CHAPTER 9

RESULTS OF GENE EXPRESSION AND IMI RESIDUE ANALYSIS

9.1 Gene expression analysis

9.1. 1 Stress markers

Chlorophyllase (*CHLASE*)

It was observed that EBR seed soaking decreased the expression of *CHLASE* to 1.07 folds, as compared to 2.66 fold in seedlings raised from untreated seeds and grown under IMI toxicity (Table 9.1.1, Fig. 9.1.1.1)

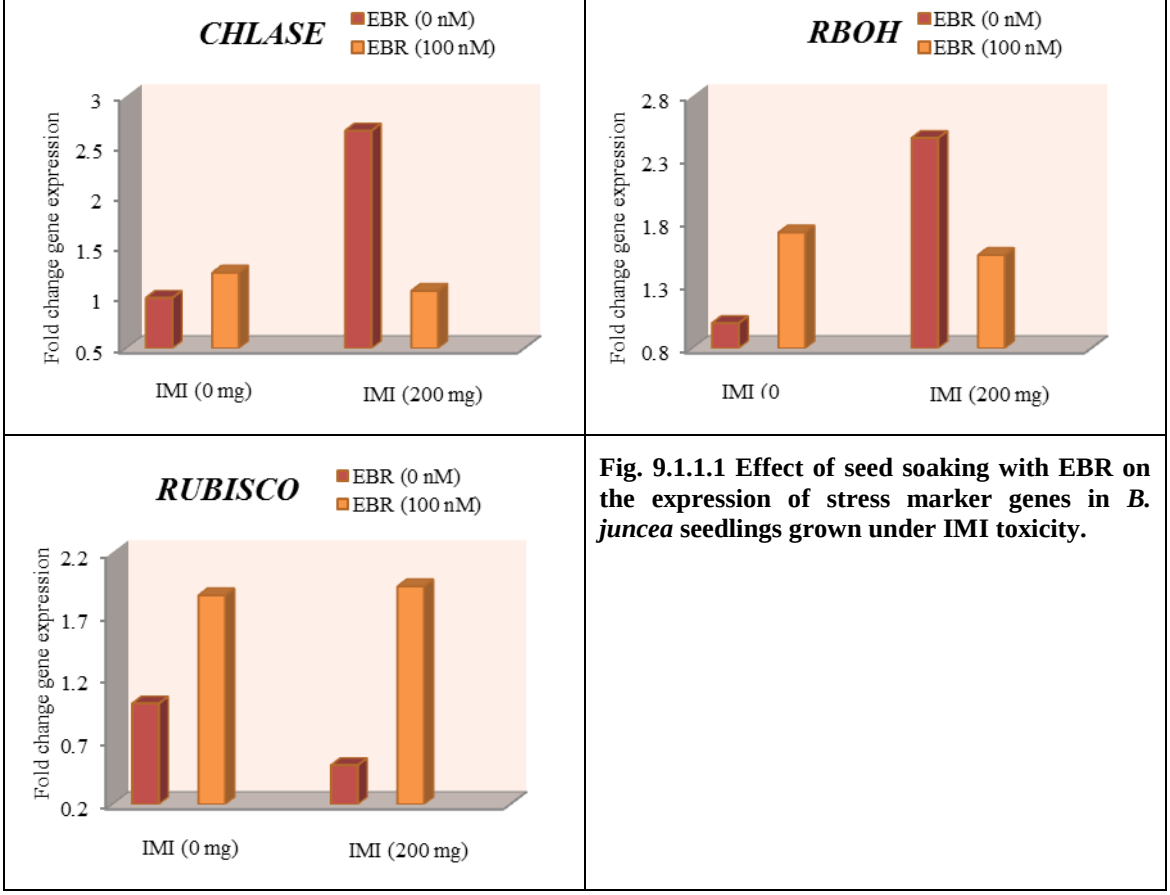


Fig. 9.1.1.1 Effect of seed soaking with EBR on the expression of stress marker genes in *B. juncea* seedlings grown under IMI toxicity.

Respiratory burst oxidase (*RBO*)

Seed soaking with EBR down-regulated the expression of *RBO* gene (1.54 fold) under IMI toxicity, when compared to *RBO* gene expression (2.47 fold) in seedlings raised from untreated seeds and grown in the presence of IMI (Table 9.1.1, Fig. 9.1.1.1).

Table 9.1.1 Effect of EBR seed soaking on gene expression in 10 days old *B. juncea* seedlings grown in IMI containing Petri-plates. Data are Mean±SD (n=3), Two-way ANOVA, Tukey's HSD and multiple linear regression analysis (MLR).

IMI (mg L^{-1})	EBR (nM L^{-1})	Expression of enzymes (fold change)												
		CHLASE	***RBO***	***RUBISCO***	***SOD***	***CAT***	***POD***	***GR***	***GST-1***	***GST-2***	***GST-3***	***GST-4***	***GST-5***	***GST-6***
0	0	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00
0	100	1.25±0.12	1.72±0.43	1.86±0.67	1.39±0.16	2.19±0.15	2.69±0.23	2.64±0.20	1.19±0.05	2.4±0.09	2.72±0.52	0.61±0.03	3.56±0.39	4.83±0.77
200	0	2.66±0.66	2.47±0.22	0.51±0.14	1.04±0.10	0.85±0.05	3.11±0.44	1.85±0.22	1.14±0.08	1.37±0.27	1.44±0.15	0.92±0.31	1.18±0.20	1.68±0.31
200	100	1.07±0.13	1.54±0.15	1.93±0.47	1.45±0.07	1.52±0.30	4.09±0.81	2.28±0.09	1.87±0.29	2.61±0.24	3.71±0.42	0.75±0.11	3.58±0.68	4.28±0.40
Two way ANOVA														
F_{IMI}		15.54**	19.06**	0.77	0.78	16.66**	40.71***	7.05*	21.14**	6.86*	12.82**	0.09	0.19	0.06
F_{EBR}		13.29**	0.46	22.26**	48.57***	87.07***	23.44**	124.3***	26.40***	143.7***	99.63***	8.38*	110.1***	143.7***
$F_{IMI \times EBR}$		25.72***	31.10***	1.33	0.01	6.89*	1.66	42.83***	8.68*	0.52	1.85	1.07	0.11	5.28
HSD		0.82	0.66	1.09	0.26	0.45	1.24	0.41	0.40	0.49	0.90	0.43	1.06	1.21

