EFFECTS OF BRASSINOSTEROIDS AND NITRIC OXIDE AGAINST CADMIUM STRESS IN *BRASSICA JUNCEA*

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BOTANY

BY

MOHD IRFAN

DEPARTMENT OF BOTANY
ALIGARH MUSLIM UNIVERSITY
ALIGARH (U.P.) INDIA

2013

DEDICATED

TO

MY PARENTS

"My Lord! Bestow on them Your Mercy as they did bring me up when I was young."

(Qur'aan: 17:24)

ANNEXURE-I

CANDIDATE'S DECLARATION

I, **Mohd Irfan**, Department of Botany, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out by me under the supervision of **Prof. Aqil Ahmad** at Aligarh Muslim University, Aligarh. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree.

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Date: 26/12/2013

Mohd Irfan

Dr. AQIL AHMAD
Professor

Plant Physiology Section
Department of Botany
Aligarh Muslim University
Aligarh-202002, U.P., India
e-mail: aqilahmad@rediffmail.com
Phone: +91-571-2702016

Dated.....

Certificate

This is to certify that the thesis entitled, "**Effects of Brassinosteroids and Nitric Oxide against Cadmium Stress in *Brassica juncea***", submitted for the degree of **Doctor of Philosophy** in Botany is a faithful record of the bonafide research work carried out at Aligarh Muslim University, Aligarh, India, by **Mr. Mohd Irfan** under my guidance and supervision and that no part of it has been submitted for any other degree or diploma.

(Aqil Ahmad)
Research Supervisor

Phones: Ext. : 2702016
Int. : 3300, 3301
Fax : 0571-2702016

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Mohd Irfan

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LIST OF ABBREVIATION

$^1O_2$*	Singlet oxygen
^{3}Chl*	Triplet state chlorophyll
ABA	Abscisic acid
ALA	Alpha aminolaevulinate
APX	Ascorbate peroxidase
BIN2	Brassinosteroid Insensitive-2
BL	Brassinolide
BR(s)	Brassinosteroid(s)
BRI1	Brassinosteroid Response Insensitive-1
BZR1	Brassinazole1
CA	Carbonic anhydrase
CAT	Catalase
Cd	Cadmium
CN	Campestenol
cPTIO	(2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide
CR	Campesterol
CS	Castesterol
DAS	Days After Sowing
DDW	Double Distilled Water
DHAR	Di Hydro Ascorbate Reductase
DHE	Dihydroethidium
DPI	Diphenylene iodonium
EBL	Epi-brassinolide
GA3	Gibberellic acid-3
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione reduced
GSNO	S-nitrosoglutathione
HBL	Homo-brassinolide
IAA	Indole Acetic Acid

MAPK	Mitogen Activated Protein Kinase
MDA	Melandoaldehyde Acid
MSI	Membrane Stability Index
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NR	Nitrate Reductase
NiR	Nitrite Reductase
$NO^{\cdot}$	Nitroxyl anion
NO^{+}	Nitrosonium cation
NOHA	N-Hydroxy-Arginine
NOR	Nitric Oxide Reductase
NOS	Nitric Oxide Synthase
NRRC	Northern Regional Research Centre
$O_2^{\cdot-}$	Superoxide anion
$^1O_2$*	Singlet oxygen
$ONOO^{-}$	Peroxinitrile
OST1	OPEN STOMATA1
OXI1	Oxidative Signal-Inducible1
PAMP	Pathogen Associated Molecular Patterns
PC	Phytochelatin
PCD	Programmed Cell Death
POX	Peroxidase
PSII	Photosystem II
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RuBPCase	Ribulose-1,5 bis phosphate carboxylase
RWC	Relative Water Content
SNAP	Ribulose-N-Acetyl Penicillinamine
SNP	Sodium NitroPrusside
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid
USDA	United States Department of Agriculture
WUE	Water Use Efficiency
XETH	Xyloglucan Endo-Transglucosylase/Hydrolase
XOR	Xanthene Oxide Reductase

Chapter 1

INTRODUCTION

CHAPTER-1

INTRODUCTION

Indian mustard or *Brassica juncea* [L] Czern. & Coss. belongs to the Brassicaceae (formerly Cruciferae) family of flowering plants, commonly known as the mustard family. The name "mustard" is thought to have originated from the Latin mustum-ardens (Weiss, 2002). The ground seed was often added to unfermented grape juice, called "must", and the second part of the word is derived from the Latin ardere, meaning "to burn", hence, "burning must". The plant is erect green annual herb of one to two meter height. Foliages are pale green with few hairs (pubescent) on first leaves, leaf blades up to petioles. The lower leaves are deeply notched while upper leaves are narrow and entire; flowers are small yellow with petals arranged diagonally as Greek cross for which the family is named earlier as "Cruciferae". The inflorescence is raceme and flowers open progressively upwards down. The fruit dehisces by two valves from the base known as siliqua. Seeds are medium sized, round and dark brown in colour as compared to other species.

The mustard family is one of the largest dicot families of angiosperms includes nearly 3500 species and 350 genera. It is amongst the ten most economically important plant families with members used particularly for oil, vegetables, condiments and fodder. The natural habitat of *Brassica* species encompasses temperate to moderate subtropical zone which includes Northern India, Pakistan, Myanmar and Nepal etc. The abundant occurrence of Brassica led scientists to incline their research on brassica species after *Arabidopsis*, the model plant. Mustard is rabi crop and is mainly cultivated on irrigated land of Indo-Gangetic plains in India. They prefer moderate temperature of 24-28°C with an optimum of 20°C. Brassica grow well in areas receiving 350-550 mm of rainfall. Plants are grown on wide range of soils as alluvial, medium loam, sandy loam or heavy loam soils. Plant matures in 90-100 days. The plant height approaches 90-200 cm and flowers in the months of November, December, January and February and the harvest season is in the months of March, April and May. Of the Brassica family the seven important oilseed plants are grown annually in India with the name rapeseed. These include Indian mustard (*Brassica juncea* [L.] Czern & Coss.), commonly called rai (raya or laha), the three ecotypes of Indian rape, *B. campestris* L. ssp. oleifera viz., toria, brown sarson (lotni

and tora types) and yellow sarson, Swede rape or gobhi sarson (*B. napus* L.), Ethiopian mustard or karan rai (*B. carinata* Braun.) and taramira or tara (*Eruca sativa* Mill.). *B. juncea* is the dominating species and considered as of native origin along with *B. campestris* L. and *B. napus* L. which are the important sources of edible oil in India. The projected demand for oilseeds in India is around 34 million tonnes by 2020, of which about 14 million tonnes (41%) is to be met by rapeseed mustard. Mustard seeds are sown after the harvest of kharif crop in the month of November thorough out the India.

The heavy metals are toxic elements mostly having high atomic weight and specific gravity greater than $5g/cm^3$ with respect to water ($1g/cm^3$). However, there is no correlation of either density or chemical property of these heavy metals with the physio-toxicological effect in metabolism. There are 53 heavy metals in periodic table (Weast, 1984). Duffus (2002) earlier reviewed different works cited the range of 'heavy' metals from 3.5-7 g/cm^3. Nevertheless, plants never show ability to detect metals on the basis of their density. Some lighter metals and metalloids are also toxic (marked with asterisk; *), therefore, incorporated under heavy metals (Table 1), while some others (e.g. Au†) are not toxic, hence there is no standard definition of heavy metals and they surpass metalloids, transition metals, basic metals, lanthanides and actinides. Heavy metals, like any other metal, are known for their tendency to lose electrons to form positive ions indicating their active participation in redox systems in trace (<1000 mg Kg^{-1}). These include divalent and monovalent cations depending upon their ability to accept free electron (Lewis acid). Biological redox systems appropriately and finely utilized this property of heavy metals in certain very important biochemical reactions in well regulated manner with protein moieties. Based on the stability constants of the metal complexes, metal acceptors are suggested to be classified as 'hard', 'soft' or 'intermediate'. Nieboer and Richardson (1980), depending upon ionic indices and covalent indices categorized metal acceptors explain their ability to react with –SH and imidazole groups of proteins to impart cellular toxicity.

Pesticide and heavy metal contamination of food stuffs is increasing problem of the grains and vegetable production. Sulphur rich crops, for instance, brassicas considerably accumulate toxic doses of heavy metals in their consumable tissues.

Table 1. Classification of heavy metals/semimetals (with density) based on their roles in plant growth

S. No.	Essential metals	Density (g/mg^3)	S. No.	Beneficial metals	Density (g/mg^3)	S. No.	Nonessential metals	Density (g/mg^3)
1.	Zn	7.13	7.	Na	0.97	13.	*As	5.72
2.	Mn	7.43	8.	*B	2.34	14.	*Cr	7.19
3.	Fe	7.87	9.	*Al	2.70	15.	*Cd	8.65
4.	*Ni	8.90	10.	Si	2.65	16.	*Pb	11.35
5.	*Cu	8.96	11.	V	6.11	17.	Ag	10.50
6.	Mo	10.22	12.	Co	8.90	18.	*Hg	13.55
						19.	†Au	19.32

These heavy metals, besides their toxic magnification in upper tropic level of food chain, perturb the metabolic machinery of plants rendering their growth and yield below potential level. Cd falls under the category of most abundant and easily accessible heavy metal to plants. Mining, fossil fuel combustion, urban wastes, industrial effluents, municipal solid waste and phosphate fertilizers are key enrichment factors of different forms of Cd in soil (Angelone and Bini, 1992; Kevresan et al., 1998). Toxic level accumulation of Cd suppresses primary metabolism through deactivating key enzymes and basic physiological plant processes, thereby curtailing growth and dry weight of tissue. The growth phase of the crop should synchronize with optimum environmental conditions for optimal expression of housekeeping genes. It is a fact that specified genotypes does not exhibit the same phenotypic characteristics in all environmental conditions. The different genotypes, growth response varies to different environment and their relative ranking usually differ (Eberhort and Russel, 1966).

Judicial application of plant hormones plays an important role to fully exploit the genetic potentiality of a crop variety supporting optimum growth conditions such as temperature, light, humidity and rainfall. Recently recognized group of steroidal plant hormones well known as 'Brassinosteroids' (BRs) gained acceleration in scientific research and field applications after potentiating their diverse role in regulating key plant physiological processes under normal (Bajguz, 2007) and stressed regimes (Bajguz and Hayat, 2009; Fariduddin et al., 2013a).

Brassinosterods is a new class of polyhydroxysteroids reported in plants which play essential role in plant growth and development at very low concentrations. Nearly 69 BRs have been identified from different groups of plant kingdom (Bajguz, 2010) and from different organs (Hayat et al., 2003b). Brassinosteroids have a common 5α-cholestan skeleton, and their structural variations come from the kind and orientation of oxygenated functions in rings A and B. Their roles have been implicated in a range of physiological and biochemical responses from seed germination stem elongation, leaf expansion, bending and epinastic movements, vascular differentiation, regulation of flower development and pollen tube growth etc. (Sasse, 2003). The identification of BRs biosynthetic and response deficient mutants in *Arabidopsis* has elucidated its essential components of signaling and connections in plant growth and development (Szekeres et al., 1996; Clouse, 1996).

Brassinosteroids potentially confer tolerance against osmotic stress (Sairam, 1994; Vardhini and Rao, 2003), temperature stress (Wilen et al., 1995; Fariduddin et al., 2011), salinity (Hayat et al., 2010a; Ali et al., 2007b), pesticide stress (Xia et al., 2009b) and various heavy metal stresses like, cadmium (Hayat et al., 2007a), nickel (Alam et al., 2007; Yusuf et al., 2011), aluminium (Ali et al., 2008a) and copper (Fariduddin et al., 2009b).

Nitric oxide (NO) is a well-established regulator of animal physiological functions and a widespread pollutant (Stohr and Ullrich, 2002). This inorganic free molecule has been described as gaseous phytohormone and inter- intracellular messenger at a very low concentration, (Leshem, 2000; Beligni and Lamittina, 2000; Stohr and Stremlau, 2006). Its production in plants is first reported in 1975 by Klepper. Later both non-enzymatic and enzymatic pathways have been proposed. NO exert growth promotion at very low concentrations (Leshem, 1996) and defense responses at slight higher concentrations when produced endogenously or applied exogenously. Therefore, NO characteristically manifests concentration dependent dual responses based upon physiological state of plant against prevailing environment and plant growth stage. Seed germination, root organogenesis, root hair induction, hypocotyl growth and stomatal conductance are the growth responses assigned to NO. It also acts against oxidative stress (Neill et al., 2003) promoting antioxidant responses under abiotic stresses. The higher concentration of Nitric oxide, however,

inhibits plant growth and negatively effects growth responses. The role of NO has also been implicated in biotic stress responses and program cell death. The processes regulated by NO also include defense responses, stomatal closure, apoptosis, hypersensitive response and phytoalexin production (Zhang et al., 2005; Besson-Bard et al., 2008; Chaki et al., 2009).

Application of NO donor chemicals such as sodium nitroprusside (SNP), S-nitroso-N-acetyl-penicillamine (SNAP), S-nitrosoglutathione (GSNO) has shown countering effects against diverse abiotic stress responses. SNP ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$) is the inorganic compound. SNP is used as the source of NO. In the solution beside NO, it releases ferricyanide, and cyanide (CN^-) molecules. Potassium ferricyanide [$K_3Fe(CN)_6$] is used as a control to test the effects of NO, which does not releases NO in the solution. Nitric oxide countering effects have been favorably studied in salinity and heavy metal stress, ultraviolet radiation, ozone and mechanical wounding. The augmenting actions of NO against different abiotic stresses in various cereal and vegetative crops are still left to be studied. Which mechanism regulates it to commit positive and detrimental decisions and effects on plant physiological functions need to be addressed in future researches.

Considering the Cd hazard to the important cash crop of India, *B. juncea*, and potential roles assigned to BRs in overcoming toxic responses of Cd to two well-grown varieties of Indian mustard in the subcontinent; Varuna and RH-30. The selected physiological and biochemical marker assays have been taken into account besides growth attributes to fulfill following objectives:

1. Comparative analysis of responses of two varieties of Indian mustard for graded concentrations of Cd through soil.
2. To compare the excellence of EBL or HBL on test plants applied through foliar application.
3. To screen the effective concentration of sodium nitroprusside solutions for optimum response on the selected varieties.
4. To compare the ameliorative effect of two BR analogues against soil applied Cd toxicity doses.
5. To analyze the ameliorative effect of effective SNP concentration against soil applied Cd toxicity doses.

6. To observe the responses of combination of effective BR and SNP concentration against soil applied Cd toxicity doses.

The metabolic and growth parameters showing optimum response to the treatments may be marked as a scale for forecasting further growth and productivity in crops.

Chapter 2

REVIEW OF LITERATURE

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CHAPTER – 2

REVIEW OF LITERATURE

2.1 INDIAN MUSTARD (*Brassica Juncea* L. Czern & Coss.)

The mustard is the most important oilseed crops cultivated in 53 countries spreading over the six continents across the globe under tropical as well as the temperate zones. In response to the regular increase in demand for edible oils and its products the world production of mustard has been increasing at a rapid rate in several countries. India occupies the first position in area and second position in production of rapeseed-mustard in the world (Kumar, 1999). It is cultivated on 6.86 million ha area of the country, particularly in Northern plains. Haryana, Madhya Pradesh, Rajasthan and Uttar Pradesh are the major states of India cultivating *Brassica juncea* representing 81% of the total acreage and contributing 82.9 per cent to the national rapeseed-mustard production. India recorded a spectacular production of oilseeds (well known as Yellow Revolution) when country witnessed increased production of mustard from 2.7 mt (1985-86) to 8.1 mt (2005-06) (Singh et al., 2008).

The integrated oilseeds policy of the Government of India lies in the adoption of high yielding varieties, improved agro production and protection technologies and price incentives that has opened up new vistas to earn valuable foreign exchange through export of oil meal and value added productions. One of the new vistas in the remunerative cultivation of oilseed brassicas in the non-traditional areas is to select appropriate species and variety suited to particular agro climatic situations which can yield more per unit of water and nutrients used.

2.2 HEAVY METALS IN BIOLOGICAL SYSTEMS

It is not well understood that why plants have selected only some of the mono/-divalent cations to signal and shape up metabolism. However, relying on the facts these were classified as "essential" or "non-essential", the role of non-essential with particular respect to heavy metals sought more attention when these were perceived to accelerate mortality and reduce potential survival of the organism, out of them cadmium (Cd) reaches into the soil through natural (lethogenic and pedogenic factors) and anthropogenic processes (Table 2). Dose dependent data of several metal

cations suggest that heavy metals are stored/metabolized at low concentrations but are toxic at higher concentrations, therefore following bell-shaped-relationship of toxicity (Marshner, 2012). Soil contaminated with the heavy metals above the permissible limit leads to a decline in agricultural yields (Nellessen and Fletcher, 1993; Salt and Rauser, 1995; Akinola and Ekiyoyo, 2006). Alternatively, those of essential heavy metals also proved to be toxic when readily acquired above a threshold limit. Plant species have developed various avoiding and countering mechanisms to safeguard themselves from the heavy metals.

Table 2. Sources of Cd contamination to different domains of environment

	S. No.	Sources	Contaminated horizons
1. Natural processes	a).	Lethogenic	Soil pollution
	b).	Pedogenic	Soil pollution
2. Anthropogenic processes	a).	Industrial effluents	Water pollution
	b).	Phosphate fertilizers	Soil pollution
	c).	Pesticides	Soil pollution
	d).	Vehicular traffic	Air pollution
	e).	Municipal Sewage Waste	Water pollution
	f).	Mining and fossil fuel	Soil and water pollution

2.3 CADMIUM

Cadmium falls under the category of non-essential divalent cations, most abundant and readily available to plant body eliciting toxic responses in aerial parts (Kabata-Pendias, 2011). The nontoxic level of Cd in soil ranges from 0.04 mM to 0.32 mM. It antagonizes Zn (Lachman et al., 2004) and blocks the Ca-channels (Swandulla and Armstorng, 1989) that share the chemical and electronic properties with Cd. The uptake of different forms of Cd in Chinese cabbage follow the pattern $CdSO_4$>$CdCl_2$>CdO>$CdCO_3$ while in rice as $CdCl_2$>$CdSO_4$>CdO>CdS>$CdCO_3$ in loamy-sand drab soil with pH 8.2 (Jikai et al., 1982). In leafy and fruity vegetables Cd is reported at the level of 0.6 μg/g tissue fresh mass (Sharma et al., 2008). Cereals and vegetables are most susceptible to increased contamination through raised levels of Cd in the soil. Besides being very toxic to plant metabolism and growth, the enrichment ratio of Cd is more comparing to other toxic heavy metals. This section of

the review preferably deals with responses of soil Cd uptake and its manifestation on plant metabolism and growth.

2.3.1 Effect of Cd on plant growth attributes

Growth morphology provides primary scale and a very important parameter to conclude the gross effect of toxicants. The parameters of fresh weight of shoot and root with respect to their length and other visible appearances directly indicate the efficiency of carbon fixation and water use efficiency. Being not an essential nutrient, added in higher concentrations, Cd potentially inhibits plant growth (Aery and Rana, 2003), therefore, reports say that even at relatively low concentrations it alters plant metabolism (Van Assche and Clijisters, 1990). Decline of plant dry weight suggests the reduced carbon fixation and nutrient uptake efficiency. The damaging impact of excessive uptake of Cd on plant growth was marked in various plant species. The presence of Cd in the soil retarded the growth of soybean (Dewdy and Ham, 1997), pea (Sandalio et al., 2001), *Corchorus olitorius* (Mazen, 2004), *Medicago sativa* (Drazic et al., 2006), maize (Krantev et al., 2008) and chickpea plants (Hasan et al., 2008). Higher concentrations of Cd decreased the growth of the whole plant (Prasad, 1995). The interaction of *Rhizobium* in the nodules of chickpea was found to be very sensitive to heavy metals resulting in a decrease in dry mass of chickpea and green gram (Rana and Ahmad, 2002). An increase in Cd concentration decreased the fresh mass in mung-bean (Wahid et al., 2007), *Medicago sativa* (Drazic et al., 2006) and *Zea mays* (Ekmekci et al., 2008). Moreover, a marked decrease in root and shoot mass was observed when treated with lower concentration of Cd in *Vigna ambacensis* (Al-Yemeni, 2001) and wheat (Milone et al., 2003).

2.3.2 Effect of Cd on photosynthesis and associated attributes

Photosynthesis is the major drawing force of carbon fixation on earth enriching the biomass at very primary level of the ecosystem. The efficiency of producers (plants) to run the complex metabolism of this physiological process relies on multiple factors viz. availability of water and mineral nutrients (as NPK); CO_2 and solar radiation etc. Cd competes with soil nutrients availability, causes water stress; checks stomatal conductance. Moreover, it inactivates membrane LHC (light harvesting complex) proteins, H^+ pump, and photosynthetic enzymes to restrict efficiency of photosynthesis. Cd preferentially accumulates in the chloroplasts and

disrupts chloroplast functions (Bi et al., 2009), therefore, it is an effective inhibitor of photosynthesis (Vassilev et al., 2005). A linear relationship between photosynthesis and inhibition of transpiration was observed in clover, lucerne, and soybean that suggested Cd inhibits stomatal opening (Huang et al., 1974). Cd damages the photosynthetic apparatus, in particular the light harvesting complexes of photosystems I and II (Siedlecka and Baszynsky, 1993; Siedlecka and Krupa, 1996). The inhibition of root Fe (III) reductase induced by Cd leads to Fe(II) deficiency which seriously affects photosynthesis (Alcantara et al., 1994). Cd causes stomatal closure in higher plants (Poschenreider et al., 1989) and an overall inhibition of photosynthesis in pigeon pea (Sheoran et al., 1990), radish (Krupa et al., 1993), *Pisum sativum* (Chugh and Sawhney, 1999), tomato (Dong et al., 2005), soybean (Shamsi et al., 2007), *Brassica juncea* (Hayat et al., 2007a), maize (Ekmekci et al., 2008).

The presence of Cd decreased the contents of chlorophyll and carotenoids, and increased non-photochemical quenching in *Brassica napus* (Larsen et al., 1998) favoring excitation of chlorophyll to triplet state ($^3Chl^*$) and generation of singlet oxygen ($^1O_2^*$). Similarly, a decreased in the rate of synthesis and level of chlorophyll is reported in certain plant species under the influence of the Cd e.g. cress and lettuce (Czuba and Ormond, 1973), barley (Stiborova et al., 1986), *Hydrilla verticillata* (Garg et al., 1997), maize (Ferretti et al., 1993), *Cupressus arizonica* (Griffiths et al., 1995), *Salvinia cucullate* (Phetsombat et al., 2006), *Catharanthus roseus* (Pandey et al., 2007) and wheat (Bishnoi et al., 1993; Amani, 2008).

2.3.3 Effect of Cd on metabolic enzymes

Loss of plant growth, associated with Cd treatment was probably caused by the inhibition of protein synthesis (Foy et al., 1978). Phyto-toxicity of the metal in crop plants has been observed in the form of a decrease in protein level (Krantev et al., 2008). Moreover, the grains developed on the plants grown under Cd stress had lower protein content (Salgare and Acharekar, 1992; Hasan et al., 2008).

Higher Cd concentration inhibited the activity of carbonic anhydrase (CA) and the rate of photosynthesis. The enzyme CA reversibly hydrates CO_2 to continuously channelize it to RuBPCase in the grana of the chloroplast (Majeau and Coleman, 1994; Price et al., 1994; Stemler, 1998a). Otherwise the declining concentration of surrounding inorganic carbon would restrict the activity of RuBPCase (Majeau and

Coleman, 1994). Low concentration of Cd stimulates the activity of CA; however, its accumulations at higher concentration inhibit CA activity (Siedlecka and Krupa, 1996; Hasan et al., 2007). Therefore, Cd effect on CA seems to be dose dependent. Cd induced changes in CA are also reflected at the level of photosynthesis (Khan et al., 2008) probably because as CA is also hypothesized to be involved in photosynthetic electron transport system (Stemler, 1997) and in maintaining chloroplast pH during rapid changes in light intensity (Reed and Graham, 1981). Stemler (1986) showed the association of CA with thylakoids and PSII in maize. Also the activity of PSII depends upon the Ca^{2+} (Stemler, 1998b) where Cd competes with the uptake of divalent cations, including Ca^{2+} (Swandulla and Armstorns, 1989).

Nitrate reductase (NR), the primary enzyme in the nitrate assimilation pathway, is the rate limiting step in determining plant growth and development (Solomonson and Barber, 1990) and its cellular level is influenced by a variety of environmental factors (Andrew, 1986; Murphy et al., 1997). Garg et al. (1997) observed a decline in the activity of NR in *Hydrilla verticillata* with an increase of Cd concentration. The presence of Cd in the soil retarded the assimilation of NO_3 in *Silene vulgaris* (Mathys, 1975), maize (Nassbaum et al., 1988; Hernandez et al., 1996), pea (Burzynski, 1988), tomato (Quariti et al., 1997), bean (Gouia et al., 2003) and in *Cicer arietinum* (Hasan et al., 2008).

2.3.4 Effect of Cd on oxidative stress and anti-oxidation response

Plants possess a number of antioxidant molecules and proteins that protect them from potential oxidative damage (Smeets et al., 2005; Pal et al., 2006) of which superoxide dismutase (SOD) is the first enzyme in the detoxifying process that converts O_2^- radicals to H_2O_2 at a very rapid rate (Polle and Rennenberg, 1994). Cd results in oxidative stress (Hendy et al., 1992; Somashekaraiah et al., 1992) either by inducing the production of free oxygen radicals (Balakhnina et al., 2005; Demirevska-Kepova et al., 2006) or by decreasing the concentration of enzymatic or non-enzymatic antioxidants (Somashekaraiah et al., 1992; Stohs and Bagchi, 1995; Shaw, 1995; Gallego et al., 1996; Sandalio et al., 2001; Balestrasse et al., 2001; Fornazier et al., 2002; Cho and Seo, 2005; Mohan and Hosetti, 2006). This defense system against the stress is composed of metabolites such as ascorbate, glutathione, tocopherol etc., and enzymatic scavengers of activated oxygen such as peroxidases (POX), catalases

(CAT) and SOD (Noctor and Foyer, 1998; Asada, 1999; Sandalio et al., 2001; Khan et al., 2002; Bor et al., 2003; Panda and Khan, 2003; Chaoui et al., 2004; Demiral and Turkan, 2005; Mandhania et al., 2006).

Peroxidase induction is a general response of higher plants after the uptake of toxic quantities of metals (Van Assche and Clijsters, 1990). In *Halianthus annus* leaves, Cd enhanced lipid peroxidation, increased lipoxygenase activity and decreased the activity of SOD (Sandalio et al., 2001; Khan et al., 2002; Panda and Khan, 2003), glutathione reductase, catalase, ascorbate peroxidase, and dehydro-ascorbate reductase (Gallego et al., 1996). Cd induced the activity of POX in soybean (Fuhrer, 1982), in roots and leaves of *Oryza sativa* (Reddy and Prasad, 1993), bean leaves (Lee et al., 1996), *Brassica juncea* (Singh and Tiwari, 2003; Hayat et al., 2007a), *Bacopa monniera* (Mishra et al., 2006) and in the leaves of *Calamus tenuis* (Khan and Patra, 2007), cucumber (Goncalves et al., 2007), *Cicer arietinum* (Hasan et al., 2008), maize (Ekmekci et al., 2008). In *Phaseolus aureus*, Cd ions decreased CAT activity and increased the activities of guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) (Shaw, 1995; John et al., 2007). However, an increase in CAT activity was observed under Cd stress in wheat (Milone et al., 2003), *Oryza sativa* (Panda and Patra, 2007), *Cicer arietinum* (Hasan et al., 2008). In maize plants CAT activity was not affected by Cd treatment (Krantev et al., 2008). Superoxide dismutase is the first line of defense against oxidative stress, Cd induced the activity of SOD in *Brassica juncea* (Hayat et al., 2007a), cucumber (Goncalves et al., 2007) and maize (Ekmekci et al., 2008).

Membrane damage due to lipid peroxidation caused by metals is mediated by activated oxygen radicals (hydrogen peroxidase, hydroxyl and superoxide radicals) but could be quenched by the induction of specific enzymes like POX, SOD and CAT (De Vos and Schat, 1981). *Phaseolus vulgaris* roots exposed to 5 mM Cd had higher activities of GPX and APX and elevated levels of lipid peroxidation (Chaomi et al., 1997). Cd treatment also increased lipid peroxidation (Lazono-Rodriguez et al., 1997; Sandalio et al., 2001; Astolfi et al., 2004; Chaoui et al., 2004; Srivastava et al., 2004) whereas no impact on lipid peroxidation was noticed in the roots of *Daucus carota* plants, exposed to Cd (Sanita di Toppi et al., 1998).

In addition to these antioxidant molecules, thiols also possess strong antioxidative properties and are able to counteract oxidative stress imposed by Cd (Pichorner et al., 1993; Shanthala et al., 2006). Germinating pigeon pea seedlings exposed to Cd^{2+} altered the enzyme activity and thus mobilization of food reserves (Bishnoi et al., 1993).

2.3.5 Role of phytohormones under heavy metal stress

Phytohormones are biochemical signals produced to regulate plant growth metabolism under prevailing external environmental conditions. These signals work synchronously in a growing plant body to shape and regulate the metabolism. However, in plants there exists no precise network of hormones like that of animal endocrine system. The plant signals may be originated in response to edaphic (nutritional, toxic level of elements/salts, depletion of key growth factor such as water; oxygen, or presence of micro-organisms) or aerial (temperature, light, pathogens) environmental cues. A consistent or flash of stress through either of the media often supports the internal rise of specific plant hormones (abscisic acid, salicylic acid, nitric acid, jasmonic acid etc.) while growth and maturation responses may be favored by a different set of plant hormones (e.g. auxins, brassinosteroids, gibberellins, cytokinins or ethylene). A phytohormone regulates the metabolism to save or promote plant growth in coordination with other phytohormones. In case of soil mediated heavy metal stress the plant defense strategy includes the avoidance of heavy metals uptake and its further check to aerial transport (Irfan et al., 2012). However, heavy metals escaped to aerial plant parts may damage the plant metabolic and physiological responses which needs further defense and repair activation. Phytohormones assist sequestration of heavy metals to metabolically less active parts as well as the expression of defense genes. The role of ABA, nitric oxide and BRs has recently been well studied in plant resistant against heavy metal resistance, suggesting the existence of their cross talk to overcome stress at various levels discussed above (DalCorso et al., 2010; Ergün and Öncel, 2012). The formation of NO in various plant tissues on exposure Cd has been a matter of conflict regarding the role of NO in stress modulation (Arasimowicz-Jelonek et al., 2011). It was recently reported that NO counteract Cd induced ROS cytotoxicity in mustard plants with the regulation of antioxidant responses (Verma et al., 2013). Also the role of NO is reviewed as Cd

stress modulator in plants in assistance with other plant hormones (Gill et al., 2013). The positive interactive effect of NO with BRs is earlier studied by several workers (Hayat et al., 2010b; Zhang et al., 2011; Villiers et al., 2012).

2.3.6 Function of nitric oxide under heavy metal stress

The exogenous application of NO has been documented to reduce the destructive action of heavy metals on plants. Kopyra and Gwózdz (2003) reported that sodium nitroprusside (SNP; the donor of NO in an aqueous solution) stimulates seed germination and root growth of lupin (*Lupinus luteus* L. cv. Ventus). This promoting effect of NO on seed germination persisted even in the presence of heavy metals (Pb, Cd) and sodium chloride. However, inhibitory effect of heavy metals on root growth was accompanied by increased activity of SOD that increased further significantly in the roots which were pre-treated with SNP. Positive changes in the activity of other antioxidant enzymes, POX and CAT, were also detected. Using the superoxide anion (O_2^-) specific indicator, dihydroethidium (DHE), these authors reports intense DHE-derived fluorescence in heavy metal-stressed roots, whereas in those pretreated with SNP the fluorescence level was very low and was comparable to that of the unstressed roots. On this basis they concluded that the protective effect of NO in stressed lupin roots is partly due to the stimulation of SOD activity and/or direct scavenging of the superoxide anion. Singh et al. (2008) investigated the ameliorative role of SNP against Cd-induced oxidative damage in plant roots and thus a protective role against Cd toxicity. Supplementation of Cd with SNP significantly reduced Cd-induced lipid peroxidation, H_2O_2 content and electrolyte leakage in wheat roots. The results indicated the ROS scavenging activity of NO. Another study evaluated the protective effect of NO against Cd-induced oxidative stress in sunflower leaves (Laspina et al., 2005). Sunflower leaves were found to significantly attenuate Cd-induced oxidative damage. This effect was mainly attributed to the prevention of growth inhibition and chlorophyll degradation, recovery of CAT activity and GSH levels, and enhancement of ascorbate content and APX activity, as components of the antioxidant machinery that allowed the plants to cope better with metal stress. These studies indicate that NO may effectively reduce the level of ROS generated during stress and, thus, limit the oxidative damage in plant cells.

The increase of proline content is positively correlated with plant resistance to stress (Hayat et al., 2012a). At low Cd concentrations, a stimulatory effect on seedling growth (Lin et al., 2007) and flowering was observed (Wang et al., 2012). However, an increase in Cd concentrations and exposure duration to plant tissues showed a rise in proline content (Zhao, 2011; Khatamipour et al., 2011) and antioxidant activity (Dinkar et al., 2008; 2009). The higher Cd toxicity, overwhelms the proline and antioxidant system mediated detoxification of ROS causing inhibitory effects, for instance; decline in the activity of metabolic enzymes (Lin et al., 2007; Dinkar et al., 2008, 2009) and reduced growth (Nikolic et al., 2008; Khatamipour et al., 2011). Proline reduces the free metal ion toxicity due to the formation of metal-proline complexes (Sharma et al., 1998).

In *Arabidopsis*, Cd suppressed NO accumulation in leaves and promoted flowering. Supplementation with SNP delayed flowering while the application of cPTIO; the scavenger of NO, further promoted the transition from vegetative to reproductive stage, under Cd stress (Wang et al., 2012). Exogenous NO alleviated Cd toxicity in rice by increasing pectin and hemicellulose contents in root cell walls, increasing the Cd deposition in root cell walls and decreasing Cd accumulation in soluble fractions of leaves (Xiong et al., 2009). Nitric oxide may participate in maintaining the auxins equilibrium by reducing IAA oxidase activity in the roots of *Medicago truncatula* when subjected to Cd stress, therefore, alleviating the inhibitory effect of Cd on root growth (Xu et al., 2010). After a long-term treatment, NO levels were inversely related to nitrite concentrations that originated from NR activity, suggesting conversion of nitrite to NO by known enzymatic pathways.

