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Full Length Research Paper

Induction of systemic resistance in soybean plants against *Fusarium* wilt disease by seed treatment with benzothiadiazole and humic acid

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The ability of benzothiadiazole (BTH), humic acid (HA) and their combination when used in seed soaking to induce systemic resistance against a pathogenic strain *of Fusarium oxysporum* was examined in four soybean cultivars under greenhouse conditions. Both inducers and their combination were able to protect soybean plants against damping-off and wilt diseases compared with check treatment. These results were confirmed under field conditions in two different locations that is Minia and New Valley governorates, Egypt. The tested treatments significantly reduced damping-off and wilt diseases and increased growth parameters, except number of branches plant^{-1}, and seed yield. Application of BTH (0.25) + HA (4 g/L) was the most potent in respect to this treatment. Soybean seed soaking in BTH+HA recorded the highest activities of oxidative enzymes followed by BTH in four soybean cultivars. Whereas, HA treatment recorded the lowest increase of these oxidative enzymes. Also, similar results were obtained in case of total phenol but HA increased the total phenol more than BTH in all tested cultivars.

Key words: Induce systemic resistance, soybean, wilt disease, benzothiadiazole (BTH), humic acid **(**HA).

INTRODUCTION

Soybean (*Glycine max L.*) is one of the world's most important sources of oil and protein. It has the highest protein content among leguminous crops (El-Abady et al., 2008). Soybean plants are subjected to attack by several fungal, bacterial and viral diseases that cause great losses in the yield. Wilt disease of soybean caused by *Fusarium oxysporum* is one of the most destructive diseases of the crop and is a very common soil-borne fungus (Hashem et al., 2009; Fayzalla et al., 2009). This pathogen is difficult to control because of their persistence in the soil and wide host range. Some chemicals are effective in controlling this disease but these chemicals are expensive and not environmental friendly. Therefore alternative measures are being tested, including induce resistance by using biotic and abiotic treatments. The phenomenon of systemic induced resistance (SIR), in which resistance to disease is enhanced in tissues distant from the site of the prior inducing treatment, has been extensively reported for a number of plant/pathogen systems and has been the subject of recent reviews (Hammerschmidt, 1999).

The majority of these studies have been conducted under controlled environment conditions. However, it has been demonstrated under field conditions for a limited number of plant/pathogen interactions. Induced resistance in some plants against root diseases was reported by Sarwar et al. (2005), and Abd-El-Kareem (2007). A new product, promoted as a safe, reliable, and nonphytotoxic plant protection agent, benzothiadiazole (BTH), was recently identified by scientists at Novartis as a novel disease-control compound. Exogenous application of BTH to tobacco, wheat, soybean, chickpea, potato, apple and Arabidopsis leaves has been shown to activate a number of SAR-associated genes, leading to enhanced plant protection against various pathogens that is *Blumeria graminis* f. sp. *tritici, Phialophora gregata*, *F. oxysporum ciceri*, *Fusarium roseum* var.*sambucinum*, *Erwinia amylovora* (Friedrich et al., 1996; Görlach et al.,

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1996; Lawton et al., 1996; Sarwar et al., 2005; Nafie and Mazen, 2008; Mejdoub-Trabelsi and Chérif, 2009; Abo-Elyousr et al., 2010). These studies provided evidence that induction of SAR gene expression by BTH did not require the contribution of salicylic acid and/or jasmonate, suggesting that this compound could act as a secondary messenger analog capable of activating the SAR signal transduction pathway independently of the accumulation of other signal molecules (Lawton et al., 1996). Benhamou and Bélanger (1998) demonstrated that application of BTH to cucumber leaves before challenging with the root pathogen *Pythium ultimum* triggered a set of plant defense reactions that resulted in the creation of a fungitoxic environment, which protected the roots by restricting pathogen growth to the outermost tissues. Dann et al. (1998) reported that severity of white mold disease in field grown soybean was significantly reduced by sprays of INA (2,6 dichloroisonicotinic acid) and BTH and increased seed yield. Sarwar et al. (2005) showed that exogenously applied salicylic acid and BTH provided protection to chickpea plants against infection with *F. oxysporum ciceri* similar to that of Benlate.

HA is a suspension, based on potassium-humates, which can be applied successfully in many areas of plant production as a plant growth stimulant or soil conditioner for enhancing natural resistance against plant diseases (Scheuerell and Mahaffee, 2004), stimulate plant growth through increased cell division, as well as optimized uptake of nutrients and water and by increasing the soil microorganisms (Chen et al., 2004). Several reports indicated the efficiency of HA in reducing some plant diseases (Abd-El-Kareem, 2007; Yigit and Dikilitas, 2008; El-Mohamedy and Ahmed, 2009). The use of inducers, BTH and HA substances would permit a reduction in the use of agrochemicals such as fungicides. So, this investigation was done to evaluate effectiveness of BTH and HA treatments on control of wilt disease, of soybean under greenhouse and field conditions as well as seed yield. Also, biochemical changes associated with the application of the two inducers were assessed.