Multiple linear regression analysis

MLR equation	β-regression coefficients		Multiple correlation coefficient
	β_{IMI}	β_{EBR}	
CHLASE (gene expression fold change) = 1.46 + 0.0037 IMI - 0.007 EBR	0.5110	- 0.4573	0.6851*
RBO (gene expression fold change) = 1.41 + 0.0032 IMI - 0.0010 EBR	0.5702	- 0.0892	0.5771
RUBISCO(gene expression fold change) = 0.86 - 0.0011 IMI + 0.0114 EBR	- 0.1547	0.8298	0.8435**
SOD (gene expression fold change) = 0.99 + 0.0003 IMI + 0.0041 EBR	0.1169	0.9201	0.9274***
CAT (gene expression fold change) = 1.13 - 0.0020 IMI + 0.0093 EBR	- 0.3748	0.8566	0.9351***
POD (gene expression fold change) = 1.17 + 0.0088 IMI + 0.0134 EBR	0.7426	0.5635	0.9322***
GR (gene expression fold change) = 1.30 + 0.0012 IMI + 0.0103 EBR	0.1968	0.8260	0.8491***
GST-1 (gene expression fold change) = 0.86 + 0.0021 IMI + 0.0046 EBR	0.5737	0.6411	0.8604***
GST-2 (gene expression fold change) = 1.04 + 0.0015 IMI + 0.0133 EBR	0.2077	0.9504	0.9728***
GST-3 (gene expression fold change) = 0.86 + 0.0036 IMI + 0.0200 EBR	0.3238	0.9025	0.9588***
GST-4 (gene expression fold change) = 0.94 + 0.0002 IMI - 0.0030 EBR	0.0747	- 0.6909	0.6949*
GST-5 (gene expression fold change) = 1.03 + 0.0005 IMI + 0.0248 EBR	0.0401	0.9643	0.9651***
GST-6 (gene expression fold change) = 1.30 + 0.0003 IMI + 0.0322 EBR	0.0195	0.9565	0.9567***

*, ** and *** indicate significant at $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (*RUBISCO*)

It was observed that IMI application resulted in down-regulation of the expression of *RUBISCO* (0.51 fold) as compared to the control seedlings. EBR seed soaking up-regulated the gene expression by 1.93 fold under IMI toxicity (Table 9.1.1, Fig. 9.1.1.1).

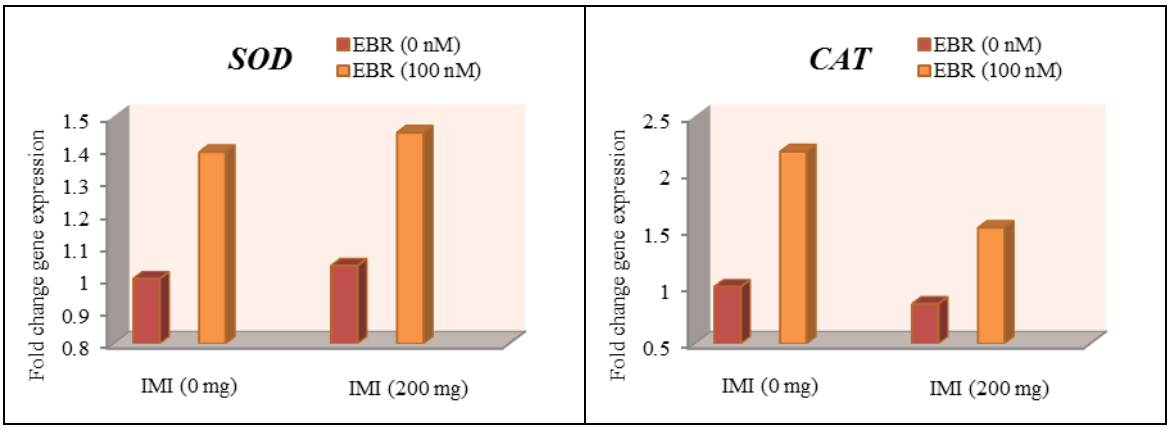
Analysis of data using two-way ANOVA and Tukey's HSD revealed significant deference for gene expression of *CHLASE*, *RBO* and *RUBISCO* genes in *B. juncea* seedlings. MLR analysis showed that IMI increased the expression of *CHLASE* and *RBO* genes (positive β_{IMI} values), and decreased the expression of *RUBISCO* (negative β_{IMI} values). EBR seed soaking decreased their expression of *CHLASE* and *RBO* as shown by negative β_{EBR} values, whereas increased the expression of *RUBISCO* (positive β_{IMI} values) (Table 9.1.1)

9.1.2 Antioxidative defense, pesticide detoxification and pigment system

Expression of genes related to antioxidative defense and pesticide detoxification system was observed to increase in seedlings raised from EBR soaked seeds and grown in Petri-plates containing IMI.

Superoxide dismutase (*SOD*)

As compared to the control, maximum increase in expression of *SOD* was 1.45 fold in seedlings raised from EBR treated seeds and grown in Petri-plates containing IMI solutions (Table 9.1.1, Fig. 9.1.2.1).



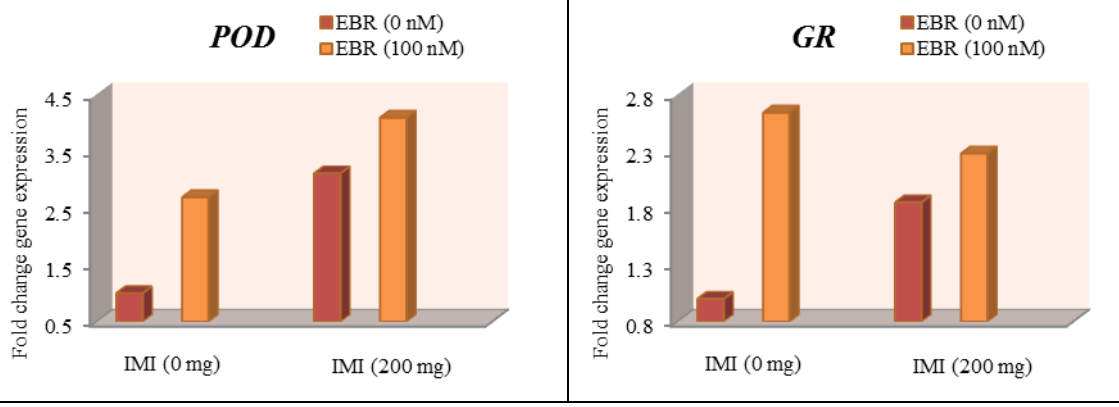


Fig. 9.1.2.1 Effect of seed soaking with EBR on the gene expression of antioxidant enzymes in *B. juncea* seedlings grown under IMI toxicity.