2.4 BRASSINOSTEROIDS

Brassinosteroids (BRs) is a class of polyhydroxysteroids, recognized as sixth group of plant hormone. Nearly 40 years back BRs were explored when Mitchell and co-workers reported the organic extract of rapeseed (*Brassica napus*) pollen that possessed stem elongation and cell division properties (Grove et al., 1979a). The first isolated BR was 'brassinolide' in 1979 as a biologically active constituent of pollen extract of rapeseed promoting cell divisions and stem elongation (Grove et al., 1979a; Grove et al., 1979b). From 230 kg of *Brassica napus* pollen only 10 mg of BRs could extracted in propanol by USDA scientists. Extracted BR was crystallized at NRRC

(Northern Regional Research Centre) and was subjected to x-ray analysis to establish its structure. This biologically active plant growth promoter was found to be steroidal lactone ($C_{28}H_{48}O_6$) and was named as "brassinolide" (BL) which was later renamed as "brassinosteroid". All natural BRs have a common 5-choleston skeleton but structural variants come from the type and the orientation of functionalities on the skeleton. Their low level in plants is not uniform throughout its body but young growing tissues have comparatively a larger share than the mature tissues (Yokota and Takahashi, 1986). The richest sources are pollen and immature seeds where its concentration ranges between 1-100 ng g^{-1} fresh mass, whereas, the shoot and leaves have about 0.01-0.1 ng g^{-1} fresh mass (Takatsuto, 1994). Since the discovery nearly 69 BRs structurally and functionally different form each other, have been characterized (Hayat et al., 2003b; Bajguz, 2007). Three of them (BL, 24-EBL and 28-HBL) are being largely applied to have an economic impact on plant metabolism, growth and productivity.

2.4.1 Biosynthesis and regulation of brassinosteroids in plants

Brassinosteroids is a group of modified sterols belonging to tri-terpenoids. In plants, the BRs are *in vivo* synthesized from campesterol. The pathway for BR biosynthesis was elucidated by Japanese researchers and later confirmed through the studies on mutants of *Arabidopsis thaliana*, *Lycopersicon esculentum* and *Pisum sativum* (Fujioka and Sakurai, 1997). In plants, the site of BR biosynthesis has not been experimentally demonstrated, however, it is hypothesized that all tissues produce BRs, as a wide range of plant organs express BR biosynthetic and signal transduction genes. This view is further strengthened by short distance activity of this class of steroid (Clouse and Sasse, 1998; Li and Chory, 1997).

Brassinosteroids are the steroidal plant hormones which can easily be transfuse through lipid bilayer. However, BRs bind to cell membrane receptors to elicit signal cascade regulating the expression of genes through cytosolic and nuclear transcription kinases and phosphatases. Contrarily, steroids hormones in animal systems have direct nuclear receptors to alter gene expression. No nuclear receptors yet have been reported in case of BRs signal transduction. A good population of receptors (BRI1) on growing tissues determines the tissue sensitivity to BRs. BR activity is down regulated through receptor endocytosis (Russinova et al., 2004)

coupled with modification/inactivation of BR molecule synthesized *in vivo*. The endogenous pool of BRs is finely tuned with the expression level of BR receptors. Endogenous BR level is negatively feedback by the population of BIN2 kinase with the mediation of BZR1, which further determines the progression of BR signal cascade after binding of BRs to its receptor (Plate 1, 2A).

Two major pathways of BL biosynthesis exist (Plate 2B); sterol specific (- squalene to campesterol) and BR-specific (campesterol to BL). Mevalonic acid, the precursor of terpenoid pathway, is condensed and cyclized to produce squalene 2,3-oxide which is subsequently modified to sitosterol and campesterol. These parent sterols serve as precursors of BL isologs such as homo- or nor-BL. Sterols, the precursors are modified to possess following functional groups: 1) saturation of a double bond at Δ^5; 2) formation of a 6-oxo-group; 3) addition of α-oriented vicinal hydroxyl groups at C-22 and C-23; 4) epimerization of a 3α-hydroxyl group to the 3β-configuration; 5) addition of a 2α hydroxyl group; and 6) a Baeyer-Villiger type oxidation in B ring. The site of BR biosynthesis and the mode of its release is released is still elusive.

The individual biosynthetic steps have been elucidated by using metabolic tests using BR-overproducing cell lines of *Catharanthus roseus* (Reid et al., 1983). These BL-overproducing lines were developed to overcome low biosynthetic activities in regular plant tissues or cell lines, which technically limits extraction and detection of BRs. BR biosynthesis proceeds through multiple branched pathways. The first branch occurs at CR and the second at campestanol (CN). Campasterol can either be C-22 hydroxylated or C-5 reduced in bifurcated pathways that are termed the early and the late C-22 oxidation pathways, respectively (Clouse, 2001) (Plate 2B).

In addition, CN proceeds to one of the two alternative pathways, the early or the late C-6 oxidation. The early C-6 oxidation pathway undergoes a two-step oxidation of C-6 position at the CN stage (Ingram et al., 1984). In the late C-6 oxidation pathway, C-6 is oxidized at the second to last step. The order of chemical substitutions other than the branching steps are conserved between the parallel pathways in such a way these reactions are performed by single enzyme acting on both the early and late intermediates (Ross et al., 2000; Brian and Hemming, 1955). The BR biosynthetic pathway that was established in the periwinkle feeding

experiments has served as a framework for further validation and modification by using the results from dwarf mutants that are defective in BR biosynthesis and signaling pathways.

2.4.2 Physiological role of brassinosteroids

Brassinosteroids are to be involved in numerous plant processes. They promote cell expansion and cell elongation (Clouse and Sasse, 1998) and interplay with auxins (Nemhauser et al., 2004). Though unclear, BRs are being suggested a role in cell division and cell wall regeneration (Clouse and Sasse, 1998). BRs signal promotes vascular differentiation (Cano-Delgado et al., 2004). BRs are also necessary for pollen tube formation and elongation (Hewitt et al., 1985). Furthermore, BRs delayed senescence in BRs deficient mutants, while accelerated senescence in dying tissue cultured cells that signifies the biological relevance of BRs action (Clouse and Sasse, 1998). BRs counteract abiotic stresses in plants (Clouse and Sasse, 1998, Sharma and Bhardwaj, 2007; Sharma et al., 2008; Bajguz and Hayat, 2009; Fariduddin et al., 2013a) while it inhibit pathogen-associated molecular pattern (PAMP)-triggered immune signaling (Albrecht et al., 2012). The chromosomal aberration assay has shown that 24-EBL treatment significantly declined maleic hydrazide (0.01%) induced genotoxicity in *Alleium cepa* (Sondhi et al., 2008). A series of experiments conducted by our group on seed pre-treatment and/or foliar spray of BR analogues noted the induction of favorable morpho-physiological and biochemical responses in crop plants that were concentration dependent, under stressed and non-stressed conditions (Table 3).

2.4.3 Effect of brassinosteroids on seed germination

Endogenous BRs have been identified in the seeds of several species, including pea (Yokota and Takahashi, 1986), *Arabidopsis thaliana* (Schmidt et al., 1997) and *Lychnis viscaria* (Friebe et al., 1999). It is well documented that BRs promote seed germination, like other hormones. The treatment of the seeds of *Lepidium sativus* (Jones-Held et al., 1996) and *Eucalyptus camaldulensis* (Sasse et al., 1995) with BL improved per cent germination. Similarly BRs promoted seed germination in case of *Brassica napus* (Chang and Cai, 1998), wheat (Sairam et al., 1996; Hayat et al., 2003a), tomato (Vardhini and Rao, 2000), tobacco (Leubner-Metzger, 2001), barley (Kartal et al., 2009) and *Brassica juncea* (Sirhindi et al.,

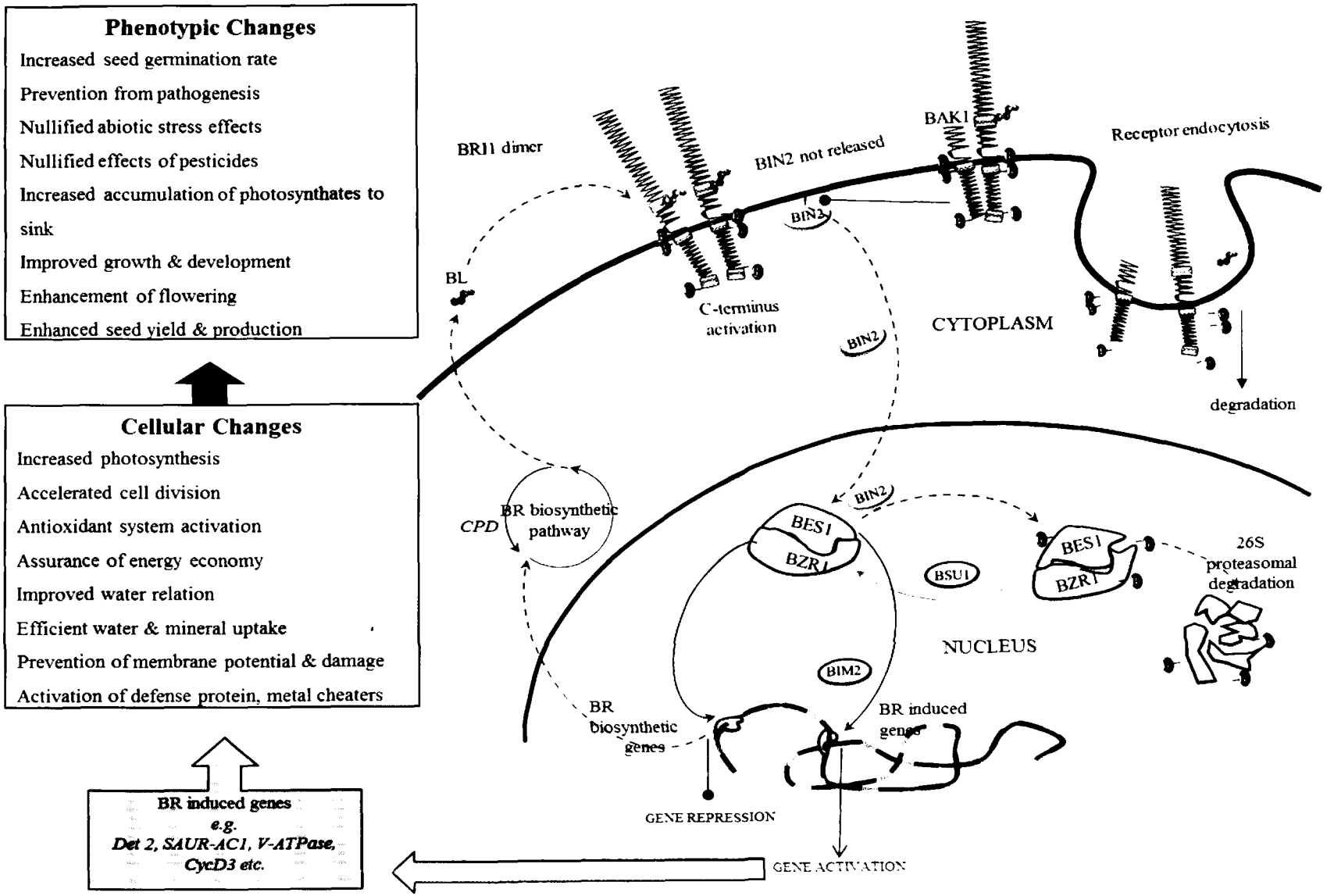


Plate 1: Brassinosteroid mediated signal transduction and its regulation. to regulate cellular and phenotypic changes (in boxes)

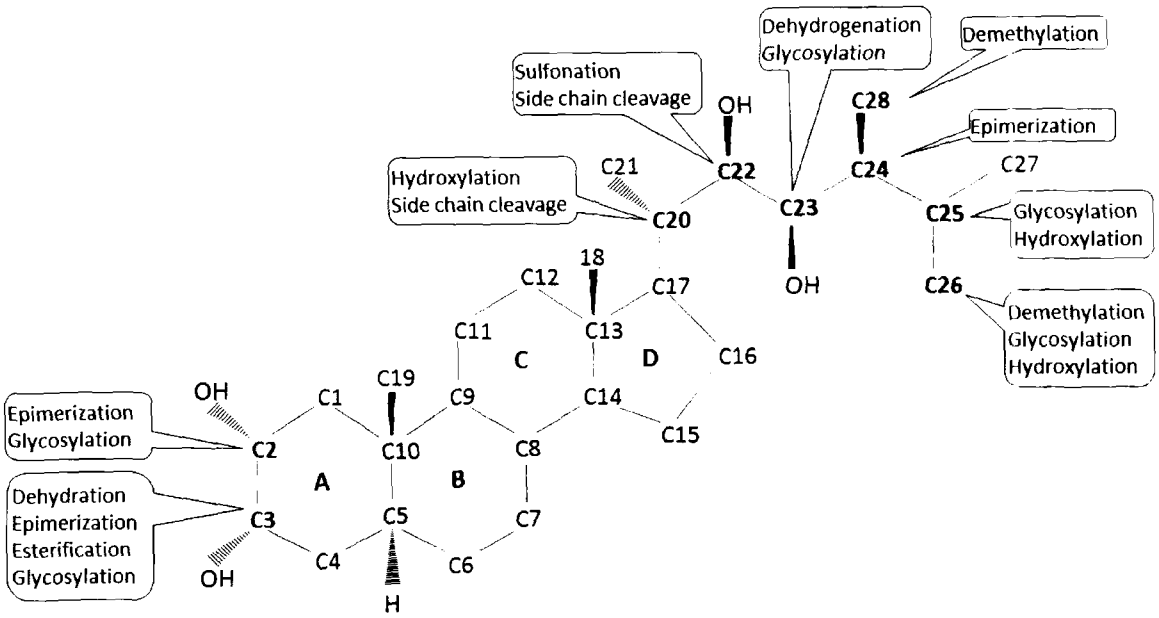


Plate 2A: Metabolism (-inactivation) of brassinosteroid molecule in different species

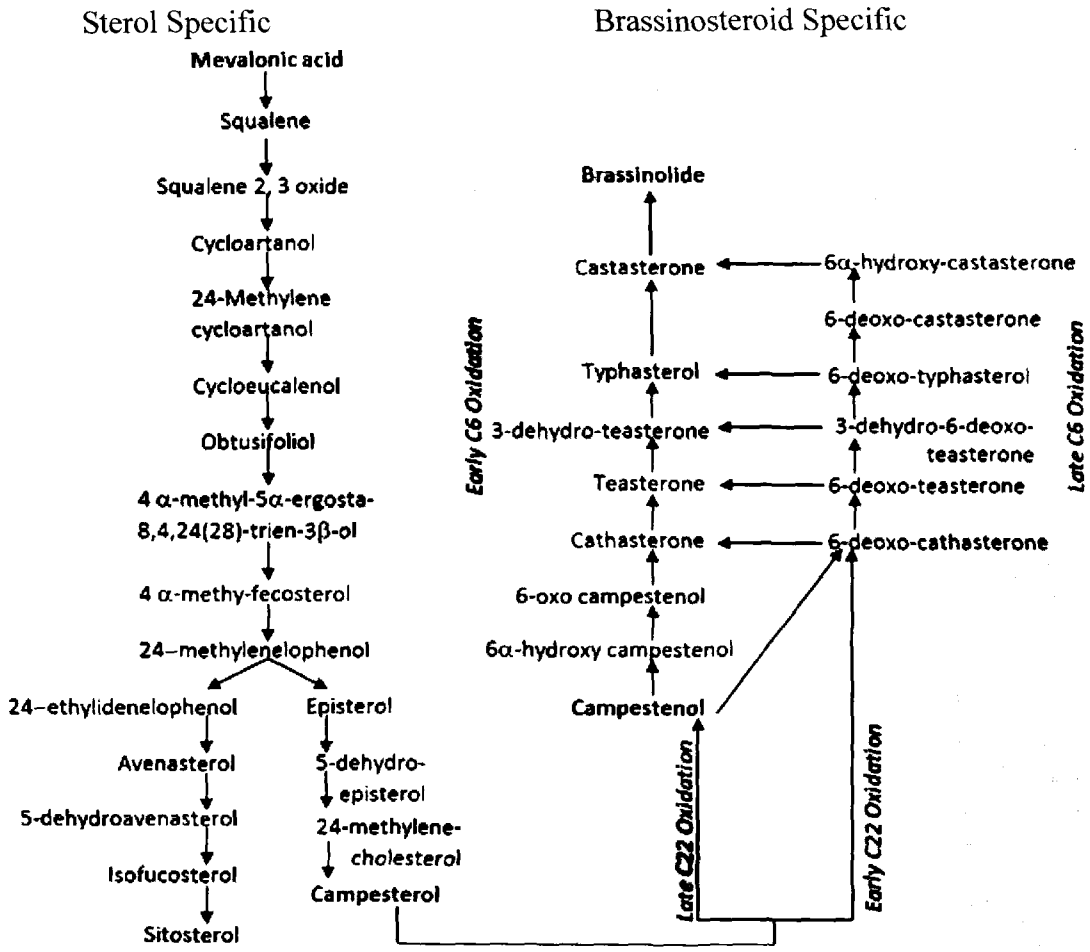


Plate 2B: Brassinosteroid biosynthesis, sterol specific and BR specific pathway; early and late C-6 oxidation pathway

2009). Moreover, BL, 24-EBL and 28-HBL promoted seed germination in groundnut (Vardhini and Rao, 1997). BR application has been reported to enhance the germination of the seeds of certain parasitic angiosperms (Takeuchi et al., 1991, 1995), cereals (Gregory, 1981; Yamaguchi et al., 1987), *Arabidopsis* (Steber and McCourt, 2001). Pretreatment with BL stimulated the germination and seedling emergence in aged rice grains (Yamaguchi et al., 1987) and seed treatment of barley accelerated subsequent seedling growth (Gregory, 1981). It is, however, not clear whether the promoting effect of BRs in cereal grains is actually manifested only at the level of seedling growth and/or also at the level of germination *per se*.

Table 3: Effect of different modes and concentrations of BR analogues on abiotically stressed and non-stressed crop plants

Crop	BR analogue	Treatments	Results	References
Wheat	28-HBL	Seeds soaked in 0, 1, 3 and 5 μM aqueous solutions for 4, 8 or 12h	Soaking for 8 or 12 h in 3 μM produced most vigorous seedlings with significantly higher leaf number, fresh and dry weight/plant, higher NR and CA activities.	Hayat et al., 2001a
Mustard	28-HBL	Foliage of 30d-old plants were sprayed with 10^{-6} M solution of IAA, GA_3, KIN, ABA or HBR	Order of response was HBR>GA_3>IAA>KIN>Control>ABA. HBR prominently affected dry mass, Chl-value, CA activity, net photosynthesis in 60d-old plants along with seed yield.	Hayat et al., 2001b
Wheat	28-HBL	Seeds imbibed in 10^{-6}, 10^{-8}, 10^{-10} M aq. solution for 4, 8 or 12 h	After 96 h, % germination, α-amylase activity increased most prominently in 10^{-10} M/4 h, followed by 10^{-8} M/4h.	Hayat et al., 2003a
Wheat	28-HBL	Seeds soaked in 10^{-10}, 10^{-8}, 10^{-6} M aq. solution for 8 h and sampled after 12, 18 or 24 h.	Significant increase in % germination, values of α-amylase, CAT, POX, soluble sugars and proteins. Effect of 10^{-10} M and 10^{-8} M proved best.	Hayat and Ahmad, 2003c
Lentil	28-HBL	Seeds soaked in 10^{-6}, 10^{-8}, 10^{-10} M aq. solution for 4, 8 or 12 h.	Soaking decreased nodule number and root length but increased NR activity and grain yield at 10^{-8} M concentration.	Hayat and Ahmad, 2003a
Lentil	28-HBL	30d-old plants sprayed with 10^{-6}, 10^{-8} or 10^{-10} M aq. solution.	Root length and nodule number decreased, while NR activity and seed yield increased with 10^{-8} or 10^{-10} M.	Hayat and Ahmad, 2003a
Mung bean	28-HBL	Seeds soaked in solution of 10^{-8}, 10^{-6} or 10^{-4} M for 4, 8 or 12 h.	10^{-6} M soaking for 8 h enhanced net photosynthesis, Chl-content, CA activity, carboxylation efficiency, stomatal conductance and seed yield.	Fariduddin et al., 2003
Mung bean	28-HBL	Leaves of 25-d-old plants were sprayed with aq. solution of	HBR excelled over KIN, increased the activities of NR and CA, chl- and total protein contents, net photosynthesis, pod number	Fariduddin et al., 2004

		0.01, 1.0 or 100 μM KIN, or 0.0001, 0.01 or 1.0 μM HBL.	and seed yield, at harvest.	
Mung bean	28-HBL	Foliage of 15d-old seedlings were sprayed with 28-HBL aq. solution at conc. 0, 10^{-10}, 10^{-8} and 10^{-6} M and sampled at 30 d stage.	10^{-8} M spray increased the activities of NR, CA, photosynthetic rate and other associated parameters and seed yield at harvest.	Fariduddin et al., 2006
Tomato	28-HBL	Roots of 20 d old seedlings were dipped in 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M 28-HBL for 15, 30 and 45 min before transplantation	Elevated NR and CA activities and Chl-contents at 30/60 d stage. Fruits had lower ascorbate while higher lycopene and β-carotene content with 10^{-8} M/15 min HBL treatment.	Ali et al., 2006
Chickpea	28-HBL	Seeds were soaked in HBR (10^{-8} M) and/or K^+ (12 μM).	HBR alone or with K^+ induced RWC, NR activity and total protein content but not that of N.	Ali et al., 2005
Chickpea	28-HBL	Chickpea seeds presoaked with 28-HBL (10^{-10} or 10^{-8} M/8 h) and/or NaCl solution (1mM-10mM/8 h)	At 90 d sampling activity of NR; CA, fresh and dry mass of plants and nodules along with their number, nitrogenase activity, carbohydrate, LegHb and N content of nodule and seed yield increased more so in response to 10^{-8} M/8 h HBL treatment. All the above parameters declined in the plants raised from NaCl treated seeds. This stress was overcome when the seeds were imbibed in HBL before or after NaCl treatment.	Ali et al., 2007
Mustard	28-HBL	One week old seedlings were	At 60 d, plants fed with $CdCl_2$ exhibited declined growth, CA	Hayat et al.,

		supplied with 50, 100 and 150 μM of Cd with nutrient solution and sprayed with 0.01mM HBL on foliage at 30 d stage	activity, photo-synthetic rate and chl-content. The carbohydrate and nitrate contents and NR activity decreased both in root and shoot. HBL countered the toxic effect of Cd. Increased activity of antioxidants (CAT, POX, SOD) and proline content.	2007a
Mustard	28-HBL	Seeds were soaked for 6 hours, in water, 50, 100 or 150 mM NaCl. 15, 30 and 45 d-old plants were sprayed with water, 10^{-10}, 10^{-8} or 10^{-6} M aq. solution of 28-HBL.	NaCl retarded growth of root and shoot at post-flowering and lowered seed yield at harvest. HBL spray at 15, 30 or 45 DAS increased these values significantly. Plants released more ethylene, under NaCl stress or HBL treatment. HBL spray (10^{-8} M) at 30d-stage has completely overcome the ill effect of lowest (50 mM) concentration of salt.	Hayat et al., 2007b
Mustard	28-HBL	Seedlings were supplied with 50-100 mM Ni 10 days after sowing and sprayed with HBL (10^{-8} M) at 20 d stage.	Plants received only Ni exhibited reduced growth and net photosynthesis, Chl-content, activity of CAT, POX and proline. HBR (10^{-8} M) partially neutralized the effects that were reflected as improved growth and boosted activity of POX and CAT in leaves, and proline in both roots and shoots.	Alam et al., 2007
Mustard (Varuna)	28-HBL	Seeds were soaked in 0, 50, 100, or 150 mM NaCl for 6 h and treated with 0, 10^{-10}, 10^{-8}, or 10^{-6} M 28-HBL at 14 d stage.	Plants received only NaCl exhibited a decrease in NR and CA activity, Chl-content and net photosynthesis at 60 d stage and a decrease of yield, at harvest. Subsequent treatment with HBL significantly increased all of the above parameters. 10^{-8} M 28-HBL remained best and it detoxified the effect of 50 mM NaCl.	Hayat et al., 2007b

Mustard	24-EBL	Plants grown with NaCl and/or $NiCl_2$ were sprayed with 1μM 24-EBL at 15 d and sampled at 30 d stage.	NaCl and/or $NiCl_2$ exhibited a significant decline in plant growth, the level of pigments and photosynthetic parameters. NaCl and/or $NiCl_2$ increased electrolyte leakage and lipid peroxidation, and decreased MSI and RWC. Follow up treatment with EBL detoxified the stress denoted as significant increase of antioxidant enzymes and proline level.	Ali et al., 2008a
Mung bean	28-HBL, 24-EBL	Seedlings were subjected to Al (0.0, 1.0 or 10.0 mM) stress in nutrient solution, at 1-week-old stage and were sprayed with 0 or 10^{-8} M of 24- EBL or 28-HBL at 14 d stage	Al caused a sharp reduction in growth attributes, activity of CA, RWC, WUE, Chl-content and photo-synthetic rate at 3 week stage. Activity of antioxidants; CAT, POX, SOD (in leaves) and proline level (in leaves and root) increased under Al stress. BRs improved the plant growth and other parameters and augmented the Al toxicity. EBL/HBL further enhanced the activity of antioxidant enzymes and proline level stimulated by Al stress	Ali et al., 2008b
Chickpea	28-HBL	15 d old seedlings were supplied with 0, 50, 100 or 150 μM of Cd as $CdCl_2$ and sprayed with 0.01 mM of 28-HBL at 30 d stage.	Increasing Cd concentration decreased proportionately plant fresh and dry mass, nodules number, their fresh and dry mass, legHb content, nodule content of N and carbohydrate, leaf Chl-content, NR and CA activity but the content of proline and the activities of CAT, POX, SOD enzymes increased. The ill effect, generated by Cd was completely overcome with HBL against minimum concentration of Cd (50 μM).	Hasan et al., 2008

Mung bean	28-HBL	HBL (1.0 μM) was used to soak the seeds and sprayed to the foliage. Samples were assessed at 30 and 50 d, after sowing	Activity of CA and NR, leaf Chl content, net photosynthesis and associated parameters, plant dry mass, number of pods per plant and seed yield at harvest increased significantly. Order of response was seed soaking + foliage application > foliage application only > seed soaking only > control	Fariduddin et al., 2008
Mustard	28-HBL	Seedlings of mustard were subjected to drought stress for 7 days at either of the two stages of growth; 8–14 (days after sowing; DS1) or 15-21 (DS2) and then returned to natural growth conditions. These seedlings were then sprayed with HBL (0.01 mM) the 30-d stage.	Plants sampled at 60 d reflected significant decrease in growth and photosynthesis. Drought at DS1 was more inhibitory than DS2. Plants recovered significantly with the follow up HBL treatment. The activity of antioxidant enzymes (CAT, POX and SOD) and leaf proline content increased in response to both the treatment factors, whereas their interaction had an additive effect.	Fariduddin et al., 2009a
Mustard	28-HBL	Seeds were soaked with H_2O, 10^{-10}, 10^{-8} or 10^{-6} M of 28-HBL and raised in sand moistened with nutrient solution with Cu (50, 100 and 150 mg Kg^{-1} of sand) and sampled at 30 DAS	Presence of Cu significantly declined the growth, chlorophyll contents and photosynthetic parameters, however, antioxidant enzymes activity (CAT, POX, SOD) and proline content increased. HBL seed treatment improved all the above parameters under Cu stress. Cu application raised leaf H_2O_2 content while HBL decreased it.	Fariduddin et al., 2009b
Chickpea	28-HBL	Plants were supplemented with 28-HBL, both in the presence or	High temperature and/or NaCl significantly declined the growth, photosynthetic parameters and maximum quantum yield of PSII.	Hayat et al., 2010a

		absence of high temperature and/or NaCl. Samples were collected 18 d, after sowing	The HBL follow-up-treatment significantly detoxified the impact of the stress and improved the plant performance. High temperature and/or NaCl increased electrolyte leakage and lipid peroxidation and decreased the MSI and LWP. The HBL treatment, in the absence of stress, improved the MSI and leaf water potential but could not influenced lipid peroxi-dation and electrolyte leakage HBL, in the presence or absence of stress, increased significantly the activity of antioxidative enzymes and the proline content.	
Tomato	28-HBL, 24-EBL	Pre-sowing seed treatment with to a NO donor, sodium nitroprusside (SNP) (0, 10^{-5} M or 1 M), for 8 h and sprayed subsequently with 10^{-8} M EBL or HBL at 30 d stage of growth.	SNP (10^{-5} M) concentration favored the growth, pigment content, photosynthetic and enzymatic activities, and also improved the antioxidant system. However, 1 M SNP had inhibitory impact on most of the indices, except the antioxidant system. EBL/HBL stimulated above indices. The inhibitory effect of SNP (1 M) was neutralized by the application of BRs, where EBL was more effective than HBL.	Hayat et al., 2010b
Cucumber	28-HBL	Cucumber seedlings were sprayed with DW, 10^{-8} or 10^{-6} M of HBL at 30-d stage and then exposed for 18 h to chilling temperature (10/8^{0}C, 5/3^{0}C) 48h	Marked reduction in plant growth, Chl content and net photo-synthesis, PS II efficiency and activity of NR and CA were noticed under chilling stress. The activity of antioxidant enzymes (CAT, POX, SOD) and proline increased. The stressed seedlings pretreated with 28-HBL maintained a higher value of antioxidant enzymes and proline content over the control and improved growth, water relations, photosynthesis	Fariduddin et al., 2011

			and maximum quantum yield of PS II with or without stress.	
Tomato	28-HBL, 24-EBL	Tomato cultivars (Sarvodya and K-25) were exposed to graded levels of Cd (0, 3, 6, 9 or 12 mg Kg^{-1} soil) and their foliage were sprayed with 0 or 10^{-8} M of HBL/EBL at 59 d stage.	Photosynthetic parameters, leaf water potential and activity of enzymes (NR and CA) declined significantly in both the cultivars, to a lesser extent in K-25 than Sarvodya with the increasing Cd level. The activity of antioxidant enzymes and proline content increased under metal stress as well as in response HBL/EBL.	Hasan et al., 2011
Tomato	28-HBL, 24-EBL	Foliage of tomato cv. K-21 was sprayed with DW, 10^{-6}, 10^{-8} or 10^{-10} M HBL or EBL 44 d after sowing.	Plants sampled at 45 d and 60 d after showed improved growth attributes CA activity and photosynthetic parameters more to EBL than HBL. 10^{-8} M of the concentrations proved best.	Hayat et al., 2011
Tomato	28-HBL, 24-EBL	Foliage of tomato cv. K-21 was sprayed with DW, 10^{-6}, 10^{-8}, or 10^{-10} M HBL or EBL.	Samples at 45 d stage of growth showed improved NR activity, proline level and antioxidant activity best at the concentration of 10^{-8} M HBL/EBL.	Hayat et al., 2012b
Mustard	28-HBL	Mustard cv. Varuna grown in the presence or absence of salinity stress. The leaves of 29 d old plants were sprayed with DW, HBL (10^{-8} M) and/or SA (10^{-5} M) and plant responses were studied at 30 and 45 DAS	Salinity significantly reduced plant growth, gas exchange parameters but increased proline content and electrolyte leakage in the leaves. The effects were more pronounced at 30 DAS than 45 DAS. Out of the two hormones HBL excelled in its effects over that of SA, both sampling stages. Toxic effects generated by salinity stress at 45 d were completely overcome by the combined effect of the two hormones (HBL and SA).	Hayat et al., 2012c

In *A. thaliana*, BR promotes the germination of pre-chilled (i.e. non-dormant) seeds of BR-deficient biosynthesis mutant *det2-1* and the BR-insensitive response mutant *bri1-1* imbibed in the light (Steber and McCourt, 2001; Zhang et al., 2009). Seed germination of *det2-1* and *bri1-1* is more strongly inhibited by ABA than in the wild type; however, BR is able to partially overcome the inhibition of germination by ABA (Finkelstein et al., 2008; Zhang et al., 2009; Xue et al., 2009). BR treatment rescues the germination in phenotype of the severe GA-deficient biosynthesis mutant *ga1-3*, which normally requires GA treatment for dormancy release and germination. BR treatment also partially rescues the germination phenotype of the severe GA-insensitive response mutant *sly1* (sleepy1), which cannot be rescued by the treatment with GA. Interestingly, a new allele for *sly1* was identified in a screen for BR-dependent germination and also proposed an interaction between BR and GA signaling in seeds (Steber et al., 1998; Steber and McCourt, 2001). This is further supported by the germination phenotype of the *gpa1* mutant of *Arabidopsis* (Ullah et al., 2002). BR promotes seedling elongation and germination of non-photodormant tobacco seeds, but do not appreciably affect testa rupture and subsequent induction of βGlu I in the micropylar endosperm (Leubner-Metzger, 2001; 2003). Treatment with BR accelerates endosperm rupture of tobacco seeds imbibed in the light. Promotion of endosperm rupture by BR is dose-dependent and 0.01 μM BL is most effective.

2.4.4 Effect of brassinosteroids on growth

Brassinosteroids application results into a broad range of plant morphological responses, e.g. increased rate of stem elongation, xylem differentiation, growth of pollen tube, epinastic bending and unrolling of grass leaves at sheath, increased fresh and dry mass of root and shoot etc. Most of these changes are facilitated by BR induced cell division, re-orientation of microfibrills, xylogenesis, proton pump activation, regulation of antioxidant system and chlorophyll biosynthesis. BR biosynthetic (e.g. *cpd, det2, dwf4, dpy* and *lk*) and perception (e.g. *bri1, bes1, bsu* and *bin2* etc.) *Arabidopsis* mutants have distinct phenotypes. For example *det2* mutants grown in light were dark green with reduced apical dominance (rosette), having small compact cell arrangement and diminished male fertility. When grown in dark these mutants shared some characteristics with light grown plants. BR application reversed this dwarf phenotype (Hayat and Ahmad, 2010c).

2.4.5 Effect of brassinosteroids on flowering

There has been limited use of these steroids in regulating flowering. In strawberry the number of flowers increased by the application of BRs on the foliage (Pipattanawong et al., 1996). However, in case of grapes, the application of BRs in autumn improved the number of flowers but inhibited if the time of application was delayed to late winter (Rao et al., 2002). BR also regulated flowering in *Arabidopsis* (Clouse, 2008; Yu et al., 2008).