MATERIALS AND METHODS

Isolation and identification of the causal pathogen

Naturally diseased soybean plants showing wilt disease symptoms were collected from different localities of Minia and New Valley governorates in summer 2009 growing season. They were thoroughly washed in tap water, cut in small pieces of 0.5 cm and surface sterilized for 2 min in 2% sodium hypochlorite solution, then rewashed several times in sterilized distilled water and dried between a number of folds of sterilized filter papers. The surface sterilized samples were plated onto Potato Dextrose Agar (PDA) medium supplemented with penicillin (20 Iu ml^{-1}) and incubated at 25±1°C for 6 days. The developed fungal colonies were purified by single spore techniques then identified according to Booth (1985).

Pathogenicity test

Pathogenicity of eight *F. oxysporum* isolates obtained from diseased soybean plants were tested on cv. Giza 21. This experiment was carried out at New Valley Agriculture Research Station, soil pots infestation was done by soil using the homogenized culture technique according to the method devised by Muthomi et al. (2007).

Preparation of fungal inoculum

Disks taken from 1 week old culture of *F. oxysporum* prepared were inoculated in 75 ml Potato Dextrose (PD) broth medium in flask (250 ml) and incubated at 25 ±1°C. The obtained fungal suspension was collected on No. 1 Whattman filter paper and rinsed with sterile distilled water, then placed in a warring blender with a small amount of sterile water and blended for 2 min at high speed. Sterile distilled water was then added to each inoculum suspension to give a final concentration of 10^6 colony forming unit (CFU/ml) and used for soil infestation 5 days before sowing. Five seeds were sown in each pot (30 cm). Percentage of damping–off was recorded 30 days after seeding. Number of plants were calculated 3 months after seeding and the severity of wilt was determined according to Abdou et al. (2001) using a rating scale of 0 to 5 on the basis of root discoloration or leaf yellowing as follows, 0 = neither root discoloration nor leaf yellowing, 1 = 1 to 25% root discoloration or one leaf yellowed, 2 = 26 to 50% root discoloration or more than one leaf yellowed, 3 = 51 to 75% root discoloration plus one leaf wilted, 4 = up to 76% root discoloration or more than one leaf wilted, and 5 = completely dead plants. For each replicate a disease severity index (DSI) similar to that described by Liu et al. (1995) was calculated as follows:

$$DSI = \frac{\Sigma d}{d\,\mathrm{max} x n} \mathrm{X} 100$$

Where: DSI is the disease rating possible, d max is the maximum disease rating and n is the total number of plants examined in each replicate. Re-isolation of the pathogen from the infected plants was also done to confirm the causal agent of wilting.

Control of damping-off and wilt diseases caused by F. oxysporum under greenhouse condition

In this experiment, benzothiadiazole (BTH, Benzo-(1, 2, 3) thiadiazole-7-carbothioic acid *S*-methyl ester wettable granule 50% WG, Bion ^{R}) and humic acid (HA, Potassium humate soluble granule 85% WSG, Humus ^{R}) were used as seed soaking for 20 min to evaluate their efficiency for controlling damping-off and wilt diseases caused by *F. oxysporum* in soybean. Five soybean (cvs. Giza 21, Giza 22, Giza 35 and Giza 111) seeds per pot were sown in 30-cm pots filled with sterilized *F. oxysporum* infested soil at the rate of 100 ml homogenized culture per pot as previously mentioned , 5 days before planting. The treatments were as follows: BTH at 0.25 and 0.5 g a.i./L, HA at 2.5 and 5 g a.i. /L as well as a combination of BTH and HA at 0.25 g BTH and 2.5 g HA, 0.25 g BTH and 5 g HA, 0.5 g BTH and 2.5 g Humic, 0.5 g BTH and 5 g HA each 1 L. The control treatment was soil infested with *F. noxysporum* and sown with untreated soybean seeds at the same rate. A set of five pots for treatment were used. Each pot received equal amounts of water. Other agricultural practices were performed according to normal schedule (El-Abady et al., 2008). Percentage of damping-off and wilt severity were recorded 30 and 90 days after seeding, respectively.

Field experiment

Field experiment was carried out at two localities that is, New Valley

Agriculture Research Station and Experimental Farm of Plant Pathology Department, Faculty of Agriculture, Minia University during the growing summer season of 2010. Soybean seeds cvs. Giza 21, Giza 22, Giza 35, and Giza 111 were soaked in the same tested treatments in greenhouse for 20 min, then dried for 30 min before seeding while in control treatment, seeds were soaked in distilled water as mentioned earlier. Treated soybean seeds were sown in the field on the 4th of May 2010 in both locations. A split plot design with three replicates was used in these experiments, the main plots represented varieties while sub-plots represented treatments.

The area of each sub–plot was 10.5 m^2 (3.0 x 3.5) containing five rows, each row was 3.5 m in length and distance between rows was 60 cm. All treatments were sown in hills 20 cm apart on both sides of row ridge and two seeds per hill (plant population = 140,000 plants/feddan). All recommended agricultural practices were adopted throughout the two locations. After 30 days from seeding date, severity of damping-off was determined. Wilt severity was also recorded on a random sample of plants of the sub-plots (20 plants) three months after seeding according to Abdou et al. (2001). At harvest stage, plant growth parameters plant height, number of branches and pods $plant^{-1}$and seed weight ton $feddan.^{-1}$ was recorded.