Catalase (*CAT*)

The maximum increase in the expression of *CAT* was by 1.52 fold in seedlings germinated from EBR treated seeds and grown in presence of IMI (Table 9.1.1, Fig. 9.1.2.1).

Guaiacol peroxidase (*POD*)

In the seedlings raised from EBR soaked seeds and grown under IMI toxicity, the *POD* expression was maximum enhanced to 4.09 fold as compared to the control (Table 9.1.1, Fig. 9.1.2.1).

Glutathione reductase (*GR*)

In comparison to the control seedlings, expression of *GR* was increased to 2.28 fold in seedlings raised from EBR soaked seeds and grown in Petri-plates containing IMI solutions (Table 9.1.1, Fig. 9.1.2.1).

Glutathione-S-transferase (*GST1-6*)

Except for *GST*-4 gene, expression of all *GST* genes was increased (*GST-1* by 1.87 fold, *GST-2* by 2.61 fold, *GST-3* by 3.71 fold, *GST-5* by 3.58 fold, *GST-6* by 4.28 fold) in seedlings raised from EBR soaked seeds and germinated in Petri-plates containing IMI solutions (Table 9.1.1, Fig. 9.1.2.2).

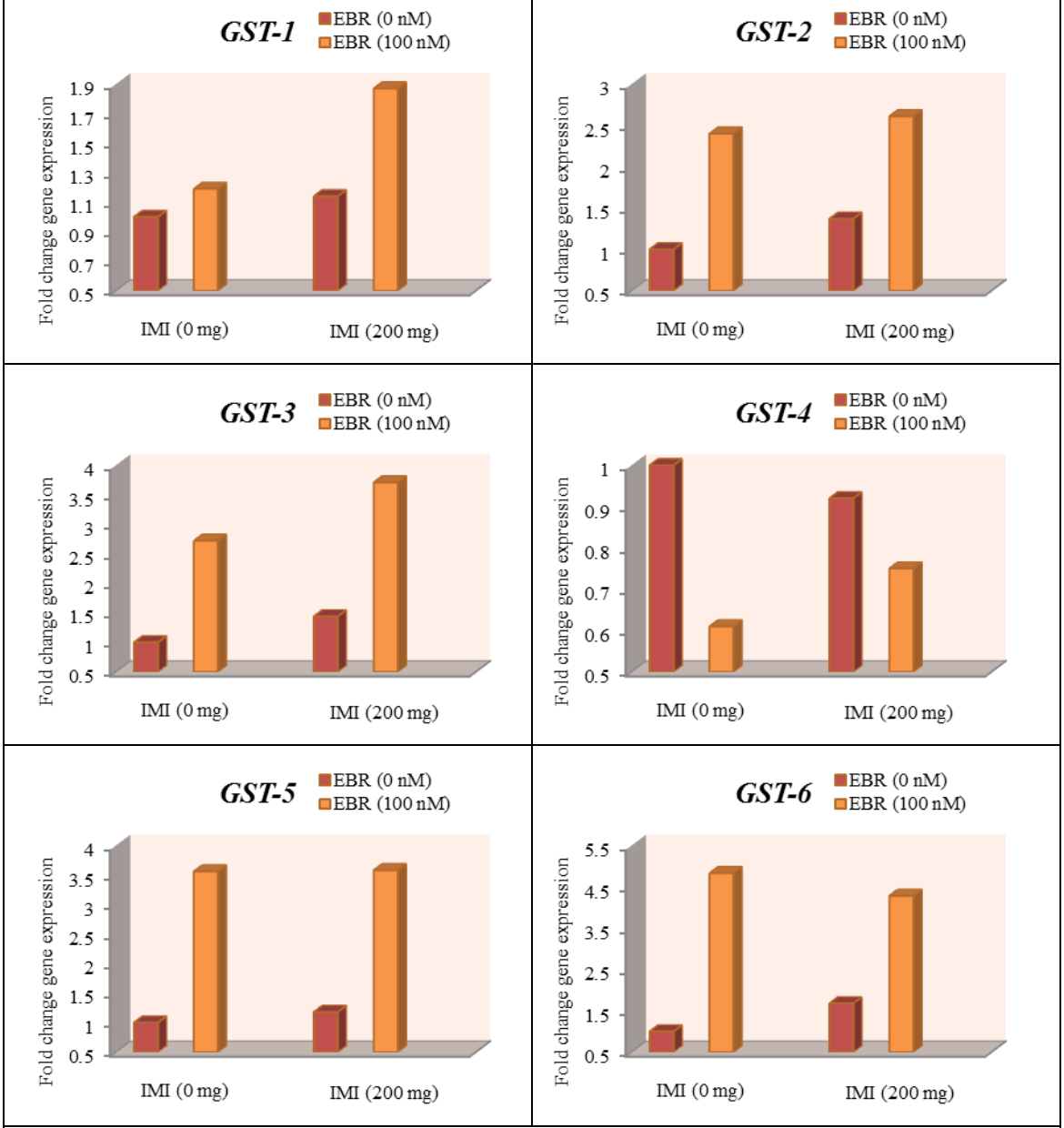


Fig. 9.1.2.2 Effect of seed soaking with EBR on the gene expression of GST enzyme in *B. juncea* seedlings grown under IMI toxicity.

Glutathione synthase (*GSH-S*)

Expression of *GSH-S* was increased by 1.44 fold in seedlings germinated from EBR treated seeds and grown under IMI toxicity, with reference to *GSH-S* expression in control seedlings (Table 9.1.2., Fig. 9.1.2.3).

Table 9.1.2 Effect of EBR seed soaking on gene expression in 10 days old *B. juncea* seedlings grown in IMI containing Petri-plates. Data are Mean±SD (n=3), Two-way ANOVA, Tukey's HSD and multiple linear regression analysis (MLR).