2.4.6 Effect of brassinosteroids on senescence

Senescence is the process, which refers to endogenously regulated deteriorative changes that lead to the natural cause of death of cells, tissues, organs or that of the whole organism (Arteca, 1997). Like other hormones (Rao et al., 2002), BRs also play a crucial role in regulating the processes leading to senescence. The BL promoted senescence in *Xanthium* and *Rumex* explants (Mandava et al., 1981). In addition to it, BRs also accelerated senescence in the detached cotyledons of cucumber seedlings (Zhao et al., 1990) and leaves of mung bean seedlings (He et al., 1996). However, BR deficient *Arabidopsis* mutants exhibited delayed senescence of chloroplast (Li et al., 1996). Similarly, the senescence of the leaves of mungbean and mustard was delayed, if supplied with 28-HBL at early stage of growth (Fariduddin, 2002). During a search of senescence associated genes, He and Gan (2001) developed a preliminary model for leaf senescence regulating network in *Arabidopsis*, where signals such as ABA, jasmonic acid, ethylene, darkness, dehydration and aging activated 147 senescence associated enhancer trap lines. 24-EBL could activate some of these but associated genes have not yet been cloned.

2.4.7 Effect of brassinosteroids on photosynthesis

The rate of photosynthesis enhanced when the aqueous solution of 28-HBL was applied to the foliage of wheat and mustard (Sairam, 1994; Hayat et al., 2000; 2001b), *Geranium* (Swamy and Rao, 2009), *Cucumis sativus* (Xia et al., 2009a), mungbean (Ali et al., 2008a) or EBL was applied alone as seed soaking to mungbean (Fariduddin et al., 2003; 2004), or in association with GA_3 to spinach (Guang-Jian et al., 1998). An increased rate of CO_2 assimilation was noted in wheat and mustard with foliar spray of BR (Braun and Wild, 1984), cucumber with EBL (Yu et al.,

2004b), rice and *Vicia faba* with brasisnolide (Fujii et al., 1991; Pinol and Simon, 2009). Likewise, the foliar application of 24-EBL enhanced the light saturated net CO_2 assimilation rate and carboxylation rate of rubisco, thereby increasing the capacity of CO_2 assimilation in the Calvin cycle (Yu et al., 2004b; Xia et al., 2009a). However, the epicotyl of cucumber, did not respond to EBL but the transport of the labeled (^{14}C) glucose towards the epicotyl was favoured (Nakajima and Toyama, 1995). Similarly, Hill activity in the foliage of *Vigna radiata* was favourably affected on being supplemented with aqueous solution of 28-HBL (Bhatia and Kaur, 1997).

2.4.8 Effect of brassinosteroids on chlorophyll content

The role of BRs signaling in regulation of plant photo-morphogenesis through the involvement of phytochrome A/B is well studied in BR biosynthetic mutants, however, its direct impact on chlorophyll in photosynthesis is not yet worked out. The chlorophyll content is an important factor and the currency of photosynthesis regulated by the destructive enzyme, chlorophyllase (Reddy and Vora, 1986), biosynthetic rate of δ-aminolevulenic acid and protochlorophyllide reductase complex (Stobart et al., 1985). BRs are reported to increase chlorophyll breakdown (Vardhini and Rao, 2002) and inhibit anthocyanin biosynthesis (Brosa, 1999).

The total chlorophyll content or its fractions increased in the leaves of *Vigna radiata* (Bhatia and Kaur, 1997) and *Brassica juncea* (Hayat et al., 2001b) by 28-HBL and in *Cucumis sativus* (Yu et al., 2004b) and *Vicia faba* (Pinol and Simon, 2009) by EBL, applied directly to their foliage. Similarly, the values for the above parameters increased in the leaves of rice (Wang, 1997), *Cicer arietinum* (Fariduddin et al., 2000), *Brassica juncea* (Hayat et al., 2003b). *Vigna radiata* (Fariduddin et al., 2003) and *Pelargonium graveolens* (Swamy and Rao, 2009) raised from the seeds given pre-sowing treatment with BRs.

2.4.9 Effect of brassinosteroids on metabolic enzymes

Carbonic anhydrase (CA) is the second most abundant soluble protein, other than RuBPcase, in C_3-chloroplast (Reed and Graham, 1981; Okabe et al., 1980). It is a zinc containing protein with a molecular weight of 180 KDa (Lawlor, 1987) and is ubiquitous enzyme, among living organisms. It catalyzes the reversible inter conversion of bicarbonates (HCO_3^-) and CO_2 (Sultemeyer et al., 1993). The rate of conversion of HCO_3^- to CO_2 is normally slow in alkaline conditions. However, CA

activates the use of HCO_3^- in the production of CO_2 (Lawlor, 1987). In C_3 plants, CA has a close association with RuBPCase where it elevates the level of CO_2 at its active site (Badger and Price, 1994). An increase in the activity of CA in the leaves was attained by the application of 28-HBL to the shoot of *Brassica juncea* (Hayat et al., 2000, 2001b). Moreover, the seedlings of wheat and mungbean, raised from the grains treated with 28-HBL, possessed high CA activity in their leaves (Hayat et al., 2001b; Fariduddin et al., 2003). Seed application of EBL reduced the toxic effect of Cd on CA activity (Anuradha and Rao, 2009).

The process of reduction of nitrate is catalyzed by the enzyme, NR, the level of which increased in the plants of rice (Mai et al., 1989), maize (Shen et al., 1990), water stressed wheat (Sairam, 1994), *Lens culinaris* (Hayat and Ahmad, 2003a, b), *Vigna radiata* (Fariduddin et al., 2004) and wheat (Hayat et al., 2001a), and in the seeds of wheat (Hayat and Ahmad, 2003c) by the application of BRs.

2.4.10 Effect of brassinosteroids on vascular tissue

Clouse and Zurek (1991) first reported the importance of BRs in the differentiation of vascular tissues where the addition of nanomolar concentration of BL caused manifold acceleration in xylem differentiation in the cells of *Helianthus tuberosus*. Moreover, a significant increase in cell numbers was observed which indicated the role for BRs in cell division and differentiation (Clouse and Zurek, 1991). *Zinnia elegans* has been used extensively to study the formation of xylem/tracheary elements, a process that has three distinct stages; here BRs have been implicated in the transition between Stage II and Stage III where secondary wall formation and cell death occurs (Fukuda, 1997). Uniconazole (a putative BR biosynthesis inhibitor) prevents the differentiation of Zinnia mesophyll cells into tracheary elements but this inhibition was overcome by exogenous BR application (Iwasaki and Shibaoka, 1991). Uniconazole appears to suppress the transcription of genes involved in the final stages of differentiation but could be recovered by BL. This clearly suggests that BRs are synthesized prior to secondary cell wall development and cell death (Yamamoto et al., 1997).

2.4.11 Effect of brassinosteroids on crop yield

Once the presence of BRs in plants was established, the next phase was to explore the possibilities of using these new chemicals in improving the yield of

economically useful plants. Meudt et al. (1983, 1984) used BL to improve the yield of lettuce, radish, bush bean and pepper. Likewise, foliar application of dilute aqueous solution of BL improved the yield in wheat and mustard (Braun and Wild, 1984), rice, corn and tobacco (Yokota and Takahashi, 1986). Brassinosteroids were also found to increase the growth and yield of sugarbeet (Schilling et al., 1991), legumes (Kamuro and Takatsuto, 1991) and rape seed (Takematsu and Takeuchi, 1989; Hayat et al., 2000; 2001b). Application of 28-HBL and 24-EBL significantly increased the yield of potato, mustard, rice and cotton (Ramraj et al., 1997), *Lens culinaris* (Hayat and Ahmad, 2003a, b), *Vigna radiata* (Fariduddin et al., 2003) and that of corn, tobacco, watermelon, cucumber and grape (Ikekawa and Zhao, 1991), respectively. Foliar application of BL, 24-EBL (Vardhini and Rao, 1997) and 28-HBL (Vardhini and Rao, 1998) was highly effective in enhancing the yield of groundnut and tomato (Vardhini and Rao, 2001). Moreover, in China, 28-HBL has been registered as a plant growth regulator in case of tobacco, sugarcane, rapeseed and tea. In India also certain products that have BRs, have been marketed by Godrej Industries to boost the growth of specific plants.

2.4.12 Effect of brassinosteroids on stress, imparted by heavy metals in plants

Brassinosteroids stimulated the synthesis of phytochelatins (PCs) in *Chlorella vulgaris* cells treated with lead. The stimulatory activity of various analogues of BRs on PC synthesis was noted in the order: brassinolide (BL) > 24-EBL > 28-HBL > castasterone (CS) > 24-ECS > 28-HCS (Bajguz, 2002). The cultures of *Chlorella vulgaris* with BRs and heavy metals showed lesser bioaccumulation of heavy metals than the cultures having metals alone. The inhibitory effect of BRs mixed with different heavy metals on their accumulation was arranged in the following order: zinc > cadmium > lead > copper. Moreover, a stimulatory effect of BRs, after blocking the accumulation of heavy metals on the growth and development of *Chlorella vulgaris* occurs. The BRs, therefore, reduced the impact of heavy metals stress on growth, prevented the loss of chlorophyll, sugars and proteins and increased PC synthesis. Concentration-dependent stimulation was observed with increasing concentration of BR and decreasing concentration of heavy metals (Bajguz, 2000a, 2002). BRs also reduced the content of Cd in the seedlings of winter rape (Janeczko et al., 2005) and copper in Indian mustard (Sharma and Bhardwaj, 2007). BR eliminates

the toxic effect of Cd on photochemical pathways in rape cotyledons, mainly by diminishing the damage in reaction centers and O_2 evolving complexes as well as maintaining efficient photosynthetic electron transport (Janeczko et al., 2005). The remedial impact of BRs on the accumulation of heavy metals (Cd, Cu, Pb and Zn) under the influence of BR has been studied for different crop plants such as barley, tomato, radish and sugar beet. It was found that the application of 24-EBL significantly reduced the metal uptake by the roots; for example, in beet roots lead content was reduced by more than 50% (Khripach et al., 1999). Moreover, Bilkisu et al. (2003) reported that BL, during aluminium-related stress stimulated growth in *Phaseolus aureus*. The application of BRs also improved the performance of mustard (Hayat et al., 2007a), chickpea (Hasan et al., 2008) subjected to Cd stress and also of mungbean (Ali et al., 2008a) and mustard (Alam et al., 2007) to aluminium and nickel, respectively. Hasan et al. (2008) reported that BRs enhanced the activity of the antioxidant enzymes (CAT, POX and SOD) and proline content. A significant correlation of BRs concentration (0.01μM) with the degree of improvement, in terms of nodulation, nitrogen fixation, pigment composition, CA and NR activities was noted. A similar pattern of response together with an elevation in the photosynthesis was noted in the plants of mustard, exposed to Cd fed through the nutrient solution (Hayat et al., 2007a). The foliar spray of either 24-EBL or 28-HBL significantly enhanced the growth, photosynthesis, antioxidant enzymes and proline content in aluminium stressed mungbean plants (Ali et al., 2008a). The activities of the enzymes CAT, POX, CA and NR also exhibited a significant enhancement in mustard plants, grown under nickel stress (Alam et al., 2007). These plants also exhibited an elevation in the relative water content and photosynthetic performance. Ali et al. (2008b) also reported that 24-EBL improved the level of antioxidant system (SOD, CAT, POX, glutathione reductase and proline), both under stress and stress-free conditions. The influence of 24-EBL on the antioxidant system was more pronounced under stress, suggesting that the elevated level of antioxidant system, at least in part, increased the tolerance of mustard plants to saline and/or nickel stress, and thus protected the photosynthetic machinery and the plant growth.

One of the mechanisms for the metal removal is biosorption. The mechanisms of biosorption are generally based on physico-chemical interactions between metal ions and the functional groups present on the cell surface, such as electrostatic

interactions, ion exchange and metal ion chelation or complexation. The functional groups most commonly implicated in such interactions include carboxylate, hydroxyl, amine and phosphoryl groups present within cell wall components such as polysaccharides, lipids and proteins. The binding process is largely independent on the metabolism and hence, is of a physical nature, which is also usually rapid and reversible. In addition, biosorption can be modified by pH and the presence of other ions in the medium. This may cause precipitation of heavy metals as insoluble salts, but remains unaffected by metabolic inhibitors or light/dark cycles (Vilchez et al., 1997). Water pH is an important factor directly affecting the toxicity of metals in algae, for example unicellular *Chlorella sp.* It is known that heavy metal toxicity decreases with decreasing pH. The pH of the medium can, in turn, moderate the toxicity of heavy metals; however, pH may increase the bioavailability of metal ions resulting in increased toxicity. BR-induced *Chlorella vulgaris* growth stimulation depends largely on acid-induced wall loosening as the apoplast pH decreases. The effects of BRs on proton secretion are associated with an early hyperpolarization of the transmembrane electrical potential which in further stimulated by the presence of K^+ in the medium (Bajguz, 2000a).

2.5 NITRIC OXIDE

Koshland (1992) recognized the biological significance of NO and named this free radical, as "Molecule of Year". In 1998 the Nobal Prize in physiology and medicine was awarded for the discovery of NO as a biological mediator produced by mammalian cell. However, the role of NO is not confined only to the animal kingdom but plants also have the ability to accumulate and metabolize atmospheric NO. Nitric oxide regulates diverse physiological processes of seed germination, rhizogenesis, stomatal closing, and adaptive responses to biotic and abiotic stress (Lamattina et al., 2003; Desikan et al., 2004; Wendehenne et al., 2004; Delledonne, 2005). Klepper (1975) was the first to observed the production of NO in soybean plants, treated with photosynthetic inhibitor herbicides (Klepper, 1978, 1979) or other chemicals (Klepper, 1991) and also under anaerobic conditions (Klepper, 1987). In plants NO can be generated via enzymatic and non-enzymatic pathways. The enzymatic pathway is catalyzed by cytosolic nitrate reductase (cNR), NO synthase (NOS) or NOS-like enzymes and nitrite: NO reductase (Ni-NOR) respectively. Non-enzymatic pathway is

the nitrite dismutation to NO and nitrate at acidic pH value (Neill et al., 2003; Graziano and Lamattina, 2005). Besides, NO has been established as a novel biological messenger in plants and animals, it has received special attention from almost all the branches of science including medicine, bio-chemistry, physiology and genetics.

With the above background the matter was raised regarding whether or not NO could be placed in the category of phytohormones. The classical concept for the categorization of the hormone includes three norms (Devies et al., 1995) (i) localized site of biosynthesis (ii) transport to target cells specially separated from the place of synthesis (iii) control of responses through changes in endogenous levels. The activity of NO is restricted had been found mainly in the actively growing tissues viz. embryonic axes and cotyledons, and the contents decreased in mature and senescing organs (Leshem et al., 1998; Caro and Puntarulo, 1999). Secondly, its small size, the hydrophobic nature and active diffusibility through biological membranes evidenced that NO is easily transportable. Regarding the third criteria, it is the sensitivity of the target cells, rather than the concentration of the plant hormone, that defines the magnitude of a response (Trewaras and Malho, 1997), because of this concept some scientists decided to substitute the term hormone with a wider term 'plant growth regulator'. Finally, NO was described as a non-traditional regulator of plant growth (Belligni and Lamattina, 2001).

Further investigations lead to the conformation that NO is soluble in water and lipids. It can exist in three interchangeable forms: the radical ($NO^{\cdot}$), nitrosonium cation (NO^{+}); and nitroxyl anion (NO^{-}). Due to its lipophilic nature, NO may diffuses through the membranes (Leshem, 1996) and acts as inter- and intracellular messenger in many physiological functions. It plays a significant role in plant growth and development, seed germination, flowering, ripening of fruit and senescence of the plant organs (Arasimowicz and Wiczorek, 2007). Moreover like other phytohormones, NO acts in a concentration dependent manner (Hayat et al., 2010b).

Considerable number of evidences in recent years has been obtained to assign important roles to NO in plants. Therefore, in this review an effort has been made to cover the recent advances in its chemical properties, mechanism of its bio-synthesis with special emphasis on the role of endogenous and exogenous NO on the

physiological and biochemical operations that occur in the plants along with the cross-talk between NO and other phytohormones.

2.5.1 Biosynthesis of nitric oxide in plants

In plants there could be four possible routs of NO production (Plate 3).

2.5.1.1 Through Nitric oxide synthase

There have been a number of reports on the presence of nitric oxide synthase (NOS) like activity in bacteria (Sudhamsu and Crane, 2009), unicellular eukaryotes (Ninnemann and Maier, 1996; Messner et al., 2009) and plants (Besson-Bard et al., 2008). Corpas et al. (2006) in pea seedlings using the chemi-luminescence assay showed arginine-dependent NOS activity, which was constitutive, sensitive to an irreversible inhibitor of animal NOS and dependent on the plant organ and its developmental stage. Tossi et al. (2009) showed that apocyanin induces the accumulation of NO in the leaves of maize seedling through a NOS-like activity. Gene encoding NOS-like proteins AtNOS1 was isolated from the *Arabidopsis* genome. It was involved in the process of growth and hormonal signaling (Guo et al., 2003), in defense response induced by a lipopolysaccharide (Zeidler et al., 2004) and was expected to control flowering (He et al., 2004).

2.5.1.2 Through plasma membrane-bound nitrate reductase

Another enzyme that can generate NO from nitrite, is a plasma membrane-bound enzyme in tobacco roots (Ni-NOR) (Stohr et al., 2001). This enzyme has a higher molecular weight than that of NR but still has to be characterized. The major origin of NO production in plants is probably through the action of NAD(P)H-dependent nitrate or nitrite reductases (NR and NiR) (Yamasaki et al., 1999). Nitrate reductase provided the first known mechanism to generate NO in plants. This enzyme normally reduces nitrate to nitrite, but it can also further reduce nitrite to NO (Crawford, 2006). Nitrate reductase is the only enzyme whose NO-producing activity has been rigorously confirmed both *in vivo* and *in-vitro* (Courtois et al., 2008; Kaiser et al., 2002). Transformation of NO^{2-} to NO occurs most probably on a molybdenum cofactor, similar to other NO-generating enzyme with a Molybdenum-Cobalt (MoCo) centre, xanthine oxidoreductase (Harrison, 2002). Xnthene oxidoreductase (XOR)

occurs in two interconvertible forms: the superoxide producing xanthine oxidase and xanthine dehydrogenase (Palma et al., 2002).

Other reasonable candidates for enzymatic generation of NO include: horseradish peroxidase (Huang et al., 2002), cytochrome P450 (Boucher et al., 1992a), CAT and hemoglobin (Boucher et al., 1992b). The production of NO and citrulline by horseradish peroxidase from N-hydroxy-arginine (NOHA) and H_2O_2 was reported a decade ago (Boucher et al., 1992a). More recently, horseradish peroxidase was also demonstrated to generate NO from hydroxyurea and H_2O_2 (Huang et al., 2002). This source of NO should be carefully considered, taking into account that peroxidases are widespread enzymes involved in important physiological processes of plant cells (Veitch, 2004).

2.5.1.3 Through mitochondrial electron transport chain

Heme proteins that have been proposed as good candidates for the enzymatic generation of NO are cytochrome P450. These plant have been shown to catalyze the oxidation of NOHA by NADPH and O_2 with the generation of NO (Boucher et al., 1992b; Mansuy and Boucher, 2002; Igamberdiev and Hill, 2009). Hemoglobin and CAT are also reported to produce NO and other nitrogen oxides by catalyzing the oxidation of NOHA by cumyl hydroperoxide (Boucher et al., 1992a).

2.5.1.4 Through nonenzymatic reactions

In plants, NO can also be generated by non-enzymatic mechanisms. Nitrification/de-nitrification cycles provide NO as a by-product of N_2O oxidation into the atmosphere (Wojtaszek, 2000). It is known that the non-enzymatic reduction of nitrite can lead to the formation of NO, and this reaction is favoured at acidic pH when nitrite can dismutate the NO and nitrate (Stohr and Ullrich, 2002). Nitrite can also be chemically reduced by ascorbic acid at pH 3-6 to yield NO and dehydroascorbic acid (Henry et al., 1997). This reaction could occur at microlocalized pH conditions in the chloroplast and apoplastic space where ascorbic acid is known to be present (Horemans et al., 2000). In barley aleurone cells, NO can also be synthesized by the reduction of nitrite by ascorbate at acidic pH (Beligni and Lamattina, 2002). Another non-enzymatic mechanism proposed for NO formation is the light mediated reduction of NO_2 by carotenoids (Cooney et al., 1994).

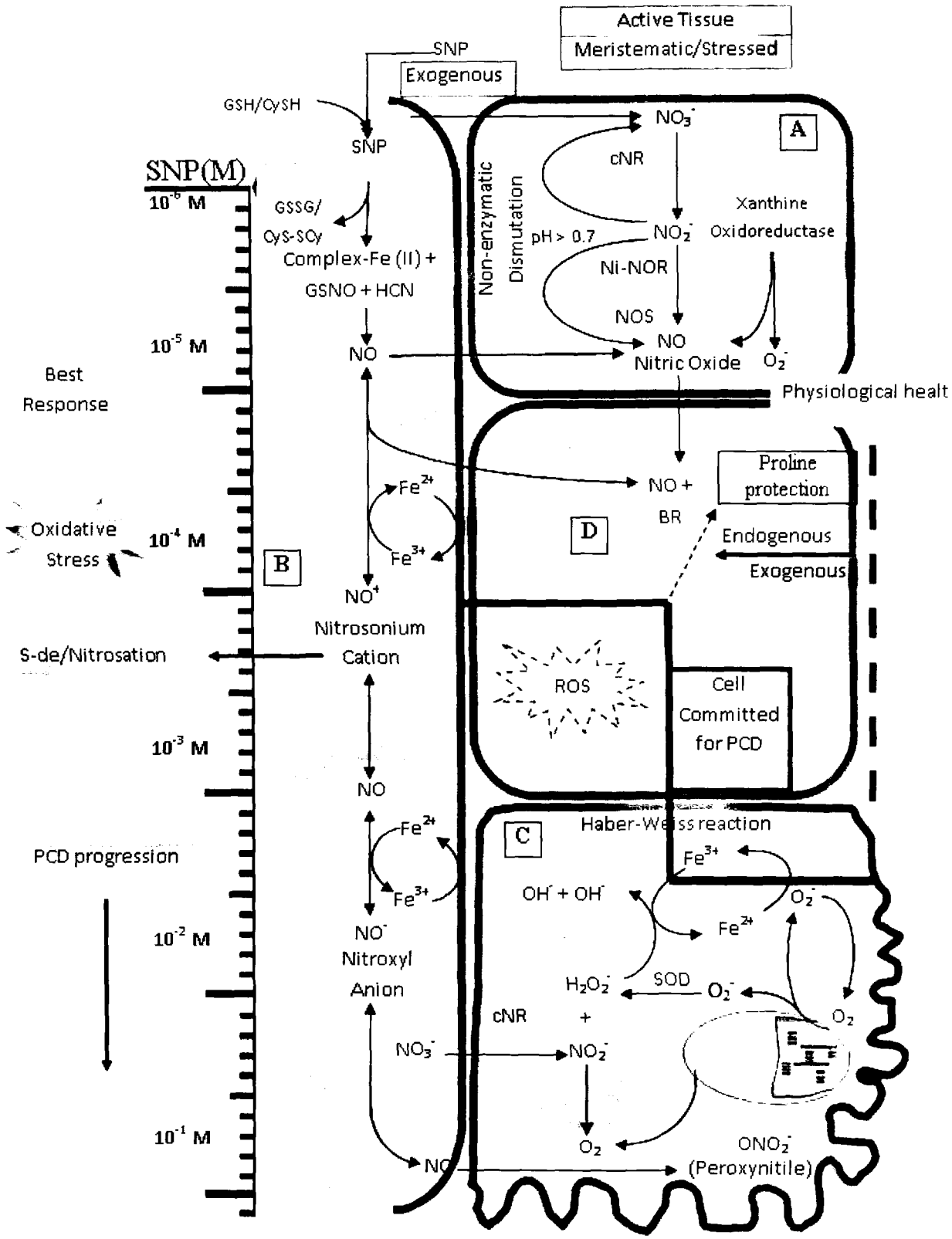


Plate 3: A. Biosynthesis of nitric oxide; B. Formation of reactive nitrogen species (RNS); C. Programmed cell death (PCD) progression; D. Interaction with BR

2.5.2 Physiological roles of nitric oxide

Nitric oxide has emerged as an important signaling molecule associated with many biochemical and physiological processes of plants (Pagnussat et al., 2002; Lamattina et al., 2003; Stohr and Stremlau, 2006). Nitric oxide may be considered as a phytohormone which might function as a gaseous endogenous plant growth regulator (Leshem, 2000) and also as a non-traditional regulator of plant growth (Beligni and Lamattina, 2001). It has a capability to regulate diverse physiological processes, in a concentration dependent manner (Anderson and Mansfield, 1979; Gouvea et al., 1997) such as root organogenesis, hypocotyl growth, defense responses, stomatal movement, apoptosis, hypersensitive responses growth and development, and phytoalaxin production etc. (Noritake et al., 1996; Delledonne et al., 1998; Durner et al., 1998; Kim et al., 1998; Durner and Klessig, 1999; Magalhaes et al., 2000; Belgini and Lamatinna, 2000; Wendehenne et al., 2001; Pagnussat et al., 2002; Neill et al., 2003; Chaki et al., 2009) under different environmental conditions. Therefore, in the recent years, the role of NO in regulating various physiological and biochemical activities in plants has become an important area of research. In this section we discussed the role of NO on different processes of plants under normal (stress-free) condition because so many articles regarding the role of NO in plant under abiotic and biotic stresses are already available.

2.5.3 Effect of nitric oxide on seed germination

The seed germination is sometimes prevented by the dormancy under the influence of certain hormones that check metabolic activity. Several endogenous substances have been reported to break seed dormancy, including nitrogen-containing compounds. For instance; nitrate, nitrite, hydroxylamine, azide and NO releasing compounds e.g. sodium nitroprusside (SNP). Besides curtailing the prolonged seed dormancy in *Arabidopsis* (Batak et al., 2002; Bethke et al., 2004; Bethke et al., 2006), barley (Bethke et al., 2004), and lettuce (Beligni and Lamattina, 2000), NO (as SNP) stimulates seed germination in crop plants (Zhang et al., 2004). In *Paulonica tomentosa* NO speeded up seed germination under normal conditions (Giba et al., 1998) while in *Suaeda salsa,* under salt stress (Li et al., 2005). SNP application facilitated seed germination in lupin during early hours up to a concentration of 0.8 mM (Kopyra and Gwozdz, 2003) and 0.05-0.5 mM concentration in canola, whereas,

at higher concentrations (1-2 mM) it was inhibited in canola (Zanardo et al., 2005). The seed germination in maize was also promoted with the application of NO analogue (Zhang et al., 2004). SNP (the donor of NO) at 0.1 mM concentration inhibited the hypocotyl growth in potato, lettuce and *Arabidopsis* (Beligni and Lamattina, 2000), whereas, in cucumber it induced the root development (Pagnussat et al, 2002).

2.5.4 Effect of nitric oxide on growth morphology

Growth of pea seedlings expressed dual behavior where an increased rate of leaf expansion was recorded at lower (μm) concentration of NO but not at higher concentration (Leshem and Haramaty, 1996). Similarly the growth of tomato, lettuce and pea inhibited at high concentrations of NO (40-80 ppm) while the low concentrations (0-20 ppm) stimulated (Hufton et al., 1996; Leshem and Haramaty, 1996). In maize root segments NO activated the growth comparable to that of IAA (Gouvea et al., 1997), while supplementing NO to maize seedlings it inhibited the mesocotyl elongation (Zhang et al., 2003). Interestingly leaf biomass of maize seedlings increased by the exogenous application of NO or due to its endogenous production (An et al., 2005). The concentration dependent responses of NO have been seen on plant growth (Anderson and Mansfield, 1979; Gouvea et al., 1997). At low concentration NO promotes plant growth while at higher concentrations it has negative or no impact on plant growth. This duality in NO behavior reported in several plants; for example in wheat, tomato and canola seedlings (Tian and Lei, 2006; Correa-Aragunde et al., 2006; Zanardo et al., 2005), maize and cucumber root growth (Gouvéa et al., 1997; Pagnussat et al., 2002) and hypocotyl growth of potato, lettuce and *Arabidopsis* (Beligni and Lamattina, 2000).

2.5.5 Effect of nitric oxide on flowering

NO donor compounds (sodium nitroprusside, S-nitroso-N-acetyl penicillamine and 3-morpholinosydnonimine) induce flowering in *Lemna aequinoctialis* and *L. aequinoctialis* under non-inductive conditions (Khurana et al., 2011). With H_2O_2 NO promote the reproductive growth (Zhou et al., 2010) as well as the expression of flower related gene *LFY* (Blazquez et al., 1997; Zhou et al., 2010). In a genetic screen of *A. thaliana* Wang et al. (2010) identified a mutant defective in H_2O_2-induced NO accumulation. In *A. thaliana* floral transition and the emission of NO is regulated by

the activity of NR (Seligman et al., 2008) suggesting direct role of NR (and NO) in anthesis. NO is expected to generate signals for the regulation of the initiation of floral primordial like that of cytokinins (Corbesier et al., 2003; Eckardt, 2003) and polyamines (Galston et al., 1997; Kakkar and Sawhney, 2002; Martin-Tanguy et al., 1990) whose endogenous level is correlated with this phenomenon.

2.5.6 Effect of nitric oxide on senescence

Senescence is a process characterized by the programmed desiccation and drying up of plant tissues. Reports suggested that NO has anti-senescence properties. Application of NO to senescing pea leaves promoted conditions that decrease ethylene synthesis (Leshem and Haramaty, 1996; Leshem et al., 1998; Leshem 2000). However, in *Arabidopsis* the level of ethylene enhanced significantly after exposure to NO gas (Magalhaes et al., 2000). Moreover, NO emission decreased as the ethylene level increased from anthesis to senescence (Kopyra and Gwozdz, 2004). Nitric oxide donors exert a protective effect against ABA-induced senescence of rice leaves by diminishing ABA-dependent effects such as leaf senescence, enhanced H_2O_2 and melondialdehyde (MDA) content, reduction in GSH, ascorbic acid level and antioxidant enzymes activity (Hung and Kao, 2003). The protective effect was reversed by NO-scavenger (cPTIO) suggesting that the observed phenomenon may be attributed to that of NO. Exogenous NO can protect naturally senescing soybean cotyledons (Jasid et al., 2009).

2.5.7 Effect of nitric oxide on photosynthesis

Photosynthesis is one of the most important physiological processes in plants, whole metabolism of plants directly or indirectly depends on this process, therefore, any change in photosynthetic rate automatically affects the rest of the processes in plants. The role of NO in photosynthesis is poorly understood by the available modest number of *in-vivo* and *in vitro* studies in this area expresses mixed results (Takahashi and Yamasaki, 2002; Yang et al., 2004). Nitric oxide and its donors such as SNP, S-nitroso-N-acetylpenicillinamine, (SNAP), S-nitrosoglutathione (GSNO) regulate photosynthetic rate differentially (Hayat et al., 2010b). Nitric oxide gas decreases net photosynthetic rate in *Avena sativa* and *Medicago sativa* (Hill-Bennet, 1970) whereas, NO donor SNP decrease the level of the enzymes involved in photosynthesis in wheat

(Tu et al., 2003), *Phaseolus aureus* (Lum et al., 2005) and *Pisum sativum* (Wodala et al., 2005; 2008).

Nitric oxide is able to influence the photosynthetic electron transport chain directly. PS II is an important site of NO action (Wodala et al., 2008). Within PS II complex, important binding sites for NO are the non-heme iron between Q_A and Q_B binding sites (Petrouleas and Diner, 1990), the Y_D Tyr residue of D2 protein (Sanakis et al., 1997) and the manganese (Mn) cluster of the water-oxidizing complex (Schansker et al., 2002).

Nitric oxide donor SNAP does not modify the maximal quantum efficiency (Fv/Fm) but inhibits the linear electron transport rate, light induced pH formation (ΔpH) across the thylakoid membrane, and decreases the rate of ATP synthesis (Takahashi and Yamasaki, 2002). Another NO donor SNP reduces Fv/Fm in the intact potato leaves but causes no difference in ΔpH dependent non-photochemical quenching (NPQ) (Yang et al., 2004). A moderate decrease in Fv/Fm was also observed by SNP treatment in pea leaves (Wodala et al., 2008). Moreover, NO donor slows down the electron transfer between the primary and secondary quinone electron acceptor *in-vivo*, in a concentration dependent manner (Petrouleas and Diner, 1990; Wodala et al., 2008). NO slows down the electron transfer from Q_A to Q_B (Wodala et al., 2005). It was also shown that NO inhibits steady state photochemical and non-photochemical quenching modulating reaction center associated with non-photochemical quenching. This leads to significant decrease in the values of maximum quantum efficiency of PSII (i.e. Fv/Fm) in intact pea leaves (Wodala et al., 2008).

2.5.8 Effect of nitric oxide on chlorophyll content

NO donor (SNP) enhances chlorophyll content in potato, lettuce, maize and *Arabidopsis* (Beligni and Lamattina, 2000). Leshem et al. (1998) observed an increase in chlorophyll content in pea and potato and also proposed a role to NO in its protective effect on chlorophyll retention by having an impact on the availability of iron. Strong evidence supporting a role of NO in plant iron nutrition were presented by Graziano et al. (2002).

2.5.9 Effect of nitric oxide on stomatal movement

Nitric oxide plays a role in stomatal movement being, together with H_2O_2, an indispensable component of ABA-induced stomatal closure (Leckie et al., 1998; Garcia-Mata and Lamattina, 2002; Garcia-Mata et al., 2003; Desikan et al., 2002; 2004). The exogenous application of NO to both monocot- and dicotyledonous epidermis strips induced stomatal closure, through a Ca^{2+} dependent process (Gracia-Matta and Lamattina, 2001). In *Pisum sativum* and *Vicia faba* plants, ABA induced an increase in endogenous NO production that was suggested as a reason for ABA-induction stomatal closure (Neill et al., 2002). There are also some convergent evidences that support the involvement of nitrate reductase through the production of NO in guard cell metabolism and stomatal movement (Gracia-Mata and Lamattina, 2002) leading to their closure (Neill et al., 2002; Gracia-Mata and Lamattina, 2002; Neill et al., 2008; Wilson et al., 2009).