Effect of soybean seed treatment with inducer chemicals on oxidative enzymes activity and phenol content

Activity of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) enzymes and total phenol contents (TPC) was studied in tissue extracts of soybean plants emerged from treated with BTH at 0.5 g/L, HA at 5 g/L as well as a combination of BTH 0.25 g/L and HA 4 g/L and untreated seeds. All treatments were grown in soil infested with *F. oxysporum* pathogen. 1 g of plant tissue was homogenized in 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.8) containing 1 M NaCl, 1% polyvinylpyrrolidone, (PVP),1 mM EDTA and 10 mM β-mercaptoethanol (Biles and Martyn, 1993).

After filtration through cheese-cloth, the homogenates were centrifuged at 8000 rpm at 4°C for 25 min. The supernatants (crude enzyme extract) were stored at −20°C or immediately used for determination POX, PPOX and PAL enzymes activities and total protein. In the case of every enzyme under investigation, each treatment consisted of four replicates (3 plants/ replicate) and two spectrophotometric readings using Milton Roy Spectrophotometer (Milton Roy spectronic1201) were taken per replicate. The experiment for bioassays was repeated twice in time.

Peroxidase activity

The enzyme activity of PO was determined using spectro-photometer (Hammerschmidt et al., 1982) using guaiacol as common substrate for peroxidases. The reaction mixture consisted of 0.2 ml crude enzyme extract and 1.40 ml of a solution containing guaiacol, hydrogen peroxide (H_2O_2) and sodium phosphate buffer (0.2 ml 1% guaiacol+0.2 ml 1% H_2O_2+1 ml 10 mM potassium phosphate buffer), was incubated at 25°C for 5 min and the initial rate of increase in absorbance was measured over 1 min at 470 nm using spectrophotometer. Peroxidase activity was expressed as units of POX/mg protein (Urbanek et al., 1991).

Polyphenoloxidase activity

The activity of PPO was determined by adding 50 µl of the crude extract to 3 ml of a solution containing 100 mM potassium phosphate buffer, pH 6.5 and 25 mM pyrocatechol. The increase of absorbance at 410 nm, for 10 min at 30°C, was measured (Gauillard et al., 1993). One PPOX unit was expressed as the variation of absorbance at 410 nm per mg of soluble protein per min.

Phenylalanine ammonia layse activity

Phenylalanine ammonia-layse (PAL) activity was determined following the direct spectrophotometric method adapted by Cavalcanti et al. (2007). 200 µl of the crude enzyme extract previously dialyzed overnight with 100 mM Tris- HCl buffer, pH 8.8, were mixed to obtain a solution containing 200 µl 40 mM phenylalanine, 20 µl 50 mM β-mercaptoethanol and 480 µl 100 mM Tris-HCl buffer, pH 8.8. After incubation at 30°C for 1 h, the reaction was stopped by adding 100 µl 6 N HCl. Absorbance at 290 nm was measured and the amount of trans-cinnamic acid formed was evaluated by comparison with a standard curve (0.1 to 2 mg trans-cinnamic acid/ml) and expressed as units of PAL min^{-1} mg $protein^{-1}$.

Protein concentration

Total protein content of the samples was quantified according to the method described by Bradford (1976).

Determination of phenolic compounds

To assess phenolic content, 1 g of fresh plant sample was homogenized in 10 ml 80% methanol and agitated for 15 min at 70°C. 1 ml of the extract was added to 5 ml of distilled water and 250 µl of 1 N Folin-Ciocalteau reagent and the solution was kept at 25°C. The absorbance was measured with a spectrophotometer at 725 nm. Catechol was used as a standard. The amount of phenolic content was expressed as phenol equivalents in mg g^{-1} fresh tissue (Saikia et al., 2006).

Statistical analysis

All experiments were performed twice. Analyses of variance were carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test for significant difference between treatments at P ≤0.05 (Gomez and Gomez, 1984).

RESULTS

Pathogenicity test and identification of the causal organism(s)

The eight fungal isolates obtained from different naturally infected soybean plants showing wilt symptoms were able to cause damping-off and wilt symptoms on artificially inoculated soybean. Data presented in Table 1 show that the highest percentage of damping-off and wilt were caused by isolate FO1 followed by isolate FO6, while the least infection was expressed by isolate FO5. All the obtained isolates were identified as *F. oxysporum* according to the descriptions of Booth (1985) and confirmed by Assuit University Mycological Center (AUMC), Assuit Governorate, Egypt.

Table 1. Pathogenicity of *Fusarium oxysporum* isolates on soybean plants.

