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BIOCHEMICAL DEFENSE INDUCTION IN INDIAN MUSTARD (*Brassica juncea* L.) AND RAPESEED (*B. napus* L.) BY SALICYLIC ACID AND BENZOTHIADIAZOLE

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ABSTRACT

Alternaria blight, caused by *Alternaria brassicae* (Berk.) Sacc., is one of the serious diseases of *Brassica* species. A field experiment was conducted to evaluate the effects of two elicitors, salicylic acid (SA) and benzothiadiazole (BTH), on defense response induction in *Brassica juncea* and *B. napus* against Alternaria blight. Four different treatments of SA and BTH were given to 10 week old plants upto 4 consecutive weeks. Chitinase and β-1,3-glucanase enzyme activities; and ascorbic acid and pigment contents were assessed 72 hr after treatment and disease incidence recorded a week after spray. Hydrolytic enzymes activities attained maximum level after 2nd spray in treatment combinations BTH (3 ppm) + SA (33 ppm) and BTH (7 ppm) + SA (17 ppm). An increase in ascorbic acid and pigment contents was observed in all the treatments after 1st, 2nd and 3rd spray followed by a decrease after 4th spray. Maximum increase was seen in treatment combinations of elicitors. These treatments also reduced Alternaria blight severity in both the species. The elicitors, applied in combinations, showed synergistic effect on the induction of hydrolytic enzyme activities as well as ascorbic acid and pigment contents in comparison to the individual applications. The study showed that the elicitor combinations can be used effectively to combat Alternaria blight in *Brassica* species.

Key words: Ascorbic acid, β-1,3-glucanase, benzothiadiazole, *Brassica*, chitinase, chlorophyll, salicylic acid

INTRODUCTION

Brassica juncea (Indian mustard or raya) and *B. napus* (rapeseed) contribute 32% of total oilseed production in India. Amongst the several diseases affecting *B. juncea* and *B. napus*, Alternaria blight is the most serious disease causing up to 47% yield loss (Meena *et al.*, 2010). It is caused by *Alternaria brassicae* (Berk.) Sacc., which is a necrotrophic pathogen that produces lesions on leaves, stem and siliquae affecting seed quantity and quality by reducing the oil content, seed size and seed colour. Various options are available to the farmers to protect their crop from disease including development of resistant cultivars, biological control, crop rotation, tillage and use of chemical fungicides. Fungicides check the disease spread but have poor compliances including health hazards. The pathogen constantly changes its nature and soon the resistant cultivars become susceptible (Chaudhry *et al.*, 2001). Therefore, there is need to alleviate infection by activating plant's own defense system with the aid of low molecular weight synthetic molecules called

elicitors (Cohen *et al.*, 1999). The use of elicitors lacks environmental and toxicological side-effects and is effective against a variety of plant pathogens.

Pre-treatment of plants with avirulent pathogens (biotic elicitors) or chemicals (abiotic elicitors) can enhance resistance to subsequent attack not only at the treatment site, but also in tissues distant from the initial infection sites. Previously, the spray of elicitors has been reported to effectively manage Alternaria blight of *B. juncea* (Meena *et al.*, 2004; Patni and Kolte, 2006). Salicylic acid (SA) and benzothiadiazole (BTH) [S-methylbenzo-1,2,3-thiadiazole-7-carbothiate], also known as acibenzolar-S-methyl (ASM) or Bion® or Actigard™, are chemical elicitors that have been used successfully to induce resistance against a wide range of diseases in field crops (Abdel-Monaim *et al.*, 2011; Farouk and Osman, 2012; Mandavia *et al.*, 2012). Treatment with these elicitors sensitize plants to defend themselves against pathogenic attack by triggering various defense mechanisms including production of phytoalexins, accumulation of acid soluble pathogenesis-related proteins (PR-proteins) and deposition of structural barriers. The PR-proteins have been classified into 17 families based on amino acid sequences and enzymatic activity. Some of the PR-proteins such as chitinases (PR-3) (Legrand *et al.*, 1987) and β-1,3-glucanases (PR-2) (Kauffmann *et al.*, 1987) have the potential to hydrolyze chitin and β-1,3-glucan, respectively, which are major components of fungal cell walls, leading to the inhibition of fungal growth (Leah *et al.*, 1991). Further, chitin and glucan oligomers released during degradation of fungal cell wall by the action of these hydrolytic enzymes act as elicitors that elicit various defense mechanisms in plants (Karthikeyan *et al.*, 2005). The application of elicitors also increases the production of antioxidative compounds that protect the plants from oxidative damage due to biotic and abiotic stresses. Ascorbic acid is the most abundant antioxidant of plants that scavenge superoxide, hydroxyl radicals, and singlet oxygen (Smirnoff *et al.*, 2001). Elicitors stimulate pigment formation and enhance the efficacy of photosynthetic apparatus with a better potential for resistance from pathogen infection (Amaresh and Bhatt, 1998). Therefore, the present study was aimed to assess the effect of SA and BTH alone and in combinations on hydrolytic enzyme activities, ascorbic acid and pigment content in *B. juncea* and *B. napus* cultivars for inducing resistance against Alternaria blight.