IMI (mg L^{-1})	EBR (nM L^{-1})	Expression of enzymes (fold change)												
		GSH-S	***GSH-T***	***PSY***	***CHS***	***PAL***	***CXE***	***P450***	***NADH***	***CS***	***SUCLG1***	***SDH***	***FH***	***MS***
0	0	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00	01±0.00
0	100	1.16±0.08	1.69±0.22	1.27±0.43	1.37±0.14	2.12±0.54	1.99±0.24	2.96±0.63	2.18±0.19	3.7±0.58	4.72±0.12	2.98±0.85	4.18±0.45	3.88±0.97
200	0	0.92±0.20	1.25±0.35	3.71±0.57	4.32±0.86	1.97±0.51	1.39±0.35	0.72±0.16	1.65±0.04	2.35±0.85	1.57±0.51	2.01±0.51	1.57±0.65	1.91±0.13
200	100	1.44±0.28	1.65±0.19	5.22±0.80	4.54±0.65	6.68±1.92	2.77±0.06	1.82±0.30	1.95±0.29	2.61±0.27	4.18±0.33	2.55±0.54	3.73±0.19	4.03±0.70
Two way ANOVA														
F_{IMI}		0.93	0.59	113.8***	105.1***	21.55**	21.64**	11.03*	4.16	0.20	0.02	0.79	0.06	2.35
F_{EBR}		10.57*	17.10**	8.26*	0.87	23.94**	88.35***	51.62***	51.72***	22.72**	306.5***	14.81**	126.0***	51.37***
$F_{IMI \times EBR}$		2.86	1.14	3.93	0.06	9.05*	2.26	4.06	18.29**	15.51**	9.47*	4.88	4.72	1.21
HSD		0.46	0.60	1.41	1.43	2.70	0.56	0.96	0.46	1.38	0.81	1.48	1.08	1.56

Multiple linear regression analysis

MLR equation	β-regression coefficients		Multiple correlation coefficient
	β_{IMI}	β_{EBR}	
GSH-S (gene expression fold change) = 0.91 + 0.0005 IMI + 0.0034 EBR	0.2040	0.6875	0.7170*
GSH-T (gene expression fold change) = 1.07 + 0.0005 IMI + 0.0055 EBR	0.1483	0.7983	0.8120**
PSY (gene expression fold change) = 0.69 + 0.0166 IMI + 0.009 EBR	0.9216	0.2483	0.9544***
CHS (gene expression fold change) = 1.04 + 0.0162 IMI + 0.003 EBR	0.9600	0.0877	0.9639***
PAL (gene expression fold change) = 0.10 + 0.0139 IMI + 0.0292 EBR	0.5869	0.6187	0.8528***
CXE (gene expression fold change) = 0.90 + 0.0029 IMI + 0.0118 EBR	0.4242	0.8571	0.9563***
P450 (gene expression fold change) = 1.21 - 0.0036 IMI + 0.0154 EBR	- 0.3842	0.8312	0.9157***
NADH (gene expression fold change) = 1.22 + 0.0011 IMI + 0.0074 EBR	0.2250	0.7933	0.8246**
CS (gene expression fold change) = 1.62 + 0.0005 IMI + 0.0152 EBR	0.0462	0.7003	0.7018*
SUCLG1 (gene expression fold change) = 1.27 + 0.0001 IMI + 0.0.0316 EBR	0.0072	0.9726	0.9725***
SDH (gene expression fold change) = 1.36 + 0.0015 IMI + 0.0126 EBR	0.1665	0.7210	0.7401**
FH (gene expression fold change) = 1.25 + 0.0003 IMI + 0.0267 EBR	0.0224	0.9528	0.9530***
MS (gene expression fold change) = 1.19 + 0.0027 IMI + 0.025 EBR	0.1936	0.9034	0.9239***

*, ** and *** indicate significant at $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

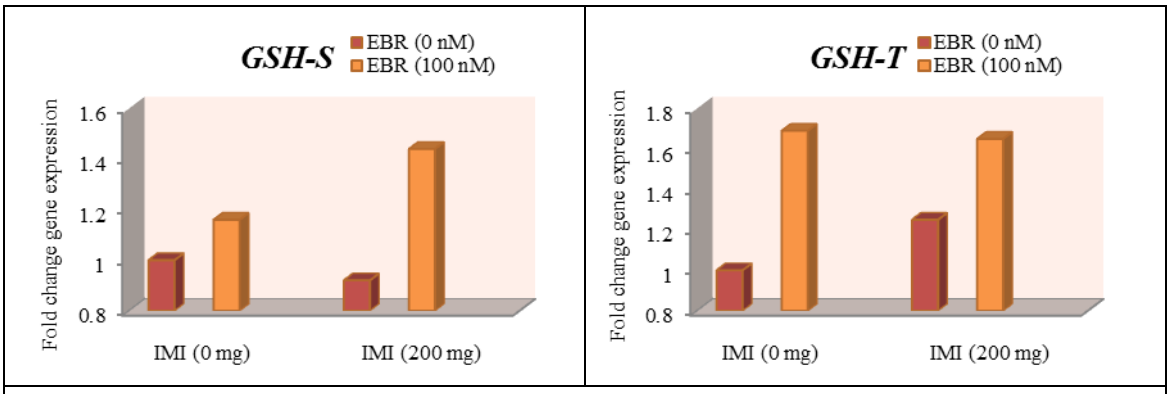


Fig. 9.1.2.3 Effect of seed soaking with EBR on the expression of genes related to glutathione regulation in *B. juncea* seedlings grown under IMI toxicity.

Glutathione transporter-1 (*GSH-T*)

In comparison to the control seedlings, expression of *GSH-T* gene was increased by 1.65 fold in seedlings from raised EBR treated seeds and grown on filter papers containing IMI solutions (Table 9.1.2., Fig. 9.1.2.3).

Phytoene synthase (*PSY*)

It was observed that the expression of gene *PSY* was increased by 5.22 fold in seedlings raised from seeds treated with EBR and grown in Petri-plates containing IMI solutions (Table 9.1.2., Fig. 9.1.2.4).