2.5.10 Effect of nitric oxide on metabolic enzymes

Nitrate reductase activity is one of the NO sources in plant roots. Exogenous application of SNP significantly enhanced the activity of NR in the leaves of maize (Zhang et al., 2004) and tomato plants (Jin et al., 2009). However, in case of pea and wheat roots SNP did not influence NR activity (Kolbert et al., 2005). GSNO and SNP at the concentrations of 10-50 μM strongly inhibited NR activity in wheat leaf segments (Rosales et al., 2011). Jin et al. (2009) suggested NO regulates NR activity differently depending upon different levels of nitrate availability. He has demonstrated that NO stimulated the NR activity in plant roots supplied with a low level of nitrate, while at higher concentration of nitrate inhibitory effect appeared. Zhang and Shangguan (2007) also showed increased NR activity with the increasing nitrogen application in winter wheat leaves. Also and there was a significant linear correlation between NR activity and NO content at tillering and jointing stages. NO given as SNP (10^{-5} M) to tomato plants completely recovered the NR activity under salinity stress given as 50-100 mM NaCl solution seed soaking (Hayat et al., 2010b).

Seeds of tomato soaked in 10^{-5} M SNP had shown increased activity of CA at 45 and 60 DAS (Hayat et al., 2011). However, *in-vitro* and *in-vivo* study by Puscas and Coltau (1995) regarding the effect of NO on CA suggested that NO reversibly effects CA activity. L-arginine (-source of NO) activated CA *in vitro*, while N-G-

monomethyl-L-arginine (-an inhibitor of NO synthesis) did not modify its activity. *In vivo*, L-arginine and N-G-monomethyl-L-arginine increased CA activity.

2.5.11 Effect of nitric oxide on antioxidant system

It is now commonly accepted that NO acts as a second messenger in plants. Now one of the most intriguing issues in NO biology is its dual function of this molecule as a potent oxidant and an effective antioxidant (Beligni and Lamattina, 1999b). This dual role of NO might depend on its concentration as well as on the environment. Oxidative stress, is the common result of the action of many environmental stressing factors, which manifest in the cell by an increased level of ROS (Mittler, 2002). The cytoprotective role of NO is mainly based on its ability to maintain the cellular redox homeostasis and to regulate the concentration and toxicity status of ROS.

The ability of NO to exert a protective function against oxidative stress caused by factors such as; a) reaction with lipid radicals, which stops the propagation of lipid oxidation, b) scavenging the superoxide anion and formation of peroxynitrite ($ONOO^-$) which is toxic to plants but can be neutralized by ascorbate and glutathione, and c) activation of antioxidant enzymes (SOD, CAT and POX).

One of the fastest reaction of NO within a biological system is its combination with superoxide anion (O_2^-) that leads to the formation of strong oxidant peroxinitrile ($ONOO^-$) (Wendehenne et al., 2001; Neill et al., 2003), the major toxic reactive nitrogen species (Stamler et al., 1992). It exerts deleterious effects on DNA, lipids and proteins (Stamler et al., 1992; Pryor and Squardrito, 1995; Yamasaki et al., 1999). The exogenous application of NO stimulated the super-oxide dismutase (SOD) activity and/or diverts the scavenging of the superoxide anion (Kopyra and Gwowdz, 2003).

The studies on the effect of NO on peroxidase are still scarce and somewhat controversial, depending on its concentration. The lower concentration of NO donor increase the peroxidase activity in *Brassica*, however, higher concentration proved inhibitory (Zanardo et al., 2005). Similarly, it was shown earlier that ascorbate peroxidase activity was inhibited by higher SNP concentration in tobacco and canola (Clark et al., 2000). Moreover, higher concentration of SNP is also reported to inhibit

coniferyl alcohol peroxidase activity in *Zinnia elegans* (Ferrer and Ros-Barcelo, 1999).

High reactivity of NO allows it to scavenge reactive intermediates and end chain-propagated reactions (Kopyra and Gwózdz, 2003). The interaction of NO to lipid peroxyl radicals breaks the self-perpetuating chain propagation during lipid peroxidation (Beligni and Lamattina, 1999a; Van Breusegem et al., 2001). Nitric oxide decreased Thiobarbituric acid reactive substances (TBARS) content in wheat seedlings (Tian and Lei, 2006). The NO counteraction with diquat or paraquat induced generated ROS was shown by Beligni and Lamattina (1999c) in potato and by Hung et al. (2002) in rice. Also NO checks jasmonic acid induced H_2O_2 production in tomato leaves (Orozco-Cárdenas and Ryan, 2002). Treatment of wheat plants with lower concentration of SNP decreased H_2O_2 content but antioxidant activity was enhanced (Tian and Lei, 2006; Hayat et al., 2010b). Nitric oxide production confers antioxidant protection in maize leaves (Tossi et al., 2009).

2.5.12 Nitric oxide signal interactions with other signaling molecules under abiotic stress

The generation of NO and ROS, such as superoxide and H_2O_2, is a regular phenomenon in response to a similar stimuli and with a similar kinetics where they interact in various ways. In several situations, such as during pathogen challenge and ABA induced stomatal closure, both H_2O_2 and NO appear to be generated and function concurrently (Desikan et al., 2004). Moreover, all these signals can induce the generation of antioxidant activity that amelioration of oxidative stress (Neill, 2007). Water stress or, strictly speaking, water deficit stress, which is often referred to as drought stress, is a major abiotic condition that dramatically affects plant growth and yield. Water stress occurs in plants growing in drying soil as the water lost from the leaves exceeds that taken up by the roots and results in cellular dehydration, damage, and ultimately death. Cellular dehydration can also occur during exposure of plants to other abiotic stresses that restrict water availability, such as cold and salt stress or during anaerobic conditions resulting from root flooding. Several defense responses are activated by water stress. One of the most important of these is stomatal closure induced by ABA redistribution and synthesis.

H_2O_2 induces NO generation by NR and NOS-like enzyme(s) via as yet to be fully characterized signaling pathway that may include OXI1 protein kinase and may also involve Ca^{2+}. Nitric oxide induces stomatal closure through the steps that require MAPKs, cGMP, and Ca^{2+}. It is also likely that NO-independent signaling from ABA and H_2O_2 also occur to cause stomatal closure during certain conditions. Nitric oxide also enhances antioxidant genes and enzyme activity via MAPK and other unidentified signaling pathways. For example, SOD activity may increase along with that of CAT and APX to combat the increase in ROS and proteins such as the dehydrins that may be produced to ameliorate the effects of cell dehydration. Other abiotic stresses induce oxidative stress, the generation of H_2O_2 and NO, and also activate enhanced antioxidant defenses. The signals of NO may also induce conformational changes in proteins as a result of S-nitrosylation or nitration. However, the exact role of these processes in stomatal closure and stress amelioration awaits clarification.

Zhang et al. (2008) provided the link between ABA, H_2O_2 and NO by showing that in maize (*Zea mays*) leaves, endogenous ABA, synthesized in response to dehydration induces H_2O_2 production that in turn synthesizes NO and a subsequent increase in the activity of antioxidant enzymes. Moreover, the effects on antioxidant enzyme gene expression and activity require the activation of MAPK signaling enzyme. ABA synthesis and action are essential for plant survival during water stress. ABA is an endogenous anti-transpirant signal that induces stomatal closure and is an activator of various processes that enhance the cell survival against in cellular dehydration (Zhu, 2002). ABA signaling in guard cells is especially complex, with H_2O_2, NO and MAPKs all playing significant roles (Neill et al., 2008). ABA-induced NO production in guard cells depends on H_2O_2 generation (Bright et al., 2006). In their previous work, Zhang et al. (2006) demonstrated that in maize leaves water stress-induced ABA activates H_2O_2 generation via the activation of an NADPH oxidase-like enzyme, similar to that generated by H_2O_2 in response to ABA in *Arabidopsis* guard cells. In another study, NO was found to be an essential intermediate in ABA-regulated processes during water stress in leaf mesophyll cells as well as in guard cells of plant. Zhang et al. (2007) used the fluorescent dye DAF-2DA to show that both ABA and H_2O_2 induce NO generation in maize mesophyll cells. The NO scavenger cPTIO and the non-NO reactive 4AF-DA were used to

demonstrate that fluorescence increases were indeed attributable to NO. ABA- and H_2O_2-induced increases in fluorescence were rapid and dose-dependent and the induction by ABA was prevented by DPI, a known inhibitor of NADPH oxidase. Rapid removal of any ABA-induced H_2O_2 by pretreatment with H_2O_2 scavengers also prevented NO increase, demonstrating that H_2O_2 generation and action require NO production. Furthermore, osmotic stress (induced by the incubation in polyethylene glycol) similarly induced NO generation that was also prevented by pretreatment with H_2O_2 scavengers. Nitric oxide generation was not induced in the ABA-deficient *vp5* mutant by osmotic stress but could be activated by ABA, thereby confirming that endogenous ABA required for H_2O_2-mediated NO production.

ABA also modulates the expression of gene networks that control other ameliorative responses. These include the maintenance of root water uptake, synthesis of osmoprotective proteins such as dehydrins, and various metabolic changes (Neill et al., 2008). Oxidative stress is a common feature of several abiotic stresses including water stress (Bailey-Serres and Mittler, 2006). During oxidative stress, the redox balance of cells is disturbed by increases in the rate of generation of reactive oxygen species (ROS) such as superoxide anion (O_2) and hydrogen peroxide (H_2O_2) above that of their removal by antioxidant enzymes or by reaction with antioxidant molecules. Cell functions are altered during oxidative stress not only because of oxidative damage *per se* but also because of ROS themselves that are centrally important signaling molecules. Thus, excessive quantities of ROS result in aberrant cell signaling (Bailey-Serres and Mittler, 2006). The activation of cellular antioxidant systems is a common feature of oxidative stress, and there is increasing evidence indicating that NO is a critical factor in such responses.

Zhang et al. (2006) reported that osmotic stress, ABA, and H_2O_2 enhance the expression of several antioxidant genes such as *CAT1*, cytosolic ascorbate peroxidase (*cAPX*) and plastidial glutathione reductase (*GR1*), and the total enzyme activities of CAT, APX, GR, and SOD. In another study (Zhang et al., 2007), they demonstrated that NO is an essential intermediate in the enhancement of ABA and H_2O_2. Pretreatment with NO-scavenger c-PTIO substantially prevented an increase in gene expression and enzyme activity. Moreover, treatment with NO donor, sodium nitroprusside essentially reproduced the effects of ABA or H_2O_2. Importantly, the

removal of NO released from SNP with c-PTIO prevented the increase, but treatment with sodium ferricyanide (a molecule similar to SNP which does not release NO) had no effect. A number of studies have already shown that exogenously applied NO can impart protective antioxidant properties. Recent work has indicated that endogenous NO induces antioxidant defenses, potentially via ABA signaling (Song et al., 2006, Zhou et al., 2005).

The results of Zhang et al. (2007) showed that MAPK activation is targeted both by H_2O_2 and NO in mesophyll cells and that this MAPK activation is required for downstream signaling to enhance antioxidant gene expression and enzyme activity. Both ABA and H_2O_2 activate an MAPK enzyme in maize leaves (or at least an enzyme with properties characteristic of MAPKs), but this activation is largely prevented by the removal of NO, through the NO scavenger cPTIO. Moreover, as with the enhancement of antioxidant activity, the MAPK is activated by treatment with NO (supplied via SNP). Finally, inhibition of MAPK activation by treatment with PD98059 (an inhibitor of mitogen-activated protein kinase kinase, MAPKK) and U0126, a compound that inhibits MAPK kinases and upstream activators of MAPKs, inhibits increase in antioxidant gene expression. She et al. (2004) indicated that both 20-amino-30-methoxyflavone (PD98059) and trifluoperazine (TFP) (a specific inhibitor of CDPK) reduced the level of NO in guard cells and significantly reversed darkness-induced stomatal closure, implying that MAPKK/CDPK mediates darkness-induced stomatal closure by enhancing NO levels in guard cells. In addition, as with NO scavenger cPTIO, but not with L-NAME, PD98059 and TFP reduced, NO level in guard cells was not only induced by SNP in light but also increased during the dark period. They were able to also reverse the stomatal closure by using SNP and darkness, suggesting that MAPKK and CDPK are probably involved in restraining the NO-scavenging to elevate NO levels in guard cells during darkness-induced stomatal closure. The results also showed that both PD98059 and TFP restricted stomatal closure through SNP, implying that the possibility of MAPKK and CDPK acting as the target downstream of NO. There may be a causal and interdependent relationship between MAPKK/CDPK and NO in darkness-induced stomatal closure, and in the process a cross talk may lead to the formation of a self-amplification loop about them.

The available data indicate a key ABA–H_2O_2–NO–MAPK–antioxidant survival cycle. It suggests that during water stress ABA has several ameliorative functions that involve NO as a key signaling intermediate and which include the rapid induction of stomatal closure to reduce transpirational water loss and the activation of antioxidant defense to combat oxidative damage.

Desikan et al. (2004) suspected the interaction of NO and H_2O_2 in the regulation of stomatal closure in response to ABA signaling with the mediation of NADPH-oxidase and nitrate reductase, respectively. NO may function in the downstream of H_2O_2 in ABA-induced stomatal closure in *Vicia faba* (Zhang et al., 2005). In *Arabidopsis* ABA-induced NO generation and stomatal closure are dependent on H_2O_2 synthesis (Bright et al., 2006). Ye et al. (2011) suggested that water stress-induced ABA prevents the excessive accumulation of H_2O_2, through the induction of the expression of catalase (*CATB* gene) in rice. This was further confirmed by Jannat et al. (2011) in *Arabidopsis* guard cells where ABA-inducible cytosolic H_2O_2 elevation functions in ABA-induced stomatal closure. Functions of many other possible components of ABA– H_2O_2–NO–cellular response signal transduction chain still require clarification. These include the protein kinases OST1 (Mustilli et al., 2002) and OXI1 (Rentel et al., 2004) and many others as-yet-unknown proteins and signaling molecules.

2.6 Future prospects of brassinosteroids and nitric oxide based researches

Forty years of research on BRs has brought into light several vital functions of this class of phytohormones in the regulation of plant growth, development and productivity. Further progress in the investigation of mechanism of BRs action in plants, on one hand, and elaboration of economically feasible schemes of the synthesis of natural BRs and their analogs, on the other hand, will surely make a basis for the inclusion of this new class of plant hormones in the regular package of chemicals used for optimizing agricultural production. Hopefully, as the research will progress, much more knowledge will be added to the present literature. It has been stated earlier that the application of these steroids to plants generates varied physio-morphological changes by involving the genome and also do not initiate co-evolution of pests, enriching our arsenal of plant protection strategies, in the twenty first century. Moreover, the knowledge of the physical and chemical properties of these steroids is tempting us to consider them highly promising, environment friendly and

promoter of agricultural productivity. One of the major constraints, to employ BRs at larger scale, in the field conditions is their high cost. However, recent progress, in chemical synthesis of BRs and their analogues has led us to economically feasible approaches that may bring large scale application very near to the reach of the farmers.

The mechanisms by which NO is generated are still largely unresolved. Elucidation of how NO is generated by different plant cells, in different situations, is clearly a research priority. The mechanism(s) by which NO is perceived by plant cells is another question, to be resolved. Transient elevation of the second messenger cyclic GMP via activation of a guanylyl cyclase enzyme is a possibility, as is directs the activation by reversible S-nitrosylation of cysteine residues; however, there could be specific receptors for this molecule. Functions of many other components of the ABA–H_2O_2–NO–cellular response signal transduction chain also require clarification. The research regarding the network, regulation, metabolism and biosynthesis of NO is still elusive and need substantial work.

Chapter 3

MATERIALS AND METHODS

CONTENTS

CHAPTER-3

MATERIALS AND METHODS

Six pot experiments were conducted on Indian mustard (*Brassica juncea* L.; Czern & Coss.) crop, during the years 2009-12 to attain the objectives mentioned in Chapter-1. The seeds of two varieties of *B. juncea* viz. Varuna and RH-30 were sown in the winter season to observe the effects of foliage application of aqueous solution of selected concentration (10^{-8} M) of brassinosteroids (BRs; 28-HBL and 24-EBL) and/or that of sodium nitroprussside (SNP; 10^{-6} M), as a donor of nitric oxide (NO), on Cd stressed and non-stressed mustard plants at 30 and 60 day after sowing (DAS). The comprehensive details of the material used and the methodologies adopted, during the course of the present investigation are presented in this chapter.

3.1 Seeds

The authentic seeds of two varieties of Indian mustard, Varuna and RH-30 were procured from National Seed Corporation Ltd. New Delhi, India. Uniform sized, healthy seeds were tested for their per cent viability before the start of each experiment. Mercuric chloride solution (0.01%) was used for the seeds disinfection. Seeds were rinsed thrice with double distilled water (DDW) following the disinfection; to remove the toxic mercuric chloride adhered to the seed coat.

3.2 Preparation of pots

Earthen pots were filled with sandy loam soil mixed with farmyard manure in a ratio of 9:1. Each pot was amended with a recommended dose of fertilizers (urea, single superphosphate and muriate of potash, at the rate of 40 mg, 138 mg and 26 mg, respectively). The pots were arranged in a simple randomized block design and the plants were raised under the natural environmental conditions in the net house.

3.3 Hormones and their preparation

28-homobrassinolide (HBL) and 24-epibrassinolide (EBL) were obtained from Sigma-Aldrich India Ltd. Chemicals, USA. Stock solution (10^{-4} M) was prepared by

dissolving the required quantity of the hormone in 5 cm^3 of ethanol, in a 100 cm^3 volumetric flask and final volume was made up to the mark, using DDW. The desired concentration of HBL or EBL was prepared by diluting the above stock solution using DDW. Surfactant "Tween-20" (0.5%) was added to each flask prior to the foliar application.

Sodium nitroprusside (SNP) was used as the source of NO. SNP in aqueous solution also releases Fe and cyanide (CN^-) therefore potassium ferricyanide [$K_3Fe(CN)_6$] solution was used as a donor of equimolar molecules of ferricyanide and (CN^-) only, to see that effects of SNP are mediated by NO and not Fe or (CN^-). The required concentrations (10^{-4}, 10^{-5}, 10^{-6} M) of each of the SNP and potassium ferricyanide (10^{-4} M) were prepared by dissolving their requisite quantities in DDW.

3.4 Experiment 1

The first experiment was laid down according to a simple randomized block design in the pots (25 x 25 cm) during the winter season (September-February) of year 2009-10. The surface sterilized seeds of Indian mustard cv. Varuna and RH-30 were sown at the rate of 8 seeds per pot amended with graded concentrations of $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil), so that each pot received 0, 15.33 mg, 30.66 mg or 61.32 mg Cd Kg^{-1} of soil, respectively. After a week plants were thinned to three plants per pot.

Each treatment had five pots as replicate where three plants per pot were maintained. Irrigation was done with tap water as and when required. The plants were sampled at 30 and 60 DAS to study the vegetative characteristics of the plants and at 120 DAS to assess yield parameters listed below:

Length of root and shoot per plant

Fresh and dry mass of root and shoot per plant

Leaf area

Chlorophyll level (SPAD value)

Net photosynthetic rate (P_N)

Stomatal conductance (g_s)

Internal CO_2 concentration (C_i)

Transpiration rate (E)

Maximum quantum yield of photosystem II (Fv/Fm)

Leaf water potential (LWP)

Leaf nitrate reductase (NR) activity

Leaf carbonic anhydrase (CA) activity

Leaf peroxidase (POX) activity

Leaf catalase (CAT) activity

Leaf superoxide dismutase (SOD) activity

Leaf proline content

Cadmium accumulation in root and shoot

Number of pods per plant

Number of seeds per pod

Seed yield per plant

100 seed mass

3.5 Experiment 2

This experiment was set up in a simple randomized design, during the winter season of 2009-10, side by side the Experiment 1. The surface sterilized seeds of Indian mustard cv. Varuna and RH-30 were sown in Cd non-treated soil. The foliage of 29 day old plants was sprayed with DDW, tween-20 (0.5%), ethanol (5%), 28-HBL (10^{-8} M) or 24-EBL (10^{-8} M). Each plant was sprayed thrice at an interval of 2 min so that foliage would get sufficient time to adsorb solution. The nozzle of the sprayer was adjusted to pump ~1 ml solution in one sprinkle. Therefore, each plant received 3 ml (15 µl, 150 µl, 0.0148 µg or 0.0144 µg, respectively) of each solution.

Each treatment had five replicates. Irrigation was done with tap water as and when required. Five plants per treatment were sampled at 30 and 60 DAS to assess the selected parameters (Experiment 1) and the remaining plants were allowed to grow up to maturity (120 DAS) to study yield characteristics (Experiment 1).

3.6 Experiment 3

This experiment was laid simultaneously along with Experiments 1 and 2, in a net house under the same environmental conditions of the winter season of year 2009-10 as per simple randomized block design. The healthy disinfected seeds of *B. juncea* cv. Varuna and RH-30 were sown in Cd non-treated soil. The foliage of 29 day old plants was sprayed thrice with ~1 ml DDW, potassium ferricyanide (10^{-4} M) or three concentrations of SNP (10^{-4}, 10^{-5} or 10^{-6} M) at an interval of 10 min so that each plant would have sufficient time to receive 3 ml (111.00 µg of potassium ferricyanide or 78.57 µg, 7.86 µg or 0.79 µg of SNP, respectively) of each solution.

Each treatment had five replicates. Plants were irrigated with tap water as and when required. Sampling was done at 30 and 60 DAS to assess vegetative characteristics and at 120 DAS for yield attributes, as mentioned in experiment 1.

3.7 Experiment 4

This experiment was performed according to simple randomized block design in the winter season of the year (2010-11) under the similar environmental conditions as in previous experiments, in a net house. The healthy and disinfected dry seeds of *B. juncea* cv. Varuna and RH-30 were sown in Cd amended soil and sprayed (3.00 ml) at 29 DAS.

Treatments	***Cadmium added ($CdCl_2$) to the soil (mg/Kg)***	***Solution applied to the foliage (10^{-8}M)***
A	0.00	DDW
B	25.00	DDW
C	50.00	DDW
D	100.00	DDW
A_1	0.00	28-homobrassinolide
B_1	25.00	28-homobrassinolide
C_1	50.00	28-homobrassinolide
D_1	100.00	28-homobrassinolide
A_2	0.00	24-epibrassinolide
B_2	25.00	24-epibrassinolide
C_2	50.00	24-epibrassinolide
D_2	100.00	24-epibrassinolide

Each treatment had five replicates. Irrigation was done with tap water as and when required. The plants were sampled at 30 and 60 DAS to study vegetative characteristics and at 120 DAS to compute the yield parameters as in Experiment 1.

3.8 Experiment 5

This experiment was laid down according to simple randomized block design during the winter season of the year 2010-11 under similar environmental conditions as in previous experiments. The healthy and disinfected seeds of mustard, varieties Varuna and RH-30 were sown in Cd amended soil and foliage was sprayed (3.00 ml) with SNP (10^{-5} M) at 29 DAS, under the following scheme:

Treatments	***Cadmium ($CdCl_2$) added in the soil (mg/Kg)***	***Solution applied to the foliage ($10^{-5}M$)***
A	0.00	DDW
B	25.00	DDW
C	50.00	DDW
D	100.00	DDW
A_1	0.00	*Sodium nitroprusside*
B_1	25.00	*Sodium nitroprusside*
C_1	50.00	*Sodium nitroprusside*
D_1	100.00	*Sodium nitroprusside*

Each treatment had five replicates. Irrigation was done with tap water as and when required. The plants were sampled at 30 and 60 DAS to assess the characteristics of vegetative phase and at 120 DAS for yield characteristics, as listed in Experiment 1.

3.9 Experiment 6

This experiment was conducted in a simple randomized block design during the winter season of the year 2011-12 in a net house. The SNP and EBL and their concentrations were selected on the basis of the observations made in Experiments 3 and 4, respectively to study their interactive effects. The healthy disinfected seeds of mustard varieties Varuna and RH-30 were grown in Cd amended soil and sprayed (3.00 ml each) with 10^{-5} M of SNP (28 DAS) and 10^{-8} M of EBL (29 DAS).

Treatments	*Cadmium ($CdCl_2$) added in the soil (mg/Kg)*	*Solution applied to the foliage (10^{-5} M) of EBL and (10^{-8} M) of SNP*
A	0.00	DDW
B	25.00	DDW
C	50.00	DDW
D	100.00	DDW
A_1	0.00	SNP + EBL
B_1	25.00	SNP + EBL
C_1	50.00	SNP + EBL
D_1	100.00	SNP + EBL

Each treatment had five replicates. Irrigation was done with tap water as and when required. The plants were sampled at 30 and 60 DAS to asses various vegetative characteristics and at maturity (120 DAS) for yield characteristics as listed in Experiment 1.

3.10 Parameters

The methods executed to assess each parameter are described in details below:

3.10.1 Growth parameters

3.10.1.1 Shoot and root length per plant

The plants with intact roots were dig out of the pots carefully with the adhering soil which was removed with gentle shake in the bucket, filled with water. The roots were then gently stirred to remove the rest of the adhering soil particles followed by washing under running tap water. The shoot and root length (cm) were measured using a meter scale.

3.10.1.2 Fresh and dry mass of shoot and root

The plants were wrapped in blotting sheets to soak the water. Shoot and root of each plant were separated and weighed individually using electronic balance to record their fresh mass separately. The samples were then subsequently transferred in an oven set at 70°C for 72 h. The dry mass of shoots and roots were recorded.

3.10.1.3 Leaf area

Leaf area was ascertained with the help of millimeter graph sheet. The fully expanded third upper leaf was taken from each replicate to trace its outline on the graph sheet. The squares falling within outline were counted. The average leaf area was expressed as cm^2.

3.10.2 Physiological characteristics

3.10.2.1 Chlorophyll content (SPAD value)

The leaf chlorophyll level at each sampling stage was measured in fully expanded leaves of the plants by using Minolta chlorophyll meter (SPAD-502; Konica Minolta Sensing Inc. Japan).

3.10.2.2 Photosynthesis and related attributes

Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and internal CO_2 concentration (C_i) at two growth stages (30 DAS and 60 DAS) were measured in fully expanded leaves of the plants by using portable photosynthesis system (LI-COR 6400, LI-COR, Lincoln, NE, USA). The air temperature, relative humidity, CO_2 concentration and photosynthetic photon flux density (PPFD) were maintained at 25°C, 85%, 600 μmol mol^{-1} and 800 μmol mol^{-2} s^{-1}, respectively. All the measurements were made between 11:00 and 12:00 h, under clear sun light.

3.10.2.3 Maximum quantum yield of Photosystem II

Maximum quantum yield of Photosystem II (Fv/Fm) was measured by using Leaf Chamber Fluorometer (LI-COR 6400-40, Portable photosynthesis system, LI-COR, Lincoln, NE, USA). All the measurements were carried out at a PPFD of 1500 μmol m^{-2} s^{-1}, with a constant airflow rate of 500 μmol s^{-1}. The sampled leaves were dark adapted for 30 min, prior to measurements.

3.10.2.4 Leaf water potential

Leaf water potential, at each selected stage, was measured in fresh, detached leaves by using PSYPRO water potential system (WESCOR Inc. Longman, USA).

3.10.3 Biochemical analyses

3.10.3.1 Nitrate reductase (NR) activity

The nitrate reductase (E.C. 1.6.6.1) activity in fresh leaves was measured following the method of Jaworski (1971). The leaves were cut into small pieces (~1 cm^2). These samples were weighed (200 mg) and transferred to plastic vials. To each vial 2.5 cm^3 of phosphate buffer (Appendix 1.1) and 0.5 cm^3 of potassium nitrate solution (Appendix 1.2) was added followed by the addition of 2.5 cm^3 of 5% isopropanol (Appendix 1.3). These vials were incubated in a BOD incubator for 2 h at 30±2°C, in dark. Incubated mixture (0.4 ml) was taken in a test tube to which 0.3 cm^3 each of sulphanilamide solution (Appendix 1.4) and NED-HCl (Appendix 1.5) were added and left for 20 minutes to attain maximum colour development. The mixture was diluted to 5 cm^3 with DDW. The absorbance was read at 540 nm on spectrophotometer (Milton & Roy, USA). A blank was run simultaneously with each sample. Standard curve was plotted by using known graded concentrations of sodium nitrite solution. The absorbance of each sample was compared with the calibration curve to record NR activity [n mol $NO_2\ g^{-1}\ h^{-1}$] on fresh mass basis.

3.10.3.2 Leaf carbonic anhydrase (CA) activity

The carbonic anhydrase (E.C. 4.2.1.1) activity in fresh leaves was measured by following the method of Dwivedi and Randhawa (1974). Fresh mustard leaves were cut into small pieces and weighed to 200 mg at a temperature below 25°C. These samples were transferred to petri plates and further cut into fine pieces in 10 cm^3 of 0.2 M cystein hydrochloride (Appendix 2.1) and were left at 4°C for 20 min. The leaf pieces were blotted and transferred to a test tube containing 4 cm^3 of phosphate buffer of pH 6.8 (Appendix 2.2). To the test tube, 4 cm^3 of 0.2 M sodium bicarbonate (Appendix 2.3) solution and 0.2 cm^3 of 0.002% bromothymol blue (Appendix 2.4) were added. The test tube was shaken gently and left at 4°C for 20 min. CO_2 liberated by the catalytic action of CA on $NaHCO_3$ was estimated by titrating the reaction mixture against 0.05 N HCl (Appendix 2.5) using methyl red as an indicator (Appendix 2.6). The volume of HCl used to develop light purple colour, persisting for at least five seconds was noted. A blank

consisting of all the above components of the reaction mixture, except the leaf sample, was run simultaneously with each set of the samples. The activity of enzyme was calculated by putting the values in the formula:

$$\text{Carbonic anhydrase activity} = \frac{V \times 22 \times N}{W} \text{ [mol } (CO_2) \text{ Kg}^{-1} \text{ FM s}^{-1}]$$

Where,

V = Difference in volume (cm^3 of HCl used, in control and test sample titrations)

22 = Equivalent weight of CO_2

N = Normality of HCl

W = Fresh mass of tissue used

3.10.3.3 Estimation of antioxidative enzymes activity

Leaf tissue (500 mg) was homogenized in 5 cm^3 of 50 mM phosphate buffer (pH 7.0) containing 1% polyvinyl pyrrolidone. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 5°C and the supernatant was used for the estimation of peroxidase, catalase and superoxide dismutase activities.

3.10.3.3.1 Leaf peroxidase (POX) activity

The POX activity was measured following the method of Chance and Maehley (1956) in fresh leaf samples. Three cm^3 of pyrogallol phosphate buffer (Appendix 3.1), 0.1 cm^3 of enzyme extract and 0.5 cm^3 of 1% H_2O_2 were mixed in a cuvette. Change in the absorbance, at 20 s interval for a period of 3 minutes was recorded at 420 nm on a spectrophotometer. The control set was prepared by boiling the enzyme extract.

3.10.3.3.2 Leaf catalase (CAT) activity

The activity of CAT was estimated by permanganate titration method (Chance and Maehly, 1956). Five cm^3 of phosphate buffer (Appendix 4.1), 1 cm^3 of 0.1 M H_2O_2 (Appendix 4.2) and 1 cm^3 of enzyme extract were mixed and incubated at 25°C for 1 minute. Then 10 cm^3 of 2% H_2SO_4 (Appendix 4.3) was added. The mixture was titrated against 0.1 N potassium permanganate (Appendix 4.4) to find the residual H_2O_2 until a

purple colour persists for at least 15 s. Similarly, a control set was maintained in which the enzyme activity was stopped by the addition of H_2SO_4, prior to the addition of the enzyme extract.

3.10.3.3.3 Leaf superoxide dismutase (SOD) activity

The activity of SOD was measured by the method of Beauchamp and Fridovich (1971). Reaction mixture contained 1 cm^3 of 50 mM phosphate buffer (Appendix 5.1), 0.5 cm^3 of 13 mM methionine (Appendix 5.2), 0.5 cm^3 of 75 mM NBT (Appendix 5.3), 0.5 cm^3 of 0.1 mM EDTA (Appendix 5.5) and 0.1 cm^3 of the enzyme extract and at last 0.5 cm^3 of 2 µM riboflavin (Appendix 5.4) was added. The absorbance of the reaction mixture was read at 560 nm on a spectrophotometer.

3.10.3.4 Leaf proline content

The proline content in fresh leaves was estimated following the procedure used by Bates et al. (1973). Fresh sample (0.5 g) was homogenized in a mortar with 5 cm^3 of 3% sulphosalicylic acid (Appendix 6.1). The homogenate was filtered through Whattman filter paper No. 2 and collected in a test tube with two washings. Five cm^3 of sulphosalicyclic acid, 2 cm^3 each of glacial acetic acid and acid ninhydrin (Appendix 6.2) were added to 2 cm^3 of the above extract. This mixture was heated in boiling water bath for 1 hour. The reaction was terminated by transferring the test tubes to ice box. Four cm^3 of toluene was mixed to the reaction mixture with vigorous shaking for 20-30 s. The chromophore (toluene) layer was aspirated and warmed to room temperature. The absorbance of red colour was read at 520 nm against a reagent blank. The amount of proline in the sample was calculated by using a standard curve prepared from pure proline (range, 0.1-36 µmol) and expressed on fresh mass basis of the sample.

$$\mu \text{ moles of proline } g^{-1} \text{ tissue} = \frac{\mu g \text{ proline } cm^{-3} \times cm^{-3} \text{ toluene}}{115.5} \times \frac{5}{g \text{ (sample)}}$$

Where, 115.5 is the molecular mass of proline

3.10.3.5 Cadmium accumulation in root and shoot

For Cd determination, the root and shoot samples were immersed for 10 min in ice cold 5 mM $CaCl_2$ solution (Appendix 7.1) to displace extracellular Cd, rinsed with distilled water. Tissue is oven dried (Meuwly and Rauser, 1992). Cd concentration in shoots and roots was estimated after digesting the samples in nitric acid:perchloric acid (3:1, v/v). Cd concentration was determined by atomic absorption spectrophotometer (Perkin-Elmer A, Analyst, 300).

3.12 Yield parameters

3.12.1 Number of pods per plant

At harvest (120 DAS), 5 plants from each treatment (representing five replicates) were randomly sampled and counted for the number of pods per plant.

3.12.2 Number of seeds per pod

From each treatment, 25 pods were randomly selected and computed to get number of seeds per pod.

3.12.3 Seed yield per plant and 100 seed mass

The pods from each replicate were cleaned, crushed, and computed to assess seed yield per plant. 100 seeds were subsequently randomly picked and weighed to record 100 seed mass in mg.