MATERIALS AND METHODS

The seeds of Indian mustard (*Brassica juncea* cv. PBR-91) and rapeseed (*B. napus* cv. GSC-6) were procured from the Oilseeds Section, Department of Plant Breeding & Genetics, PAU, Ludhiana (India). A field experiment was conducted in *rabi* (winter) season 2012-13 at the Experimental Farm, PAU, Ludhiana. The seeds were sown in field at a plant spacing of 30 x 10 cm in case of *B. juncea* and 45 x 10 cm in case of *B. napus* in plot size of 3 x 4 m^2 for each treatment. The treatments comprised of benzothiadiazole (BTH) @ 10 ppm, salicylic acid (SA) @ 50 ppm, BTH (3 ppm) + SA (33 ppm), BTH (7 ppm) + SA (17 ppm), copper oxychloride 50% WP @ 0.25% (fungicide) and a control (water-spray). The treatments were replicated three times for both the cultivars. The first foliar spray of elicitors/fungicide was given to 10 week old plants no sooner the first symptom of blight appeared in the field. A total of four foliar sprays of elicitors and three sprays of fungicide (as per recommended agronomic practices) were given on consecutive weeks. Leaves were randomly sampled 72 hr after each treatment and brought to the laboratory under cold conditions for studying hydrolytic enzymic activities, ascorbic acid content and pigment content.

Hydrolytic enzyme assays

Fresh leaf samples (0.5 g) were homogenized in 6 mL of 0.1M sodium acetate buffer (pH 4.8) containing 15mM β-mercaptoethanol and 4% polyvinylpyrrolidone. After centrifugation at 10,000 rpm for 15 min. at 4ºC, the supernatant was used for enzyme assays of chitinase and β-1,3-glucanase.

Chitinase assay: Chitinase activity was assayed by measuring the absorbance of the release of N-acetylglucosamine (NAG) by chitin from crab shells (Sigma) as per the method of Boller and Mauch (1988). A reaction mixture containing 300 µL enzyme extract and 200 µL chitin (0.5%) in 0.1M sodium acetate buffer (pH 4.8) was incubated in a water bath for 1 hr at 37ºC. The reaction was stopped by heating the reaction mixture at 100ºC for 5 min. The released reducing ends were then measured by the method of Reissig *et al.* (1955). Chitinase activity was expressed as µM NAG released min^{-1} g^{-1} fresh weight.

β-1,3-glucanase assay**:** The β-1,3-glucanase activity was assayed by measuring the rate of reducing sugar production using laminarin (Sigma) as substrate as per the method of Kauffmann *et al.* (1987). The reaction mixture was prepared by mixing 500 µL 4% laminarin and 500 µL enzyme and incubated at 40ºC for 1 hr. The reaction was stopped by adding 375 µL dinitrosalicylic acid reagent with subsequent heating in boiling water bath for 5-15 min. The resulting red-brown colour of the solution was stabilized by adding 1 mL 40% potassium sodium tartarate and vortexed. The absorbance of released glucose was measured at 575 nm and enzyme activity expressed as µM glucose released min^{-1} g^{-1} fresh weight.

Determination of ascorbic acid

Ascorbic acid was estimated according to the method of Roe and Kuether (1943). Fresh leaf sample (1 g) was homogenized with 10 mL 4% trichloroacetic acid and centrifuged at 2000 rpm for 10 min. Bromine water was added to the supernatant drop-wise with constant mixing. To the aliquot (0.5 mL), 1.5 ml 4% trichloroacetic acid was added, followed by 0.5 mL 2, 4-dinitrophenyl hydrazine reagent (2% in 9N sulphuric acid) and a few drops of 10% thiourea. Reaction mixtrure thus prepared was incubated at 37°C for 3 hr. Osazone crystals formed were dissolved in 2.5 mL 85% sulphuric acid and incubated at room temperature for 30 min. Absorbance of the colour developed was read at 540 nm. Ascorbic acid content (mg 100 g^{-1} fresh weight) was calculated by using standard ascorbate (10 mg 100 mL^{-1} 4% trichloroacetic acid).

