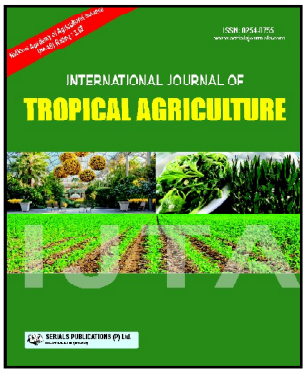

INTERNATIONAL JOURNAL OF TROPICAL AGRICULTURE

ISSN : 0254-8755

available at http: www.serialsjournal.com

Volume 35 • Number 2 • 2017

Brassinolide Seed Treatment Modulates the Enzymatic Antioxidative Defense System in Indian Mustard under Imidacloprid Toxicity

Anket Sharma[1,2*], Vinod Kumar[1,2], Shagun Bali[1], Ashwani Kumar Thukral[1], Renu Bhardwaj[1*]

[1]Plant Stress Physiology Lab, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, India.
[2]Department of Botany, DAV University, Sarmastpur, Jalandhar-144012, India.
**Corresponding author E-mail: anketsharma@gmail.com; renubhardwaj82@gmail.com*

Abstract: The effect of seed-soaking with 24-epibrassinolide (EBR) on enzymatic antioxidative defense system was studied in the leaves of 90 days old *Brassica juncea* plants grown under imidacloprid (IMI) toxicity. It was observed that IMI treatment resulted in increase of the contents of oxidative stress markers including superoxide anion ($O^{\cdot}_2^-$), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). Maximum reduction in the contents of these stress markers was noticed when seeds treated with 100nM EBR were grown in soils amended with IMI. The activities of the antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), guaiacol peroxidase (POD), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione-S-transferase (GST) were noticed to increase with IMI treatment. In comparison to plants germinated from untreated seeds and grown under IMI toxicity, a further enhancement in the activities of SOD (22.33%), CAT (51.02%), APX (50.00%), GPX (58.06%), POD (10.24%), DHAR (33.33%), GR (54.76%) and GST (32.34%) was noticed when 100nM EBR treated seeds were germinated in pots containing 350 mg IMI/Kg soil. Data analysis using multiple linear regression (MLR) also revealed that EBR seed-soaking before sowing, reduced the contents of oxidative stress markers and increased the activities of antioxidative enzymes.

INTRODUCTION

Brassica juncea L., commonly known as Indian mustard, is an important vegetable as well as oil yielding crop. It is widely attacked by sucking insect resulting in its yield loss. Without the application of pesticides, the yield loss of 6.5 to 26.4 % has been reported in Punjab, India (*Brassica* spp.) [1]. Imidacloprid (IMI), a neonicotinoid and a systemic

insecticide is mostly applied to check sucking insects. IMI is generally applied via soil to crop plants like rape, maize and sunflower, and due to its systemic nature, it helps in controlling aerial sucking insects [2]. Pesticides also cause toxicity to crop plants and as a consequence of pesticide toxicity, generation of reactive oxygen species (ROS) takes place which have negative impact on growth and development of plants. However, in response to pesticide toxicity, the internal defense system of plants known as antioxidative defense system gets activated to counterattack pesticide toxicity. Brassinosteroids (BRs) are steroidal plant hormones which are generally present in pollens, seeds and young vegetative tissues [3]. They play key roles in providing resistance to plants against various abiotic stresses like temperature, salt, drought, pesticides and heavy metals [4-8]. BR application further triggers the antioxidative defense system of the plants which results in increasing their resistance against pesticide stress [4, 8, 9]. Keeping in mind the roles of BRs in boosting the antioxidative defense system of plants, the present study aimed to investigate the role of 24-epibrassinolide (EBR) on enzymatic antioxidative defense system of *B. juncea* grown under IMI stress.

MATERIALS & METHODS

Certified seeds of *Brassica juncea* variety RLC-1 were procured from Punjab Agricultural University, Ludhiana, Punjab, India.

Raising of Plants: Seeds of *B. juncea* were given pre-sowing treatment for 8h with EBR solutions (0, 0.1, 1 and 100 nM). Pots were filled with 8 Kg soil (clay, sand and manure in the ratio of 2:1:1). Soils filled in pots were given pre-sowing treatment of IMI (0, 250, 300, 350 mg IMI/Kg soil). Seeds soaked with EBR were rinsed using distilled water before sowing in IMI treated soils. Plants were irrigated with ground water as per need. Leaves of the plants were harvested for further analysis after 90 days of seed sowing. All the analysis was done in triplicates.