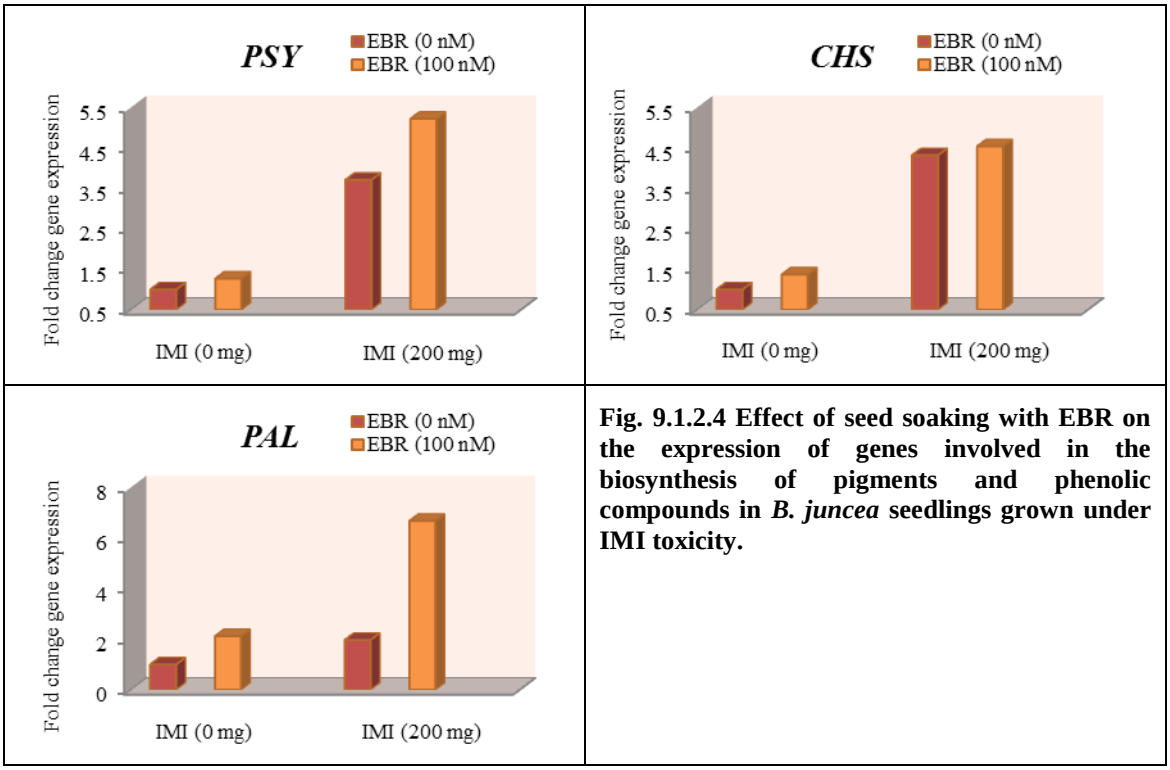


Fig. 9.1.2.4 Effect of seed soaking with EBR on the expression of genes involved in the biosynthesis of pigments and phenolic compounds in *B. juncea* seedlings grown under IMI toxicity.

Chalcone synthase (*CHS*)

Expression of *CHS* was observed to increase by 5.54 fold in seedlings germinated from EBR treated seeds and grown under IMI toxicity (Table 9.1.2., Fig. 9.1.2.4).

Phenylalanine ammonialyase (*PAL*)

Expression of *PAL* gene was increased by 6.68 fold in *B. juncea* seedlings raised from seeds soaked with EBR followed by growing them in IMI containing Petri-plates (Table 9.1.2., Fig. 9.1.2.4).

Carboxylesterase (*CXE*)

It was observed that expression of *CXE* was up-regulated by IMI as well as EBR. Maximum increase in *CXE* expression was by 2.77 fold in seedlings raised from EBR treated seeds and growing under IMI toxicity (Table 9.1.2., Fig. 9.1.2.5).

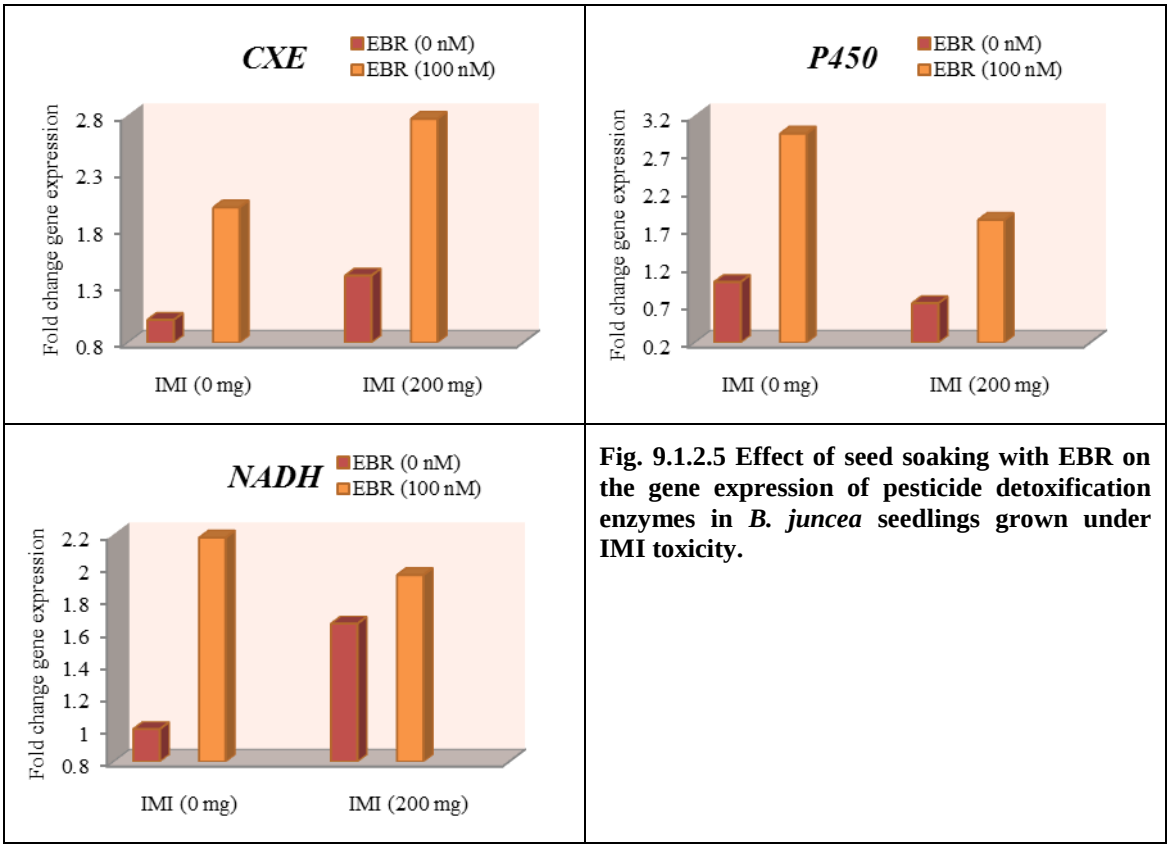


Fig. 9.1.2.5 Effect of seed soaking with EBR on the gene expression of pesticide detoxification enzymes in *B. juncea* seedlings grown under IMI toxicity.