3.13 Statistical analysis

The experiments were conducted according to simple randomized design. Each treatment was represented by five pots where each pot was considered as a replicate. Three plants were maintained per pot. The treatment means were compared by analysis of variance using SPSS ver. 17 Inc., Chicago, USA. Least significant difference (LSD) was calculated at 5% level of probability.

Chapter 4

EXPERIMENTAL RESULTS

CONTENTS

CHAPTER-4

RESULTS

4.1 EXPERIMENT 1

This experiment was conducted to study the comparative response of the two varieties of *Brassica juncea* (L.) Czern & Coss (Varuna and RH-30) to soil applied $CdCl_2$ (now Cd onwards) (0, 25, 50 or 100 mg Cd Kg^{-1}). The experiment was laid under simple randomized block design during the winter season of 2009-10 in earthen pots (25 × 25 cm). Soil was amended with uniform basal dose of N, P and K. Ten seeds per pot were sown, the seedlings were thinned to 3 plants per pot after a week (Chapter 3.2). Each treatment was represented by 5 pots. Plants were irrigated with tap water as and when required. Samples were randomly collected at 30 and 60 days after sowing (DAS). The crop was harvested finally at 120 DAS. The growth and yield characteristics are explained below:

4.1.1 Root and shoot length

The data in table 1 indicates that Cd at all its levels, in a concentration dependent manner (25, 50 or 100 mg Cd Kg^{-1}) significantly reduced the root and shoot length in both the varieties i.e. Varuna and RH-30. The reduction was more prominent in RH-30 than Varuna at the two growth stages. At the highest concentration (100 mg Cd Kg^{-1}) the per cent loss was 65% and 39% (RH-30) and 57% and 35% (Varuna) for root and shoot length, respectively, at 30 day stage of growth, compared with the control plants (0 mg Cd Kg^{-1}). However, a slight recovery was noted at day 60.

4.1.2 Fresh and dry mass of root

With the growth advancement from 30 to 60 DAS, the fresh and dry mass of root increased (Table 2). The plants raised in the soil with different doses of Cd exhibited a concentration dependent inhibition of root fresh and dry mass. The minimum decline was noted against the lowest concentration (25 mg Cd Kg^{-1} soil) where root fresh mass decreased by 34% and 40% at 30 DAS and by 25% and 35% at 60 DAS, in Varuna and RH-30 respectively, as compared to the controls (0 mg Cd Kg^{-1}). Similarly, the decline of root dry mass at the same concentration of Cd was 18% and 23% and 13% and 26% in

Varuna and RH-30 respectively, compared with their controls, at the two growth stages (30 and 60 DAS). The losses in RH-30 were more prominent than Varuna.

4.1.3 Fresh and dry mass of shoot

It is evident from table 3 that the fresh and dry mass of shoot was significantly reduced by Cd at the two growth stages (30 and 60 DAS) in both the mustard varieties. This loss was in proportion to the Cd concentration. The lowest level (25 mg Cd Kg^{-1} of soil) of Cd decreased the shoot fresh and dry mass by 36% and 49%, whereas it was 55% and 65% against the highest level of (100 mg Cd Kg^{-1} of soil) in RH-30 at 30 DAS. However, in Varuna, the loss of fresh and dry mass was comparatively less than RH-30 both at 30 and 60 DAS. Moreover, as the growth progressed (60 DAS) the per cent loss in fresh and dry mass of shoot decreased. RH-30 was more sensitive to Cd as reflected by greater loss in root fresh and dry mass.

4.1.4 Leaf area

The leaf area increased as the growth progressed from 30 to 60 days (Table 4). However, the values decreased with the amendment of Cd, in a concentration dependent manner in both the varieties. The loss was more significant in RH-30 than Varuna. The decrease was more prominent at early stage (30 DAS) of growth than at the latter stage (60 DAS) which was 54% and 48% in RH-30 and Varuna respectively, compared with their controls against the highest level of Cd (100 mg Kg^{-1} of soil).

4.1.5 SPAD chlorophyll value

The chlorophyll content increased with plant age (Table 4). However, the plants grown under stress, generated by Cd (0, 25, 50 or 100 mg Kg^{-1}) possessed significantly lower values of SPAD chlorophyll than the unstressed control plants (0 mg Cd Kg^{-1}). The loss was in proportion to the Cd concentration. The Cd (100 mg Kg^{-1}) generated a loss of 51% and 72% at 30 DAS and 35% and 55% at 60 DAS in Varuna and RH-30, respectively, as compared to the controls (0 mg Cd Kg^{-1}). Therefore, RH-30 exhibited greater loss than Varuna at the two growth stages.

Table 1. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root and shoot length (cm) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root length						Shoot length					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	15.89	14.58	15.24	24.06	21.96	23.01	29.10	25.76	27.43	74.50	69.20	71.85
$CdCl_2$ (25mg)	11.11	09.17	10.14	18.02	15.85	16.94	25.16	22.26	23.71	61.56	55.34	58.45
$CdCl_2$ (50mg)	08.99	06.01	07.50	15.84	12.59	14.22	22.11	18.91	20.51	56.38	48.42	52.40
$CdCl_2$ (100mg)	06.89	05.05	05.97	13.41	10.62	12.02	18.86	15.70	17.28	52.06	42.49	47.28
Mean	10.72	08.70		17.83	15.26		23.81	20.66		61.13	53.86	
LSD at 5%												
Varieties	V = 0.84(Sig.)			V = 0.76(Sig.)			V = 1.93(Sig.)			V = 2.41(Sig.)		
Treatment	T = 0.60(Sig.)			T = 1.08(Sig.)			T = 1.16(Sig.)			T = 3.41(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 2. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root fresh mass						Root dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	2.79	2.43	2.61	5.38	4.57	4.98	0.77	0.60	0.69	1.45	1.07	1.26
$CdCl_2$ (25mg)	1.83	1.45	1.64	4.03	2.99	3.51	0.63	0.46	0.55	1.26	0.78	1.02
$CdCl_2$ (50mg)	1.36	1.13	1.25	3.47	2.67	3.07	0.49	0.33	0.41	1.14	0.65	0.90
$CdCl_2$ (100mg)	1.06	0.80	0.93	3.10	2.12	2.61	0.35	0.19	0.27	1.03	0.53	0.78
Mean	1.76	1.45		4.00	3.09		0.56	0.39		1.22	0.76	
LSD at 5%												
Varieties	V = 0.13(Sig.)			V = 0.19 (Sig.)			V = 0.04(Sig.)			V = 0.06(Sig.)		
Treatment	T = 0.08(Sig.)			T = 0.24(Sig.)			T = 0.03(Sig.)			T = 0.07(Sig.)		
Var. x Treat.	V x T = 0.16(Sig.)			V x T = 0.37(Sig.)			V x T = (NS)			V x T = 0.12(Sig.)		

Sig = Significant; NS = Non-significant

Table 3. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on shoot fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Shoot fresh mass						Shoot dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	6.24	5.49	5.87	11.70	9.84	10.77	1.63	1.40	1.52	3.08	2.54	2.81
$CdCl_2$ (25mg)	4.81	3.49	4.15	09.79	6.27	08.03	1.14	0.71	0.93	2.65	1.92	2.29
$CdCl_2$ (50mg)	4.30	2.91	3.61	09.12	5.64	07.38	1.01	0.60	0.81	2.31	1.55	1.93
$CdCl_2$ (100mg)	3.74	2.47	3.11	08.24	5.01	06.63	0.89	0.49	0.69	1.97	1.22	1.60
Mean	4.77	3.59		09.91	6.94		1.17	0.80		2.50	1.81	
LSD at 5%												
Varieties	V = 0.25(Sig.)			V = 0.10(Sig.)			V = 0.13(Sig.)			V = 0.14(Sig.)		
Treatment	T = 0.30(Sig.)			T = 0.14(Sig.)			T = 0.05(Sig.)			T = 0.13(Sig.)		
Var. x Treat.	V x T = 0.51 (Sig.)			V x T = 0.20(Sig.)			V x T = 0.17(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 4. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on leaf area (cm^2) and SPAD value of chlorophyll of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Leaf area						SPAD value of chlorophyll					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	25.00	23.17	24.09	38.75	35.26	37.01	36.70	29.87	33.29	44.83	35.34	40.09
$CdCl_2$ (25mg)	19.26	15.74	17.50	32.87	25.83	29.35	29.03	19.51	24.27	38.52	25.63	32.08
$CdCl_2$ (50mg)	16.08	13.34	14.71	28.39	23.53	25.96	23.12	12.88	18.00	33.62	20.55	27.09
$CdCl_2$ (100mg)	12.98	10.55	11.77	25.25	18.22	21.74	17.81	08.38	13.10	29.20	15.96	22.58
Mean	18.33	15.70		31.32	25.71		26.67	17.66		36.54	24.37	
LSD at 5%												
Varieties	V = 1.04(Sig.)			V = 1.47(Sig.)			V = 0.78(Sig.)			V = 2.26(Sig.)		
Treatment	T = 1.05(Sig.)			T = 2.10(Sig.)			T = 1.10(Sig.)			T = 1.37(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 1.56(Sig.)			V x T = 5.24(Sig.)		

Sig = Significant; NS = Non-significant

4.1.6 Photosynthetic parameters

The photosynthetic parameters improved as the growth progressed from day 30 to 60. However, the soil contaminated with Cd (25, 50 or 100 mg Kg^{-1} of soil) significantly decreased the net photosynthetic rate (P_N) and its related parameters (stomatal conductance; g_s, internal CO_2 concentration; C_i, transpiration rate; E, and maximum quantum yield of PSII; Fv/Fm) in the leaves of mustard plants, compared to the control (0 mg Cd Kg^{-1}), in a manner dependent on its concentration. Even at the lowest level (25 Cd mg Kg^{-1} of soil) the per cent decrease in P_N; g_s; Ci; E and Fv/Fm at 30 and 60 DAS was 12% and 22%; 41% and 17%; 10% and 8%; 1% and 5%, 6% and 4% for Varuna as compared to its stress free control (Tables 5-7). Moreover, this loss was of a higher order in all the parameters in the other cultivar (RH-30), which looked like to be less resistant to Cd-stress.

4.1.7 Leaf water potential (LWP)

The increase in plant age (30 to 60 DAS) expressed an increase in per cent LWP (Table 7). However, the stress, induced by Cd significantly reduced the LWP in a concentration dependent manner. Therefore, maximum loss was recorded in the plants cultured with highest concentration of Cd (100 mg Kg^{-1}). The values were lesser in variety RH-30, compared to Varuna. At early growth stage (30 DAS), against the minimum concentration of Cd (25 mg Kg^{-1} of soil) the decline in LWP in Varuna was 16% while in RH-30 it was 19%, compared to their non-stressed plants (0% Cd).

4.1.8 Nitrate reductase (NR) and carbonic anhydrase (CA) activity

The activity of NR and CA (Table 8) increased with plant age (30 to 60 DAS) but exhibited a significant decrease under Cd-stress (25, 50 or 100 mg Kg^{-1} of soil). Out of the concentrations used, 25 mg Kg^{-1} was least toxic and induced a loss in NR and CA activity by 14% and 24% (Varuna) and 29% and 27% (RH-30), respectively, when compared to their respective controls (0 mg Cd Kg^{-1}), at 30 DAS. The activity of both the enzymes decreased further as the concentration of Cd was increased. The loss of enzymes activity was more in RH-30 than Varuna.

Table 5. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on net photosynthetic rate (P_N; μ mol CO_2 m^{-2} sec^{-1}) and stomatal conductance (g_s; mol H_2O m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Net photosynthetic rate						Stomatal conductance					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	14.08	12.03	13.06	19.54	16.28	17.91	0.061	0.053	0.057	0.089	0.073	0.081
$CdCl_2$ (25mg)	12.42	09.73	11.08	15.29	10.81	13.05	0.036	0.036	0.036	0.073	0.056	0.065
$CdCl_2$ (50mg)	10.49	07.28	08.89	12.62	07.86	10.24	0.028	0.026	0.027	0.056	0.040	0.048
$CdCl_2$ (100mg)	09.28	06.01	07.65	10.94	05.64	08.29	0.018	0.017	0.017	0.040	0.024	0.032
Mean	11.57	08.76		14.60	10.15		0.036	0.033		0.065	0.048	
LSD at 5%												
Varieties	V = 0.80(Sig.)			V = 0.91(Sig.)			V = 0.001(Sig.)			V = 0.004(Sig.)		
Treatment	T = 0.60(Sig.)			T = 1.09(Sig.)			T = 0.002(Sig.)			T = 0.004(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 1.82(Sig.)			V x T = 0.003(Sig.)			V x T = 0.006(Sig.)		

Sig = Significant; NS = Non-significant

Table 6. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on internal CO_2 concentration (C_i; ppm) and transpiration rate (E; m mol m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Internal CO_2 concentration						Transpiration rate					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	319	297	308	366	323	345	3.09	2.97	3.03	4.63	4.35	4.49
$CdCl_2$ (25mg)	286	236	261	337	269	303	2.82	2.50	2.66	4.39	3.57	3.98
$CdCl_2$ (50mg)	264	214	239	322	245	284	2.58	1.99	2.29	4.09	3.25	3.67
$CdCl_2$ (100mg)	243	193	218	313	224	269	2.32	1.76	2.04	3.79	3.01	3.40
Mean	278	235		335	265		2.70	2.30		4.23	3.55	
LSD at 5%												
Varieties	V = 9.55(Sig.)			V = 5.78(Sig.)			V = 0.17(Sig.)			V = 0.27(Sig.)		
Treatment	T = 8.50(Sig.)			T = 8.18(Sig.)			T = 0.11(Sig.)			T = 0.10(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 0.14(Sig.)		

Sig = Significant; NS = Non-significant

Table 7. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on maximum quantum yield of PSII (F_v/F_m) and leaf water potential (MPa) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Maximum quantum yield of PS II						Leaf water potential					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.774	0.762	0.768	0.810	0.780	0.795	-0.65	-0.72	-0.69	-0.46	-0.53	-0.50
$CdCl_2$ (25mg)	0.728	0.698	0.713	0.776	0.735	0.756	-0.75	-0.85	-0.80	-0.53	-0.60	-0.56
$CdCl_2$ (50mg)	0.661	0.619	0.640	0.729	0.682	0.706	-0.82	-0.94	-0.88	-0.59	-0.65	-0.62
$CdCl_2$ (100mg)	0.584	0.534	0.559	0.662	0.624	0.643	-0.89	-1.03	-0.96	-0.66	-0.73	-0.69
Mean	0.687	0.653		0.744	0.705		-0.78	-0.89		-0.56	-0.63	
LSD at 5%												
Varieties	V = 0.003(Sig.)			V = 0.005(Sig.)			V = 0.09(Sig.)			V = 0.05(Sig.)		
Treatment	T = 0.004(Sig.)			T = 0.002(Sig.)			T = 0.06(Sig.)			T = 0.03(Sig.)		
Var. x Treat.	V x T = 0.002(Sig.)			V x T = 0.001(Sig.)			V x T = 0.08(Sig.)			V x T = 0.09(Sig.)		

Sig = Significant; NS = Non-significant

Table 8. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on the activities of nitrate reductase [NR; n mole NO_2 $g^{-1}s^{-1}$ (FM)] and carbonic anhydrase [CA; mole (CO_2) g^{-1} (FM) s^{-1}] in the leaves of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Nitrate reductase activity						Carbonic anhydrase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	420	395	408	433	415	424	2.02	1.94	1.98	2.34	2.23	2.29
$CdCl_2$ (25mg)	359	279	319	392	302	347	1.53	1.42	1.48	2.08	1.86	1.97
$CdCl_2$ (50mg)	327	219	273	366	253	310	1.43	1.28	1.36	1.79	1.32	1.56
$CdCl_2$ (100mg)	285	186	235	337	204	271	1.26	1.06	1.16	1.56	0.97	1.27
Mean	348	270		382	294		1.56	1.43		1.94	1.60	
LSD at 5%												
Varieties	V = 14.04(Sig.)			V = 8.08(Sig.)			V = 0.56(Sig.)			V = 0.12(Sig.)		
Treatment	T = 19.86(Sig.)			T = 11.42(Sig.)			T = 0.10(Sig.)			T = 0.14(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 16.15(Sig.)			V x T = 0.51(Sig.)			V x T = 0.13(Sig.)		

Sig = Significant; NS = Non-significant

4.1.9 Activity of antioxidant system

The activity of antioxidant enzyme system i.e. CAT, POX and SOD (Tables 9) increased with the increasing level of Cd in the soil (0, 25, 50 or 100 mg Kg^{-1}). Against the different concentrations of the metal (25, 50 or 100 mg Cd Kg^{-1}) at 30 day stage, the increase in the activity of POX was 30%, 42% and 57%, of CAT it was 22%, 28% and 36% and that of SOD it was 41%, 55% and 81% in Varuna, compared to the control (0 mg Cd Kg^{-1}). Higher level of these enzymes was maintained even at the latter stage of growth (60 DAS) in stressed plants. The per cent activity of all the three enzymes was lower in RH-30 than Varuna, at both the stages of growth (30 and 60 DAS).

The leaf proline level also showed an increasing trend with increasing level of the metal (Table 10). The highest concentration of Cd (100 mg Kg^{-1}) induced maximum proline accumulation which was 82% and 75% at 30 DAS and 66% and 63% at 60 DAS in Varuna and RH-30, respectively, compared with their controls (0 mg Cd Kg^{-1}). The Varuna therefore accumulated higher proline level as compared to RH-30.

4.1.10 Cd accumulation in root and shoot

The data presented in table 11 revealed that increase in Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) led to the accumulation of more and more metal both in root and shoot where former tissue had more than the latter, irrespective of the variety. At the highest Cd concentration (100 mg Cd Kg^{-1}), the roots of RH-30 possessed 170µg and 221µg Cd g^{-1} dry mass compared with dry mass of the control (0 mg Cd Kg^{-1}) plants. Against the same level of Cd, shoot accumulated only 83µg and 106µg metal g^{-1} of dry mass as compared with control plants whose shoot accumulated Cd 0.50µg and 0.54µg g^{-1} dry mass, at 30 and 60 DAS, respectively. The accumulation of Cd in Varuna was significantly lower than RH-30.

4.1.11 Yield characteristics

It is evident from the data presented in table 12 that the exposure of the plants to the metal-stress (0, 25, 50 or 100 mg Cd Kg^{-1} of soil) reduced all the yield characteristics significantly. The per cent loss in RH-30, against all the three concentrations of Cd (25,

Table 9. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on peroxidase [POX; units (g FM)$^{-1}$] and catalase [CAT; µ mol H_2O_2 decomposed (g FM)$^{-1}$] activity of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Peroxidase activity						Catalase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	12.62	06.84	09.73	16.97	08.87	12.92	417	380	399	437	391	414
$CdCl_2$ (25mg)	16.41	08.08	12.25	20.93	10.14	15.54	509	419	464	485	429	457
$CdCl_2$ (50mg)	17.99	09.33	13.66	22.47	11.43	16.95	534	445	490	507	451	479
$CdCl_2$ (100mg)	19.78	10.61	15.20	24.19	12.77	18.48	567	469	518	535	474	505
Mean	16.70	08.72		21.14	10.80		507	428		491	436	
LSD at 5%												
Varieties	V = 0.45(Sig.)			V = 1.01(Sig.)			V = 14.20(Sig.)			V = 16.62 (Sig.)		
Treatment	T = 0.27(Sig.)			T = 1.21(Sig.)			T = 13.05(Sig.)			T = 7.50(Sig.)		
Var. x Treat.	V x T = 0.90(Sig.)			V x T = 2.02(Sig.)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 10. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on superoxide dismutase [SOD; units g^{-1} (FM)] activity and proline content [μ mol (g FM)$^{-1}$] of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Superoxide dismutase activity						Proline content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	129	112	121	161	136	149	13.60	12.07	12.84	21.04	15.81	18.43
$CdCl_2$ (25mg)	183	132	157	212	157	185	19.26	14.98	17.12	27.96	19.11	23.54
$CdCl_2$ (50mg)	200	155	177	243	179	211	21.97	17.93	19.95	31.32	22.38	26.85
$CdCl_2$ (100mg)	233	192	212	289	202	245	24.79	21.08	22.94	35.04	25.72	30.38
Mean	186	148		226	168		19.91	16.52		28.84	20.76	
LSD at 5%												
Varieties	V = 9.60(Sig.)			V = 4.81(Sig.)			V = 1.32(Sig.)			V = 1.76(Sig.)		
Treatment	T = 13.57(Sig.)			T = 6.80(Sig.)			T = 0.76(Sig.)			T = 1.51(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 9.62(Sig.)			V x T = 3.21(Sig.)			V x T = 3.13(Sig.)		

Sig = Significant; NS = Non-significant

Table 11. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root and shoot Cd content (µg g^{-1} DW) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root Cd content						Shoot Cd content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.48	0.57	0.53	0.60	0.75	0.68	0.37	0.50	0.44	0.47	0.54	05.05
$CdCl_2$ (25mg)	47.11	58.02	52.57	49.63	62.90	56.27	10.28	14.83	12.56	12.13	20.40	16.27
$CdCl_2$ (50mg)	97.48	118.70	108.09	103.20	150.40	126.80	25.95	37.53	31.74	30.38	48.66	39.52
$CdCl_2$ (100mg)	148.30	169.60	158.95	146.09	220.80	183.45	59.81	83.48	71.65	66.96	106.23	86.60
Mean	73.34	86.72		74.88	108.71		24.10	34.09		27.49	43.96	
LSD at 5%												
Varieties	V = 0.07(Sig.)			V = 0.05(Sig.)			V = 0.12(Sig.)			V = 0.06(Sig.)		
Treatment	T = 15.18(Sig.)			T = 6.64(Sig.)			T = 6.28(Sig.)			T = 7.79(Sig.)		
Var. x Treat.	V x T = 15.20(Sig.)			V x T = (NS)			V x T = 8.24(Sig.)			V x T = 9.11(Sig.)		

Sig = Significant; NS = Non-significant

Table 12. Effect of different concentrations of soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on yield characteristics of *Brassica juncea* cv. Varuna and RH-30 at harvest

Treatment	Pods plant^{-1}			Seeds pod^{-1}			100 seed mass (mg)			Seed yield plant^{-1} (g)		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	222	213	218	12.63	11.97	12.30	311	303	307	8.72	7.73	8.22
$CdCl_2$ (25mg)	195	176	186	12.05	11.17	11.61	298	283	291	7.00	5.56	6.28
$CdCl_2$ (50mg)	182	161	172	11.73	10.86	11.30	285	269	277	6.08	4.70	5.39
$CdCl_2$ (100mg)	170	150	160	11.11	09.98	10.55	272	255	264	5.14	3.82	4.48
Mean	192	175		11.88	11.00		292	278		6.74	5.45	
LSD at 5%												
Varieties	V = 7.24(Sig.)			V = 0.56(Sig.)			V = 14.24(Sig.)			V = 0.16(Sig.)		
Treatment	T = 10.23(Sig.)			T = 0.30(Sig.)			T = 12.78(Sig.)			T = 0.22(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.11(Sig.)			V x T = (NS)			V x T = 0.32(Sig.)		

Sig = Significant; NS = Non-significant

50 or 100 mg Kg^{-1}) was significantly higher than in Varuna. The maximum loss, as compared to the control (0 mg Cd Kg^{-1}), was noticed in RH-30 at the highest concentration of Cd (100 mg Kg^{-1} of soil) which was 30%, 17%, 16% and 51% for the number of pods per plant, number of seed per pod, mass of 100 seeds and seed yield per plant, at harvest, respectively.

4.2 EXPERIMENT 2

To study the impact of BR analogues (HBL/EBL; 10^{-8} M) on two varieties of *B. juncea* L., Czern & Coss; Varuna and RH-30, this experiment was laid in a simple randomized block design during the winter season of 2009-10. All the agricultural practices to raise the plants were kept the same as in Experiment 1. The foliage of 29 days old plants was sprayed with double distilled water (control), tween-20 (0.05%), ethanol (5%), HBL (10^{-8} M) or EBL (10^{-8}M) solutions. Samples were collected randomly at 30 and 60 DAS to study the growth parameters and at 120 DAS for yield parameters as in Experiment 1. The growth and yield characteristics are explained below:

4.2.1 Root and shoot length

The length of root and shoot increased with the advancement of plant age (Table 13). Both the varieties of mustard that received BR application showed significant increase, at latter stage (60 DAS) of growth in their root and shoot length which was 39% and 41%, and 34% and 33% by HBL and 54% and 49%, and 36% and 37% by EBL in Varuna and RH-30, respectively, compared to the water sprayed control plants. At 30 DAS, BRs generated the values comparable with water sprayed control. However, foliar spray of tween-20 or ethanol had no effect on the length of root or shoot, both at 30 and 60 DAS, compared to control plants, treated with water only. Out of the two BRs, EBL excelled in its effect over HBL. Varuna exhibited higher values both for root and shoot, compared to RH-30.

4.2.2 Fresh and dry mass of root

With age of the plants, the values for fresh and dry mass of root and shoot (Table 14) improved. Moreover, the application of BRs (10^{-8} M HBL/EBL) significantly

increased these values further, noted at 60 DAS, whereas, tween-20 and ethanol had the impact comparable with water-sprayed controls. Plants that received 10^{-8} M of HBL or EBL showed 33% and 40% (Varuna) and 31% and 38% (RH-30) increase in root fresh mass, whereas the root dry mass increased by 38% and 46% (Varuna) and 29% and 39% (RH-30) at 60 DAS, compared to water sprayed plants. EBL, therefore, proved better than HBL. Varuna was more responsive to the foliar spray of BRs than RH-30.

4.2.3 Fresh and dry mass of shoot

Fresh and dry mass of shoot (Table 15) followed a similar pattern to that of the root (Section 4.2.2). Plant foliage that received 10^{-8} M solution of HBL or EBL possessed significantly higher fresh and dry mass, at 60 DAS. EBL improved the fresh and dry mass of shoot more than HBL where the per cent increase in shoot fresh mass was 39% and 36% and dry mass was 45% and 44% in Varuna and RH-30, as compared to control (sprayed with DDW). The increase was more prominent in Varuna than RH-30.

4.2.4 Leaf area

Leaf area increased with the advancement of age from 30 to 60 days, in both the varieties (Table 16). The impact of tween-20 and ethanol was comparable with that of the water sprayed control plants. The treatment with HBL/EBL (10^{-8} M) significantly increased the leaf area at 60 DAS. The per cent increase of leaf area for Varuna and RH-30 was 22% and 19% with HBL and was 26% and 25% with EBL, at 60 DAS, compared to the water-sprayed control. EBL excelled in promoting leaf area over HBL. Moreover, Varuna was more responsive than RH-30 and possessed larger leaf area.

4.2.5 SPAD chlorophyll value

The SPAD values of chlorophyll significantly increased with the plant age (Table 16) and had comparable values with water, tween-20 or ethanol. Foliar spray of 10^{-8} M solution of HBL or EBL elevated the level at both the stages of growth (30 and 60 DAS). The per cent increase of leaf chlorophyll content by brassinosteroid was more at 30 DAS as compared to 60 DAS. Application of HBL improved the chlorophyll level by 19% and 15%, whereas, EBL by 23% and 21% in Varuna and RH-30 at 30 DAS, respectively, compared to their controls (sprayed with water). EBL generated more promising response

Table 13. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on root and shoot length (cm) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root length						Shoot length					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	16.71	15.00	15.86	25.39	22.40	23.90	28.09	25.21	26.65	73.21	68.59	70.90
Tween-20 (0.5%)	16.68	14.90	15.79	25.46	22.45	23.96	28.12	25.09	26.61	74.30	67.90	71.10
Ethanol (5%)	16.70	15.03	15.87	25.62	22.59	24.11	28.15	25.15	26.65	72.40	69.20	70.80
HBL (10^{-8}M)	16.72	15.07	15.90	41.98	38.13	40.06	28.27	25.29	26.78	111.06	103.22	107.14
EBL (10^{-8}M)	16.79	15.08	15.94	55.06	43.97	49.52	27.72	25.32	26.52	115.41	107.80	111.61
Mean	16.72	15.02		34.70	29.91		28.07	25.21		89.28	83.34	
LSD at 5%												
Varieties	V = 0.75(Sig.)			V = 1.69(Sig.)			V = 2.80(Sig.)			V = 6.49(Sig.)		
Treatment	T = (NS)			T = 2.09(Sig.)			T = (NS)			T = 4.26(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 1.55(Sig.)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 14. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on root fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root fresh mass						Root dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	2.61	2.34	2.48	5.08	4.51	4.80	0.77	0.60	0.68	1.54	1.17	1.36
Tween-20 (0.5%)	2.70	2.30	2.50	5.12	4.45	4.79	0.77	0.60	0.68	1.55	1.19	1.37
Ethanol (5%)	2.57	2.38	2.48	5.02	4.55	4.79	0.78	0.60	0.69	1.52	1.15	1.34
HBL (10^{-8}M)	2.64	2.37	2.51	7.62	6.53	7.08	0.78	0.59	0.69	2.50	1.66	2.08
EBL (10^{-8}M)	2.69	2.39	2.54	8.51	7.16	7.84	0.77	0.60	0.68	2.89	1.96	2.43
Mean	2.64	2.36		6.27	5.44		0.77	0.60		2.00	1.43	
LSD at 5%												
Varieties	V = 0.13(Sig.)			V = 0.50(Sig.)			V = 0.04(Sig.)			V = 0.12(Sig.)		
Treatment	T = (NS)			T = 0.62(Sig.)			T = (NS)			T = 0.16(Sig.)		
Var. x Treat.	V x T = 0.29(Sig.)			V x T = 1.02(Sig.)			V x T = (NS)			V x T = 0.20(Sig.)		

Sig = Significant; NS = Non-significant

Table 15. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on shoot fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Shoot fresh mass						Shoot dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	6.27	5.56	5.92	11.98	9.84	10.91	1.60	1.49	1.55	3.12	2.69	2.91
Tween-20 (0.5%)	6.20	5.52	5.86	12.00	9.92	10.96	1.57	1.43	1.50	3.14	2.66	2.90
Ethanol (5%)	6.33	5.52	5.93	11.86	9.78	10.82	1.63	1.42	1.53	3.09	2.72	2.91
HBL (10^{-8}M)	6.32	5.60	5.96	18.28	14.27	16.28	1.64	1.44	1.54	5.37	4.47	4.92
EBL (10^{-8}M)	6.34	5.65	6.00	19.76	15.58	17.67	1.69	1.51	1.60	5.71	4.76	5.24
Mean	6.29	5.57		14.78	11.88		1.63	1.46		4.09	3.46	
LSD at 5%												
Varieties	V = 0.32(Sig.)			V = 1.08(Sig.)			V = 0.13(Sig.)			V = 0.27(Sig.)		
Treatment	T = (NS)			T = 1.29(Sig.)			T = (NS)			T = 0.26(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.40(Sig.)			V x T = (NS)			V x T = 0.37(Sig.)		

Sig = Significant; NS = Non-significant

Table 16. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on leaf area (cm^2) and SPAD value of chlorophyll of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Leaf area						SPAD value of chlorophyll					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	26.40	23.77	25.09	37.50	34.60	36.05	35.96	29.14	32.55	43.78	34.56	39.17
Tween-20 (0.5%)	26.42	23.60	25.01	37.38	34.49	35.94	35.95	29.10	32.53	43.77	34.52	39.15
Ethanol (5%)	26.48	23.71	25.10	37.42	34.52	35.97	35.98	29.16	32.57	43.81	34.57	39.19
HBL (10^{-8}M)	26.39	23.76	25.08	48.11	42.56	45.34	44.28	34.38	39.33	52.58	38.97	45.78
EBL (10^{-8}M)	26.43	23.79	25.11	50.72	46.07	48.40	46.97	37.04	42.01	54.74	41.70	48.22
Mean	26.42	23.73		42.23	38.45		39.83	31.76		47.74	36.86	
LSD at 5%												
Varieties	V = 1.14(Sig.)			V = 3.21(Sig.)			V = 2.38(Sig.)			V = 2.15(Sig.)		
Treatment	T = (NS)			T = 2.32(Sig.)			T = 3.17(Sig.)			T = 3.14(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.47(Sig.)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

than HBL. Varuna exhibited higher chlorophyll content than RH-30 at the two stages of growth.

4.2.6 Photosynthetic parameters

It is evident from tables 17-19 that the foliage of plants, that received brassinosteroid analogues (10^{-8} M, HBL/EBL) had significant increase in net photosynthetic rate (P_N) and the associated parameters (stomatal conductance, g_s internal CO_2 concentration, C_i; transpiration rate, E and maximum quantum yield of PSII. Fv/Fm) at both the growth stages (30 and 60 days). However, application of tween-20 or ethanol generated values comparable with that of the water sprayed control. Out of two brassinosteroid analogues EBL excelled in its effect over HBL both at 30 and 60 day stages. At 30 DAS, the leaves of Varuna and RH-30 sprayed with EBL exhibited maximum increase in P_N (10% and 9%), g_s (54% and 44%), Ci (20% and 21%), E (34% and 23%) and Fv/Fm (5% and 4%) with respect to water sprayed control plants, however, the impact declined at 60 DAS. Varuna was more responsive to brassinosteroid spray than RH-30.

4.2.7 Leaf water potential (LWP)

Table 19 indicates that the LWP as the growth progressed from 30 to 60 days. Lower values of LWP were recorded in RH-30 as compared to Varuna at the two growth stages. Treatment with tween-20/ethanol had no effect on LWP as the values were comparable with the water sprayed, control. However, plants that received the foliar spray of HBL/EBL (10^{-8} M) had significantly higher LWP, compared with the control. Out of the two BRs, EBL was more promising and it increased the LWP by 56% and 47% (Varuna) and 44% and 38% (RH-30) at 30 and 60 DAS.