Oxidative Stress Markers: Contents of superoxide anion ($O^{\cdot}_2^-$), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were determined by using methods given by Wu *et al.* [10], Patterson *et al.* [11] and Heath and Packer [12] respectively.

Antioxidative enzymes: Activities of antioxidative enzymes were estimated according to the methods given in table 1.

Table 1
Methods followed to estimate activities of antioxidative enzymes

Antioxidative Enzyme	*Reference*
SOD	Kono [13]
CAT	Aebi [14]
APX	Nakano and Asada [15]
GPX	Flohé and Günzler [16]
POD	Putter [17]
DHAR	Dalton *et al.* [18]
GR	Carlberg and Mannervik [19]
GST	Habig and Jacoby [20]

SOD (superoxide dismutase), CAT (catalase), APOX (ascorbate peroxidase), GPX (glutathione peroxidase), POD (guaiacol peroxidase), DHAR (dehydroascorbate reductase), GR (glutathione reductase), GST (glutathione-S-transferase).

Statistical Analysis of Data: Data was statistically analyzed by using Two-way analysis of variance (ANOVA) and multiple linear regression (MLR) using self-coded softwares in MS-Excel 2010 [21-23].

RESULTS & DISCUSSION

In comparison to untreated plants (control), the contents of all oxidative stress markers ($O^{\cdot}_2^-$, H_2O_2, MDA) were enhanced with the increasing concentration of IMI in soil. Seed treatment with EBR resulted in reduction of the contents of all the stress markers. It has been observed that, as

compared to untreated plants, contents of $O_2^{\cdot-}$, H_2O_2, MDA were increased by 226.60%, 79.475 and 74.53% respectively in plants germinated form untreated seeds and grown under highest IMI toxicity (Table 2). However, a significant reduction in the contents of all these stress markers was noticed in plants raised from EBR treated seeds and grown in IMI amended soils (Table 2). The activities of all the antioxidative enzymes were noticed to enhance with EBR seed application as well as IMI soil treatment (Table 3). Data analysis using Two-way ANOVA revealed that the contents of oxidative stress markers and activities of antioxidative enzymes were significantly different in the control plants, and plants raised from EBR treated/untreated seeds and grown under IMI toxicity (Table 2, 3). MLR analysis

Table 2
Effect of EBR seed soaking on the contents of oxidative stress markers in the leaves of 90-days old *B. juncea* plants grown under IMI toxicity.

Treatments		*Contents of Oxidative Stress Markers (µmole g^{-1} fr. wt.)*		
IMI (mg Kg^{-1})	*EBR(nM L^{-1})*	$O_2^{\cdot-}$	H_2O_2	*MDA*
0	0	0.94±0.15	12.91±1.03	7.46±0.25
0	0.1	0.87±0.24	13.07±0.74	7.56±0.23
0	1	0.92±0.13	12.78±1.12	6.83±0.15
0	100	0.86±0.25	12.37±0.56	6.77±0.16
250	0	1.65±0.27	15.19±1.90	9.49±0.07
250	0.1	1.40±0.24	14.31±3.39	8.74±0.29
250	1	1.30±0.29	12.52±1.51	8.26±0.21
250	100	1.14±0.23	12.35±2.17	7.78±0.21
300	0	2.53±0.23	18.83±0.39	10.16±0.23
300	0.1	2.15±0.43	17.59±3.67	9.71±0.32
300	1	2.01±0.32	17.19±0.73	9.17±0.34
300	100	1.55±0.20	12.78±3.15	8.18±0.51
350	0	3.07±0.36	23.17±0.35	13.02±0.67
350	0.1	2.86±0.25	21.78±1.55	10.06±0.12
350	1	2.85±0.43	21.50±0.31	10.28±0.36
350	100	2.14±0.46	17.74±3.61	8.06±0.21
Two-way ANOVA				
F_{IMI}		87.71***	41.32***	230.1***
F_{EBR}		9.16***	7.47***	118.8***
$F_{IMI \times EBR}$		1.25	1.04	19.57***
HSD		0.90	6.11	0.93

Multiple linear regression

MLR equation	*β-regression coefficients*		
	β_{IMI}	β_{EBR}	*r*
$O_2^{\cdot-}$ content = 0.87 + 0.0045 IMI - 0.0050 EBR	0.8199	- 0.2675	0.8625***
H_2O_2 content = 12.59 + 0.0185 IMI - 0.0290 EBR	0.6848	- 0.3501	0.7692***
MDA content = 7.38 + 0.0082 IMI - 0.0150 EBR	0.7145	- 0.4303	0.8341***

*** indicates significant at p<0.001. r = correlation coefficient. Superoxide anion ($O_2^{\cdot-}$), Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA)

Table 3
Effect of EBR seed soaking on the activities of antioxidative enzymes in the leaves of 90-days old *B. juncea* plants grown under IMI toxicity.