Cytochrome P450 monooxygenase (*P450*)

Expression of *P450* was observed to increase by 1.82 fold in seedlings germinated from EBR treated seeds and grown in Petri-plates containing IMI solutions (Table 9.1.2., Fig. 9.1.2.5).

NADH-ubiquinone oxidoreductase (*NADH*)

Application of EBR to seeds and growing them in Petri-plates containing IMI solutions resulted in increase of *NADH* expression by 1.95 fold when compared to control *B. juncea* seedlings (Table 9.1.2., Fig. 9.1.2.5).

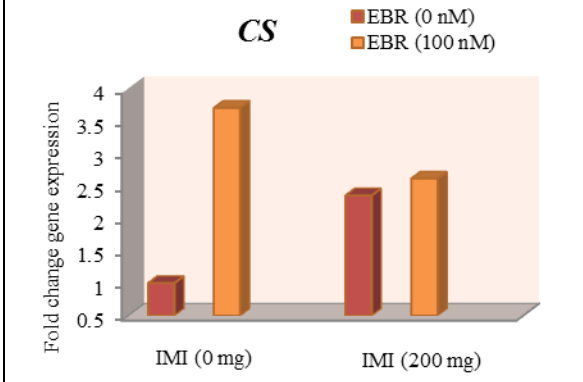
Significant difference for expression of genes encoding antioxidative enzymes, antioxidative non-enzymes and pesticide detoxification enzymes in *B. juncea* seedlings was observed after analyzing data using two-way ANOVA and Tukey's HSD. Positive β_{EBR} values obtained from MLR analysis revealed that EBR seed soaking up-regulated expression of all the genes (encoding antioxidative enzymes, antioxidative non-enzymes and pesticide detoxification enzymes) except *GST-4* (Tables 9.1.1 and 9.1.2).

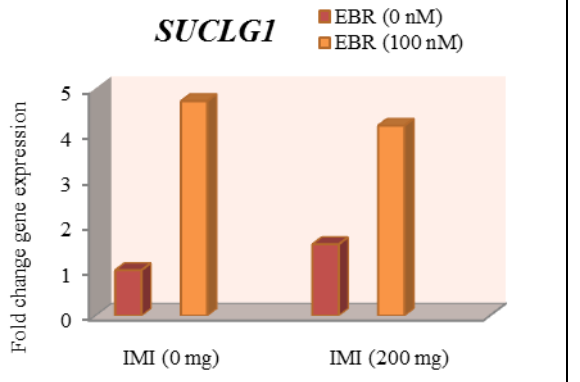
9.1.3 Organic acid metabolism

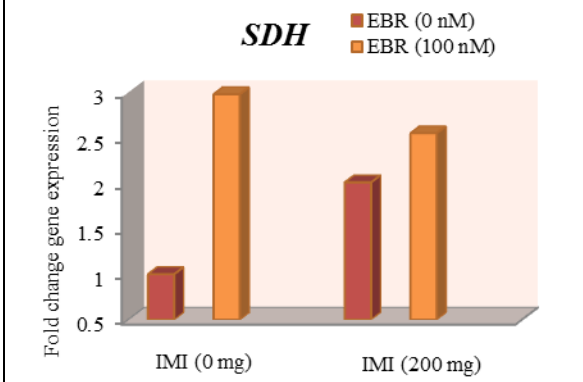
Expression of genes involved in organic acid biosynthesis was observed to increase with the application of IMI as well as EBR.

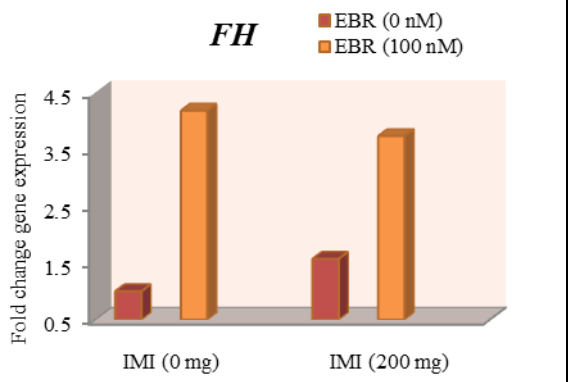
Citrate synthase (*CS*)

There was a 2.61 fold increase in the expression of *CS* in seedlings raised from seeds treated with EBR before sowing in IMI containing Petri-plates (Table 9.1.2., Fig. 9.1.3.1).









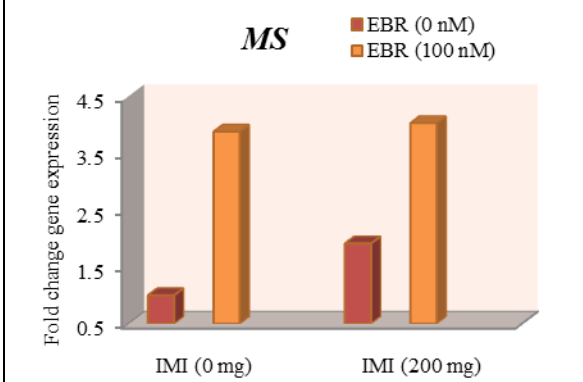


Fig. 9.1.3.1 Effect of seed soaking with EBR on the gene expression of enzymes involved in organic acid metabolism in *B. juncea* seedlings grown under IMI toxicity.

Succinyl Co-A ligase (*SUCLG1*)

Expression of *SUCLG1* was increased by 4.18 fold in *B. juncea* seedlings germinated from EBR treated seeds and grown in Petri-plates containing IMI solutions (Table 9.1.2., Fig. 9.1.3.1).

Succinate dehydrogenase (*SDH*)

As compared to control seedlings, expression of *SDH* was increased by 2.55 fold in seedlings germinated from EBR treated seeds and grown in the presence of IMI (Table 9.1.2., Fig. 9.1.3.1).

Fumarate hydratase (*FH*)

It has been observed that the expression of gene *FH* was increased by 3.73 fold in *B. juncea* seedlings raised from seeds soaked with EBR and grown in Petri-plates containing IMI solutions (Table 9.1.2., Fig. 9.1.3.1).

Malate synthase (*MS*)

Maximum increase in the expression of *MS* occurred by 4.03 fold in *B. juncea* seedlings germinated from EBR treated seeds and grown under IMI toxicity, as compared to its expression in control the seedlings (Table 9.1.2., Fig. 9.1.3.1).

