f	18.46 f
2	3.64 e	3.24 e	39.26 e	37.43 e
4	4.32 d	4.04 d	42.65 d	40.73 d
6	5.39 c	5.03 c	51.40 c	48.58 c
8	6.22 b	6.04 b	62.86 b	58.83 b
10	7.35 a	7.09 a	70.06 a	66.10 a
Method (M)				
Foliar Application	4.96 a	4.64 a	48.97 a	47.00 a
Seedling root dipping	4.76 b	4.47 b	46.99 b	43.05 b
Treatments (T)				
Control	4.58 f	4.37 f	36.15 f	31.46 f
Control + *A. solani*	4.73 e	4.47 e	39.28 e	37.14 e
Bion	4.99 b	4.64 b	57.24 b	54.01 b
Salicylic acid	4.83 D	4.52 D	44.47 D	42.20 D
Bion + *A. solani*	5.10 A	4.75 A	60.72 A	57.97 A
Salicylic acid + *A. solani*	4.92 C	4.60 C	49.99 C	47.36 C
LSD ($p \leq 0.05$)				
D	0.03	0.02	1.37	0.98
M	0.02	0.01	0.79	0.57
T	0.03	0.02	1.55	0.98
D × M	**	**	NS	**
D × T	**	**	**	**
M × T	*	**	NS	**
D × M × T	**	**	NS	NS

Any two means sharing same letters are not significant at p ≤ 0.05. ** = Highly Significant at $p \leq 0.01$; * = Significant at $p \leq 0.05$

Impact of Bion and Salicylic Acid on Peroxidase activity (Change in absorbance/min/mg of protein): Bion and salicylic acid applied through foliar and seedling root dipping method significantly ($p \leq 0.05$) increase the peroxidase activity at various growth intervals during both years. Maximum POD activity in tomato plants was observed at 10th day followed by 8th day during both years. While minimum POD activity was observed in control in both years of study. Regarding the application methods of resistance inducers by foliar application improved the POD activity as compared to the seedling root dipping method. Significant increase was observed in POD activity in inoculated plants as compared to un-inoculated plants (Table 2). Regarding the interactive effect of various factors, chemicals × days, days × application method, chemicals × application methods and chemicals × days × application methods significantly ($p \leq 0.05$) enhanced the POD activity in both years. In the interactive effect of chemicals × days, maximum POD activity was observed after 10th day when Bion applied to inoculated plants, while minimum POD activity was observed in control treatment during both year trials (Fig. 5). In the interactive effect of day's × application method, maximum POD activity was observed on 10th day when resistance inducers were applied through foliar application in both year (Fig. 6). In the interactive effect of chemicals × application methods, in both years maximum POD activity was observed in Bion treated inoculated plants through foliar application (Fig. 7). In the interactive effect of chemicals × days × application methods, maximum POD activity was observed in Bion treated