Treatments		*Activities of Antioxidative Enzymes (Mean ± S.D.)*							
IMI (mg Kg^{-1})	*EBR (nM L^{-1})*	*SOD (Units mg^{-1} protein)*	*CAT (µmole min^{-1} mg^{-1} protein)*	*APX (µmole min^{-1} mg^{-1} protein)*	*GPX (µmole min^{-1} mg^{-1} protein)*	*POD (µmole min^{-1} mg^{-1} protein)*	*DHAR (µmole min^{-1} mg^{-1} protein)*	*GR(µmole min^{-1} mg^{-1} protein)*	*GST (µmole min^{-1} mg^{-1} protein)*
0	0	13.9±2.3	2.6±0.4	3.3±0.5	1.6±0.4	40.9±5.1	3.1±0.5	3.8±0.5	19.9±1.9
0	0.1	16.2±1.8	3.1±0.7	4.5±0.4	1.6±0.3	42.6±0.7	3.5±0.2	3.9±0.4	20.1±1.7
0	1	18.8±2.8	3.2±0.3	4.5±0.6	1.7±0.4	46.6±2.6	3.6±0.2	4.5±0.6	21.5±2.7
0	100	20.6±3.6	4.1±0.6	5.0±0.8	2.2±0.2	46.9±4.8	4.0±0.4	4.9±0.2	22.9±2.7
250	0	18.3±2.4	3.2±0.6	4.9±0.9	3.4±0.4	41.5±6.0	4.8±0.3	4.3±0.8	24.1±1.5
250	0.1	21.0±2.2	3.8±0.8	6.5±0.7	3.8±0.4	43.7±3.3	5.9±0.5	5.9±0.6	24.7±4.8
250	1	24.4±1.7	5.4±0.3	6.9±0.5	4.1±0.5	45.3±3.8	6.5±0.2	5.7±0.8	26.1±3.5
250	100	33.0±3.9	5.7±0.3	7.0±0.6	5.0±0.5	48.1±6.3	6.8±0.9	6.1±0.6	27.2±2.4
300	0	25.1±3.4	5.5±0.8	6.3±0.7	4.0±0.6	47.1±5.0	5.7±0.6	5.8±0.4	26.9±1.2
300	0.1	26.7±4.1	6.1±0.7	7.2±0.7	4.1±0.6	49.6±9.2	5.9±0.4	6.0±1.2	28.6±1.0
300	1	27.2±3.5	7.4±0.5	8.8±0.4	5.2±0.3	53.9±5.7	7.6±0.6	6.2±0.8	28.8±3.6
300	100	34.2±1.9	8.2±1.1	9.1±0.5	5.8±0.4	59.5±4.6	8.9±0.9	7.8±0.8	30.7±4.9
350	0	20.6±2.8	4.9±0.5	4.4±0.5	3.1±0.9	42.0±4.6	4.5±0.8	4.2±0.5	20.1±2.7
350	0.1	23.1±3.0	6.3±0.6	5.3±0.9	3.3±0.5	42.6±5.0	4.7±0.7	5.3±1.1	22.9±1.3
350	1	23.4±4.9	6.8±0.7	5.7±1.1	3.4±0.4	45.9±3.3	5.4±1.1	5.4±0.9	24.6±6.5
350	100	25.2±6.2	7.4±0.5	6.6±1.3	4.9±0.3	46.3±5.3	6.0±0.5	6.5±0.9	26.6±1.9
Two-way ANOVA									
F_{IMI}		21.33***	82.32***	48.99***	90.39***	7.89***	70.76***	16.87***	12.44***
F_{EBR}		14.53***	30.87***	20.36***	22.06***	5.15**	23.96***	11.37***	3.66*
$F_{IMI \times EBR}$		1.22	1.16	0.80	1.30	0.30	2.33*	0.83	0.16
HSD		5.59	1.04	1.21	0.79	8.35	1.00	1.25	5.25