Analysis of data using two-way ANOVA and Tukey's HSD showed significant differences for the expression of *CS, SUCLG1, SDH, FH* and *MS* in *B. juncea* seedlings. Positive β-regression coefficients for IMI and EBR obtained from MLR analysis revealed that application of IMI as well as EBR up-regulated the genes involved in organic acid biosynthesis (Table 9.1.2).

9.2 IMI residue analysis

A reduction in IMI residues was observed in seedlings, leaves and pods of *B. juncea* plants raised from EBR soaked seeds and grown in substratum amended with IMI.

In 10 days old seedlings, maximum IMI residues detected were 15.79 mg Kg^{-1} fr. wt. in seedlings raised from untreated seeds and grown in Petri-plates amended with 250 mg IMI L^{-1}. Seed soaking with 100 nM EBR and growing under IMI toxicity (250 mg IMI L^{-1}) resulted in decreasing the IMI residues to 7.63 mg Kg^{-1} fr. wt. (Table 9.2.1, Fig. 9.2.1).

Table 9.2.1 Effect of EBR seed soaking on IMI residues in 10 days old *B. juncea* seedlings grown in IMI containing Petri-plates. Data are Mean±SD (n=3), Two-way ANOVA, Tukey's HSD and multiple linear regression analysis (MLR).

Treatments		IMI residues (mg Kg^{-1} fr. wt.)
IMI (mg Kg^{-1})	EBR (nM L^{-1})	
150	0	10.08±0.82
150	100	7.24±0.37
200	0	11.32±0.62
200	100	7.29±0.20
250	0	15.79±0.40
250	100	7.63±0.84
F_{IMI}		44.21***
F_{EBR}		322.6***
$F_{IMI \times EBR}$		33.31***
HSD		1.62

Multiple linear regression analysis			
MLR equation	β-regression coefficients		r
	β_{IMI}	β_{EBR}	
IMI residues = 6.29 + 0.03 IMI - 0.05 EBR	0.4031	- 0.8117	0.9062***

*** indicates significant at p<0.001. r = multiple correlation coefficient.

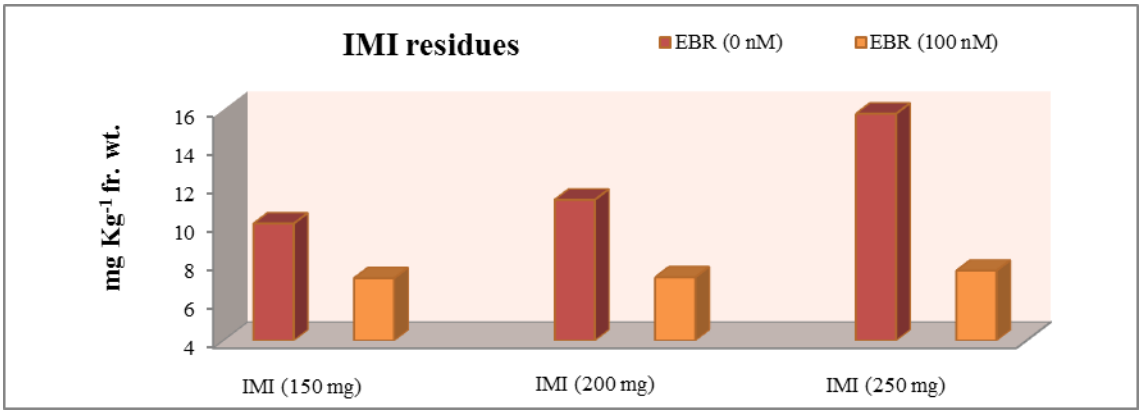


Fig. 9.2.1 Effect of seed soaking with EBR on IMI residue content in *B. juncea* seedlings grown under IMI toxicity.

IMI residues detected were 88.66 mg Kg^{-1} fr. wt. in the leaves of 65 days old plants raised from untreated seeds and grown in soils amended with 350 mg IMI Kg^{-1} soil. Decrease in IMI residues to 35.31 mg Kg^{-1} fr. wt. was observed in plants raised from 100 nM EBR soaked seeds and grown in soils amended with 350 mg IMI Kg^{-1} soil (Table 9.2.2, Fig. 9.2.2).

Table 9.2.2 Effect of EBR seed soaking on IMI residues in the leaves and pods of *B. juncea* plants grown in IMI amended soils. Data are Mean±SD (n=3), Two-way ANOVA, Tukey's HSD and multiple linear regression analysis (MLR).

Treatments		IMI residues (mg Kg^{-1} fr. wt.)	
IMI (mg Kg^{-1})	**EBR (nM L^{-1})**	65 DAS (Leaves)	80 DAS (Pods)
250	0	27.36±1.84	7.57±0.66
250	0.1	25.69±0.83	7.0±0.54
250	1	15.26±0.06	6.69±0.73
250	100	12.40±0.34	5.63±0.26
300	0	48.26±3.12	10.94±0.15
300	0.1	34.75±2.86	9.62±0.53
300	1	26.53±1.91	8.86±0.61
300	100	26.10±0.18	7.46±0.63
350	0	88.66±0.36	17.05±0.95
350	0.1	45.58±0.74	11.42±0.19
350	1	39.22±0.25	11.04±0.30
350	100	35.31±0.96	10.35±0.87
F_{IMI}		1347.9***	280.4***
F_{EBR}		733.7***	74.92***
$F_{IMI \times EBR}$		137.1***	14.97***
HSD		4.46	1.75

Multiple linear regression analysis

MLR equation	β-regression coefficients		r
	β_{IMI}	β_{EBR}	
IMI (leaves) = -56.92 + 0.320 IMI - 0.146 EBR	0.6842	- 0.3308	0.7600**
IMI (pods) = - 7.14+ 0.057 IMI - 0.022 EBR	0.7980	- 0.3299	0.8635***