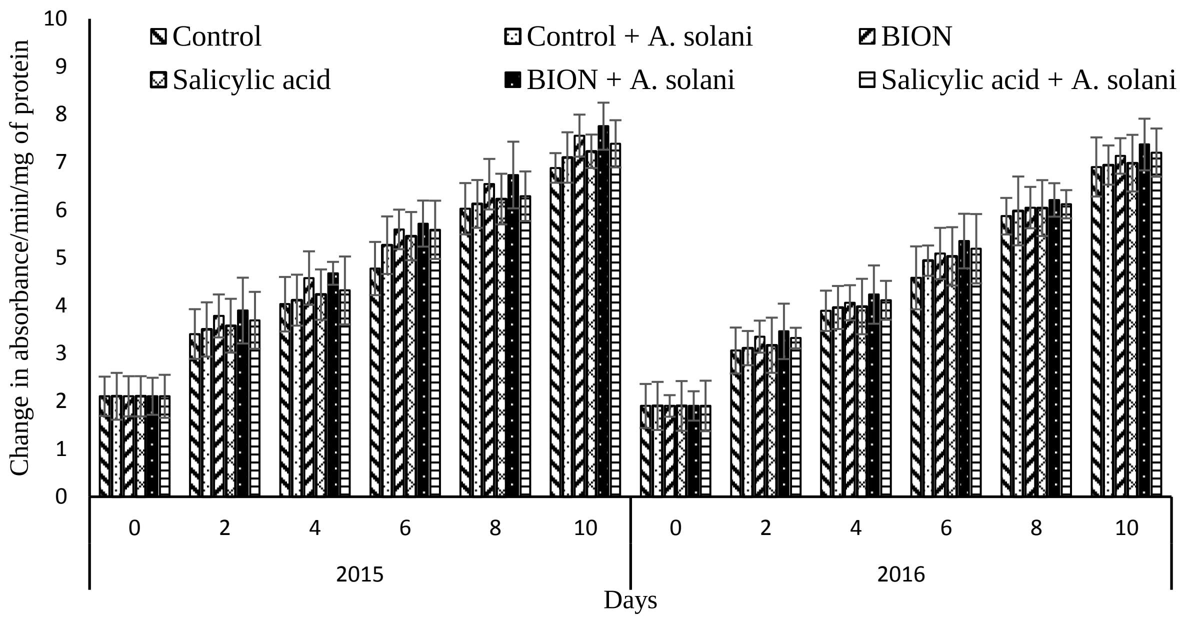

Figure 5. Interactive effect of resistance inducers × days on peroxidase activity as influenced by the different resistance inducers applied through various methods.

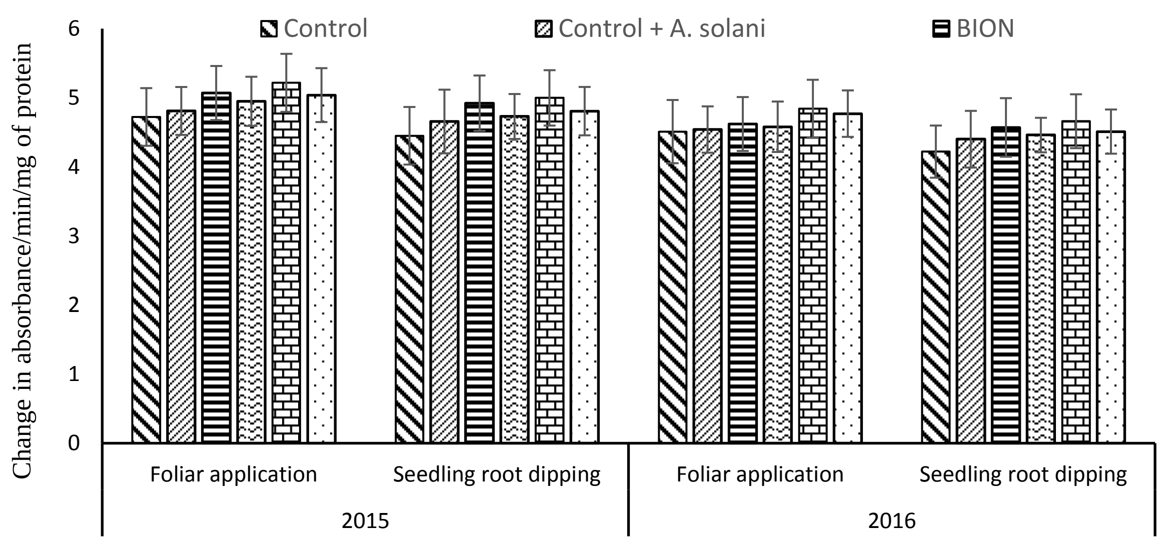

Figure 6. Interactive effect of resistance inducers × application method on peroxidase activity as influenced by the different resistance inducers applied through various methods.