Determination of photosynthetic pigments

The pigments content was estimated as per the method of Singh (1977). Fresh leaf sample (250 mg) was homogenized in 10 mL 80% acetone and centrifuged at 2000 rpm for 20 min. Total volume of the supernatant was made up to 20 mL with 80% acetone and absorbance recorded at 645, 652 and 663 nm against 80% acetone as blank. The amount of chlorophyll in the extract was calculated according to the following equations:

$$\text{Total chlorophyll (mg g}^{-1}\text{ fresh weight)} = \frac{A_{652nm} \times 1000 \times \text{Volume of extract}}{34.5 \times 1000 \times \text{Weight of fresh sample}}$$

$$\text{Chlorophyll a (mg g}^{-1}\text{ fresh weight)} = \frac{12.7 \times A_{663nm} - 2.69 \times A_{645nm} \times \text{Volume of extract}}{1000 \times \text{Weight of fresh sample}}$$

$$\text{Chlorophyll b (mg g}^{-1}\text{ fresh weight)} = \frac{22.9 \times A_{645nm} - 4.68 \times A_{663nm} \times \text{Volume of extract}}{1000 \times \text{Weight of fresh sample}}$$

The carotenoid content was estimated by the method of Davies (1976). Absorbance of the extract was measured at 480, 645 and 663 nm. The carotenoids were determined by equation:

$$\text{Carotenoids (mg g}^{-1}\text{ fresh weight)} = A_{480nm} + 0.114 \times A_{663nm} - 0.638 \times A_{645nm}$$

Assessment of Alternaria blight severity

The Alternaria blight disease was recorded every week after spray. Twenty plants from each plot were chosen at random and used to estimate the leaf area affected. Disease severity index (DSI) was calculated for each plot by summing the scores of twenty leaves and analyzing using rating scale

described by Conn *et al.* (1990). The disease scale (0-9) was used for calculating severity with 0 = no infection; 1 = upto 5% leaf area covered with Alternatia blight infection; 3= 5-10% leaf area covered with infection; 5 = 11-25% leaf area covered with infection; 7 = 26-50% leaf area covered with infection 9 = $\geq$ 50% leaf area covered with infection.

The data was subjected to analysis of variance (ANOVA) using SPSS (version 16). Tukey's test was used to test the significance of difference between the treatment means.

RESULTS AND DISCUSSION

In both the cultivars, the combination application of elicitors resulted in significantly higher chitinase and β-1,3-glucanase activities (Tables 1 and 2). The increase in enzyme activities was observed in all the treatments after 1st and 2nd spray, thereafter a decline was noticed up to 4th spray. In *B. juncea*, BTH (3 ppm) + SA (33 ppm) and in *B. napus*, BTH (7 ppm) + SA (17 ppm) showed maximum chitinase activity. In *B. juncea*, BTH (3 ppm) + SA (33 ppm) showed 1.41- and 1.44-fold increase in chitinase activity than control after 1st and 2nd spray, respectively. However, after 4th spray of the same treatment in *B. juncea*, 1.81-fold increase in chitinase activity over control was observed. In *B. napus*, BTH (7 ppm) + SA (17 ppm) caused 1.67-, 1.60-, 1.61- and 1.38-fold increase in chitinase activity after 1st, 2nd, 3rd and 4th spray, respectively, than control. In both the cultivars, 1st spray of BTH (7 ppm) + SA (17 ppm) resulted in significantly higher β-1,3-glucanase activity (1.74- and 1.69-fold, respectively) than control. In *B. juncea*, BTH (3 ppm) + SA (33 ppm) showed 1.52-, 1.73- and 1.67-fold increase in β-1,3-glucanase activity after 2nd, 3rd and 4th spray, respectively, over control. In *B. napus*, 2nd spray of BTH (7 ppm) + SA (17 ppm) exhibited 1.84-fold increase in β-1,3-glucanase activity, whereas 3rd and 4th spray of BTH (3 ppm) + SA (33 ppm) exhibited 1.50- and 1.70-fold increase in β-1,3-glucanase activity, respectively, as compared to the control.

In general, the activities of chitinase and β-1,3-glucanase were strongly induced by combined sprays of elicitors. The perception of elicitors by plants lead to the activation of signal transduction pathways which results in the production of active oxygen species, synthesis of defense enzymes and accumulation of PR-proteins. Yao and Tian (2005) showed that the combinations of SA or

Table 1: Effect of foliar spray of elicitors on chitinase activity in leaves of *B. juncea* & *B. napus*