4.2.8 Nitrate reductase (NR) and carbonic anhydrase (CA) activity

It is evident from table 20 that the activity of NR and CA enzymes increased with plant age (30 to 60 day stage) and foliar spray of HBL/EBL (10^{-8}M) further increased these values. Out of the two brassinosteroid analogues, EBL was better as compared to HBL which induced the NR activity 22% and 15% and CA activity 28% and 21% more

Table 17. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on net photosynthetic rate (P_N; μ mol CO_2 $m^{-2}sec^{-1}$) and stomatal conductance (g_s; mol H_2O m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Net photosynthetic rate						Stomatal conductance					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	14.05	12.02	13.04	19.53	16.26	17.90	0.061	0.053	0.057	0.088	0.073	0.081
Tween-20 (0.5%)	14.06	12.02	13.04	19.51	16.23	17.87	0.060	0.054	0.057	0.088	0.070	0.079
Ethanol (5%)	14.09	12.05	13.07	19.54	16.26	17.90	0.061	0.052	0.057	0.086	0.071	0.079
HBL (10^{-8}M)	15.28	13.01	14.15	20.84	16.89	18.87	0.100	0.074	0.087	0.140	0.110	0.125
EBL (10^{-8}M)	15.61	13.17	14.39	21.02	17.12	19.07	0.130	0.096	0.113	0.155	0.123	0.139
Mean	14.62	12.45		20.09	16.55		0.082	0.066		0.110	0.089	
LSD at 5%												
Varieties	V = 1.05(Sig.)			V = 0.79(Sig.)			V = 0.004(Sig.)			V = 0.006(Sig.)		
Treatment	T = 0.26(Sig.)			T = 0.24(Sig.)			T = 0.011(Sig.)			T = 0.012(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 1.76(Sig.)			V x T = 0.014(Sig.)			V x T = 0.015(Sig.)		

Sig = Significant; NS = Non-significant

Table 18. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on internal CO_2 concentration (C_i; ppm) and transpiration rate (E; m mol m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Internal CO_2 concentration						Transpiration rate					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	312	288	300	357	319	338	3.20	2.97	3.09	4.65	4.26	4.46
Tween-20 (0.5%)	317	284	301	352	319	336	3.16	2.99	3.08	4.63	4.30	4.47
Ethanol (5%)	312	289	301	359	322	341	3.19	2.96	3.08	4.66	4.28	4.47
HBL (10^{-8}M)	378	338	358	389	342	366	4.42	3.47	3.95	5.94	4.87	5.41
EBL (10^{-8}M)	399	360	380	407	354	381	4.77	3.88	4.33	6.31	5.08	5.70
Mean	344	312		373	331		3.75	3.25		5.24	4.56	
LSD at 5%												
Varieties	V = 20.16(Sig.)			V = 4.71(Sig.)			V = 0.28(Sig.)			V = 0.06(Sig.)		
Treatment	T = 20.12(Sig.)			T = 7.45(Sig.)			T = 0.34(Sig.)			T = 0.09(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 0.13(Sig.)		

Sig = Significant; NS = Non-significant

Table 19. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on maximum quantum yield of PSII (F_v/F_m) and leaf water potential (MPa) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Maximum quantum yield of PS II						Leaf water potential					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.775	0.766	0.771	0.806	0.778	0.792	-0.63	-0.70	-0.67	-0.47	-0.52	-0.50
Tween-20 (0.5%)	0.769	0.764	0.767	0.798	0.775	0.787	-0.64	-0.69	-0.67	-0.47	-0.51	-0.49
Ethanol (5%)	0.773	0.762	0.768	0.789	0.772	0.781	-0.63	-0.69	-0.66	-0.46	-0.51	-0.49
HBL (10^{-8}M)	0.796	0.781	0.789	0.818	0.790	0.804	-0.47	-0.54	-0.51	-0.37	-0.43	-0.40
EBL (10^{-8}M)	0.813	0.803	0.808	0.821	0.806	0.814	-0.41	-0.48	-0.45	-0.32	-0.37	-0.35
Mean	0.785	0.775		0.806	0.784		-0.56	-0.62		-0.42	-0.47	
LSD at 5%												
Varieties	V = 0.005(Sig.)			V = 0.008(Sig.)			V = 0.05(Sig.)			V = 0.04(Sig.)		
Treatment	T = 0.004(Sig.)			T = 0.003(Sig.)			T = 0.03(Sig.)			T = 0.03(Sig.)		
Var. x Treat.	V x T = 0.001(Sig.)			V x T = 0.004(Sig.)			V x T = (NS)			V x T = 0.05(Sig.)		

Sig = Significant; NS = Non-significant

than control in Varuna and RH-30, respectively, at 30 DAS which are higher than at day 60. However, plants sprayed with solutions of tween-20 or ethanol had no impact. Varuna possessed higher enzymes activity than RH-30.

4.2.9 Activity of antioxidant system

The data presented in tables 21 and 22 revealed the increasing trend of antioxidant enzymes activity (CAT, POX and SOD) with the growth progression from 30 to 60 days. Moreover, application of 10^{-8} M HBL or EBL further increased the activity of these enzymes and also the level of proline at both the stages of growth. The per cent increase in the activity of enzymes and proline level was more at 30 DAS than at 60 DAS. Out of the two brassinosteroid analogues EBL was more effective than HBL. It promoted the activity of POX by 42% and 30%, CAT 32% and 21%, SOD by 37% and 20%, and leaf proline level by 32% and 21% in Varuna that generated better response than RH-30, at 30 and 60 DAS, respectively, as compared to control plants.

4.2.10 Cd accumulation in root and shoot

Mustard plants accumulated more Cd with the increase of plant age (Table 23). At 30 DAS, no significant effect of brassinosteroids appeared on the root or shoot Cd content but the level decreased at the latter stage (60 DAS). Moreover, roots accumulated higher level of Cd as compared to shoot. EBL was more effective that decreased Cd level in Varuna and RH-30 by 17% and 11% in roots and by 14% and 13% in shoot, respectively, at 60 DAS, compared to control plants. RH-30 accumulated more Cd than Varuna.

4.2.11 Yield characteristics

The data in table 24 revealed that foliar application of either of the brassinosteroid analogues (HBL/EBL; 10^{-8} M) significantly increased the number of pods per plant and seed yield per plant with EBL excelling in its effects than HBL in both the varieties. The EBL spray increased the number of pods per plant by 32% and 26% and seed yield per plant by 38% and 32% in Varuna and RH-30 respectively, compared to their water

Table 20. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on the activities of nitrate reductase [NR; n mole NO_2 $g^{-1}s^{-1}$ FM] and carbonic anhydrase [CA; mole (CO_2) g^{-1} leaf (FM) s^{-1}] in the leaves of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Nitrate reductase activity						Carbonic anhydrase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	419	397	408	435	418	427	2.03	1.96	2.00	2.37	2.25	2.31
Tween-20 (0.5%)	417	394	406	422	420	421	2.00	1.92	1.96	2.33	2.23	2.28
Ethanol (5%)	421	396	409	419	418	419	2.04	1.96	2.00	2.38	2.27	2.33
HBL (10^{-8}M)	514	440	477	540	469	505	2.81	2.49	2.65	2.92	2.55	2.74
EBL (10^{-8}M)	537	463	500	552	499	526	2.94	2.62	2.78	3.23	2.69	2.96
Mean	562	418		474	445		2.36	2.19		2.65	2.40	
LSD at 5%												
Varieties	V = 13.48(Sig.)			V = 5.30(Sig.)			V = 0.17(Sig.)			V = 0.20(Sig.)		
Treatment	T = 22.14(Sig.)			T = 5.09(Sig.)			T = 0.12(Sig.)			T = 0.11(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.37(Sig.)			V x T = 0.15(Sig.)		

Sig = Significant; NS = Non-significant

Table 21. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on peroxidase [POX; units (g FM)$^{-1}$] and catalase [CAT; μ mol H_2O_2 decomposed (g FM)$^{-1}$] activity of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Peroxidase activity						Catalase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	13.00	07.25	10.13	17.42	09.36	13.39	419	377	398	438	388	413
Tween-20 (0.5%)	12.80	07.19	10.00	17.11	09.30	13.21	421	375	398	435	385	410
Ethanol (5%)	12.50	07.13	09.82	17.24	09.18	13.21	415	382	399	437	390	414
HBL (10^{-8}M)	18.46	09.80	14.13	22.90	11.43	17.17	507	426	467	508	421	465
EBL (10^{-8}M)	22.19	11.27	16.53	24.46	12.97	18.72	622	498	560	548	468	508
Mean	15.79	8.53		19.83	10.45		477	412		473	410	
LSD at 5%												
Varieties	V = 0.80(Sig.)			V = 0.92(Sig.)			V = 5.70(Sig.)			V = 10.91(Sig.)		
Treatment	T = 0.82(Sig.)			T = 1.34(Sig.)			T = 9.01(Sig.)			T = 17.24(Sig.)		
Var. x Treat.	V x T = 0.40(Sig.)			V x T = 0.48(Sig.)			V x T = 12.75(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 22. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on superoxide dismutase [SOD; units g^{-1} (FM)] activity and proline content [μ mol (g FM)$^{-1}$] of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Superoxide dismutase activity						Proline content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	132	113	123	162	123	143	12.90	12.00	12.45	19.21	16.04	17.63
Tween-20 (0.5%)	130	110	120	163	121	142	12.98	12.01	12.50	19.13	15.98	17.56
Ethanol (5%)	127	108	118	157	123	140	13.02	12.06	12.54	19.16	15.94	17.55
HBL (10^{-8}M)	163	133	148	191	139	165	17.95	14.45	16.20	23.03	18.09	20.56
EBL (10^{-8}M)	205	161	183	203	148	176	19.15	16.55	17.85	24.30	19.33	21.82
Mean	151	125		175	131		15.20	13.41		20.97	17.08	
LSD at 5%												
Varieties	V = 6.57(Sig.)			V = 13.37(Sig.)			V = 0.82(Sig.)			V = 1.42(Sig.)		
Treatment	T = 10.38(Sig.)			T = 8.33(Sig.)			T =1.20(Sig.)			T = 1.14(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 10.54(Sig.)			V x T = 1.17(Sig.)			V x T = 2.15(Sig.)		

Sig = Significant; NS = Non-significant

Table 23. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on root and shoot Cd content (µg g^{-1}DW) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root Cd content						Shoot Cd content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.53	0.56	0.55	0.71	0.75	0.73	0.36	0.52	0.44	0.49	0.53	0.51
Tween-20 (0.5%)	0.52	0.57	0.55	0.70	0.73	0.72	0.36	0.51	0.44	0.50	0.53	0.52
Ethanol (5%)	0.53	0.56	0.55	0.70	0.74	0.72	0.35	0.51	0.43	0.51	0.53	0.52
HBL (10^{-8}M)	0.52	0.54	0.53	0.67	0.71	0.69	0.36	0.52	0.44	0.45	0.49	0.47
EBL (10^{-8}M)	0.52	0.54	0.53	0.60	0.66	0.63	0.35	0.51	0.44	0.44	0.47	0.46
Mean	0.52	0.55		0.68	0.72		0.36	0.51		0.48	0.51	
LSD at 5%												
Varieties	V = 0.02(Sig.)			V = 0.03(Sig.)			V = 0.04(Sig.)			V = 0.03(Sig.)		
Treatment	T = (NS)			T = 0.03(Sig.)			T = (NS)			T = 0.01(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.34(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 24. Effect of tween-20, ethanol and brassinosteroids (HBL or EBL; 10^{-8} M) on yield characteristics of *Brassica juncea* cv. Varuna and RH-30 at harvest

Treatment	Pods plant^{-1}			Seeds pod^{-1}			100 seed mass (mg)			Seed yield plant^{-1} (g)		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	230	214	222	12.70	12.04	12.37	314	310	312	9.17	7.99	8.58
Tween-20 (0.5%)	227	210	219	12.60	11.99	12.30	315	307	311	9.01	7.73	8.37
Ethanol (5%)	225	212	219	12.68	12.03	12.36	314	311	313	8.96	7.93	8.45
HBL (10^{-8}M)	332	284	308	12.91	12.22	12.56	326	321	324	13.97	11.14	12.55
EBL (10^{-8}M)	340	290	315	12.98	12.29	12.64	328	322	325	14.48	11.48	12.98
Mean	271	242		12.77	12.11		319	314		11.12	9.25	
LSD at 5%												
Varieties	V = 1.30(Sig.)			V = 1.26(Sig.)			V = 3.01(Sig.)			V = 0.56(Sig.)		
Treatment	T = 3.37(Sig.)			T = (NS)			T = (NS)			T = 0. 21(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.58(Sig.)			V x T = (NS)			V x T = 0.74(Sig.)		

Sig = Significant; NS = Non-significant

sprayed control plants. Moreover, the yield improvement in Varuna was better as compared to RH-30, against the foliar spray of brassinosteroid analogues.

4.3 EXPERIMENT 3

This experiment was conducted with the objective to study the effect of sodium nitroprusside (10^{-4}, 10^{-5} or 10^{-6} M; the donor of NO) or potassium ferricyanide (10^{-4} M; non-NO donor) in the two mustard varieties. The agricultural practices and the parameters were same as in Experiment 1. Plants were sprayed either with distilled water (control), $K_3[Fe(CN)_6]$ (10^{-4} M) or SNP (10^{-4}, 10^{-5} or 10^{-6} M) at 29 day stage of growth (DAS). Samples were collected at 30 and 60 DAS to analyze various parameters. Plants were harvested at maturity (120 DAS) to study the yield attributes. The growth and yield characteristics are explained below:

4.3.1 Root and shoot length

The length of root and shoot increased with the advancement of plant age from 30 to 60 day stage (Table 25). All the concentrations of SNP (10^{-4}, 10^{-5} or 10^{-6} M) improved the root and shoot length, noted at 60 DAS. Moreover, application of 10^{-5} M SNP proved best that increased the root length by 29% and 24% and shoot length by 34% and 31% in Varuna and RH-30 respectively, compared to control plants. However, application of potassium ferricyanide had no impact. Varuna excelled in its responses over RH-30.

4.3.2 Fresh and dry mass of root

As evident from table 26, the fresh and dry mass of root increased from 30 day stage to 60 day stage. Moreover, the application of SNP further increased the values of fresh and dry mass of root at 60 DAS in both the varieties. Out of the three concentration (10^{-4}, 10^{-5} or 10^{-6} M) of SNP, 10^{-5} M proved best which increased the fresh mass by 32% and 29% and dry mass by 40% and 38% in Varuna and RH-30 respectively, compared to the control plants at 60 DAS. Varuna was more responsive to SNP treatment than RH-30 but did not show any impact to potassium ferricyanide.

4.3.3 Fresh and dry mass of shoot

Fresh and dry mass of shoot exhibited an increasing trend as that of root (Section 4.3.2) from 30 to 60 DAS. The foliar spray of potassium ferricyanide (10^{-4} M) induced no change in the fresh and dry mass of shoot and the values were comparable with the controls (Table 27). However, foliar spray of SNP improved the shoot fresh and dry mass significantly at 60 DAS, where, out of three concentrations of SNP (10^{-4}, 10^{-5} or 10^{-6} M); 10^{-5} M proved best and increased the shoot fresh mass by 36% and 33% and shoot dry mass by 38% and 35% in Varuna and RH-30 respectively compared to water sprayed control plants. The per cent SNP-induced increase of fresh and dry mass of shoot in Varuna was more than RH-30.

4.3.4 Leaf area

As evident from table 28, the leaf area of the plants increased from 30 to 60 DAS in both the varieties. SNP significantly promoted the leaf area at 60 day stage. However, potassium ferricyanide was ineffective in either of the variety. Spray with 10^{-5}M SNP solution induced maximum leaf area which was 18% more in Varuna and 16% in RH-30 over water sprayed control plants at 60 DAS.

4.3.5 SPAD chlorophyll value

The leaf chlorophyll content (SPAD value) increased with the advancement of plant age from 30 to 60 day stage of growth in both the varieties (Table 28). The foliar spray of SNP (10^{-4}, 10^{-5} or 10^{-6} M) significantly increased the SPAD value as compared to plants treated with water (control) or potassium ferricyanide (10^{-4} M) at 30 and 60 DAS. The three concentrations of SNP (10^{-4}, 10^{-5} or 10^{-6} M) increased the SPAD value of chlorophyll in Varuna, by 11%, 16% or 6% while in RH-30 by 9%, 14% or 5%, respectively, over the water sprayed controls at 30 DAS. However, as the growth progressed the differences became less sharp. Varuna possessed higher values of chlorophyll level than RH-30.

Table 25. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on root and shoot length (cm) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root length						Shoot length					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	16.68	14.96	15.82	25.21	22.17	23.69	28.00	25.18	26.59	72.98	68.30	70.64
$K_3Fe(CN)_6 10^{-4}$M	16.65	14.97	15.81	25.23	22.20	23.72	28.03	25.21	26.62	72.96	68.32	70.64
SNP 10^{-4}M	16.70	15.01	15.86	33.28	28.15	30.72	28.06	25.24	26.65	100.13	89.42	94.78
SNP 10^{-5}M	16.72	15.03	15.88	35.29	29.25	32.26	28.10	25.26	26.68	110.92	99.22	105.07
SNP 10^{-6}M	16.68	14.99	15.84	32.51	27.49	30.00	28.05	25.22	26.64	105.09	94.15	99.62
Mean	16.69	14.99		30.30	25.85		28.05	25.22		92.42	83.88	
LSD at 5%												
Varieties	V = 0.68(Sig.)			V = 1.32(Sig.)			V = 2.02(Sig.)			V = 3.10(Sig.)		
Treatment	T = (NS)			T = 1.09(Sig.)			T = (NS)			T = 4.90(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 26. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on root fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root fresh mass						Root dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	2.59	2.33	2.46	5.01	4.48	4.75	0.74	0.58	0.66	1.50	1.12	1.31
$K_3Fe(CN)_6 10^{-4}$M	2.64	2.31	2.48	5.16	4.54	4.85	0.73	0.56	0.65	1.72	1.19	1.46
SNP 10^{-4}M	2.78	2.38	2.58	6.86	5.82	6.34	0.75	0.58	0.67	2.26	1.60	1.93
SNP 10^{-5}M	2.81	2.42	2.62	7.33	6.35	6.84	0.77	0.60	0.69	2.50	1.82	2.16
SNP 10^{-6}M	2.66	2.31	2.49	6.56	5.64	6.10	0.76	0.59	0.68	2.18	1.53	1.86
Mean	2.70	2.35		6.18	5.37		0.75	0.58		2.03	1.45	
LSD at 5%												
Varieties	V = 0.19(Sig.)			V = 0.52(Sig.)			V = 0.04(Sig.)			V = 0.13(Sig.)		
Treatment	T = (NS)			T = 0.45(Sig.)			T = (NS)			T = 0.12(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.80(Sig.)			V x T = (NS)			V x T = 0.20(Sig.)		

Sig = Significant; NS = Non-significant

Table 27. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on shoot fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Shoot fresh mass						Shoot dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	6.22	5.28	5.75	11.79	9.79	10.79	1.55	1.43	1.49	3.09	2.76	2.93
$K_3Fe(CN)_6 10^{-4}$M	6.32	6.05	6.19	11.96	9.81	10.89	1.78	1.55	1.67	3.29	2.84	3.07
SNP 10^{-4}M	6.52	5.60	6.06	17.21	13.80	15.51	1.80	1.57	1.69	4.72	4.07	4.40
SNP 10^{-5}M	6.83	6.01	6.42	18.39	14.68	16.54	1.82	1.59	1.71	4.95	4.25	4.60
SNP 10^{-6}M	6.47	5.34	5.91	16.97	13.60	15.29	1.80	1.56	1.68	4.60	3.86	4.23
Mean	6.47	5.66		15.26	12.34		1.75	1.54		4.13	3.56	
LSD at 5%												
Varieties	V = 0.53(Sig.)			V = 1.13(Sig.)			V = 0.13(Sig.)			V = 0.31(Sig.)		
Treatment	T = (NS)			T = 0.87(Sig.)			T = (NS)			T = 0.18(Sig.)		
Var. x Treat.	V x T = 0.74 (Sig.)			V x T = 2.13(Sig.)			V x T = (NS)			V x T = 0.52(Sig.)		

Sig = Significant; NS = Non-significant

Table 28. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on leaf area (cm^2) and SPAD value of chlorophyll of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Leaf area						SPAD value of chlorophyll					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	24.03	22.07	23.05	37.30	34.42	35.86	35.72	29.02	32.37	43.49	34.38	38.94
$K_3Fe(CN)_6 10^{-4}M$	24.00	22.12	23.06	37.44	34.57	36.01	35.74	29.07	32.41	43.49	34.42	38.96
SNP $10^{-4}M$	24.09	22.19	23.14	44.27	39.58	41.93	40.24	31.76	36.00	48.41	37.18	42.80
SNP $10^{-5}M$	24.10	22.04	23.07	45.52	40.79	43.16	42.58	33.82	38.20	50.92	39.65	45.29
SNP $10^{-6}M$	24.02	21.98	23.00	43.52	38.70	41.11	38.22	30.70	34.46	47.11	35.99	41.55
Mean	24.05	22.08		41.61	37.61		38.50	30.87		47.68	36.32	
LSD at 5%												
Varieties	V = 1.22(Sig.)			V = 3.12(Sig.)			V = 1.95(Sig.)			V = 2.22(Sig.)		
Treatment	T = (NS)			T = 1.19(Sig.)			T = 2.05(Sig.)			T = 2.34(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 3.26(Sig.)			V x T = 2.13(Sig.)			V x T = 2.49(Sig.)		

Sig = Significant; NS = Non-significant

4.3.6 Photosynthetic parameters

A progressive increase in photosynthetic parameters (net photosynthetic rate; P_N, stomatal conductance; g_s, internal CO_2 concentration; C_i, transpiration rate; E and maximum quantum yield of PSII; Fv/Fm) was observed with the increase of plant age from 30 to 60 DAS (Tables 29-31). The foliar spray of three concentrations (10^{-4}, 10^{-5} or 10^{-6}M) of SNP increased the values of photosynthetic parameters in both the varieties, the per cent increase being more at 30 DAS than at 60 DAS, compared to water or potassium ferricyanide sprayed plants which had similar values. The medium concentration 10^{-5} M SNP generated maximum responses that increased P_N, g_s, C_i, E and Fv/Fm by 5%, 35%, 16%, 23% and 3% in Varuna and 4%, 51%,14 %, 29% and 2% in RH-30, compared to the control plants at 30 DAS. Varuna was more responsive than RH-30.

4.3.7 Leaf water potential (LWP)

With the plant age, the LWP increased in both the varieties (Table 31). The foliar spray of water or potassium ferricyanide generated comparable values of LWP. However, application of SNP to the plant foliage increased LWP values both at 30 and 60 DAS. Out of the three concentration (10^{-4}, 10^{-5} or 10^{-6} M) of SNP, 10^{-5} M proved best and increased the LWP values by 27% and 24% in Varuna and by 25% and 18% in RH-30 at 30 and 60 DAS, respectively, compared to their control plants. Varuna exhibited higher values than RH-30.

4.3.8 Nitrate reductase (NR) and carbonic anhydrase (CA) activity

As the plant age progressed from 30 to 60 DAS, the activity of NR and CA increased in both the varieties (Table 32). There was no shift in the activity of these enzymes in the plants, sprayed with potassium ferricyanide, and the values were comparable with that of the controls. However, the application of SNP (10^{-4}, 10^{-5} or 10^{-6} M) increased the activity of NR and CA both at 30 and 60 DAS. The medium concentration (10^{-5} M) of SNP proved the best, in terms of per cent; it increased NR activity by 12% and 13% in Varuna and by 9% and 11% in RH-30 at 30 and 60 DAS, respectively, compared with water sprayed controls. Similarly for CA, the per cent increase was 22% and 24% in Varuna and 21% and 12% in RH-30 at 30 and 60 DAS, respectively. Varuna was more responsive to SNP than RH-30.

Table 29. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on net photosynthetic rate (P_N; μ mol CO_2 m^{-2} sec^{-1}) and stomatal conductance (g_s; mol H_2O m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Net photosynthetic rate						Stomatal conductance					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	14.06	12.03	13.05	19.51	16.25	17.88	0.063	0.043	0.053	0.095	0.072	0.084
$K_3Fe(CN)_6 10^{-4}M$	14.08	12.01	13.05	19.55	16.28	17.92	0.079	0.054	0.067	0.094	0.073	0.084
SNP $10^{-4}M$	14.59	12.52	13.56	19.94	16.58	18.26	0.082	0.070	0.076	0.119	0.096	0.108
SNP $10^{-5}M$	14.84	12.57	13.71	20.16	16.61	18.39	0.097	0.087	0.092	0.123	0.100	0.112
SNP $10^{-6}M$	14.51	12.43	13.47	19.92	16.56	18.24	0.081	0.068	0.075	0.117	0.094	0.106
Mean	14.42	12.31		19.82	16.46		0.080	0.064		0.110	0.087	
LSD at 5%												
Varieties	V = 0.13(Sig.)			V = 0.17(Sig.)			V = 0.004(Sig.)			V = 0.007(Sig.)		
Treatment	T = 0.17(Sig.)			T = 0.08(Sig.)			T = 0.005(Sig.)			T = 0.003(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.16(Sig.)			V x T = 0.006(Sig.)			V x T = 0.010(Sig.)		

Sig = Significant; NS = Non-significant

Table 30. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on internal CO_2 concentration (C_i; ppm) and transpiration rate (E; m mol m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Internal CO_2 concentration						Transpiration rate					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	318	290	304	359	319	339	3.55	2.69	3.12	4.53	3.97	4.25
$K_3Fe(CN)_6 10^{-4}$M	330	289	310	354	318	336	3.68	2.70	3.19	4.78	4.01	4.40
SNP 10^{-4}M	362	321	342	398	341	370	4.39	3.17	3.78	5.25	4.39	4.82
SNP 10^{-5}M	378	336	357	416	354	385	4.62	3.32	3.97	5.63	4.77	5.20
SNP 10^{-6}M	356	316	336	387	334	361	3.29	3.12	3.71	5.11	4.32	4.72
Mean	349	310		383	333		4.11	3.00		5.06	4.29	
LSD at 5%												
Varieties	V = 15.26(Sig.)			V = 8.61(Sig.)			V = 0.25(Sig.)			V = 0.40(Sig.)		
Treatment	T = 14.13(Sig.)			T = 6.61(Sig.)			T = 0.13(Sig.)			T = 0.35(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 0.52(Sig.)		

Sig = Significant; NS = Non-significant

Table 31. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on maximum quantum yield of PSII (F_v/F_m) and leaf water potential (MPa) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Maximum quantum yield of PSII						Leaf water potential					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.774	0.764	0.769	0.808	0.781	0.795	-0.66	-0.75	-0.71	-0.46	-0.53	-0.50
$K_3Fe(CN)_6 10^{-4}M$	0.773	0.764	0.769	0. 808	0.778	0.793	-0.65	-0.71	-0.68	-0.47	-0.54	-0.51
SNP $10^{-4}M$	0.787	0.773	0.780	0.813	0.785	0.799	-0.58	-0.65	-0.62	-0.40	-0.48	-0.44
SNP $10^{-5}M$	0.795	0.780	0.788	0.817	0.789	0.803	-0.52	-0.60	-0.56	-0.37	-0.45	-0.41
SNP $10^{-6}M$	0.784	0.771	0.778	0.811	0.784	0.798	-0.57	-0.66	-0.62	-0.42	-0.49	-0.46
Mean	0.783	0.770		0.811	0.783		-0.60	-0.67		-0.42	-0.50	
LSD at 5%												
Varieties	V = 0.006(Sig.)			V = 0.007(Sig.)			V = 0.03(Sig.)			V = 0.03(Sig.)		
Treatment	T = 0.005 (Sig.)			T = 0.003(Sig.)			T = 0.04(Sig.)			T = 0.02(Sig.)		
Var. x Treat.	V x T = 0.006(Sig.)			V x T = 0.004(Sig.)			V x T = 0.08(Sig.)			V x T = 0.07(Sig.)		

Sig = Significant; NS = Non-significant

Table 32. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on the activity of nitrate reductase [NR; n mole NO_2 g^{-1} s^{-1}(FM)] and carbonic anhydrase [CA; mole (CO_2) g^{-1} (FM) s^{-1}] in the leaves of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Nitrate reductase activity						Carbonic anhydrase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	424	393	409	435	412	424	2.00	1.95	1.98	2.32	2.21	2.27
$K_3Fe(CN)_6 10^{-4}$M	436	391	414	438	411	425	2.02	1.96	1.99	2.35	2.25	2.30
SNP 10^{-4}M	454	415	435	475	442	459	2.35	2.23	2.29	2.78	2.40	2.59
SNP 10^{-5}M	480	433	457	498	461	480	2.58	2.46	2.52	3.04	2.52	2.78
SNP 10^{-6}M	433	398	416	454	423	439	2.28	2.17	2.23	2.71	2.32	2.52
Mean	445	406		460	430		2.25	2.15		2.64	2.34	
LSD at 5%												
Varieties	V = 31.09(Sig.)			V = 32.83(Sig.)			V = 0.14(Sig.)			V = 0.06(Sig.)		
Treatment	T = 15.77(Sig.)			T = 18.91(Sig.)			T = 0.22(Sig.)			T = 0.10(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.32(Sig.)			V x T = 0.14(Sig.)		

Sig = Significant; NS = Non-significant

4.3.9 Activity of antioxidant system

The activity of peroxidase (POX), superoxide dismutase (SOD) and catalase (CAT) and the proline level increased as the growth progressed (Table 33 and 34). Moreover, the application of SNP (10^{-4}, 10^{-5} or 10^{-6} M) to foliage had an additive effect but potassium ferricyanide generated values comparable with the controls. Out of the three concentrations (10^{-4}, 10^{-5} or 10^{-6} M) of SNP, 10^{-5} M proved best in increasing the activity of antioxidant enzymes and the proline level. In terms of per cent, the increase was 27% and 25% (POX), 19% and 16% (CAT), 11% and 9% (SOD) and 23% and 21% (proline level) in Varuna and RH-30 respectively, compared to water sprayed plants at 30 DAS. However, at 60 DAS, the per cent increase by SNP treatment was less sharp than at 30 DAS. Varuna possessed higher values for antioxidant system than RH-30.

4.3.10 Cd accumulation in root and shoot

Data in table 35 indicates that Cd level increased with the age of plant (30 to 60 DAS). Plants, in general accumulated more Cd in roots than in shoot. Application of SNP (10^{-4}, 10^{-5}, or 10^{-6} M) decreased Cd level significantly in both the tissues only at 60 DAS, compared to their respective controls (sprayed with water). However, no effect of potassium ferricyanide (10^{-8} M) was evident in either of the variety. Plants that received SNP treatment (10^{-4}, 10^{-5} or 10^{-6} M) recorded significantly lower level of Cd in their tissues in the order of $10^{-5} > 10^{-4} > 10^{-6}$. The root and shoot of the plants treated with 10-5 M of SNP exhibited a decline of 3% and 12% in Varuna and 9% and 8% in RH-30, compared to the respective controls at 60 day stage. RH-30 accumulated higher levels of Cd, irrespective of the treatment.

4.3.11 Yield characteristics

The foliage of the plants that received SNP (10^{-4}, 10^{-5} or 10^{-6} M) treatments produced more pods and seeds per plant (Table 36). 10^{-5} M, of all the concentrations, of SNP proved most effective which significantly enhanced the number of pods by 22% and 20% and seed yield by 26% and 24% in Varuna and RH-30, respectively, over the water sprayed controls. The yield responses of Varuna were, therefore, better than RH-30.

Table 33. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on peroxidase [POX; units (g FM)$^{-1}$] and catalase [CAT; μ mol H_2O_2 decomposed (g FM)$^{-1}$] activity of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Peroxidase activity						Catalase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	12.97	6.81	09.89	16.74	08.99	12.87	411	377	394	438	390	414
$K_3Fe(CN)_6 10^{-4}$M	13.21	7.17	10.19	16.82	09.14	12.98	409	382	396	430	390	410
SNP 10^{-4}M	17.15	8.35	12.75	20.42	10.01	15.22	477	407	442	477	409	443
SNP 10^{-5}M	17.83	9.04	13.44	21.40	10.34	15.87	509	449	479	494	423	459
SNP 10^{-6}M	15.67	8.10	11.89	19.60	10.00	14.80	460	399	430	468	401	435
Mean	15.37	7.89		19.00	9.70		453	403		461	403	
LSD at 5%												
Varieties	V = 0.70(Sig.)			V = 0.90(Sig.)			V = 12.36(Sig.)			V = 2.61(Sig.)		
Treatment	T = 1.57(Sig.)			T = 1.24(Sig.)			T = 11.54(Sig.)			T = 8.12(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 2.33(Sig.)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 34. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on superoxide dismutase [SOD; units g^{-1} (FM)] activity and proline content [μ mol $(g\ FM)^{-1}$] of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Superoxide dismutase activity						Proline content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	139	127	133	160	144	152	13.66	12.50	13.08	19.22	15.98	17.60
$K_3Fe(CN)_6 10^{-4}M$	142	128	135	158	137	148	13.90	12.54	13.22	19.10	15.87	17.49
SNP $10^{-4}M$	150	132	141	179	157	168	17.34	15.00	16.17	21.70	17.57	19.64
SNP $10^{-5}M$	164	145	155	185	162	174	17.76	15.74	16.75	22.83	18.34	20.59
SNP $10^{-6}M$	152	134	143	177	154	166	16.42	14.76	15.59	22.21	18.11	20.16
Mean	149	133		172	151		15.82	14.11		21.01	17.17	
LSD at 5%												
Varieties	V = 12.87(Sig.)			V = 14.22(Sig.)			V = 1.19(Sig.)			V = 1.12(Sig.)		
Treatment	T = 4.93(Sig.)			T = 1.95(Sig.)			T = 1.30(Sig.)			T = 0.72(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 1.42(Sig.)			V x T = 1.78(Sig.)		