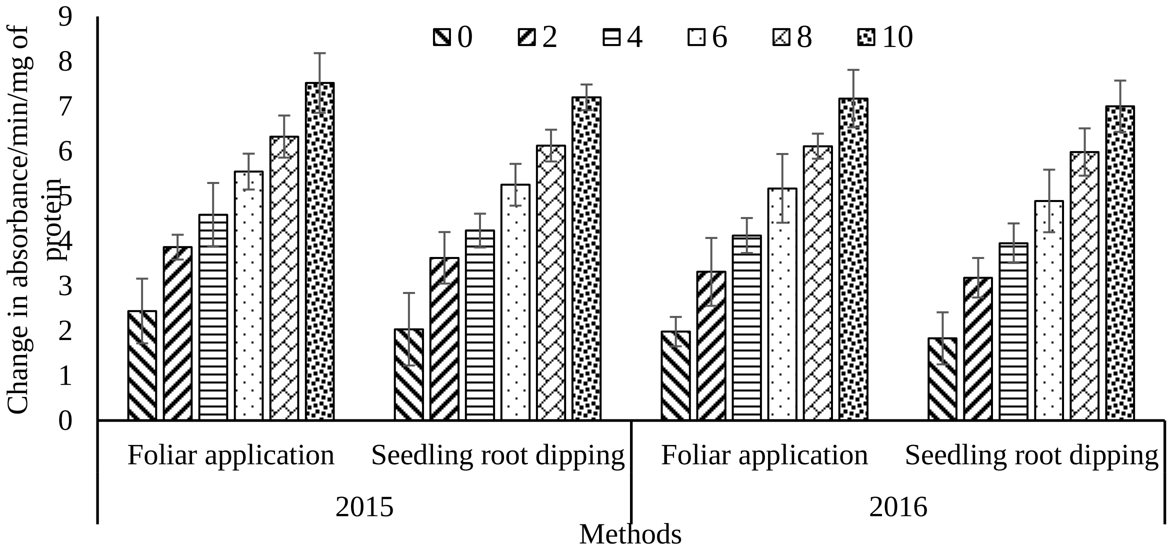

Figure 7. Interactive effect of application method × days on peroxidase activity as influenced by the different resistance inducers applied through various methods.

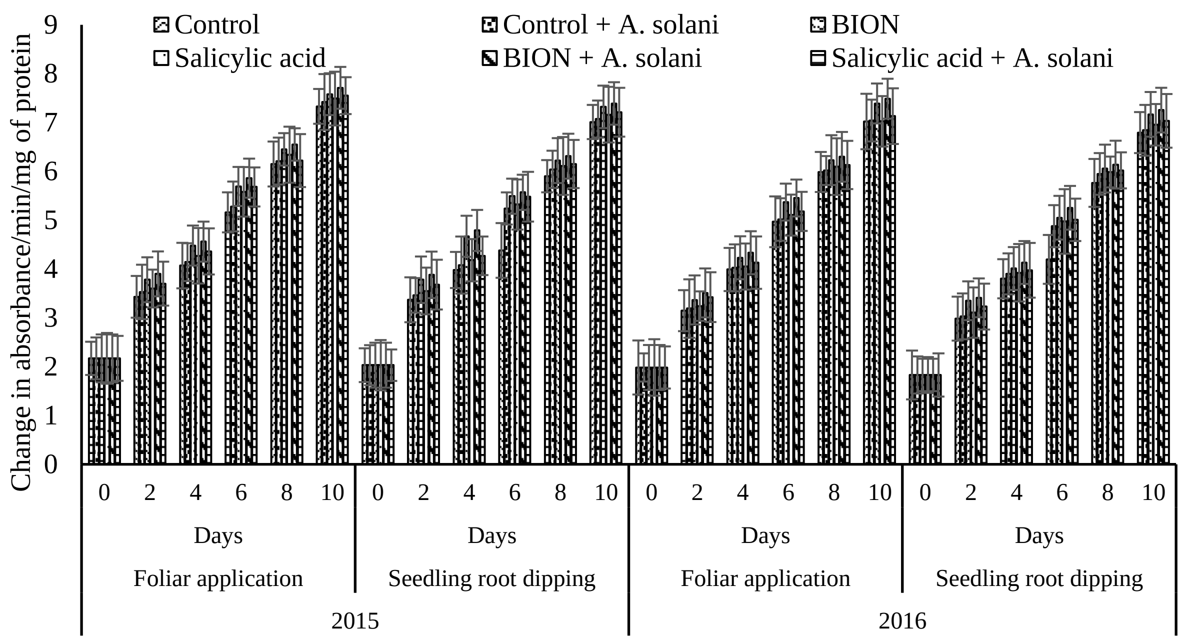

Figure 8. Interactive effect of resistance inducers × days × application method on peroxidase activity as influenced by the different resistance inducers applied through various methods in both years.

inoculated plants through foliar application on 10th day as compared to seedling root dipping in both years (Fig. 8).