Elicitor/fungicide sprays	Chitinase activity (μM NAG released min^{-1} g^{-1} fresh weight)			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	181.84 ± 7.6^{bcd}	266.19 ± 8.0^{c}	179.81 ± 10.7^{b}	133.30 ± 10.8^{bc}
SA (50 ppm)	172.21 ± 6.5^{bc}	256.04 ± 11.4^{bc}	170.57 ± 11.1^{b}	129.99 ± 8.2^{bc}
BTH (3 ppm) + SA (33 ppm)	204.15 ± 8.7^{d}	277.22 ± 14.5^{c}	189.04 ± 11.9^{b}	148.12 ± 7.7^{c}
BTH (7 ppm) + SA (17 ppm)	193.59 ± 9.5^{cd}	275.74 ± 11.1^{c}	184.12 ± 15.2^{b}	143.56 ± 11.3^{bc}
Blitox (0.25%)	160.71 ± 7.7^{ab}	227.14 ± 8.1^{b}	160.75 ± 12.2^{ab}	121.86 ± 7.8^{b}
Control (water-spray)	144.49 ± 10.2^{a}	192.99 ± 8.6^{a}	126.89 ± 15.9^{a}	81.73 ± 9.5^{a}
B. napus (cv. GSC-6)				
BTH (10 ppm)	69.60 ± 3.9^{b}	289.92 ± 8.3^{b}	145.77 ± 9.6^{bc}	132.59 ± 9.0^{b}
SA (50 ppm)	66.70 ± 4.1^{b}	287.52 ± 12.5^{b}	138.20 ± 10.3^{b}	130.10 ± 6.7^{b}
BTH (3 ppm) + SA (33 ppm)	76.61 ± 6.9^{c}	301.93 ± 12.4^{bc}	155.46 ± 12.5^{bc}	142.30 ± 12.0^{b}
BTH (7 ppm) + SA (17 ppm)	85.25 ± 9.2^{c}	332.41 ± 13.6^{c}	172.08 ± 10.5^{c}	143.47 ± 8.2^{b}
Blitox (0.25%)	57.67 ± 5.7^{ab}	280.98 ± 14.5^{b}	127.95 ± 10.5^{ab}	128.17 ± 10.4^{b}
Control (water-spray)	50.98 ± 5.7^{a}	207.62 ± 14.3^{a}	106.79 ± 11.2^{a}	103.89 ± 9.2^{a}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

Table 2: Effect of foliar spray of elicitors on β-1,3-glucanase activity in leaves of *B. juncea* and *B. napus*

Elicitor/fungicide sprays	β-1,3-glucanase activity (μM glucose released min^{-1} g^{-1} fresh weight)			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	93.74 ± 5.9^{c}	192.42 ± 5.2^{b}	150.78 ± 3.1^{d}	81.72 ± 2.6^{bc}
SA (50 ppm)	83.62 ± 4.7^{b}	184.23 ± 3.5^{b}	129.70 ± 5.0^{c}	78.05 ± 6.1^{b}
BTH (3 ppm) + SA (33 ppm)	106.79 ± 4.2^{d}	223.23 ± 4.6^{c}	154.04 ± 2.6^{d}	103.37 ± 4.9^{d}
BTH (7 ppm) + SA (17 ppm)	107.07 ± 4.0^{d}	216.74 ± 2.2^{c}	148.56 ± 4.4^{d}	92.38 ± 5.8^{cd}
Blitox (0.25%)	74.38 ± 4.4^{b}	192.11 ± 5.9^{b}	104.75 ± 2.8^{b}	72.77 ± 3.0^{b}
Control (water-spray)	61.56 ± 2.6^{a}	146.81 ± 3.8^{a}	88.80 ± 6.1^{a}	61.84 ± 4.5^{a}
B. napus (cv. GSC-6)				
BTH (10 ppm)	70.10 ±7.8^{bc}	180.00 ±7.7^{c}	78.68 ± 5.8^{bc}	75.98 ± 5.2^{bc}
SA (50 ppm)	58.19 ± 5.7^{b}	155.99 ±7.2^{b}	76.40 ± 5.8^{b}	70.39 ± 4.2^{b}
BTH (3 ppm) + SA (33 ppm)	85.89 ± 7.2^{c}	179.92 ± 4.7^{c}	91.66 ± 4.1^{c}	83.07 ± 5.2^{c}
BTH (7 ppm) + SA (17 ppm)	78.65 ±7.2^{c}	189.07 ±9.3^{c}	85.66 ± 5.7^{bc}	80.28 ± 5.3^{c}
Blitox (0.25%)	58.05 ± 4.9^{b}	146.45 ± 6.2^{b}	72.20 ± 5.0^{ab}	61.63 ± 4.7^{b}
Control (water-spray)	46.54 ± 5.2^{a}	102.79 ± 6.7^{a}	61.19 ± 5.2^{a}	48.72 ± 3.3^{a}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

jasmonic acid (JA) and bioagent *Cryptococcus laurentic* strongly induced β-1,3-glucanase and chitinase in sweet cherry. Cheng-Peng and Li (2006) observed that BTH spray enhanced the activities of chitinase and β-1,3-glucanase in wheat leaves. El-Gamal *et al.* (2007) sprayed ascorbic acid, dichloroisonicotinic acid, ethylene diamine tetra acetic acid and calcium chloride on potato plants and observed increase in chitinase and β-1,3-glucanase activities under greenhouse and field conditions providing resistance against *Phytophthora infestans* and *Alternaria solani*. Cavalcanti *et al.* (2008) studied the effect of spray of acibenzolar-S-methyl (0.2 g L^{-1}) and chitosan suspension from *Crinipellis perniciosa* mycelium on cacao plants and observed increase in chitinase and β-1,3-glucanase activities, 4-18 days after spray.