F. oxysporum isolates	Locations	Percentage damping-off	Percentage wilt
FO1	El-Minia	36	57.67
FO2	El-Minia	28	40.25
FO3	El-Minia	20	35.28
FO4	New Valley	16	32.67
FO5	New Valley	12	15.38
FO6	New Valley	28	50.67
FO7	New Valley	28	44.25
FO8	New Valley	20	38.84
LSD at 0.05		3.79	5.51

Effect of BTH, HA on damping-off and wilt diseases caused by *F. oxysporum*

Under greenhouse conditions

Data present in Table 2 reveal that both the tested chemical inducers individually or combinations, in most cases, were significantly effective in reducing infection with *F. oxysporum* under greenhouse conditions compared with the check treatment (control). This reduction reached its maximum when combination between BTH and HA was used at 0.25 and 4 g/L followed by 0.5 and 2 g/L for all the tested cultivars. BTH and HA at 0.25 and 4 g/L reducing the average damping-off and wilt for the four soybean cvs, that is Giza 21, Giza 22 , Giza 35 and Giza 111 from 36, 28, 36, 20% damping-off and 57.33, 30.20, 49.18 and 24.37% wilt in control to 8, 8, 8, 4% damping-off and 8.81, 6.33, 11.33 and 4.67% wilt, respectively.

On the other hand, soybean seed treated with HA at 2 g/L recorded the lowest reduction of damping-off for all the tested cultivars, while seed treated with BTH at 0.25 g/L recorded the lowest wilt for all the tested cultivars. Also, the obtained results show considerable differences in the response of different soybean cultivars to infection with *F. oxysporum.* Generally, soybean Giza 21 cv. revealed to be more susceptible to *F. oxysporum* followed by Giza 35 and Giza 22 cvs., respectively. Whereas, Giza 111 cv. was the least affective one, where it resulted in less damping-off and wilt.

Under field conditions

The effect of the resistance inducers HA and BTH individually or in combination at different concentrations on damping-off and wilt of four soybean cultivars under field conditions are shown in Tables 3 and 4. The obtained data showed that all treatments, in most cases, caused significant reduction in the percentage of damping-off and wilt severity compared with control in both locations and the combination between HA and BTH decreased the percentage of damping-off and wilt severity in both locations more than using them separately on the tested soybean cultivars. Soybean treated with a combination of BTH and HA at a concentration of 0.25+4 g/L caused the highest protection against infection with damping-off and wilt diseases, where it led to decreased damping-off in New Valley location from 18.33, 14.25, 23.67, 9.33% in control to 5.33, 6.67, 6 and 2.67% and in Minia location, this treatment reduced damping-off from 22.33, 18.25, 25.67 and 11.33% in control to 6.67, 6.67, 7 and 3% for the four soybean tested cultivars that is Giza 21, Giza 22, Giza 35 and Giza 111, respectively.

Also, the wilt symptoms were reduced from 21.41, 10.67, 27.67 and 10.20% in control to 3, 2.67, 7.33 and 2.15% in New Valley location and from 25.41, 13.67, 26.67 and 12.20% in control to 5, 3.33, 10.33 and 2.56% in Minia location for the tested soybean cultivars, respectively. On the other hand, soybean treated with HA at 2 g/L recorded the least reduction and non significance of damping-off disease compared with control in both locations, while soybean treated with BTH at 0.25 g/L recorded the lowest wilt severity for the tested cultivars in both locations compared with control. In general, BTH was highly effective to reduce the incidence of damping-off in seedling stage than HA but in contrary in case of wilt disease. Also, the four tested cultivars were infected with damping-off and wilt diseases either in New Valley or in El-Minia governorates and Giza 35 cv. was the most susceptible to infection followed by Giza 21 and Giza 22 cvs., respectively. Meanwhile, cv. Giza 111 was the least susceptible one.

Effect of BTH and HA on growth parameters and seed yield under field conditions

Data present in Tables 5 and 6 demonstrate that the various responses of the four tested soybean cultivars at their growth parameters (plant height, and no. of pods/

Table 2. Effect of BTH and HA on damping-off and wilt diseases caused by *F. oxysporum* isolate FO1 of the four soybean cultivars under greenhouse conditions

Treatments	Con. (g a.i./L)	Cultivars							
		Giza 21		Giza 22		Giza 35		Giza 111	
		% Damping-off	% Wilt	% Damping-off	% Wilt	% Damping-off	% Wilt	% Damping-off	%Wilt
BTH	0.25	32	20.00	20	28.00	28	34.72	12	17.85
	0.50	16	17.94	16	12.40	16	15.33	8	12.11
HA	2	36	16.33	24	17.00	32	21.90	20	14.40
	4	20	10.15	16	11.55	20	13.43	12	11.00
BTH +HA	0.25 + 2	16	16.40	12	10.63	20	17.62	16	8.73
	0.25 + 4	8	8.81	8	6.33	8	11.33	4	4.67
	0.50 +2	12	10.42	8	9.37	12	12.39	8	6.25
	0.50 +4	20	12.95	12	10.93	20	13.22	8	7.84
Control		36	57.33	28	30.20	36	49.18	20	24.73
LSD at 5%		4.02	4.63	3.40	4.51	3.88	5.14	2.09	2.94

Table 3. Effect of BTH and HA on damping-off and wilt diseases of the four soybean cultivars under field conditions in New Valley governorate at summer season 2010.