Impact of Bion and Salicylic Acid on Total phenolics (µm/g of plant tissues): Bion and salicylic acid applied through foliar and seedling root dipping method significantly ($p \leq 0.05$) increase the total phenolics at various growth intervals during both years. Maximum phenolics content in tomato plants was observed at 10th day followed by 8th day during 1st year of the experiment. Similar trend was also observed during the 2nd year. While minimum total phenolics contents were noticed in control during both years of study. Regarding the application methods of resistance induced by foliar application improved the total phenolics as compared to the seedling root dipping method. Significant increase was observed in total Phenolics contents in inoculated plants as compared to un-inoculated plants. Ten days after treatment, compared with controls, total phenolics were higher in both Bion treated inoculated plants and salicylic acid-treated inoculated plants, but total phenolics in Bion treated plants were significantly greater than in the salicylic acid treated plants during both years. Decreasing trend was observed in total phenolics during in 2nd year of study (Table 2). In the interactive effect of chemicals × days, maximum total phenolic contents were observed after 10th day when Bion + *A. solani* was applied, while minimum total phenolic contents were observed in control during both year of study (Fig. 9).

In the interactive effect of day's × application method, maximum total phenolic contents were observed on 10th day when resistance inducers were applied through foliar application in 2nd year (Fig. 10). In the interactive effect of chemicals × application methods, maximum total phenolic contents were witnessed in Bion treated inoculated plants through foliar application in 2nd year of study (Fig. 11).

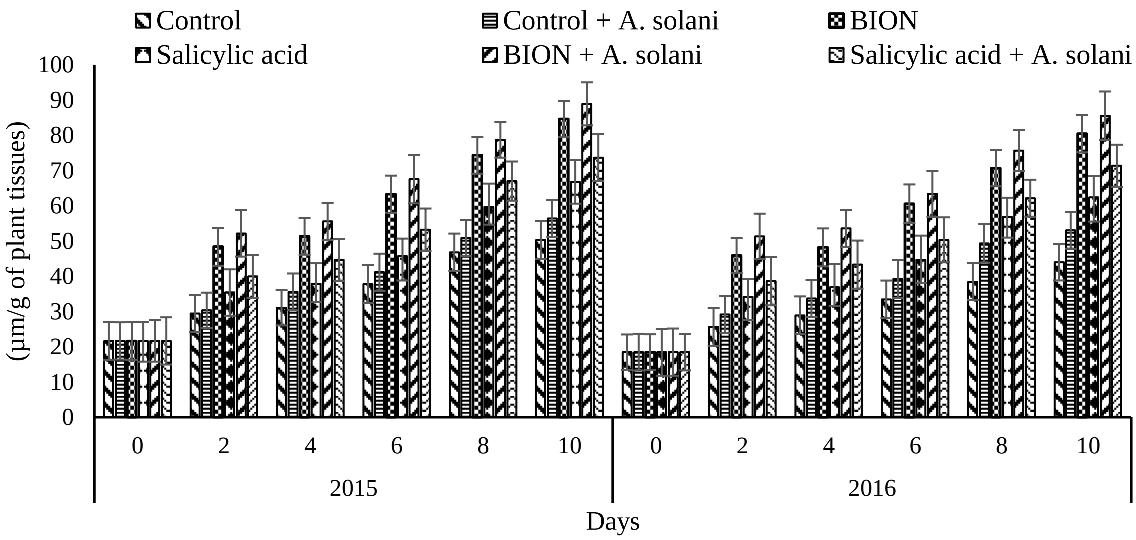

Figure 9. Interactive effect of resistance inducers × days on total phenolics influenced by the different resistance inducers applied through various methods.