An increase in ascorbic acid content was observed in all the treatments after every spray in *B. juncea*. But in *B. napus*, ascorbic acid content increased upto 3rd spray and declined after 4th spray (Table 3). In both the cultivars, combination of elicitors showed maximum ascorbic acid content as compared to other treatments.

Table3: Effect of foliar spray of elicitors on ascorbic acid content in leaves of *B. juncea* and *B. napus*

Elicitor/fungicide sprays	Ascorbic acid content (mg 100 g^{-1} leaf fresh weight)			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	69.83 ± 5.6^{bc}	73.36 ± 4.2^{b}	179.52 ± 5.2^{c}	197.61 ± 4.6^{c}
SA (50 ppm)	58.04 ± 4.6^{b}	63.30 ± 5.1^{b}	151.11 ± 4.2^{b}	183.17 ± 3.5^{b}
BTH (3 ppm) + SA (33 ppm)	85.00 ± 4.0^{e}	87.44 ± 4.7^{c}	189.12 ± 4.5^{c}	239.52 ± 3.8^{e}
BTH (7 ppm) + SA (17 ppm)	77.53 ± 4.6^{cd}	86.10 ± 3.5^{c}	181.78 ± 5.6^{c}	218.37 ± 5.2^{d}
Blitox (0.25%)	61.26 ± 4.8^{b}	63.12 ± 5.4^{a}	141.47 ± 3.1^{ab}	176.16 ± 4.9^{ab}
Control (water-spray)	45.87 ± 3.7^{a}	47.61 ± 4.6^{a}	125.89 ± 4.5^{a}	167.68 ± 5.1^{a}
B. napus (cv. GSC-6)				
BTH (10 ppm)	43.35 ± 4.9^{b}	72.37 ± 5.4^{c}	97.19 ± 3.2^{b}	84.04 ± 4.8^{bc}
SA (50 ppm)	41.62 ± 5.2^{b}	68.16 ± 4.3^{bc}	114.95 ± 4.8^{c}	92.67 ± 2.7^{cd}
BTH (3 ppm) + SA (33 ppm)	45.70 ± 5.6^{c}	77.98 ± 4.4^{c}	115.41 ± 3.9^{c}	98.22 ± 5.0^{d}
BTH (7 ppm) + SA (17 ppm)	48.57 ± 4.2^{c}	77.22 ± 5.1^{c}	114.49 ± 3.8^{c}	94.38 ± 3.8^{cd}
Blitox (0.25%)	38.02 ± 3.6^{ab}	66.60 ± 3.8^{bc}	94.97 ± 3.2^{b}	77.58 ± 4.9^{b}
Control (water-spray)	31.47 ± 5.2^{a}	57.87 ± 3.4^{ab}	74.53 ± 3.6^{a}	64.76 ± 3.7^{a}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

Table 4: Effect of foliar spray of elicitors on total chlorophyll content in leaves of *Brassica juncea* and *B. napus*

Elicitor/fungicide sprays	Total chlorophyll content (mg g^{-1} fresh weight)			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	1.18 ± 0.05^{c}	1.40 ± 0.03^{c}	1.61 ± 0.03^{c}	1.42 ± 0.04^{cd}
SA (50 ppm)	1.12 ± 0.03^{c}	1.39 ± 0.03^{c}	1.57 ± 0.04^{c}	1.33 ± 0.03^{c}
BTH (3 ppm) + SA (33 ppm)	1.29 ± 0.03^{d}	1.52 ± 0.02^{d}	1.74 ± 0.02^{d}	1.49 ± 0.03^{de}
BTH (7 ppm) + SA (17 ppm)	1.40 ± 0.04^{e}	1.61 ± 0.03^{e}	1.80 ± 0.03^{d}	1.55 ± 0.04^{e}
Blitox (0.25%)	1.01 ± 0.02^{b}	1.24 ± 0.01^{b}	1.44 ± 0.02^{b}	1.18 ± 0.02^{b}
Control (water-spray)	0.88 ± 0.03^{a}	1.12 ± 0.02^{a}	1.25 ± 0.02^{a}	1.05 ± 0.04^{a}
B. napus (cv. GSC-6)				
BTH (10 ppm)	1.41 ± 0.04^{cd}	1.53 ± 0.03^{cd}	1.56 ± 0.03^{b}	1.46 ± 0.04^{c}
SA (50 ppm)	1.33 ± 0.04^{bc}	1.44 ± 0.02^{bc}	1.46 ± 0.02^{a}	1.44 ± 0.03^{ab}
BTH (3 ppm) + SA (33 ppm)	1.48 ± 0.03^{d}	1.53 ± 0.04^{cd}	1.64 ± 0.06^{bc}	1.56 ± 0.04^{c}
BTH (7 ppm) + SA (17 ppm)	1.51 ± 0.06^{d}	1.55 ± 0.03^{d}	1.68 ± 0.03^{c}	1.57 ± 0.04^{c}
Blitox (0.25%)	1.23 ± 0.03^{b}	1.36 ± 0.03^{b}	1.44 ± 0.02^{a}	1.37 ± 0.03^{ab}
Control (water-spray)	1.04 ± 0.04^{a}	1.25 ± 0.03^{a}	1.37 ± 0.03^{a}	1.35 ± 0.03^{a}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