Treatments	Con. (g a.i./L)	Cultivars							
		Giza 21		Giza 22		Giza 35		Giza 111	
		% Damping-off	% Wilt	% Damping-off	% Wilt	% Damping-off	% Wilt	% Damping-off	% Wilt
BTH	0.25	12.33	9.33	11.67	9.00	17.33	16.33	7.33	6.53
	0.50	10.33	7.30	10.67	7.33	11.33	14.33	5.33	4.36
HA	2	15.00	8.36	12.33	7.67	20.33	14.25	9.00	5.24
	4	10.67	6.00	8.33	6.00	14.33	10.67	7.33	4.23
BTH+HA	0.25 + 2	7.33	6.44	7.33	4.67	8.33	9.67	3.33	3.12
	0.25 + 4	5.33	3.00	6.67	2.67	6.00	7.33	2.67	2.15
	0.50 +2	5.67	4.29	7.00	3.00	7.67	8.00	3.00	2.56
	0.50 +4	9.33	7.20	7.67	5.14	10.33	8.39	4.33	3.82
Control		18.33	21.41	14.25	10.67	23.67	27.67	9.33	10.2
LSD at 5%		3.47	3.40	3.17	2.54	3.97	4.38	2.33	2.07

Table 4. Effect of BTH and HA on damping-off and wilt diseases of the four soybean cultivars under field conditions in Minia governorate at summer season 2010.

Treatments	Con. (g/L)	Cultivars							
		Giza 21		Giza 22		Giza 35		Giza 111	
		% Damping-off	% Wilt	% Damping-off	% Wilt	% Damping-off	% Wilt	% Damping-off	% Wilt
BTH	0.25	16.67	12.23	15.00	12.00	23.67	19.33	10.33	9.53
	0.50	12.33	9.20	9.67	8.14	16.33	13.67	6.33	6.82
HA	2	19.00	10.46	17.67	9.67	26.33	18.25	12.00	8.00
	4	13.33	8.33	13.00	10.23	18.33	17.33	8.33	4.68
BTH+HA	0.25 + 2	11.33	7.41	10.33	5.67	11.33	12.00	4.33	3.25
	0.25 + 4	6.67	5.00	6.67	3.33	7.00	10.33	3.00	2.56
	0.50 +2	7.33	6.29	7.00	5.00	8.67	11.67	3.00	3.03
	0.50 +4	10.00	7.00	12.00	7.17	16.33	12.39	9.33	5.53
Control		22.33	25.41	18.25	13.67	25.67	26.67	11.33	12.2
LSD at 5%		4.01	3.57	4.66	3.04	4.50	5.17	2.70	2.37

plant) and seed yield/feddan as affected by different concentrations of BTH and HA either individually or in combination under Minia or New Valley governorates conditions. Results indicate that all treatments significantly improved plant height and increased number of pods plant^{-1} and seed yield fed.$^{-1}$ in comparison with those of check treatment for all the tested cultivars, while the increase of number of branches/plant was non-significant in all tested cultivars in both locations. In this respect, the combination between BTH and humic acid at concentrations of 0.25 and 4 g/L followed by 0.5 and 2 g/L were the superior treatments, while BTH when used individually at 0.25 g/L were the least effective ones. On the other hand, soybean Giza 111 cv. resulted with the best results for growth parameters and seed yield in case of seed treated or untreated in both locations.

Biochemical changes associated with inducer compounds

Activity of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase enzymes

The effect of BTH and HA individually or in combination as inducer chemicals on the activity of oxidative enzymes that is peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) of four soybean cvs. grown in soil infested with *F. oxysporum* was studied and the obtained data are shown in Figures 1 to 3. Both the tested inducers increased the activity of PO, PPO, PAL enzymes in the four soybean tested cultivars used either individually or in combination, compared with untreated plants (control). The combination between BTH and HA possessed the highest change in oxidative enzymes followed by BTH when used individually in the four soybean cultivars. Whereas, HA treatment recorded the lowest increase of these oxidative enzymes. On the other hand, the susceptibility of the four soybean cvs. was positively correlated with the activity of these enzymes, where cv. Giza 111 (more resistant to *F. oxysporum*) as it recorded the highest enzymes activity and cv. Giza 21 (highly susceptible) recorded the lowest enzymes activity either in treated or untreated plants.

Phenolic compounds content

The total phenol compounds content were highly increased in plants treated with inducers compared with untreated ones in all the tested cultivars and the combination between HA acid

Table 5. Effect of BTH and HA on growth parameters of the four soybean cultivars under field conditions on New Valley governorate at summer season 2010.