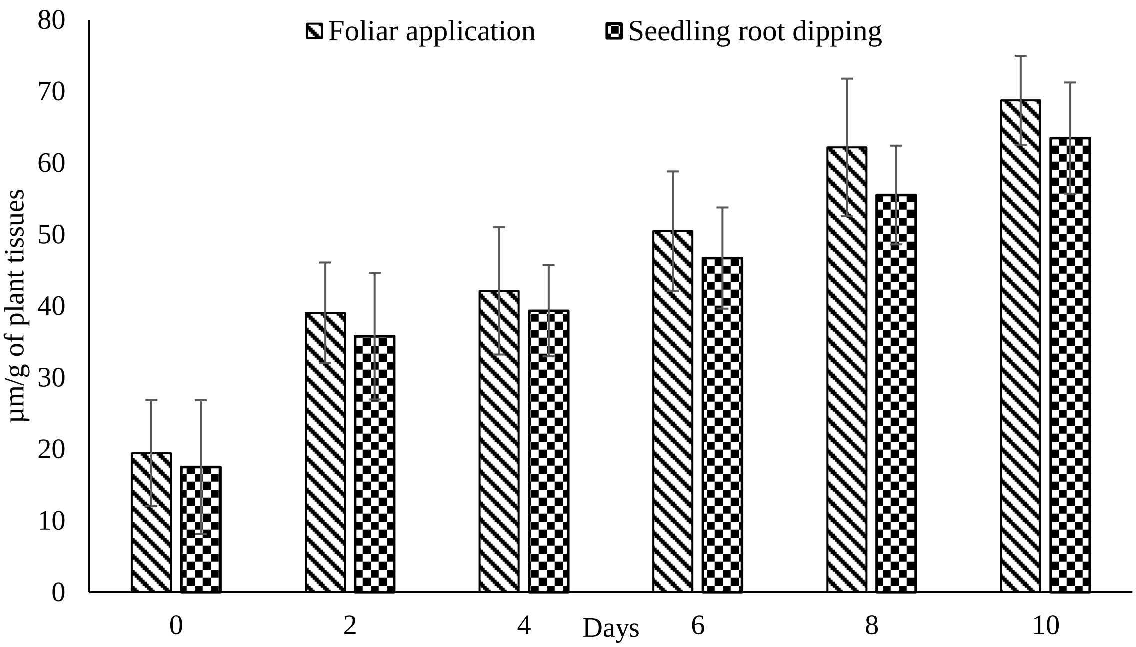

Figure 10. Interactive effect of application method × days on total phenolics influenced by the different resistance inducers applied through various methods in 2016.

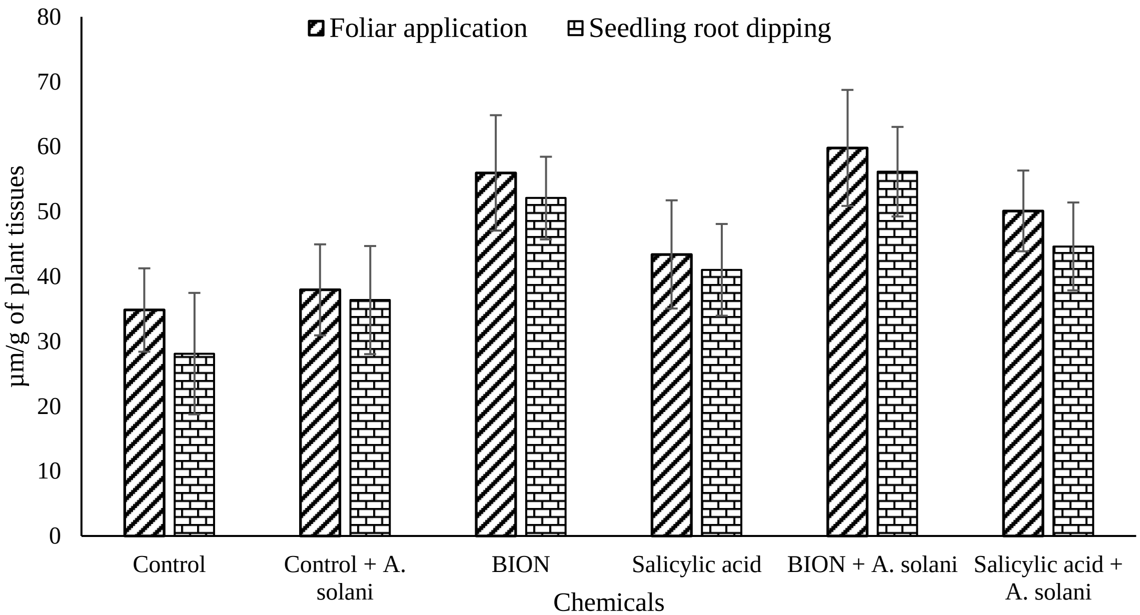

Figure 11. Interactive effect of application method × chemicals on total phenolics influenced by the different resistance inducers applied through various methods in 2016.