After 1st and 2nd spray, BTH (3 ppm) + SA (33 ppm) exhibited higher ascorbic acid content (85.0 and 87.4 mg 100 g^{-1}, respectively) as compared to other treatments in *B. juncea*. After 3rd and 4th spray, the same treatment showed 1.50- and 1.43-fold higher ascorbic acid content, respectively, over control. After 1st spray, *B. napus* plants sprayed with BTH (7 ppm) + SA (17 ppm) showed maximum ascorbic acid content (48.57 mg 100 g^{-1}) as compared to other treatments. After 2nd, 3rd and 4th spray, BTH (3 ppm) + SA (33 ppm) exhibited 1.35-, 1.55- and 1.52-fold higher ascorbic acid content over control. The results are in line with Lee *et al.* (2005) who showed that chitosan treatment caused 10% increase in ascorbic acid content in soybean sprouts as compared to control. Jaleel *et al.* (2009) investigated the effect of plant growth regulators *viz*., paclobutrazol, gibberellic acid and *Pseudomonas fluorescens* on *Catharanthus roseus* and observed increase in ascorbic acid content than control. Farouk and Osman (2012) observed that SA (100 mg L^{-1}) significantly increased the ascorbic acid content compared to control or untreated infested bean plants.

Table 5: Effect of foliar spray of elicitors on foliar chlorophyll 'a' content in *B. juncea* and *B. napus*

Elicitor/fungicide sprays	Chlorophyll 'a' content (mg g^{-1} fresh weight)			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	0.56 ± 0.03^{cd}	0.64 ± 0.03^{c}	0.88 ± 0.03^{cd}	0.65 ± 0.04^{cd}
SA (50 ppm)	0.51 ± 0.04^{bc}	0.62 ± 0.01^{c}	0.82 ± 0.04^{c}	0.60 ± 0.04^{bc}
BTH (3 ppm) + SA (33 ppm)	0.60 ± 0.04^{d}	0.72 ± 0.03^{d}	0.83 ± 0.04^{c}	0.74 ± 0.03^{de}
BTH (7 ppm) + SA (17 ppm)	0.64 ± 0.03^{d}	0.78 ± 0.02^{d}	0.94 ± 0.01^{d}	0.83 ± 0.02^{e}
Blitox (0.25%)	0.44 ± 0.03^{b}	0.53 ± 0.03^{b}	0.68 ± 0.03^{b}	0.56 ± 0.03^{ab}
Control (water-spray)	0.39 ± 0.02^{a}	0.46 ± 0.03^{a}	0.59 ± 0.02^{a}	0.49 ± 0.03^{a}
B. napus (cv. GSC-6)				
BTH (10 ppm)	0.45 ± 0.01^{cd}	0.55 ± 0.01^{bc}	0.59 ± 0.01^{cd}	0.55 ± 0.01^{cd}
SA (50 ppm)	0.43 ± 0.01^{c}	0.53 ± 0.01^{b}	0.58 ± 0.01^{c}	0.53 ± 0.01^{c}
BTH (3 ppm) + SA (33 ppm)	0.46 ± 0.02^{cd}	0.56 ± 0.01^{bc}	0.64 ± 0.01^{de}	0.58 ± 0.01^{d}
BTH (7 ppm) + SA (17 ppm)	0.48 ± 0.02^{d}	0.58 ± 0.01^{c}	0.66 ± 0.01^{e}	0.59 ± 0.01^{d}
Blitox (0.25%)	0.37 ± 0.01^{b}	0.51 ± 0.01^{b}	0.56 ± 0.01^{b}	0.49 ± 0.01^{b}
Control (water-spray)	0.31 ± 0.01^{a}	0.45 ± 0.01^{a}	0.48 ± 0.01^{a}	0.40 ± 0.02^{a}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

Table 6: Effect of foliar spray of elicitors on foliar chlorophyll 'b' content in *B. juncea* and *B. napus*