Treatments	Con (g/L)	Cultivars															
		Giza 21				Giza 22				Giza 35				Giza 111			
		Plant height (cm)	No. of branches Plant $^{-1}$	No. of Pods Plant $^{-1}$	Seed yield (Ten f $^{-1}$)	Plant height (cm)	No. of branches plant $^{-1}$	No. of Pods plant $^{-1}$	Seed yield (Ten/f $^{-1}$)	Plant height (cm)	No. of branches Plant $^{-1}$	No. of Pods/ plant	Seed yield (Ten/f $^{-1}$)	Plant height (cm)	No. of branches Plant $^{-1}$	No. of Pods Plant $^{-1}$	Seed yield (Ten/f $^{-1}$)
BTH	0.25	58.3	2.8	55.2	1.428	46.6	6.8	50.4	1.410	54.2	6.4	42.4	0.972	67.2	6.8	63.2	1.783
	0.5	62.8	2.9	62.8	1.615	50.2	6.6	53.4	1.486	54.3	6.8	45.6	1.055	71.2.	7.2	70.4	1.908
HA	2	60.5	3.0	65.4	1.566	47.3	7.2	55.8	1.562	55.3	6.4	46.0	1.062	69.2	7.0	66.8	1.826
	4	64.3	3.0	67.4	1.699	55.3	7.4	58.2	1.635	56.2	7.0	50.2	1.154	76.5	7.4	74.2	1.987
BTH+HA	0.25 + 2	62.5	3.2	68.4	1.661	49.3	7.0	57.0	1.599	55.2	6.6	49.2	1.135	71.3	7.0	69.4	1.89
	0.25 + 4	67.3	3.4	72.6	1.825	58.2	7.4	59.4	1.663	57.2	6.6	55.2	1.278	77.6	7.4	79.2	2.247
	0.5+2	66.2	3.1	70.4	1.796	56.5	7.2	57.2	1.601	60.2	6.8	53.4	1.225	75.4	7.4	76.8	2.097
	0.5+4	63.4	2.9	68.2	1.732	54.1	6.8	56.4	1.579	59.0	6.4	51.2	1.172	72.3	7.4	75.6	2.026
Control		53.5	2.8	50.1	1.195	41.8	6.6	40.2	1.118	50.3	6.2	39.2	0.826	64.5	7.0	56.5	1.502
LSD at 5%		4.35	NS	3.99	0.251	3.58	NS	3.08	0.204	3.25	NS	2.47	0.208	3.81	NS	3.19	0.232

Table 6. Effect of BTH and HA on the growth parameters of the four soybean cultivars under field conditions Minia governorate at summer season 2010.

Treatments	Con (g/L)	Cultivars															
		Giza 21				Giza 22				Giza 35				Giza 111			
		Plant height (cm)	No. of branches Plant $^{-1}$	No. of Pods Plant $^{-1}$	Seed yield (Ten f $^{-1}$)	Plant height (cm)	No. of branches plant $^{-1}$	No. of pods plant $^{-1}$	Seed yield (Ten/f $^{-1}$)	Plant height (cm)	No. of branches Plant $^{-1}$	No. of Pods/ plant	Seed yield (Ten/f $^{-1}$)	Plant height (cm)	No. of branches Plant $^{-1}$	No. of Pods Plant $^{-1}$	Seed yield (Ten/f $^{-1}$)
BTH	0.25	54.3	2.6	52.2	1.317	43.1	6.5	48.8	1.41	49.2	6.0	40.4	0.912	64.2	6.4	60.4	1.647
	0.5	59.8	2.8	60.4	1.245	47.2	6.1	50.9	1.486	51.0	6.5	43.2	1.010	69.2.	7.0	67.8	1.825
HA	2	55.5	2.8	62.7	1.397	45.1	6.9	53.8	1.562	52.0	6.3	44.1	1.000	68.2	6.8	64.2	1.787
	4	60.3	2.9	65.9	1.587	53.4	7.1	55.9	1.635	53.4	6.5	48.9	1.127	75.5	7.2	70.2	1.927
BTH+HA	0.25 + 2	60.5	3.1	66.8	1.601	48.1	6.8	54.0	1.599	53.0	6.4	48.2	1.105	70.3	6.9	67.3	1.827
	0.25 + 4	66.3	3.5	70.1	1.775	56.4	7.3	58.6	1.663	54.2	6.2	54.2	1.218	75.6	7.3	77.2	2.137
	0.5+2	63.2	3.0	68.4	1.724	54.2	7.0	56.4	1.601	52.2	6.8	52.8	1.200	73.1	7.2	75.5	2.002
	0.5+4	61.4	2.5	66.0	1.654	51.3	6.4	53.0	1.579	54.0	6.3	49.8	1.151	70.0	7.0	74.6	1.979
Control		48.5	2.4	45.3	1.009	36.4	6.0	37.2	0.997	50.0	5.7	37.2	0.806	61.8	6.5	53.5	1.142
LSD at 5%		3.17	NS	2.84	0.209	2.97	NS	2.57	0.261	NS	NS	2.04	0.244	3.66	NS	2.33	0.314

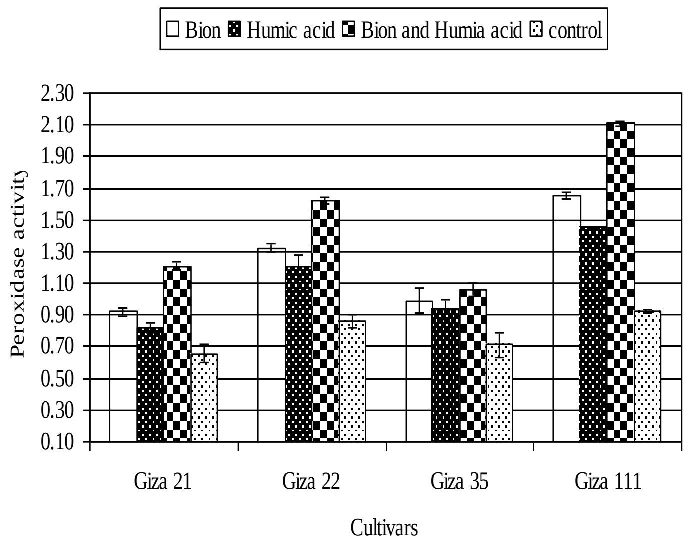

Figure 1. Activity of peroxidase enzyme (enzyme unit mg protein-1 min-1) of soybean cultivars as affected by BTH (0.5 g/l), HA (4 g/l) individually or combination (0.25+4 g/l). Means of standard deviation for nine plants per treatment are shown.