Multiple linear regression

MLR equation	*β-regression coefficients*		
	β_{IMI}	β_{EBR}	*r*
SOD activity = 16.26 + 0.0234 IMI + 0.0672 EBR	0.5941	0.5471	0.8077***
CAT activity = 2.71 + 0.0095 IMI + 0.0151 EBR	0.7532	0.3843	0.8457***
APX activity = 4.24 + 0.0064 IMI + 0.0124 EBR	0.5555	0.3444	0.6535**
GPX activity = 1.64 + 0.0073 IMI + 0.0119 EBR	0.7642	0.4020	0.8635***
POD activity = 43.17 + 0.0086 IMI + 0.0516 EBR	0.2453	0.4735	0.5332*
DHAR activity = 3.46 + 0.0072 IMI + 0.0137 EBR	0.6374	0.3883	0.7463**
GR activity = 4.06 + 0.0045 IMI + 0.0123 EBR	0.5852	0.5082	0.7751***
GST activity = 20.97 + 0.0135 IMI + 0.0285 EBR	0.5654	0.3822	0.6825**

SOD (superoxide dismutase), CAT (catalase), APX (ascorbate peroxidase), GPX (glutathione peroxidase), POD (guaiacol peroxidase), DHAR (dehydroascorbate reductase), GR (glutathione reductase), GST (glutathione-S-transferase). *, ** and *** indicate significant at $p<0.05$, $p<0.01$ and $p<0.001$ respectively. r = multiple correlation coefficient.

indicated that seed treatment with EBR before sowing resulted in reduction of the contents of all oxidative stress markers as indicated by negative β-regression values (Table 2). Positive β-regression values for the activities of all antioxidative enzymes revealed that EBR seed soaking as well as IMI soil treatment resulted in enhanced activities of these enzymes (Table 3).

As a result of oxidative stress caused by pesticide toxicity, the antioxidative defense system gets activated in order to efficiently scavenge the ROS and ultimately help in reducing the oxidative stress. In the present study, the activities of enzymatic antioxidants including SOD, CAT, APX, GPX, POD, DHAR, GR and GST were observed to increase with IMI toxicity as well as with the EBR seed application. SOD and CAT convert superoxide anions to non-toxic water and molecular oxygen. Additionally, ascorbate-glutathione cycle is involved in detoxification of H_2O_2, which consists of four antioxidative enzymes named APX, monodehydroascorbate reductase (MDHAR), DHAR and GR [24, 25]. Peroxidase enzymes are involved in the detoxification of H_2O_2 into metabolites which are less toxic [26]. The activities of antioxidative enzymes which are involved in pesticide detoxification (DHAR, POD and GPX) were also observed to enhance after IMI application.

In the present study, it has been noticed that EBR seed application further enhanced the activities of all these antioxidative enzymes under IMI toxicity. The regulation of the activities of all these enzymes might be due to the EBR modulated enzyme kinetics and synthesis of various proteins [27, 28]. Moreover, it has also been reported that expression of the genes for *SOD*, *CAT*, *GR*, *POD* and *GST* was enhanced in seedlings raised from EBR soaked seeds and grown under IMI toxicity [8]. It suggested that the enhanced expression of the genes encoding these antioxidative enzymes might be one of the possible reasons for regulation of antioxidative defense system in *B. juncea* plants raised from EBR treated seeds and grown under IMI toxicity.

CONCLUSIONS

From the present study, it has been concluded that the seed-soaking with EBR resulted in reduction of the contents of oxidative stress markers by modulating the enzymatic antioxidative defense system of *B. juncea* plants grown under IMI toxicity.

ACKNOWLEDGEMENTS

Department of science and technology, Government of India, is duly acknowledged for providing INSPIRE fellowship to Anket Sharma.

REFERENCES

Aebi, H. (1984), Catalase in vitro. Methods Enzymol. 105: 121-126.

Asada, K. and Takahash, M. (1987), Production and scavenging of active oxygen in photosynthesis. In: *Photoinhibition* (Eds. D.J. Kyle, C.B. Osmond and C.J. Arntzen) Elsevier, Amsterdam, pp. 227–287.

Bonmatin, J.M., Marchand, P.A., Charvet, R., Moineau, I., Bengsch, E.R. and Colin, M.E. (2005), Quantification of imidacloprid uptake in maize crops. J. Agric. Food Chem. 53: 5336-5341.

Bowler, C., Montagu, M.V. and Inze, D. (1992), Superoxide dismutase and stress tolerance. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 43: 83-116.

Carlberg, I. and Mannervik, B. (1975), Purification of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* 250: 5475-5480.

Chew, O., Whelan, J. and Millar, A.H. (2003), Molecular definition of the ascorbate-glutathione cycle in *Arabidopsis* mitochondria reveals dual targeting of antioxidant defenses in plants. *J. Biol. Chem.* 47: 46869–46877.

Clouse, S.D. and Sasse, J.M. (1998), Brassinosteroids: essential regulators of plant growth and development. Ann. Rev. Plant Physiol. Plant Mol. Bio. 49: 427-451.