Sig = Significant; NS = Non-significant

Table 35. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on root and shoot Cd content (μg g^{-1} DW) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root Cd content						Shoot Cd content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.54	0.56	0.55	0.69	0.76	0.73	0.38	0.51	0.45	0.50	0.52	0.51
$K_3Fe(CN)_6 10^{-4}$M	0.53	0.57	0.55	0.70	0.75	0.73	0.36	0.50	0.43	0.49	0.53	0.51
SNP 10^{-4}M	0.52	0.55	0.54	0.68	0.71	0.70	0.36	0.49	0.43	0.46	0.50	0.48
SNP 10^{-5}M	0.52	0.54	0.53	0.67	0.70	0.69	0.35	0.49	0.42	0.45	0.48	0.46
SNP 10^{-6}M	0.52	0.54	0.53	0.68	0.71	0.70	0.36	0.49	0.43	0.46	0.50	0.48
Mean	0.53	0.55		0.68	0.73		0.36	0.50		0.47	0.51	
LSD at 5%												
Varieties	V = 0.05(Sig.)			V = 0.03(Sig.)			V = 0.04(Sig.)			V = 0.04(Sig.)		
Treatment	T = (NS)			T = 0.01(Sig.)			T = (NS)			T = 0.01(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 36. Effect of potassium ferricyanide (10^{-4} M) and different concentrations of sodium nitroprusside (SNP; 10^{-4}, 10^{-5} or 10^{-6} M) on yield characteristics of *Brassica juncea* cv. Varuna and RH-30 at harvest

Treatment	Pods plant^{-1}			Seeds pod^{-1}			100 seed mass (mg)			Seed yield plant^{-1} (g)		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	225	217	221	12.83	12.08	12.46	318	312	315	9.18	8.18	8.68
$K_3Fe(CN)_6 10^{-4}M$	221	218	220	12.71	12.14	12.43	321	311	316	9.02	8.23	8.62
SNP $10^{-4}M$	272	258	265	12.92	12.37	12.65	326	319	323	11.46	10.01	10.73
SNP $10^{-5}M$	290	273	282	13.05	12.24	12.65	330	324	327	12.49	10.81	11.65
SNP $10^{-6}M$	276	264	270	12.89	12.25	12.57	324	317	321	11.53	10.15	10.84
Mean	257	246		12.88	12.15		324	317		10.73	9.48	
LSD at 5%												
Varieties	V = 2.75(Sig.)			V = 3.40(Sig.)			V = 3.01(Sig.)			V = 0.54(Sig.)		
Treatment	T = 4.12(Sig.)			T = (NS)			T = (NS)			T = 0.55(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

4.4 EXPERIMENT 4

This experiment was conducted to explore the effect of BR analogues (10^{-8} M; HBL/EBL) application on the foliage of two varieties of *B. juncea* (L.) Czern & Coss (Varuna and RH-30) against the soil applied Cd (0, 25, 50 or 100 mg Cd Kg^{-1}). The agricultural practices remained the same as in Experiment 1. The Cd was to the through soil at the time of sowing. The foliage of 29 day old plants was sprayed with distilled water/HBL/EBL in the presence (stressed) or absence (stress free) of Cd. Selected number of samples were randomly collected at 30 and 60 DAS and rest of the plants were harvested at 120 DAS to study the yield parameters. The results are briefly explained below:

4.4.1 Root and shoot length

Application of BR analogues (HBL/EBL; 10^{-8} M) to the plant foliage at day 29, significantly increased root and shoot length in both the varieties (Varuna and RH-30) at 60 day stage (Table 37). However, the presence of Cd (25, 50 or 100 mg Kg^{-1}) significantly reduced the length of root and shoot at both the stages of growth (30 and 60 DAS) in a manner determined by its concentration. The inhibitory action of the metal was more prominent at early stage (30 DAS) but had slight recovery at 60 DAS. Against different concentrations of Cd (25, 50 or 100 mg Kg^{-1}), in Varuna the per cent decrease of root and shoot length was 29%, 43% and 57% and 13%, 25% and 36% at 30 DAS, compared to the control plants (Cd, 0 mg Kg^{-1}). However, BRs (10^{-8} M) completely neutralized the damages caused by the two lower concentrations of the metal (25 and 50 mg Kg^{-1}), observed 60 DAS. Out of them EBL was more effective than HBL. The response of Varuna was better than RH-30.

4.4.2 Fresh and dry mass of root

The data presented in table 38 indicates that fresh and dry mass of root increased from 30 to 60 day, after sowing (DAS). Presence of Cd (25, 50 or 100 mg Kg^{-1} of soil) significantly decreased the fresh and dry mass of root at both the stages of growth. However, foliar spray of HBL or EBL (10^{-8} M) to the stress free plants significantly improved the growth of plants at 60 DAS, where EBL was much better than HBL. The application BRs to the stress free plants increased the fresh and dry mass of root in

Varuna by 40% and 47% whereas HBL increased it by 37% and 39%, with respect to the control plants (water sprayed), at 60 DAS. BRs (HBL or EBL, 10^{-8} M), given as a follow up treatment, completely neutralized the Cd (25 or 50 mg Kg^{-1}) mediated loss of fresh and dry mass of root in the two varieties, at the 60 day stage. Moreover, Varuna performed better than RH-30.

4.4.3 Fresh and dry mass of shoot

It is evident from table 39 that shoot fresh and dry mass followed a trend similar to that of root fresh and dry mass (Section 4.4.2). Here again the two BRs (HBL/EBL) induced a complete recovery in the plants, exposed to Cd stress (25 or 50 mg Kg^{-1} of soil) at 60 DAS, where the values were at par to the controls. EBL, out of the two analogues of BRs, excelled over HBL. Varuna performed better than RH-30.

4.4.4 Leaf area

The leaf area increased with the plant age from 30 to 60 DAS (Table 40). However, with the increase of Cd level (0, 25, 50 or 100 mg Kg^{-1}) the values decreased proportionately in both the varieties (i.e. Varuna and RH-30) at the two stages of growth. BR analogues (10^{-8} M; EBL/HBL), significantly increased the leaf area in stress free plants but also completely ameliorated the loss against Cd (25 or 50 mg Kg^{-1}), at 60 DAS. Moreover, a partial recovery was also recorded against the highest concentration (100 mg Kg^{-1}). EBL performed better than HBL. Varuna under all circumstances provided higher values of leaf area than RH-30.

4.4.5 SPAD chlorophyll value

The leaf chlorophyll content (SPAD value) of plants increased with the progress of growth from 30 to 60 DAS (Table 40). The presence of Cd in the soil had a negative impact on the chlorophyll values at the early stage (30 DAS) of growth and that persisted up to 60 DAS where the per cent loss corresponding to 25, 50 or 100 mg Kg^{-1} of Cd was 14%, 25% and 35%, in Varuna at 60 DAS, compared with the control. However, this metal induced ill effect was neutralized by the follow up treatment with HBL/EBL. It was complete against 25 or 50 mg Kg^{-1} and partial against 100 mg Kg^{-1} of soil. Varuna had higher values than RH-30.

Table 37. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) and/or on root and shoot length (cm) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root length						Shoot length					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	16.07	14.84	15.46	26.20	23.15	24.68	29.80	25.00	27.40	75.00	69.77	72.39
HBL	16.24	14.90	15.57	36.69	31.06	33.88	29.88	25.03	27.46	121.60	106.50	114.05
EBL	16.27	15.00	15.64	40.10	33.65	36.88	29.93	25.07	27.50	126.80	111.60	119.20
$CdCl_2$ (25mg)	11.41	9.35	10.38	19.65	16.90	18.28	25.84	21.58	23.71	61.75	55.82	58.79
$CdCl_2$ (50mg)	9.16	5.94	7.55	17.05	13.20	15.13	22.47	18.38	20.43	57.00	48.84	52.92
$CdCl_2$ (100mg)	6.91	5.05	5.98	14.47	11.14	12.81	19.13	15.06	17.10	52.25	43.26	47.76
$CdCl_2$ (25mg) +HBL	11.16	9.40	10.28	29.52	25.63	27.58	25.80	21.76	23.78	85.94	75.03	80.49
$CdCl_2$ (50mg) +HBL	8.83	6.15	7.49	26.54	23.28	24.91	22.42	18.44	20.43	80.66	70.18	75.42
$CdCl_2$(100mg)+HBL	6.90	5.12	6.01	20.42	16.19	18.31	19.09	15.08	17.09	62.09	56.68	59.39
$CdCl_2$ (25mg) +EBL	11.28	9.72	10.50	31.58	27.73	29.66	25.89	21.83	23.86	90.56	79.74	85.15
$CdCl_2$ (50mg) +EBL	8.99	6.45	7.72	28.89	25.34	27.12	22.50	18.52	20.51	85.43	74.88	80.16
$CdCl_2$(100mg)+EBL	7.19	5.29	6.24	22.53	18.57	20.55	19.18	15.20	17.19	67.15	61.58	64.37
Mean	10.87	8.93		26.14	22.15		24.33	20.08		80.52	71.16	
LSD at 5%												
Varieties	V = 0.87(Sig.)			V = 1.20(Sig.)			V = 2.00(Sig.)			V = 2.21(Sig.)		
Treatment	T = 0.60(Sig.)			T = 2.05(Sig.)			T = 1.70(Sig.)			T = 4.09(Sig.)		
Var. x Treat.	V x T = 1.10 (Sig.)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 38. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root fresh mass						Root dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	3.00	2.81	2.91	5.49	4.88	5.19	0.82	0.68	0.75	1.61	1.20	1.41
HBL	3.03	2.82	2.93	8.29	7.08	7.69	0.78	0.61	0.70	2.68	1.66	2.17
EBL	3.06	2.84	2.95	9.12	7.76	8.44	0.78	0.60	0.69	3.05	1.98	2.52
$CdCl_2$ (25mg)	1.98	1.67	1.83	4.12	3.20	3.66	0.67	0.52	0.60	1.40	0.87	1.14
$CdCl_2$ (50mg)	1.47	1.31	1.39	3.55	2.88	3.22	0.52	0.37	0.45	1.27	0.73	1.00
$CdCl_2$ (100mg)	1.13	0.93	1.03	3.15	2.27	2.71	0.37	0.21	0.29	1.14	0.60	0.87
$CdCl_2$ (25mg) +HBL	1.83	1.62	1.73	5.93	5.13	5.53	0.66	0.53	0.60	1.93	1.38	1.66
$CdCl_2$ (50mg) +HBL	1.49	1.26	1.38	5.00	3.89	4.45	0.51	0.37	0.44	1.68	1.22	1.45
$CdCl_2$(100mg)+HBL	1.16	0.83	1.00	4.27	3.45	3.86	0.36	0.22	0.29	1.45	1.06	1.26
$CdCl_2$ (25mg) +EBL	1.86	1.68	1.77	6.37	5.47	5.92	0.67	0.55	0.61	2.15	1.56	1.86
$CdCl_2$ (50mg) +EBL	1.52	1.29	1.41	5.85	5.11	5.48	0.51	0.39	0.45	1.93	1.36	1.65
$CdCl_2$(100mg)+EBL	1.17	0.85	1.01	4.73	3.94	4.34	0.36	0.23	0.30	1.61	1.19	1.40
Mean	1.89	1.66		5.49	4.59		0.58	0.44		1.83	1.23	
LSD at 5%												
Varieties	V = 0.15(Sig.)			V = 0.41(Sig.)			V = 0.04(Sig.)			V = 0.12(Sig.)		
Treatment	T = 0.10(Sig.)			T = 0.29(Sig.)			T = 0.03(Sig.)			T = 0.12(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.41(Sig.)			V x T = (NS)			V x T = 49(Sig.)		

Sig = Significant; NS = Non-significant

Table 39. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on shoot fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Shoot fresh mass						Shoot dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	6.00	5.08	5.54	11.09	10.00	10.55	1.84	1.77	1.81	3.16	2.63	2.90
HBL	6.27	5.21	5.74	17.64	14.72	16.18	1.87	1.79	1.83	5.50	4.31	4.91
EBL	6.12	5.14	5.63	18.41	16.00	17.21	1.92	1.81	1.87	5.87	4.74	5.31
$CdCl_2$ (25mg)	4.62	3.25	3.94	9.26	6.37	7.82	1.29	0.89	1.09	2.72	1.96	2.34
$CdCl_2$ (50mg)	4.14	2.69	3.42	8.65	5.74	7.20	1.14	0.76	0.95	2.37	1.60	1.99
$CdCl_2$ (100mg)	3.60	2.29	2.95	7.81	5.08	6.45	1.00	0.62	0.81	2.02	1.25	1.64
$CdCl_2$ (25mg) +HBL	4.68	3.20	3.94	12.52	10.97	11.75	1.27	0.90	1.09	3.64	2.98	3.31
$CdCl_2$ (50mg) +HBL	4.21	2.54	3.38	11.83	10.29	11.06	1.13	0.76	0.95	3.23	2.62	2.93
$CdCl_2$(100mg)+HBL	3.72	2.18	2.95	9.38	8.04	8.71	1.00	0.63	0.82	2.75	2.14	2.45
$CdCl_2$ (25mg) +EBL	4.74	3.34	4.04	13.18	11.65	12.42	1.28	0.89	1.09	4.14	3.35	3.75
$CdCl_2$ (50mg) +EBL	4.24	2.64	3.44	12.55	10.94	11.75	1.15	0.78	0.97	3.65	2.97	3.31
$CdCl_2$(100mg)+EBL	3.80	2.27	3.04	10.08	8.75	9.42	1.01	0.66	0.84	3.11	2.53	2.82
Mean	4.68	3.32		11.87	9.88		1.33	1.02		3.51	2.76	
LSD at 5%												
Varieties	V = 0.30(Sig.)			V = 0.60(Sig.)			V = 0.10(Sig.)			V = 0.16(Sig.)		
Treatment	T = 0.34(Sig.)			T = 0.57(Sig.)			T = 0.12(Sig.)			T = 0.24(Sig.)		
Var. x Treat.	V x T = 0.44(Sig.)			V x T = (NS)			V x T = (NS)			V x T = 0.48(Sig.)		

Sig = Significant; NS = Non-significant

Table 40. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on SPAD value of chlorophyll in *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Leaf area						SPAD value of chlorophyll					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	24.75	23.00	23.88	37.10	34.04	35.57	36.04	29.37	32.71	42.93	31.48	37.21
HBL	24.77	23.00	23.89	48.23	42.89	45.56	44.33	34.69	39.51	51.49	35.51	45.50
EBL	24.97	23.07	24.02	50.82	44.25	47.54	47.03	37.24	42.14	53.72	38.09	45.91
$CdCl_2$ (25mg)	19.06	15.64	17.35	31.46	24.95	28.21	28.44	19.20	23.82	36.90	22.85	29.88
$CdCl_2$ (50mg)	15.94	13.50	14.72	27.19	22.74	24.97	22.71	12.68	17.70	32.24	18.30	25.27
$CdCl_2$ (100mg)	12.87	10.44	11.66	24.19	17.60	20.90	17.44	7.25	12.35	27.92	14.22	21.07
$CdCl_2$ (25mg) +HBL	18.81	15.18	17.00	41.66	36.29	38.98	37.64	30.67	34.16	47.56	34.37	40.97
$CdCl_2$ (50mg) +HBL	15.34	12.65	14.00	37.67	34.11	35.89	36.28	28.99	32.64	44.94	32.33	38.64
$CdCl_2$(100mg)+HBL	12.96	9.43	11.20	33.42	29.05	31.24	29.27	22.91	26.09	33.53	27.98	30.76
$CdCl_2$ (25mg) +EBL	19.06	15.41	17.24	43.88	38.47	41.18	39.16	31.78	35.47	49.74	36.41	43.08
$CdCl_2$ (50mg) +EBL	15.59	12.71	14.15	40.08	36.27	38.18	37.48	30.12	33.80	47.16	33.37	40.27
$CdCl_2$(100mg)+EBL	13.11	9.46	11.29	35.64	31.25	33.45	31.24	24.04	27.64	42.00	30.09	36.05
Mean	18.10	15.29		37.61	32.66		33.92	25.75		42.51	29.58	
LSD at 5%												
Varieties	V = 0.17(Sig.)			V = 1.02(Sig.)			V = 2.20(Sig.)			V = 1.40(Sig.)		
Treatment	T = 0.36(Sig.)			T = 2.15(Sig.)			T = 1.10(Sig.)			T = 2.03(Sig.)		
Var. x Treat.	V x T = 0.51(Sig.)			V x T = 1.01(Sig.)			V x T = 0.32(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

4.4.6 Photosynthetic parameters

All the photosynthetic parameters (viz. net photosynthetic rate; P_N, stomatal conductance; g_s; internal CO_2 concentration; C_i; transpiration rate; E and maximum quantum yield of PSII; Fv/Fm) improved as the growth advanced from 30 to 60 day stage (Tables 41-43). Treatment with either of the BR analogues, HBL or EBL (10^{-8}M) increased the values for the aforesaid parameters. However, highest concentration (100 mg Kg^{-1}) of Cd significantly decreased the photosynthesis and values related to its attributes by 44%, 55%, 14%, 18% and 18% (Varuna), 64%, 67%, 31%, 31% and 20% (RH-30) at the 60 day stage of growth. The spray of EBL to the stress free plants increased P_N, g_s, C_i, E and Fv/Fm by 10%, 36%, 21%, 34% and 5%, whereas, HBL by 8%, 13%; 16%, 28% and 3%, respectively, at 30 DAS in Varuna that performed better than RH-30, compared to control plants (Cd, 0 mg Kg^{-1}, water sprayed). EBL excelled in its response over HBL. Moreover, follow up application of HBL to the metal stressed plants or EBL completely neutralized the decline of photosynthetic traits by 25 or 50 mg Cd Kg^{-1} of soil but the response was partial against 100 mg Cd Kg^{-1} of soil. Out of the two varieties, Varuna was more resistant than RH-30, against Cd concentrations.

4.4.7 Leaf water potential (LWP)

The tabulated data (Table 43) shows that both HBL and EBL increased the LWP both in stress free and stressed plants. On the other hand, presence of Cd in soil (25, 50 or 100 mg Kg^{-1} of soil) lowered the leaf water potential in a concentration dependent manner. However, BRs (10^{-8} M) completely recovered the loss of leaf water potential caused by 25 and 50 mg Cd Kg^{-1} of soil in both the varieties at the two stages of growth whereas, this recovery was more prominent at 30 DAS than at 60 DAS. EBL proved to be more effective than HBL. The response of Varuna was more promising than RH-30.

4.4.8 Nitrate reductase (NR) and carbonic anhydrase (CA) activity

The activity of both the enzymes, NR and CA progressed as the growth advanced from 30 to 60 day stage (Table 44). The presence of the Cd decreased the values. However, both HBL and EBL (10^{-8}M), significantly ameliorated Cd (0, 25, 50 or 100 mg Kg^{-1}) mediated decline of enzymes activity. Complete recovery was noted in the plants

grown with 25 or 50 mg Cd Kg^{-1} of soil and the values were comparable with control. However, values were only partially recovered by BRs in plants fed with 100 mg Cd Kg^{-1} of soil. EBL was a better performer than HBL. The values were higher for Varuna as compared to RH-30.

4.4.9 Activity of antioxidant system

The activity of antioxidant enzymes [peroxidase (POX), catalase (CAT)] and the proline level increased from 30 to 60 DAS (Tables 45-46). These values increased further because of the presence of Cd (0, 25, 50 or 100 mg Kg^{-1} soil) in the proportion of its concentration and/or treatment with HBL or EBL (10^{-8}M). Varuna possessed higher activity of antioxidant enzymes and proline level at both the stages of growth. Against lowest Cd level (25 mg Kg^{-1}) in Varuna, the increase in the activity of POX, CAT, SOD and of proline level was 30%, 22%, 41% and 42%, whereas, EBL enhanced it in stress free plants by 37%, 25%, 32% and 35%, respectively, at 30 DAS as compared to respective controls (Cd, 0 mg Kg^{-1}, water sprayed). The maximum enzymes activity and proline accumulation was recorded in Varuna grown with Cd 100 mg Kg^{-1} and also sprayed with BRs (10^{-8}M) which increased POX, CAT, SOD activity and proline level by 110%, 63%, 95% and 101% with HBL, whereas, EBL increased it by 120%, 76%, 113% and122% at 30 DAS.

4.4.10 Cd accumulation in root and shoot

Table 47 expresses an increase in Cd in a progressive manner both in root and shoot with its level (0, 25, 50 or 100 mg Kg^{-1} soil) amended in the soil. Roots accumulated more Cd than shoot. However, follow up treatment of Cd-stressed plants with BRs had a remedial effect on the metal accumulation. Out of the two cultivars, RH-30 accumulated more Cd than Varuna. Among the BRs, EBL more effectively retarded the Cd concentration both in root and shoot tissues, as compared to HBL at 60 DAS. Against the Cd (25 mg Kg^{-1} of soil), Varuna accumulated 67μg metal; however, on being sprayed with EBL its level decreased to 62μg Cd, g^{-1} of root dry mass. Alternatively, RH-30 accumulated 74μg Cd similar conditions and on being sprayed with EBL, it reached to 68μg Cd, g^{-1} of root dry mass. Likewise, in shoot, the accumulation of

the metal, under 25 mg Kg^{-1} Cd in Varuna and RH-30 was 12μg and 20μg, whereas, when sprayed with EBL, the values came down to 10μg and 12μg Cd g^{-1} of shoot dry mass, at 60 DAS, respectively. RH-30 accumulated higher level of Cd, whereas; shoot accumulated lower level of Cd than root, in both the varieties.

4.4.11 Yield characteristics

All the yield characteristics (number of pods per plant, number of seeds per pod, mass of 100 seeds and seed yield per plant) reduced significantly with Cd (25, 50 or 100 mg Kg^{-1} of soil) in the two cultivars of brassica; Varuna and RH-30 (Table 48). The two BR analogues (HBL or EBL; 10^{-8} M) significantly increased the values of number of seeds per plant and seed yield per plant in non-stressed plants fed with different levels of Cd. Against the lowest Cd concentration (25 mg Kg^{-1}), the loss of seed yield per plant was completely neutralized with the application of 10^{-8} M HBL/EBL, whereas, above this concentration (Cd 50 or 100 mg Kg^{-1}) BRs generated only partial recovery in Varuna and RH-30. The yield loss was more prominent in RH-30 than Varuna. Out of the two BR analogues the response of EBL was greater than HBL.

4.5 EXPERIMENT 5

This experiment was laid down to explore the response of exogenous application of SNP (10^{-5}M) against different levels of Cd (0, 25, 50 or 100 mg Kg^{-1}) in the two varieties of *Brassica juncea* (L.) Czern & Coss; Varuna and RH-30. All the agricultural practices, sampling and parameters studied were same as in other experiments. Cadmium was added into the soil at the time of sowing, whereas, aqueous solution of SNP (10^{-5}M) was applied to the foliage of 29 day old plants.

4.5.1 Root and shoot length

The data presented in tables 49 revealed that root and shoot length increased with the progress of plant age from 30 to 60 DAS. On the other hand, presence of Cd (25, 50 or 100 mg Kg^{-1}) in the soil significantly reduced the length of both root and shoot at the two stages of growth. At 60 DAS, the reduction of root and shoot length against Cd 25, 50 or 100 mg Kg^{-1} of soil in Varuna was 25% and 18%, 35% and 24% or 45% and 30%

Table 41. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on net photosynthetic rate (P_N; μ mol CO_2 m^{-2} sec^{-1}) and stomatal conductance (g_s; mol H_2O m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Net photosynthetic rate						Stomatal conductance					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	14.10	12.07	13.09	19.55	16.27	17.91	0.084	0.064	0.074	0.097	0.081	0.089
HBL	15.36	13.08	14.22	20.85	17.10	18.98	0.097	0.072	0.085	0.139	0.102	0.121
EBL	15.66	13.24	14.45	21.29	17.37	19.33	0.132	0.093	0.113	0.158	0.119	0.139
$CdCl_2$ (25mg)	12.44	9.76	11.10	15.31	10.80	13.06	0.049	0.043	0.046	0.080	0.062	0.071
$CdCl_2$ (50mg)	10.50	7.30	8.90	12.62	07.45	10.24	0.038	0.031	0.035	0.061	0.044	0.053
$CdCl_2$ (100mg)	9.29	6.03	7.66	10.95	05.80	08.38	0.025	0.020	0.023	0.044	0.027	0.036
$CdCl_2$ (25mg) +HBL	14.53	12.41	13.47	19.77	16.43	18.10	0.102	0.075	0.089	0.115	0.093	0.104
$CdCl_2$ (50mg) +HBL	14.12	12.08	13.10	19.42	16.11	17.77	0.095	0.070	0.083	0.108	0.087	0.198
$CdCl_2$(100mg)+HBL	12.06	9.98	11.02	16.33	12.81	14.57	0.078	0.058	0.068	0.188	0.072	0.080
$CdCl_2$ (25mg) +EBL	15.06	12.78	13.92	20.19	16.76	18.48	0.123	0.086	0.105	0.136	0.107	0.122
$CdCl_2$ (50mg) +EBL	14.19	12.10	13.15	19.63	16.30	17.97	0.098	0.074	0.086	0.115	0.092	0.104
$CdCl_2$(100mg)+EBL	12.47	10.37	11.42	17.78	14.58	16.18	0.085	0.064	0.075	0.094	0.077	0.086
Mean	13.32	10.93		17.81	14.02		0.084	0.063		0.103	0.080	
LSD at 5%												
Varieties	V = 0.12(Sig.)			V = 0.15(Sig.)			V = 0.005(Sig.)			V = 0.006(Sig.)		
Treatment	T = 0.32(Sig.)			T = 0.31(Sig.)			T = 0.001(Sig.)			T = 0.003(Sig.)		
Var. x Treat.	V x T = 0.14 (Sig.)			V x T = 0.12(Sig.)			V x T = 0.003(Sig.)			V x T = 0.005(Sig.)		

Sig = Significant; NS = Non-significant`

Table 42. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on internal CO_2 concentration (C_i; ppm) and transpiration rate (E; m mol m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Internal CO_2 concentration						Transpiration rate					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	309	286	298	355	312	334	3.11	3.03	3.07	4.66	4.36	4.51
HBL	369	319	344	389	337	363	4.32	3.74	4.03	5.90	5.14	5.52
EBL	389	340	365	402	349	376	4.69	3.90	4.30	6.23	5.32	5.78
$CdCl_2$ (25mg)	278	227	253	327	260	294	2.84	2.55	2.70	4.42	3.58	4.00
$CdCl_2$ (50mg)	256	206	231	316	240	278	2.60	2.03	2.32	4.12	3.26	3.69
$CdCl_2$ (100mg)	235	186	211	304	216	260	2.33	1.79	2.06	3.87	3.02	3.42
$CdCl_2$ (25mg) +HBL	343	309	326	387	334	361	3.44	3.17	3.31	5.05	4.61	4.83
$CdCl_2$ (50mg) +HBL	318	287	303	368	318	343	3.24	3.05	3.15	4.75	4.38	4.57
$CdCl_2$(100mg)+HBL	266	215	241	324	268	296	2.91	2.55	2.73	4.29	4.12	4.21
$CdCl_2$ (25mg) +EBL	365	334	350	399	345	372	3.57	3.29	3.43	5.28	4.97	5.13
$CdCl_2$ (50mg) +EBL	340	308	324	380	327	354	3.42	3.17	3.30	5.04	4.73	4.89
$CdCl_2$(100mg)+EBL	287	254	271	336	275	306	3.03	2.63	2.83	4.48	4.36	4.42
Mean	313	273		357	298		3.29	2.91		4.84	4.32	
LSD at 5%												
Varieties	V = 9.20(Sig.)			V = 4.77(Sig.)			V = 0.05(Sig.)			V = 0.26(Sig.)		
Treatment	T = 19.51(Sig.)			T = 10.12(Sig.)			T = 0.11(Sig.)			T = 0.17(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 0.40(Sig.)		

Sig = Significant; NS = Non-significant

Table 43. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on maximum quantum yield of PSII (F_v/F_m) and leaf water potential (MPa) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Maximum quantum yield of PSII						Leaf water potential					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.772	0.765	0.769	0.808	0.781	0.795	-0.64	-0.75	-0.70	-0.40	-0.56	-0.48
HBL	0.798	0.780	0.789	0.815	0.788	0.802	-0.45	-0.55	-0.50	-0.33	-0.47	-0.40
EBL	0.812	0.800	0.806	0.820	0.793	0.807	-0.41	-0.49	-0.45	-0.28	-0.40	-0.34
$CdCl_2$ (25mg)	0.726	0.701	0.714	0.774	0.736	0.755	-0.74	-0.89	-0.82	-0.46	-0.63	-0.55
$CdCl_2$ (50mg)	0.660	0. 622	0.641	0.728	0.683	0.706	-0.81	-0.98	-0.90	-0.51	-0.69	-0.60
$CdCl_2$ (100mg)	0.583	0.536	0.560	0.660	0.625	0.643	-0.88	-1.07	-0.98	-0.58	-0.77	-0.68
$CdCl_2$ (25mg) +HBL	0.786	0.774	0.780	0.821	0.789	0. 805	-0.55	-0.67	-0.61	-0.33	-0.49	-0.41
$CdCl_2$ (50mg) +HBL	0.777	0.768	0.773	0.812	0.783	0.798	-0.62	-0.74	-0.68	-0.38	-0.54	-0.46
$CdCl_2$(100mg)+HBL	0.769	0.757	0.763	0.806	0.775	0.791	-0.69	-0.85	-0.77	-0.45	-0.63	-0.54
$CdCl_2$ (25mg) +EBL	0.791	0.781	0.786	0.825	0.793	0.809	-0.48	-0.60	-0.54	-0.27	-0.44	-0.36
$CdCl_2$ (50mg) +EBL	0.782	0.773	0.778	0.817	0.787	0.802	-0.58	-0.70	-0.64	-0.33	-0.50	-0.42
$CdCl_2$(100mg)+EBL	0.776	0.761	0.769	0.811	0.776	0.794	-0.68	-0.82	-0.75	-0.41	-0.58	-0.50
Mean	0.753	0.735		0.791	0.759		-0.63	-0.76		-0.39	-0.56	
LSD at 5%												
Varieties	V = 0.01(Sig.)			V = 0.03(Sig.)			V = 0.03(Sig.)			V = 0.02(Sig.)		
Treatment	T = 0.01(Sig.)			T = 0.06(Sig.)			T = 0.06(Sig.)			T = 0.04(Sig.)		
Var. x Treat.	V x T = 0.02(Sig.)			V x T = 0.09(Sig.)			V x T = 0.09(Sig.)			V x T = 0.06(Sig.)		

Sig = Significant; NS = Non-significant

Table 44. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) and/or on the activity of nitrate reductase [NR; n mole NO_2 g^{-1} s^{-1} (FM)] and carbonic anhydrase [CA; mole (CO_2) g^{-1} (FM) s^{-1}] in the leaves of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Nitrate reductase activity						Carbonic anhydrase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	418	392	405	431	418	525	2.05	1.97	2.01	2.40	2.26	2.33
HBL	513	536	575	537	470	504	2.83	2.50	2.67	2.95	2.56	2.76
EBL	535	565	500	556	500	528	2.97	2.62	2.80	3.27	2.61	2.94
$CdCl_2$ (25mg)	358	277	318	390	304	347	1.56	1.44	1.50	2.13	1.89	2.01
$CdCl_2$ (50mg)	325	218	272	364	255	310	1.45	1.30	1.38	1.84	1.34	1.59
$CdCl_2$ (100mg)	284	185	235	336	205	271	1.28	1.08	1.18	1.60	0.98	1.29
$CdCl_2$ (25mg) +HBL	482	436	459	527	483	505	2.23	2.09	2.16	2.68	2.46	2.57
$CdCl_2$ (50mg) +HBL	450	412	431	489	457	473	2.07	1.98	2.03	2.55	2.33	2.44
$CdCl_2$(100mg)+HBL	342	298	320	396	365	381	1.66	1.19	1.43	2.42	2.02	2.22
$CdCl_2$ (25mg) +EBL	504	458	481	571	514	543	2.34	2.20	2.27	2.82	2.61	2.72
$CdCl_2$ (50mg) +EBL	464	424	444	521	479	500	2.19	2.03	2.11	2.68	2.46	2.57
$CdCl_2$(100mg)+EBL	379	336	358	435	417	426	1.92	1.65	1.79	2.55	2.15	2.35
Mean	421	361		463	406		2.05	1.83		2.49	2.14	
LSD at 5%												
Varieties	V = 28.82(Sig.)			V = 14.03(Sig.)			V = 0.16(Sig.)			V = 0.20(Sig.)		
Treatment	T = 21.13(Sig.)			T = 18.76 (Sig.)			T = 0.10(Sig.)			T = 0.12(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 42.09(Sig.)			V x T = 0.18(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 45. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on peroxidase [POX; units (g FM)$^{-1}$] and catalase [CAT; μ mol H_2O_2 decomposed (g FM)$^{-1}$] activity of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Peroxidase activity						Catalase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	12.87	7.11	9.99	17.34	9.27	13.31	413	370	392	433	380	407
HBL	18.47	8.77	13.62	22.84	10.78	16.81	503	425	464	505	409	457
EBL	20.30	10.58	15.44	24.52	12.13	18.33	548	451	500	528	432	480
$CdCl_2$ (25mg)	16.74	8.39	12.57	21.38	10.60	15.99	504	408	456	481	417	449
$CdCl_2$ (50mg)	18.35	9.71	14.03	22.95	11.94	17.45	529	433	481	502	438	470
$CdCl_2$ (100mg)	20.17	11.03	15.60	24.72	13.32	19.02	553	457	505	530	461	496
$CdCl_2$ (25mg) +HBL	20.06	9.68	14.87	25.26	12.18	18.72	562	456	509	543	442	493
$CdCl_2$ (50mg) +HBL	25.13	11.52	18.33	30.85	13.55	22.20	634	502	568	579	465	522
$CdCl_2$(100mg)+HBL	27.01	13.51	20.26	32.75	15.06	23.91	673	527	600	636	487	562
$CdCl_2$ (25mg) +EBL	21.65	11.36	16.51	26.57	13.49	20.03	616	489	553	568	465	517
$CdCl_2$ (50mg) +EBL	26.78	12.81	19.80	32.23	14.90	23.57	695	529	612	601	489	545
$CdCl_2$(100mg)+EBL	28.31	14.82	21.57	35.00	16.40	25.70	727	557	642	658	518	588
Mean	21.32	10.77		26.37	12.80		580	467		547	450	
LSD at 5%												
Varieties	V = 1.01(Sig.)			V = 1.18(Sig.)			V = 20.70(Sig.)			V = 15.29 (Sig.)		
Treatment	T = 1.27(Sig.)			T = 1.30(Sig.)			T = 23.91 (Sig.)			T = 20.44(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 46. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on superoxide dismutase [SOD; units g^{-1} (FM)] activity and proline content [μ mol (g FM)$^{-1}$] of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Superoxide dismutase						Proline content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	130	112	121	154	121	138	14.84	12.17	13.51	19.25	14.84	17.05
HBL	155	129	142	182	140	161	19.48	15.35	20.72	23.20	17.81	20.51
EBL	191	158	175	209	163	186	22.73	17.68	20.21	27.31	20.08	23.70
$CdCl_2$ (25mg)	184	132	158	203	140	172	21.02	15.12	18.07	25.58	17.94	21.76
$CdCl_2$ (50mg)	201	155	178	232	159	196	23.97	18.08	21.03	28.65	21.01	24.83
$CdCl_2$ (100mg)	234	192	213	276	180	228	27.04	21.26	24.15	32.06	24.13	28.10
$CdCl_2$(25mg) +HBL	216	164	190	231	159	195	23.12	18.25	20.69	28.84	21.15	25.00
$CdCl_2$(50mg) +HBL	232	188	210	254	179	217	26.15	21.19	23.67	32.04	24.27	28.16
$CdCl_2$(100mg)+HBL	253	212	233	293	203	248	29.86	24.20	27.03	35.13	27.85	31.49
$CdCl_2$ (25mg) +EBL	233	180	207	253	179	216	26.29	21.31	23.80	32.30	24.26	28.28
$CdCl_2$ (50mg) +EBL	250	206	228	275	201	238	29.95	24.25	27.10	35.87	27.33	31.60
$CdCl_2$(100mg)+EBL	277	228	253	316	225	271	33.02	26.32	29.67	40.84	30.92	35.88
Mean	213	171		240	171		24.79	19.60		30.09	22.63	
LSD at 5%												
Varieties	V = 7.33(Sig.)			V = 19.20(Sig.)			V = 1.85(Sig.)			V = 1.44(Sig.)		
Treatment	T = 15.55(Sig.)			T = 18.00(Sig.)			T = 2.03(Sig.)			T = 2.25(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 47. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) on soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root and shoot Cd content (µg g^{-1} DW) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root Cd content						Shoot Cd content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.53	0.72	0.62	0.81	0.88	0.84	0.37	0.51	0.44	0.47	0.54	0.51
HBL	0.52	0.56	0.54	0.67	0.74	0.71	0.35	0.49	0.42	0.37	0.44	0.40
EBL	0.52	0.56	0.54	0.54	0.61	0.57	0.35	0.48	0.41	0.26	0.33	0.30
$CdCl_2$ (25mg)	51.50	73.00	62.25	66.90	73.70	70.30	10.30	15.00	12.65	12.20	20.50	16.35
$CdCl_2$ (50mg)	106.40	149.50	127.95	135.80	175.80	155.80	26.00	38.00	32.00	30.50	48.80	39.65
$CdCl_2$ (100mg)	154.40	214.30	184.35	196.90	258.20	227.55	60.00	85.00	72.50	67.30	106.70	87.00
$CdCl_2$(25mg)+HBL	53.10	72.80	62.95	64.70	71.70	68.20	10.10	14.91	12.51	10.70	13.70	12.20
$CdCl_2$(50mg)+HBL	105.30	147.90	126.60	132.80	173.10	152.95	25.92	37.88	31.90	21.90	33.80	27.85
$CdCl_2$(100mg)+HBL	153.85	212.80	183.33	194.40	254.40	224.40	59.70	85.07	72.39	52.20	79.70	65.95
$CdCl_2$(25mg)+EBL	50.90	70.20	60.55	61.80	67.80	64.80	9.83	14.95	12.39	9.64	12.52	11.08
$CdCl_2$(50mg) +EBL	103.90	145.80	124.85	129.40	156.13	142.77	25.79	37.76	31.78	20.79	32.70	26.75
$CdCl_2$(100mg)+EBL	153.68	212.30	182.99	189.60	250.30	219.95	59.81	85.03	72.42	50.08	78.20	64.14
Mean	78.05	109.18		97.86	123.61		24.04	34.59		23.03	35.66	
LSD at 5%												
Varieties	V = 0.08(Sig.)			V = 0.06(Sig.)			V = 0.06(Sig.)			V = 0.05(Sig.)		
Treatment	T = 0.17(Sig.)			T = 0.13(Sig.)			T = 0.13(Sig.)			T = 0.10(Sig.)		
Var. x Treat.	V x T = 0.23(Sig.)			V x T = 0.19(Sig.)			V x T = 0.19(Sig.)			V x T = 0.05(Sig.)		