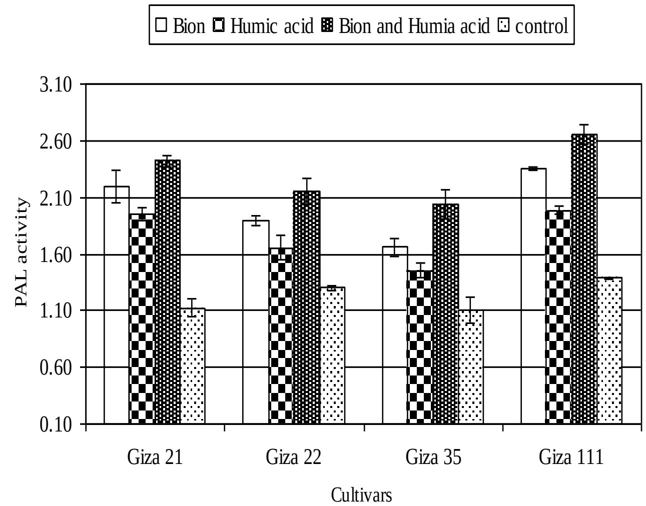

Figure 3. Activity of phenylalanine ammonia lyase enzyme (enzyme unit mg protein-1 min-1) of four soybean cultivars as affected by BTH (0.5 g/l), HA (4 g/l) individually or combination (0.25+4 g/l). Means of standard deviation for nine plants per treatment are shown.

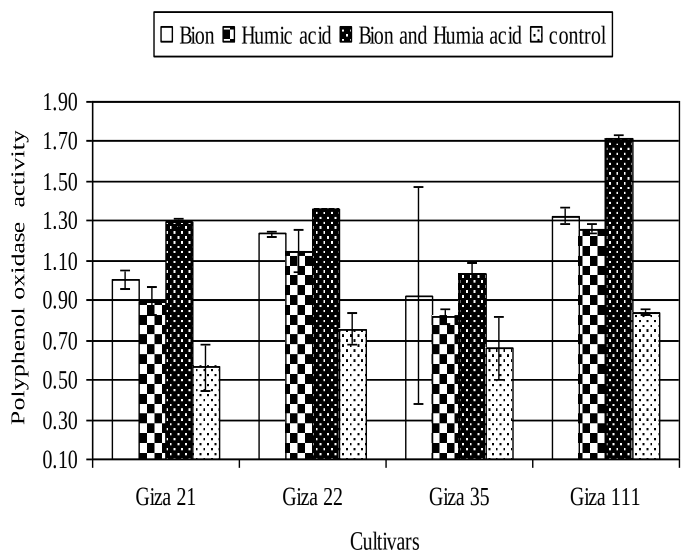

Figure 2. Activity of peroxidase enzyme (enzyme unit mg protein^{-1} min^{-1}) of four soybean cultivars as affected by BTH (0.5 g/l), HA (4 g/l) individually or combination (0.25+4 g/l). Means of standard deviation for nine plants per treatment are shown.

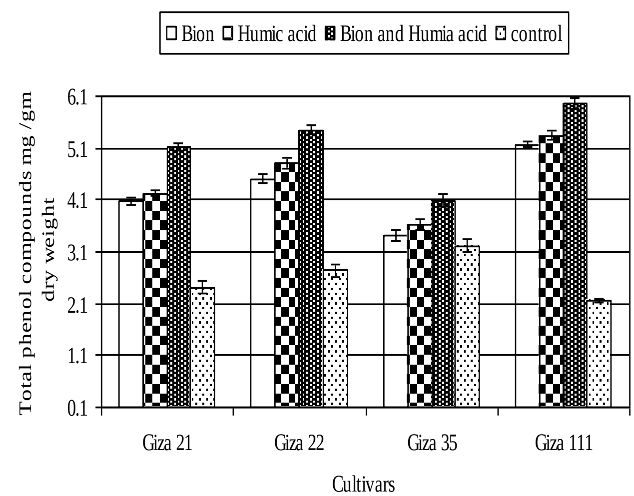

Figure 4. Total phenol compounds (TPC) content of four soybean cultivars as affected by BTH (0.5 g/l), HA (4 g/l) individually or combination (0.25+4 g/l). Means of standard deviation for nine plants per treatment are shown.

and BTH increased the phenols content than when used individually (Figure 4). Soybean cultivars were differed in phenols content in treated and untreated plants, where soybean c v. Giza 111 recorded the highest phenols content in case of treated plants followed by Giza 22, while Giza 35 recorded the lowest ones. Meanwhile, in case of untreated plants the opposite trend was recorded.