** and *** indicate significant at p<0.01 and p<0.001. r = multiple correlation coefficient. DAS = days after sowing.

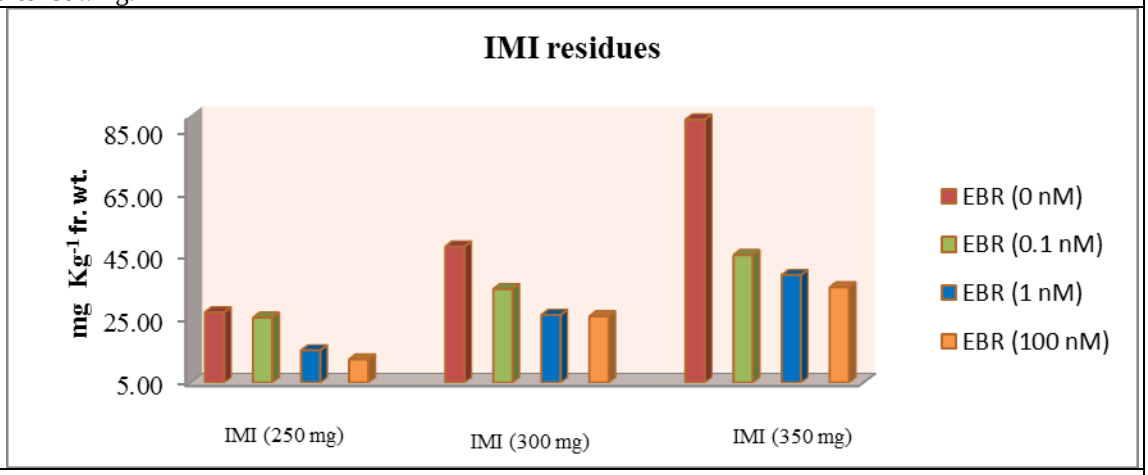


Fig. 9.2.2 Effect of seed soaking with EBR on IMI residue content in the leaves of *B. juncea* plants (65 DAS) grown under IMI toxicity.

In green pods of *B. juncea* plants (80 DAS), maximum IMI residue content (17.05 mg Kg^{-1} fr. wt.) was observed in plants raised from untreated seeds and grown in pots containing 350 mg IMI Kg^{-1} soil. Seed soaking with 100 nM EBR before sowing in pots amended with 350 mg IMI Kg^{-1} soil resulted in reduction of IMI residues to 10.35 mg Kg^{-1} fr. wt. (Table 9.2.2, Fig. 9.2.3).

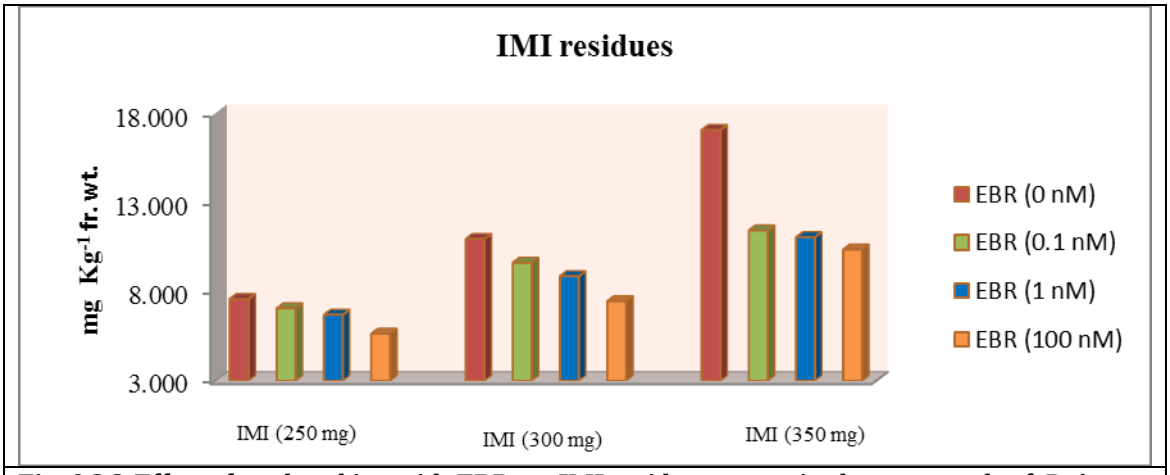


Fig. 9.2.3 Effect of seed soaking with EBR on IMI residue content in the green pods of *B. juncea* plants (80 DAS) grown under IMI toxicity.

Two-way ANOVA and Tukey's HSD showed significant differences for IMI residue contents in the seedlings, leaves and pods of *B. juncea* plants raised from EBR treated seeds and grown under IMI toxicity. MLR analysis also revealed that EBR seed soaking reduced the IMI residues in *B. juncea* as indicated by negative β-regression coefficients (Tables 9.2.1 and 9.2.2). Data analysis using ANN revealed that correlation between simulated (output) and experimental (target) values was very high as shown in Fig. 9.2.4.

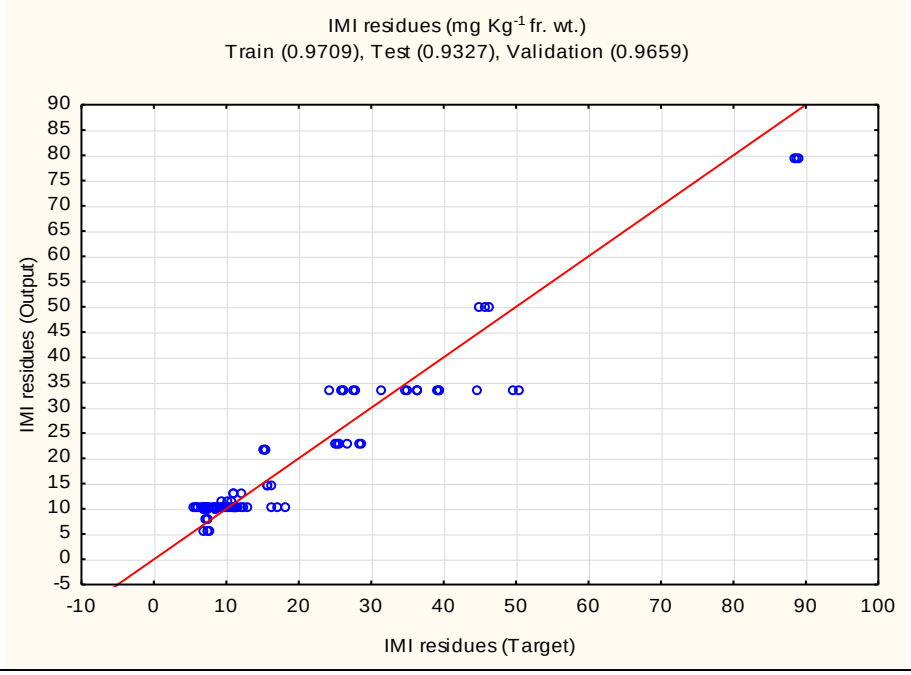


Fig. 9.2.4 Correlation between target (experimental) and output (simulated) IMI residue contents using ANN model ($p<0.001$).