Dalton, D.A., Russell, S.A., Hanus, F.J., Pascoe, G.A. and Evans, H.J. (1986), Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. Proceed. Nat. Acad. Sci. U. S. A. 83: 3811-3815.

Flohé, L. and Günzler, W.A. (1984), Assays of glutathione peroxidase. Methods Enzymol. 105: 114-21.

Habig, W.H. and Jakoby, W.B. (1981), Glutathione S-transferases (rat and human). Methods Enzymol. 77: 218-31.

Heath, R.L. and Packer, L. (1968), Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189-198.

Kono, Y. (1978), Generation of superoxide radical during autooxidation of hydroxylamine and an assay for superoxide dismutase. Arch. Biochem. Biophys. 186: 189-195.

Krishna, P. (2003), Brassinosteroid-mediated stress responses. *J. Plant Growth Regul.* 22: 289-297.

Kular, J. and Kumar, S. (2011), Quantification of avoidable yield losses in oilseed *Brassica* caused by insect pests. J. Plant Prot. Res. 51: 38-43.

Lascano, H.R., Gomez, L.D., Casano, L.M. and Trippi, V.S. (1998). Changes in gluthatione reductase activity and protein content in wheat leaves and chloroplasts exposed to photooxidative stress. Plant Physiol. Biochem. 36: 321-329.

Nakano, Y. and Asada, K. (1981), Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-880.

Patterson, B.D., Mackae, E.A. and Mackae, I. (1984), Estimation of hydrogen peroxide in plants extracts using titanium (IV). Analyt. Biochem. 139: 487-492.

Putter, J. (1974), Peroxidase. In: *Methods of Enzymatic Analysis*. (Ed. H.U. Bergmeyer), Verlag Chemie, Weinhan, pp. 685-690.

Sharma, A., Bhardwaj, R., Kumar, V. and Thukral, A.K. (2016c). GC-MS studies reveal stimulated pesticide detoxification by brassinolide application in *Brassica juncea* L. plants. Environ. Sci. Poll. Res. 23: 14518-14525.

Sharma, A., Kumar, V., Bhardwaj, R. and Thukral, A.K. (2016d), Seed pre-soaking with 24-epibrassinolide reduces the imidacloprid pesticide residues in green pods of *Brassica juncea* L. Toxicol. Environ. Chem. Doi. 10.1080/02772248.2016.1146955.

Sharma, A., Kumar, V., Kanwar, M.K., Thukral, A.K. and Bhardwaj, R. (2016), Multivariate analysis reveals the role of 24-epibrassinolide in elemental uptake by *Brassica juncea* L. under imidacloprid toxicity. Poll. Res. 35: 749-756.

Sharma, A., Kumar, V., Singh, R., Thukral, A.K., and Bhardwaj, R. (2016b), Effect of seed pre-soaking with 24-epibrassinolide on growth and photosynthetic parameters of *Brassica juncea* L. in imidacloprid soil. Ecotoxicol. Environ. Saf. 133: 195-201.

Sharma, A., Kumar, V., Thukral, A.K. and Bhardwaj, R. (2016a), Epibrassinolide-imidacloprid interaction enhances non-enzymatic antioxidants in *Brassica juncea* L. Indian J. Plant Physiol. 21: 70-75.

Sharma, A., Thakur, S., Kumar, V., Kanwar, M.K., Kesavan, A. K., Thukral, A. K., Bhardwaj, R., Alam P. and Ahmad, P. (2016), Ameliorating role of 24-Epibrassinolide against pesticide stress in *Brassica juncea* L. through the modulation of stress markers. Front. Plant Sci. DOI: 10.3389/fpls.2016.01569.

Sharma, A., Thakur, S., Kumar, V., Kesavan, A. K., Thukral, A. K. and Bhardwaj, R. (2017), 24-epibrassinolide stimulates imidacloprid detoxification by modulating the gene expression of *Brassica juncea* L. BMC Plant Biol., 17:56, DOI: 10.1186/s12870-017-1003-9.

Sharma, I., Bhardwaj, R. and Pati, P.K. (2015), Exogenous application of 28-homobrassinolide modulates the dynamics of salt and pesticides induced stress responses in an elite rice variety Pusa Basmati-1. J. Plant Growth Regul. 34: 509-518.

Thao, N.P. and Tran, L.S. (2012), Potentials toward genetic engineering of drought tolerant soybean. *Critical Rev. Biotech.* **32**: 349-362.

Wu, G.L., Cui, J., Tao, L. and Yang, H. (2010), Fluroxypyr triggers oxidative damage by producing superoxide and hydrogen peroxide in rice (*Oryza sativa*). Ecotoxicol. 19: 124-132.