Elicitor/fungicide sprays	Chlorophyll 'b' content (mg g^{-1} fresh weight)			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	0.46 ± 0.01^{c}	0.55 ± 0.01^{bc}	0.64 ± 0.01^{bc}	0.55 ± 0.01^{c}
SA (50 ppm)	0.42 ± 0.01^{b}	0.53 ± 0.01^{cd}	0.63 ± 0.01^{bc}	0.53 ± 0.01^{bc}
BTH (3 ppm) + SA (33 ppm)	0.49 ± 0.01^{c}	0.57 ± 0.01^{cd}	0.67 ± 0.02^{cd}	0.59 ± 0.01^{d}
BTH (7 ppm) + SA (17 ppm)	0.47 ± 0.01^{c}	0.59 ± 0.01^{d}	0.69 ± 0.01^{d}	0.61 ± 0.01^{d}
Blitox (0.25%)	0.40 ± 0.02^{b}	0.50 ± 0.02^{b}	0.61 ± 0.01^{b}	0.51 ± 0.01^{b}
Control (water-spray)	0.31 ± 0.01^{a}	0.42 ± 0.01^{a}	0.56 ± 0.02^{a}	0.46 ± 0.02^{a}
B. napus (cv. GSC-6)				
BTH (10 ppm)	0.45 ± 0.01^{c}	0.49 ± 0.01^{cd}	0.54 ± 0.01^{c}	0.45 ± 0.01^{bc}
SA (50 ppm)	0.45 ± 0.01^{bc}	0.48 ± 0.01^{bc}	0.53 ± 0.01^{c}	0.45 ± 0.01^{bc}
BTH (3 ppm) + SA (33 ppm)	0.48 ± 0.02^{cd}	0.52 ± 0.01^{de}	0.55 ± 0.01^{c}	0.46 ± 0.01^{c}
BTH (7 ppm) + SA (17 ppm)	0.50 ± 0.01^{d}	0.53 ± 0.01^{e}	0.59 ± 0.01^{d}	0.48 ± 0.01^{c}
Blitox (0.25%)	0.41 ± 0.01^{ab}	0.45 ± 0.01^{b}	0.50 ± 0.01^{b}	0.43 ± 0.01^{b}
Control (water-spray)	0.38 ± 0.01^{a}	0.40 ± 0.01^{a}	0.45 ± 0.01^{a}	0.39 ± 0.01^{a}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

The total chlorophyll, chlorophyll 'a', chlorophyll 'b' and carotenoid contents in all the treatments of both the cultivars increased after 1st, 2nd and 3rd spray but showed decrease after 4th spray (Table 4-7). Combination of elicitors showed higher pigment content as compared to the other treatments. Spray of BTH (7 ppm) + SA (17 ppm) resulted in significantly higher total chlorophyll content in both the cultivars. In *B. juncea*, 1.59-, 1.43-, 1.44- and 1.48-fold increase in total chlorophyll content was observed after 1st, 2nd, 3rd and 4th spray, respectively, over control. In *B. napus*, such respective increase was 1.45-, 1.24-, 1.23- and 1.16-fold.

In *B. juncea*, BTH (7 ppm) + SA (17 ppm) showed maximum increase (1.69-fold) in chlorophyll 'a' content compared to control after 2nd spray, whereas in *B. napus*, the same treatment showed maximum increase (1.55-fold) over control after 1st spray. Maximum increase in chlorophyll 'b' content (1.58-fold) was observed in *B. juncea* after 1st spray of BTH (3 ppm) + SA (33 ppm). In *B. napus*, maximum increase in chlorophyll 'b' content (1.32-fold) was observed after

Table 7: Effect of foliar spray of elicitors on carotenoid content in leaves of *B. juncea* and *B. napus*

Elicitor/fungicide sprays	Carotenoids content (mg g^{-1} fresh weight)			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	0.43 ± 0.01^{cd}	0.46 ± 0.01^{cd}	0.50 ± 0.01^{cd}	0.46 ± 0.01^{cd}
SA (50 ppm)	0.41 ± 0.01^{bc}	0.44 ± 0.01^{bc}	0.48 ± 0.01^{bc}	0.44 ± 0.01^{c}
BTH (3 ppm) + SA (33 ppm)	0.45 ± 0.01^{de}	0.49 ± 0.01^{d}	0.52 ± 0.01^{d}	0.48 ± 0.01^{de}
BTH (7 ppm) + SA (17 ppm)	0.46 ± 0.01^{e}	0.50 ± 0.01^{d}	0.56 ± 0.01^{e}	0.50 ± 0.01^{e}
Blitox (0.25%)	0.38 ± 0.01^{ab}	0.42 ± 0.01^{b}	0.45 ± 0.01^{b}	0.40 ± 0.01^{b}
Control (water-spray)	0.36 ± 0.01^{a}	0.37 ± 0.01^{a}	0.41 ± 0.01^{a}	0.35 ± 0.01^{a}
B. napus (cv. GSC-6)				
BTH (10 ppm)	0.44 ± 0.01^{cd}	0.52 ± 0.01^{bc}	0.55 ± 0.01^{bc}	0.51 ± 0.01^{cd}
SA (50 ppm)	0.42 ± 0.01^{bc}	0.50 ± 0.01^{b}	0.54 ± 0.01^{b}	0.49 ± 0.01^{c}
BTH (3 ppm) + SA (33 ppm)	0.45 ± 0.01^{de}	0.54 ± 0.01^{cd}	0.57 ± 0.01^{c}	0.51 ± 0.01^{cd}
BTH (7 ppm) + SA (17 ppm)	0.47 ± 0.01^{e}	0.56 ± 0.01^{d}	0.57 ± 0.01^{c}	0.53 ± 0.01^{d}
Blitox (0.25%)	0.40 ± 0.01^{b}	0.48 ± 0.01^{b}	0.51 ± 0.01^{b}	0.45 ± 0.01^{b}
Control (water-spray)	0.36 ± 0.01^{a}	0.46 ± 0.01^{a}	0.46 ± 0.01^{a}	0.40 ± 0.01^{a}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