DISCUSSION

Wilt disease caused by F. oxysporum was the most important disease of soybean plants during the growing season (Hashem et al., 2009; Fayzalla et al., 2009). The present study plan to investigate the possibility of minimizing the infection with damping-off, wilt diseases of soybean using benzothiadiazole (BTH) and HA (HA) as resistance inducer. The obtained data revealed that both BTH, HA and their combination caused significant reduction to both damping-off and wilt diseases in greenhouse or field, compared with the control treatment. In general the combination between BTH and HA was more efficient in reducing disease incidence than using these chemicals individually spatially at concentration of 0.25+4 g/L. Also, under field condition, these chemicals significantly increased growth parameters and seed yield fed-1 and the use of BTH+HA in combination resulted in increased growth parameters compared with check treatment. Such results agree with those reported by Benhamou and Bélanger (1998), Sarwar et al. (2005), Abd-El-Kareem (2007), Nafie and Mazen (2008), Yigit and Dikilitas (2008) and El-Mohamedy and Ahmed (2009).

Both compounds induced disease resistance and increased yield in a number of plants including soybean and other legumes (Lawton et al., 1996; Dann et al., 1998; Abd- El- Kareem, 2007; Nafie and Mazen, 2008; El-Ghamry et al., 2009; Abo-Elyousr et al., 2010), against a broad range of pathogens. These compounds have no direct antimicrobial activity against many fungal and bacterial pathogens (Dann et al., 1998; Abd-El-Kareem, 2007). We suggest, therefore, that in soybean, BTH and HA treatments may stimulate inherent defense mechanisms so that the plant can respond more quickly against the invading, colonizing fungus. It seems likely that increased activity of the enzymes involved in defense reactions may be one of the basic ways in the action of BTH and HA in inducing resistance in soybean against wilt disease. Thus, oxidative enzymes that is peroxidase (PO) polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) were increased after BTH, HA and their combination treatments on infected and untreated control plants. On the other hand, the susceptibility of the four soybean cvs. was positively correlated with the activity of these enzymes, where cv. Giza 111 (more resistant *to F. oxysporum*) as it recorded the highest enzyme activity and cv. Giza 21 (highly susceptible) recorded the lowest enzyme activity either in treated or untreated plants. Also, the total phenol compounds content were highly increased in plants treated with inducers compared with untreated ones in all the tested cultivars and the combination between BTH and HA increased the phenols content more than if used individually.

The role of BTH as a resistance inducer was reported and that induction of systemic acquired resistance (SAR) gene expression by BTH did not require the contribution of SA which suggest that this compound could act as a secondary messenger analog capable of activating SAR signal transduction pathway independently of the accumulation of other signal molecules (Lawton et al., 1996; Abo-Elyousr et al., 2010). Application of BTH to a variety of plants before challenge with the pathogens triggered a set of plant defense reactions that resulted in the creation of a fungitoxic environment, which protect them by different physical and / or chemical means (Nafie and Mazen, 2008). Also, treatment with BTH and HA led to an increase in enzymatic activity of PO, PPO and PAL and many investigators reported that the expression of resistance is often accompanied by the activation of phenol-oxidizing enzymes such as PO, PPO and PAL (Goodman and Novacky, 1994). Increase in PO and PPO activity may contribute to defense through the production of oxidized forms of quinones, which can inactivate pectinolytic enzymes produced by pathogens. This suggests that PO and PPO could play an effective role in the observed resistance. PO and PPO have been associated with induced resistance and have been involved in several plant defense mechanisms, such as lignin biosynthesis, oxidative cross-linking of plant cell walls, and also generation of AOS (Faize et al., 2004). PAL is a key enzyme of phenylpropanoid pathway that leads to a variety of defense-related plant secondary metabolites such as SA, phytoalexins, and lignin-like polymers (Cools and Ishii, 2002). It has been shown to play a critical role in BTH-mediated resistance, as its expression was primed early by BTH in Japanese pear (Faize et al., 2004) and cucumber (Cools and Ishii, 2002). However, the direct role of PAL in resistance induced by ASM comes from the work of Standik and Buchenauer (2000), who showed that chemical inhibition of PAL abolished resistance in wheat induced against *B. graminis* f. sp. *tritici*.

On the other hand, data strongly suggest that HA directly or indirectly, plays as a signal for inducing systemic resistance as proposed by Abd-El-Kareem (2007). HA is a suspension, based on potassium humates, which can be applied successfully in many areas of plant production as a plant growth stimulant or soil conditioner for enhancing natural resistance against plant diseases and pests (Scheuerell and Mahaffee, 2004) which consequently increase yield of plant. Application of HA consistently enhanced antioxidants such as α-tocopherol, β-carotene, superoxide dismutases, and ascorbic acid concentrations in turf grass species (Zhang, 1997). These antioxidants may play a role in the regulation of plant development, flowering and chilling of disease resistance (Dmitrier et al., 2003). HA is considered to increase the permeability of plant membranes and enhance the uptake of nutrients. Moreover, it (HA) is also considered to improve soil nitrogen uptake and encourage the uptake of potassium, calcium, magnesium and phosphorus, making these more mobile and available to plant root system (Piccolo et al., 1997). In conclusion, it could be suggested that combined treatment between BTH and HA as safety control method might be used commercially for controlling soybean diseases under field conditions.

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