DISCUSSION

Biochemical markers are very important to study the induced resistance in tomato plants. When a necrotrophic pathogen attacks the plants, it triggers the plant defense mechanism through different biochemical and antioxidant alteration. Several synthetic resistance inducers are available which have no direct antimicrobial activity but initiate the resistance mechanism without any hazardous effect like fungicidal control. In the current study, foliar application of Bion and SA minimized the severity of disease in 2015-16, of early blight symptoms of tomato at 5, 10 and 15 days as compared to seedling root dipping method in both years. Foliar application was found more effective than seedling root dipping method. This result is might be due signal transduction from roots to distant parts of the plant take time as compared to foliar application which directly triggers the defense genes in leaves. Achuo *et al.* (2004) narrated that Bion foliar application induced resistance in tomato against *Botrytis cinerea* has been found effective. Many studies on Bion and salicylic acid proved their impact in managing many diseases of plants caused by various pathogens including bacteria and fungi (Baysal *et al.*, 2005). These results are supported by the findings of Spletzer and Enyedi (1999) as they observed the reduction in disease severity of early blight of tomato after application of SA. Similarly, Abo-Elyousr *et al.* (2008) observed the same results in case of Bion on onion crop. Our results agree with the results of Mosa (2002) who stated that 1 mM dose of salicylic acid reduced the severity of rice blast caused by *Pyricularia grisea*. Bion is a functional derivative of salicylic acid that initiates resistance against many plant pathogens by activating the SAR signaling pathway (Vallad and Goodman, 2004). Scarponi *et al.* (2001) stated that Bion was found effective in tomato plants against *Pseudomonas syringae* pv. tomato. Soylu *et al.* (2003) obtained similar results against bacterial canker. Our results are also verified by Anfoka (2000) who describe Bion efficacy against *Cucumber mosaic virus*. Several studies supported our results by proving the efficacy of exogenous application of SA and Bion for controlling fungal and bacterial diseases (Siegrist *et al.*, 1997; Cole, 1999), the effect of which expressed in the form of induced resistance.

Foliar application of Bion and Salicylic acid (SA) on inoculated plants induced significant increases of PO and total Phenolics at all sampling intervals as compared to seedling root dipping method, Maximum activity of all the biochemical attributes was observed at tenth day of sampling. Decreasing trend in the biochemical attributes was observed during second year of study because of disease severity was less in second year.

We have chosen certain enzymes like peroxidase (PO) and total Phenolics because of their vital role in SAR induction this has been previously documented in different plant species (He *et al.*, 2002; Yasin *et al*., 2017).

Regarding PO activity significant increase was observed with Bion treated inoculated plants followed by Salicylic acid and control. PO activity in pathogen inoculated plants treated with resistance inducers was significantly higher because increase in PO activity is related to systemic acquired resistance in plants against numerous pathogens (Baysal *et al.*, 2005). It also acts as a booster of many plants resistance processes, such as lignin biosynthesis, oxidative cross linking of plant cell walls and generate reactive oxygen species (ROS) (Bestwick *et al*., 1998). Noteworthy increase in the activity of PO triggers the systemic acquired resistance against *Clavibacter michiganensis* in tomato plants treated with Bion (Baysal *et al*., 2003). Tremendous increase in PO activity was detected in soybean plants sprayed with BTH (Nafie and Mazen, 2008). Similarly, Karthikeyan *et al*. (2009) reported increased PO activity in black gram (*Vigna mungo*) sprayed with SA (100 ppm) and BTH (100 ppm) providing resistance against urdbean leaf crinkle virus. Farouk and Osman (2012) also observed enhanced PO activity in bean plants which were treated with salicylic acid and methyl ester of jasmonic acid.

Significant increase in total phenolic contents was observed in Bion and salicylic acid treated inoculated plants in both years. Phenolics compound accumulation at the site of infection restricted the pathogen due to their toxic effects. In addition, phenolic contents may also increase the mechanical strength of the host cell wall to avoid the pathogen further invasion (Benhamou *et al.*, 2000). Results of present study are further supported by Stadnik and Buchenauer (2000) described the increase in PAL activity and accumulation of cell wall-bound phenolic compounds in Bion treated wheat plants. Anttonen *et al.* (2003) documented increased level of two flavanols, i.e., quercetin and kaempferol in BTH treated berries under field conditions. Katoch (2005) observed increased o-dihydroxy phenol content in pea plants sprayed with 5mM SA compared to control plants.

***Conclusion*:** Our results showed that Bion could be an alternate to highly toxic fungicides usage against early blight. It is less toxic, quickly decomposable compound and nature friendly. But further research is needed to confirm the success of resistance inducers under field condition.

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