Table 8: Effect of foliar spray of elicitors on Alternaria blight severity on *B. juncea* and *B. napus*

Elicitor/fungicide sprays	Percent disease index			
	1st spray	2nd spray	3rd spray	4th spray
B. juncea (cv. PBR-91)				
BTH (10 ppm)	26.4 ± 0.9^{b}	27.3 ± 0.8^{bc}	27.5 ± 1.5^{c}	28.9 ± 0.5^{b}
SA (50 ppm)	27.0 ± 0.8^{b}	27.5 ± 1.8^{c}	28.5 ± 0.6^{c}	28.9 ± 0.8^{b}
BTH (3 ppm) + SA (33 ppm)	19.0 ± 1.3^{a}	20.3 ± 1.0^{a}	20.9 ± 1.2^{a}	23.0 ±1.0^{a}
BTH (7 ppm) + SA (17 ppm)	22.8 ± 1.5^{ab}	23.3 ± 1.0^{ab}	23.9 ± 1.0^{b}	28.5 ±1.1^{b}
Blitox (0.25%)	27.5 ± 1.7^{b}	28.7 ± 2.0^{c}	29.0 ± 0.5^{c}	29.3 ± 0.8^{b}
Control (water-spray)	27.4 ± 4.3^{b}	30.0 ± 1.8^{c}	30.2 ± 0.8^{c}	34.8 ± 0.8^{c}
B. napus (cv. GSC-6)				
BTH (10 ppm)	22.5 ± 1.5^{a}	26.2 ± 1.3^{a}	29.8 ± 1.8^{ab}	30.9 ± 0.8^{ab}
SA (50 ppm)	23.3 ± 1.8^{a}	26.8 ± 2.1^{a}	32.3 ± 1.6^{bc}	32.4 ± 1.0^{bc}
BTH (3 ppm) + SA (33 ppm)	21.8 ± 1.0^{a}	24.3 ± 1.8^{a}	27.2 ± 2.0^{a}	27.9 ± 1.7^{a}
BTH (7 ppm) + SA (17 ppm)	21.8 ± 0.8^{a}	22.7 ± 0.8^{a}	28.4 ± 1.8^{ab}	30.0 ± 2.5^{ab}
Blitox (0.25%)	29.8 ± 1.5^{b}	31.7 ± 1.8^{b}	35.0 ± 1.0^{cd}	36.2 ± 1.3^{cd}
Control (water-spray)	31.7 ± 0.8^{b}	36.8 ± 1.8^{c}	38.0 ± 0.9^{d}	38.5 ± 1.0^{d}

*Data represent mean ± SD of three replications. Different letters indicate significant difference between treatments at p = 0.05, according to Tukey's test.

2nd spray of BTH (7 ppm) + SA (17 ppm). After 4th spray, BTH (7 ppm) + SA (17 ppm) showed 1.43-1.43- and 1.32-fold higher carotenoid content as compared to control in *B. juncea* and *B. napus*, respectively. Our results are in agreement with those obtained by Yildirim *et al.* (2008) in cucumber and Farouk and Osman (2012) in bean plants who found that SA caused significant increase in photosynthetic pigments in the leaves.

In *B. juncea*, 4th spray of the treatment containing BTH (3 ppm) + SA (33 ppm) showed greater reduction in disease severity (33.9%) as compared to control. Second spray of the same treatment exhibited most of the reduction in disease severity (33.97%) in *B. napus* plants as compared to control. Second spray of the treatment containing BTH (7 ppm) + SA (17 ppm) also showed 38.3% decrease in disease severity in *B. napus* plants than water-sprayed control plants (Table 8). Babu *et al.* (2003) reported about 50% reduction in bacterial leaf blight caused by *Xanthomonas oryzae* in rice plants treated with BTH @ 100 µg mL^{-1}. El-Hendawy *et al.* (2010) observed that oxalic acid and ascorbic acid treatments decreased chocolate spot disease severity in faba bean upto 79.4% Exogenous application of elicitors stimulated plant defense related genes, reduced infection on plants, and thus markedly alleviated the increase in hydrolytic enzyme activities as well as ascorbic acid and pigment content as compared to control. The combinations of SA and BTH were highly effective than applied alone. Inducing the defense system of plants by prior application of elicitors is thought to be a novel plant protection strategy. In conclusion, the combined treatments of SA and BTH might be used to control Alternaria blight in *B. juncea* and *B. napus* under field conditions.

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