Sig = Significant; NS = Non-significant

Table 48. Effect of brassinosteroids (HBL or EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on yield characteristics of *Brassica juncea* cv. Varuna and RH-30 at harvest

Treatment	Pods plant^{-1}			Seeds pod^{-1}			100 seed mass (mg)			Seed yield plant^{-1} (g)		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	228	215	222	12.51	12.00	12.26	315	308	312	8.98	7.95	8.47
HBL	290	242	266	12.71	12.17	12.40	327	319	323	12.05	9.39	10.72
EBL	324	287	306	12.79	12.25	12.52	329	320	324	13.61	11.25	12.43
$CdCl_2$ (25mg)	200	178	189	11.95	11.19	11.57	302	288	295	7.23	5.74	6.48
$CdCl_2$ (50mg)	186	162	174	11.62	10.88	11.25	289	274	281	6.25	4.83	5.54
$CdCl_2$ (100mg)	174	151	163	11.00	10.01	10.51	275	260	268	5.26	3.93	4.60
$CdCl_2$(25mg)+HBL	254	236	245	12.00	11.30	11.65	305	290	298	9.30	7.73	8.52
$CdCl_2$(50mg)+HBL	240	221	231	11.66	10.99	11.33	292	276	284	8.14	6.70	7.44
$CdCl_2$(100mg)+HBL	204	189	197	11.06	10.14	10.60	278	263	271	6.27	5.04	5.66
$CdCl_2$ (25mg) +EBL	267	251	259	12.03	11.32	11.68	308	293	301	9.89	8.33	9.11
$CdCl_2$ (50mg) +EBL	249	232	241	11.69	11.01	11.35	293	278	286	8.53	7.10	7.81
$CdCl_2$(100mg)+EBL	229	203	216	11.12	10.29	10.71	281	265	273	7.16	5.54	6.35
Mean	237	214		11.85	11.13		299	286		8.56	6.96	
LSD at 5%												
Varieties	V = 9.84(Sig.)			V = 0.60(Sig.)			V = 5.69(Sig.)			V = 0.10(Sig.)		
Treatment	T = 10.87(Sig.)			T = (NS)			T = (NS)			T = 0.21(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 5.20(Sig.)		

Sig = Significant; NS = Non-significant``

and in RH-30 it was 17% and 20%, 13% and 30% or 11% and 38%. The foliar spray of SNP (10^{-5}M), significantly increased the values of both the parameters at 60 day stage. Moreover, the follow-up treatment of SNP to the plants fed with 25 or 50 mg Cd Kg^{-1} of soil completely neutralized the damages caused by the metal at 60 DAS and also partially improved the values because of the impact of 100 mg Cd Kg-^{1} of soil. Varuna recorded higher values of root and shoot length than RH-30.

4.5.2 Fresh and dry mass of root

The values for fresh and dry mass of root increased with plant age in both the varieties (Table 50). Presence of Cd (0, 25, 50 or 100 mg Kg^{-1}) in the soil significantly decreased the root fresh and dry mass in a dose dependent manner at the two stages of growth (30 and 60 DAS). The reduction of fresh and dry mass of root was more prominent in RH-30 than in Varuna. Moreover, root fresh and dry mass recorded decrease of 34% and 27%, 41% and 39% and 53% and 50% in RH-30, and 24% and 13%, 35% and 21%, 43% and 29% in Varuna, respectively, against 25, 50 or 100 mg Cd Kg^{-1}, at 60 DAS. Application of SNP (10^{-5} M) improved the values for root fresh and dry mass at 60 DAS by 31% and 27% over the control. Moreover, as a follow up treatment to the stressed plants it completely augmented the loss of root fresh and dry mass by 25 or 50 mg Cd Kg^{-1} at 60 day stage of growth and partially that of 100 mg Kg^{-1} of soil.

4.5.3 Fresh and dry mass of shoot

Foliar application of SNP (10^{-5} M) significantly increased the fresh and dry mass of shoot in the two varieties (Varuna and RH-30). However, the presence of Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) decreased the values in a concentration dependent manner (Table 51) which were regained by the follow up application of 10^{-5} M SNP at 60 day stage. The plants grown with 25 or 50 mg Cd Kg^{-1} of soil and supplemented with SNP had shoot fresh and dry mass comparable with that of the control. Moreover, the damage caused by 100 mg Cd Kg^{-1} of soil was also partially overcome by SNP. Varuna excelled in its response to SNP than RH-30.

4.5.4 Leaf area

As evident from table 52; the leaf area increased with plant age (30 to 60 days) and the application of SNP. However, Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) caused a decrease in leaf area of the two varieties in a manner dependent on its concentration.

Varuna and RH-30 raised in soil even with the lowest Cd i.e. 25 mg Kg^{-1} of soil lost the leaf area by 15% and 27% at 60 DAS, as compared to the control plants (Cd 0 mg Kg^{-1}, water sprayed). Foliar spray of SNP (10^{-5}M) improved the leaf area in the presence of Cd and could induce complete recovery of the loss against 25 or 50 mg Cd Kg^{-1} but partially that of 100 mg Kg^{-1}, at 60 DAS. The response was more prominent in Varuna than RH-30.

4.5.5 SPAD chlorophyll value

The leaf chlorophyll content (SPAD value) increased as the growth progressed from 30 to 60 DAS (Table 52). The values increased significantly by the application of SNP which were 16% and 14% at 30 DAS and 17% and 15%, at 60 DAS in Varuna and RH-30, respectively, as compared to control plants (water sprayed only). The plants exposed to Cd significantly lost chlorophyll but the application of SNP to the stressed plants completely neutralized the toxic effect of 25 or 50 mg Cd Kg^{-1} and partially that of 100 mg Kg^{-1} of Cd, at the both stages of plant growth. Higher SPAD values were recorded in Varuna than RH-30.

4.5.6 Photosynthetic parameters

Net photosynthesis (P_N) and its associated attributes (stomatal conductance; g_s, internal CO_2 concentration; C_i, transpiration rate; E, maximum quantum yield of PSII; Fv/Fm) increased with plant age from 30 to 60 DAS (Tables 53-55) but the values decreased in the presence of Cd (0, 25, 50 or 100 mg Kg^{-1}) in a concentration dependent manner. However, the foliar application of SNP (10^{-5} M) on the foliage of stress free and stressed plants improved the values to a significant level. Moreover, the toxic effect developed by 25 or 50 mg Cd Kg^{-1} of soil was completely neutralized by SNP (30 and 60 DAS) both in Varuna and RH-30. The values are therefore at par with the water sprayed control plants. The impact of 100 mg Cd Kg^{-1} was also overcome by SNP but to a non-significant level. The values for all these characteristics were higher in Varuna than RH-30.

Table 49. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root and shoot length (cm) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root length						Shoot length					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	16.02	14.64	15.33	25.82	23.00	24.41	29.56	25.19	27.38	75.08	69.53	72.31
SNP	16.08	14.67	15.38	32.42	27.94	30.18	29.62	25.06	27.34	101.00	92.20	96.60
$CdCl_2$ (25mg)	11.37	9.22	10.30	19.36	16.77	18.07	25.64	21.85	23.75	61.84	55.62	58.73
$CdCl_2$ (50mg)	9.14	5.85	7.50	16.78	13.10	14.94	22.28	18.53	20.41	57.00	48.67	52.84
$CdCl_2$ (100mg)	6.88	4.97	5.93	14.26	11.08	12.67	18.97	15.17	17.07	52.29	43.03	47.66
$CdCl_2$ (25mg) + SNP	10.86	8.99	9.93	28.45	25.22	26.84	25.38	21.74	23.56	84.34	73.76	79.05
$CdCl_2$ (50mg) + SNP	8.37	5.98	7.18	25.78	22.88	24.33	22.01	18.41	20.21	79.03	67.58	73.31
$CdCl_2$(100mg) + SNP	6.40	4.65	5.52	18.82	15.61	17.21	18.75	15.00	16.87	63.82	57.96	60.89
Mean	12.16	9.85		25.96	22.23		27.46	22.92		82.06	72.62	
LSD at 5%												
Varieties	V = 0.82(Sig.)			V = 1.85(Sig.)			V = 2.51 (Sig.)			V = 3.26(Sig.)		
Treatment	T = 0.83(Sig.)			T = 2.01(Sig.)			T = 3.08 (Sig.)			T = 4.15(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 50. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root fresh mass						Root dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	2.94	2.69	2.82	5.40	4.73	5.07	0.87	0.70	0.79	1.60	1.21	1.41
SNP	2.95	2.70	2.83	7.07	6.02	6.55	0.89	0.71	0.80	2.21	1.52	1.87
$CdCl_2$ (25mg)	1.93	1.60	1.77	4.06	3.10	3.58	0.71	0.54	0.62	1.39	0.88	1.13
$CdCl_2$ (50mg)	1.44	1.26	1.35	3.49	2.79	3.14	0.55	0.38	0.47	1.26	0.74	1.00
$CdCl_2$ (100mg)	1.11	0.89	1.00	3.10	2.20	2.65	0.39	0.22	0.31	1.13	0.60	0.87
$CdCl_2$ (25mg)+ SNP	1.76	1.52	1.64	5.84	5.05	5.45	0.69	0.38	0.53	1.71	1.28	1.50
$CdCl_2$ (50mg)+ SNP	1.42	1.18	1.30	5.49	4.74	5.12	0.53	0.33	0.43	1.58	1.15	1.37
$CdCl_2$(100mg)+SNP	1.10	0.83	0.96	5.04	4.26	4.65	0.37	0.22	0.30	1.43	1.02	1.22
Mean	2.09	1.81		5.64	4.70		0.72	0.50		1.76	1.20	
LSD at 5%												
Varieties	V = 0.06(Sig.)			V = 0.33(Sig.)			V = 0.01(Sig.)			V = 0.16(Sig.)		
Treatment	T = 0.11(Sig.)			T = 0.30(Sig.)			T = 0.02(Sig.)			T = 0.12(Sig.)		
Var. x Treat.	V x T = 0.15(Sig.)			V x T = (NS)			V x T = (NS)			V x T = 0.35 (NS)		

Sig = Significant; NS = Non-significant

Table 51. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on shoot fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Shoot fresh mass						Shoot dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	6.00	5.04	5.52	11.12	9.92	10.52	1.87	1.75	1.81	3.17	2.60	2.89
SNP	6.09	5.17	5.63	14.76	12.78	13.77	1.89	1.70	1.80	4.41	3.57	3.99
$CdCl_2$ (25mg)	4.62	3.22	3.92	9.28	6.32	7.80	1.31	0.88	1.10	2.73	1.94	2.34
$CdCl_2$ (50mg)	4.15	2.67	3.41	8.67	5.69	7.18	1.16	0.75	0.95	2.38	1.58	1.98
$CdCl_2$ (100mg)	3.59	2.27	2.93	7.81	5.04	6.43	1.02	0.61	0.82	2.03	1.24	1.63
$CdCl_2$ (25mg)+ SNP	4.76	3.09	3.93	12.65	11.14	11.90	1.29	0.87	1.08	3.51	2.83	3.17
$CdCl_2$ (50mg)+ SNP	4.20	2.52	3.36	11.39	9.76	10.58	1.13	0.74	0.93	3.21	2.53	2.87
$CdCl_2$(100mg)+SNP	3.74	2.07	2.91	9.95	8.53	9.24	1.00	0.60	0.80	2.74	2.10	2.42
Mean	5.31	3.72		12.23	9.88		1.52	1.13		3.45	2.63	
LSD at 5%												
Varieties	V = 0.75(Sig.)			V = 1.14(Sig.)			V = 0.24(Sig.)			V = 0.18(Sig.)		
Treatment	T = 0.39(Sig.)			T = 0.58(Sig.)			T = 0.12(Sig.)			T = 0.29(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 0.20(Sig.)		

Sig = Significant; NS = Non-significant

Table 52. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on leaf area (cm^2) and SPAD value of chlorophyll in *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Leaf area						SPAD chlorophyll value					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	24.86	22.96	23.91	36.00	34.12	35.06	36.27	29.27	32.77	43.32	35.56	39.44
SNP	24.92	23.00	23.96	44.19	40.68	42.44	42.14	33.44	37.79	51.55	41.13	46.34
$CdCl_2$ (25mg)	19.12	15.59	17.36	30.55	25.02	27.79	28.60	19.14	23.87	37.23	27.95	32.59
$CdCl_2$ (50mg)	15.99	13.47	14.73	26.38	22.81	24.60	22.83	12.64	17.74	32.51	20.68	26.60
$CdCl_2$ (100mg)	12.91	10.43	11.67	23.44	17.66	20.55	17.54	08.22	12.88	28.19	16.05	22.12
$CdCl_2$ (25mg)+ SNP	18.61	14.88	16.75	40.20	36.18	38.19	39.76	31.08	35.42	46.10	37.56	41.83
$CdCl_2$ (50mg)+ SNP	15.11	12.28	13.70	36.31	34.01	35.16	37.02	29.64	32.33	43.78	35.57	39.68
$CdCl_2$(100mg)+SNP	12.69	9.21	10.95	32.25	29.65	30.95	32.18	24.83	28.51	36.76	29.54	33.15
Mean	20.60	17.40		38.48	34.31		36.62	26.89		45.53	34.86	
LSD at 5%												
Varieties	V = 0.12(Sig.)			V = 1.12(Sig.)			V = 0.08(Sig.)			V = 7.99(Sig.)		
Treatment	T = 0.19(Sig.)			T = 1.76(Sig.)			T = 0.13 (Sig.)			T = 1.83(Sig.)		
Var. x Treat.	V x T = 1.26(Sig.)			V x T = (NS)			V x T = 4.19(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

4.5.7 Leaf water potential (LWP)

It was observed (Table 55) that LWP of the plants increased with the advancement of age or foliar application of SNP (10^{-5} M) at the two growth stages i.e. 30 and 60 DAS. The plants grown in the soil supplemented with Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) possessed significantly lower LWP, depending on the metal concentration. At 30 DAS, the three levels of Cd (25, 50 or 100 mg Kg^{-1}) decreased LWP in Varuna by 17%, 27% or 38%, whereas, in RH-30 by 18%, 31% or 43% in comparison to their respective controls (Cd 0 mg Kg^{-1} and sprayed with water). The loss of LWP was higher in RH-30 as compared to Varuna. However, the metal stressed plants on being sprayed with SNP lost the damage caused to its LWP by 25 or 50 mg Cd Kg^{-1} and the values were comparable with the stress free plants. Moreover, SNP also overcome the LWP of the plants exposed to 100 mg Cd Kg^{-1} of soil, partially. Leaves of Varuna had higher leaf water potential and responded more positively than EH-30.

4.5.8 Nitrate reductase (NR) and carbonic anhydrase (CA) activity

With the progress of plant growth, the activity of these enzymes increased (Table 56). However, the metal stressed plants possessed lower level of their activity, compared with the control, in a manner determined by Cd concentration. The plants that received SNP to their foliage exhibited 15% and 29% increase in Varuna whereas; in RH-30 it was 11% and 23%, compared to their respective water sprayed control plants at 60 DAS. Moreover, the loss in the activity of NR and CA, in the two cultivars, by 25 or 50 mg Cd Kg^{-1} of soil was completely regained on being supplied with SNP to their foliage both at 30 and 60 DAS. In addition to this SNP also partially overcome the impact of 100 mg Cd Kg^{-1} of soil. Varuna recorded higher level of enzyme activity as compared to RH-30.

4.5.9 Activity of antioxidant system

The activity of antioxidant enzymes i.e. peroxidase (POX), catalase (CAT), superoxide dismutase (SOD) and the level of proline increased as growth progressed from 30 to 60 DAS (Tables 57 and 58). Availability of Cd (0, 25, 50 or 100 mg Kg^{-1}) in soil, in a concentration dependent manner, has an additive effect on the antioxidant enzymes activity and proline level. Moreover, the application of 10^{-5} M SNP with

different levels of Cd further promoted their level in both the cultivars. Out of the two varieties, the higher values were recorded in Varuna as compared to RH-30 at both the stages of growth. The per cent increase was more prominent at 30 DAS than that 60 DAS. Out of the two cultivars, Varuna grown with 100 mg Cd Kg^{-1} of soil and followed with the application of 10^{-5}M SNP solution generated maximum increase in antioxidant enzymes activity which at 30 DAS was 91% (POX), 62% (CAT), 94% (SOD) and 95% (proline) higher, compared to that of control (Cd 0 mg Kg^{-1}, water sprayed).

4.5.10 Cd accumulation in root and shoot

The data presented in table 59 revealed that increasing Cd level (0, 25, 50 or 100 mg Kg^{-1}) led to higher level of metal accumulation in root and shoot tissues. Root accumulated more Cd than that of shoot in dose dependent manner. Cd accumulation increased with the plant age. SNP (10^{-5} M) significantly inhibited the Cd accumulation in root and shoot tissue at 60 day stage of growth. Varuna accumulated lesser Cd in both the tissues as compared to RH-30. The root and shoot accumulation of Cd in Varuna was 0.80μg and 0.48μg (0 mg Kg^{-1}), 70μg and 13μg (25 mg Kg^{-1}), 139μg and 31μg (50 mg Kg^{-1}), 198μg and 68μg (100 mg Kg^{-1}) of Cd g^{-1} of tissue dry mass, respectively, at 60 DAS.

4.5.11 Yield characteristics

The data presented in table 60 revealed that 10^{-5} M SNP, applied to the plant foliage, significantly increased the number of pods per plant and seed yield per plant in Varuna and RH-30 compared to the control, sprayed with water. However, the presence of Cd in the soil reduced all the yield characteristics (number of pods per plant, seeds per pod, mass of 100 seeds and seed yield) in the two varieties, more being in RH-30. The loss of number of pods and seed yield per plant generated by lowest concentration of Cd (25 mg Kg^{-1}) was completely neutralized by 10^{-5} M SNP in both the varieties and the values were comparable with that of the control. Moreover, the losses induced by 50 or 100 mg Cd Kg^{-1} of soil were partially overcome by SNP treatment.

Table 53. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on net photosynthetic rate (P_N; μ mol CO_2 $m^{-2}sec^{-1}$) and stomatal conductance (g_s; mol H_2O m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Net photosynthetic rate						Stomatal conductance					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	14.06	12.04	13.05	19.55	16.27	17.91	0.082	0.061	0.072	0.098	0.079	0.089
SNP	14.80	12.61	13.71	20.19	16.63	18.41	0.092	0.068	0.080	0.125	0.094	0.110
$CdCl_2$ (25mg)	12.41	9.73	11.07	15.30	10.81	13.06	0.049	0.041	0.045	0.081	0.060	0.071
$CdCl_2$ (50mg)	10.48	7.28	08.88	12.63	7.85	10.24	0.037	0.030	0.033	0.062	0.043	0.052
$CdCl_2$ (100mg)	09.26	6.01	07.64	10.93	5.78	8.36	0.024	0.019	0.022	0.045	0.026	0.035
$CdCl_2$ (25mg)+ SNP	14.74	12.46	13.60	20.06	16.62	18.34	0.098	0.071	0.085	0.116	0.091	0.106
$CdCl_2$ (50mg)+ SNP	14.14	11.38	12.76	18.87	15.57	17.22	0.092	0.066	0.079	0.108	0.085	0.100
$CdCl_2$(100mg)+SNP	11.53	9.65	10.59	15.67	12.73	14.20	0.075	0.055	0.065	0.089	0.070	0.080
Mean	14.49	11.59		19.03	14.61		0.079	0.059		0.103	0.078	
LSD at 5%												
Varieties	V = 0.61(Sig.)			V = 0.72(Sig.)			V = 0.002(Sig.)			V = 0.001(Sig.)		
Treatment	T = 0.24(Sig.)			T = 0.11(Sig.)			T = 0.003 (Sig.)			T = 0.001(Sig.)		
Var. x Treat.	V x T = 0.14(Sig.)			V x T = 0.16(Sig.)			V x T = (NS)			V x T = 0.005(Sig.)		

Sig = Significant; NS = Non-significant

Table 54. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on internal CO_2 concentration (Ci; ppm) and transpiration rate (E; m mol m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Internal CO_2 concentration						Transpiration rate					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	312	290	301	357	319	338	3.18	2.88	3.03	4.50	4.16	4.33
SNP	356	319	338	383	339	361	4.02	3.37	3.70	5.41	4.76	5.09
$CdCl_2$ (25mg)	281	230	256	329	266	298	2.90	2.42	2.66	4.27	3.42	3.84
$CdCl_2$ (50mg)	258	209	234	318	245	281	2.66	1.93	2.30	3.98	3.11	3.55
$CdCl_2$ (100mg)	237	189	213	306	221	264	2.38	1.70	2.04	3.69	2.88	3.28
$CdCl_2$ (25mg)+ SNP	343	311	327	388	341	364	3.48	3.07	3.28	4.79	4.37	4.58
$CdCl_2$ (50mg)+ SNP	319	289	304	368	323	346	3.25	2.93	3.09	4.56	4.14	4.35
$CdCl_2$ (100mg)+ SNP	272	217	245	328	272	300	3.12	2.78	2.95	4.33	3.91	4.12
Mean	340	293		397	332		3.57	3.01		5.08	4.39	
LSD at 5%												
Varieties	V = 2.77(Sig.)			V = 3.56(Sig.)			V = 0.08(Sig.)			V = 0.14(Sig.)		
Treatment	T = 3.83(Sig.)			T = 5.63(Sig.)			T = 0.12(Sig.)			T = 0.22(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.17(Sig.)			V x T = 0.32(Sig.)		

Sig = Significant; NS = Non-significant

Table 55. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on maximum quantum yield of PSII and leaf water potential (MPa) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Maximum quantum yield of PSII						Leaf water potential					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.774	0.764	0.769	0.809	0.779	0.794	-0.60	-0.71	-0.66	-0.40	-0.55	-0.48
SNP	0.795	0.782	0.789	0.820	0.789	0.805	-0.42	-0.49	-0.46	-0.32	-0.42	-0.37
$CdCl_2$ (25mg)	0.728	0.701	0.715	0.774	0.735	0.755	-0.70	-0.84	-0.77	-0.46	-0.62	-0.54
$CdCl_2$ (50mg)	0.662	0.621	0.642	0.727	0.681	0.704	-0.76	-0.93	-0.84	-0.51	-0.68	-0.59
$CdCl_2$ (100mg)	0.584	0.535	0.560	0.660	0.624	0.642	-0.83	-1.01	-0.92	-0.58	-0.76	-0.67
$CdCl_2$ (25mg)+ SNP	0.781	0.770	0.776	0.813	0.776	0.795	-0.51	-0.62	-0.57	-0.33	-0.48	-0.40
$CdCl_2$ (50mg)+ SNP	0.773	0.762	0.768	0.806	0.769	0.788	-0.59	-0.70	-0.65	-0.38	-0.53	-0.45
$CdCl_2$(100mg)+SNP	0.768	0.756	0.762	0.798	0.761	0.780	-0.71	-0.81	-0.76	-0.45	-0.62	-0.53
Mean	0.838	0.813		0.887	0.845		-0.73	-0.87		-0.49	-0.66	
LSD at 5%												
Varieties	V = 0.004(Sig.)			V = 0.005(Sig.)			V = 0.04(Sig.)			V = 0.03(Sig.)		
Treatment	T = 0.002(Sig.)			T = 0.005(Sig.)			T = 0.05(Sig.)			T = 0.04(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = 0.008(Sig.)			V x T = 0.10(Sig.)			V x T = 0.06(Sig.)		

Sig = Significant; NS = Non-significant

Table 56. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on the activity of nitrate reductase [NR; n mole NO_2 g^{-1} s^{-1} (FM)] and carbonic anhydrase [CA; mole (CO_2) g^{-1} (FM) s^{-1}] in the leaves of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Nitrate reductase activity						Carbonic anhydrase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	421	387	404	432	414	423	2.10	2.00	2.05	2.45	2.30	2.38
SNP	482	427	455	497	459	478	2.70	2.46	2.58	2.99	2.65	2.82
$CdCl_2$ (25mg)	360	274	317	391	301	346	1.60	1.46	1.53	2.17	1.92	2.05
$CdCl_2$ (50mg)	327	215	271	365	252	309	1.48	1.32	1.40	1.88	1.36	1.62
$CdCl_2$ (100mg)	286	183	235	337	203	270	1.31	1.10	1.21	1.73	1.00	1.32
$CdCl_2$ (25mg)+ SNP	474	429	451	488	467	478	2.34	2.14	2.24	2.84	2.64	2.74
$CdCl_2$ (50mg)+ SNP	442	395	419	476	443	460	2.12	2.01	2.07	2.52	2.34	2.43
$CdCl_2$ (100mg)+ SNP	347	278	312	389	358	374	1.74	1.17	1.46	2.13	1.91	2.02
Mean	448	370		482	414		2.20	1.95		2.66	2.30	
LSD at 5%												
Varieties	V = 29.76(Sig.)			V = 6.38(Sig.)			V = 0.07(Sig.)			V = 0.81(Sig.)		
Treatment	T = 28.68(Sig.)			T = 10.09 (Sig.)			T = 0.11(Sig.)			T = 0.17(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.16(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 57. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on peroxidase [POX; units (g FM)$^{-1}$] and catalase [CAT; μ mol H_2O_2 decomposed (g FM)$^{-1}$] activity of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Peroxidase activity						Catalase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	13.00	7.12	10.06	17.20	9.29	13.25	419	382	401	440	392	416
SNP	16.79	8.98	12.89	21.31	10.54	15.93	488	426	457	498	418	458
$CdCl_2$ (25mg)	16.91	8.40	12.66	21.21	10.62	15.92	512	421	467	489	430	460
$CdCl_2$ (50mg)	18.53	9.72	14.13	22.77	11.97	17.37	537	447	492	510	452	481
$CdCl_2$ (100mg)	20.37	11.05	15.71	24.52	13.35	18.94	561	472	517	539	476	507
$CdCl_2$ (25mg)+ SNP	20.03	9.60	14.82	24.95	12.03	18.49	566	467	517	550	458	504
$CdCl_2$ (50mg)+ SNP	24.07	11.78	17.93	30.48	13.43	21.96	637	515	576	586	482	534
$CdCl_2$(100mg)+SNP	24.81	12.79	18.80	32.37	14.77	23.57	678	539	608	644	505	574
Mean	22.07	11.35		27.83	13.71		628	524		608	516	
LSD at 5%												
Varieties	V = 1.35(Sig.)			V = 2.40(Sig.)			V = 6.09(Sig.)			V = 51.00(Sig.)		
Treatment	T = 0.71(Sig.)			T = 1. 24(Sig.)			T = 9.63 (Sig.)			T = 18.64(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 13.62(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 58. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on superoxide dismutase [SOD; units g^{-1} (FM)] activity and proline content [μ mol (g FM)$^{-1}$] of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Superoxide dismutase activity						Proline content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	131	119	125	164	132	148	14.13	11.22	12.68	18.15	13.18	15.67
SNP	151	134	143	186	148	167	17.25	13.54	15.40	20.89	15.38	18.14
$CdCl_2$ (25mg)	185	140	163	216	153	184	20.01	13.94	16.98	24.12	15.94	20.03
$CdCl_2$ (50mg)	203	165	184	247	174	210	22.68	16.67	19.68	27.02	18.66	22.84
$CdCl_2$ (100mg)	236	204	220	294	196	245	25.75	19.61	22.68	30.23	21.43	25.83
$CdCl_2$ (25mg)+ SNP	216	173	195	245	183	214	21.92	16.60	19.26	27.07	18.52	22.80
$CdCl_2$ (50mg)+ SNP	233	198	216	274	221	248	24.64	18.53	21.59	30.15	21.59	25.87
$CdCl_2$ (100mg)+ SNP	255	224	239	341	250	296	27.60	21.52	24.56	33.24	24.67	28.95
Mean	230	194		281	208		24.85	18.80		30.12	21.34	
LSD at 5%												
Varieties	V = 4.27(Sig.)			V = 32.82(Sig.)			V = 2.73(Sig.)			V = 3.20(Sig.)		
Treatment	T = 14.38(Sig.)			T = 17.39(Sig.)			T = 1.81(Sig.)			T = 1.76(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 59. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on Cd content (µg g^{-1} DW) in root and shoot (µg g^{-1} tissue dry weight) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root Cd content						Shoot Cd content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.55	0.70	0.63	0.80	0.88	0.84	0.36	0.60	0.48	0.48	0.55	0.51
SNP	0.56	0.69	0.62	0.64	0.73	0.68	0.34	0.60	0.47	0.36	0.42	0.39
$CdCl_2$ (25mg)	50.80	68.70	59.75	69.92	76.92	73.22	10.30	17.82	14.06	12.90	20.83	16.87
$CdCl_2$ (50mg)	102.50	140.50	121.50	139.20	178.96	159.08	25.00	44.83	34.92	31.05	49.16	40.11
$CdCl_2$ (100mg)	159.60	209.00	184.30	198.26	270.02	234.14	56.80	98.30	77.55	67.62	106.88	87.25
$CdCl_2$ (25mg)+ SNP	49.73	64.70	56.37	65.09	73.20	69.14	13.98	23.06	18.52	12.50	15.24	13.87
$CdCl_2$ (50mg)+SNP	91.49	139.89	115.69	135.47	176.61	156.04	23.92	41.29	32.60	23.64	32.78	28.21
$CdCl_2$(100mg)+SNP	139.50	209.08	174.10	195.71	268.24	231.97	50.68	87.25	68.97	54.25	81.71	67.98
Mean	84.96	119.04		115.01	149.31		25.91	44.82		28.97	43.94	
LSD at 5%												
Varieties	V = 0.12(Sig.)			V = 0.47(Sig.)			V = 1.27(Sig.)			V = 0.68(Sig.)		
Treatment	T = 0.01(Sig.)			T = 0.02(Sig.)			T = 0.10(Sig.)			T = 0.11(Sig.)		
Var. x Treat.	V x T = 0.18(Sig.)			V x T = (NS)			V x T = 0.28(Sig.)			V x T = 0.15(Sig.)		

Sig = Significant; NS = Non-significant

Table 60. Effect of sodium nitroprusside (SNP; 10^{-5} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on yield characteristics of *Brassica juncea* cv. Varuna and RH-30 at harvest

Treatment	Pods plant^{-1}			Seeds pod^{-1}			100 seed mass (mg)			Seeds yield plant^{-1} (g)		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	227	219	223	12.61	12.03	12.32	322	311	317	9.22	8.19	8.71
SNP	270	240	255	12.80	12.18	12.49	334	322	328	11.53	9.41	10.47
$CdCl_2$ (25mg)	199	181	190	12.04	11.22	11.63	313	301	307	7.50	6.11	6.81
$CdCl_2$ (50mg)	185	165	175	11.71	10.91	11.31	308	298	303	6.67	5.35	6.01
$CdCl_2$ (100mg)	173	154	164	11.09	10.04	10.57	299	288	294	5.74	4.51	5.13
$CdCl_2$ (25mg) + SNP	247	236	241	12.08	11.39	11.74	317	305	311	9.45	8.20	8.83
$CdCl_2$ (50mg) +SNP	228	215	221	11.75	10.94	11.35	311	300	306	8.33	7.04	7.69
$CdCl_2$(100mg) +SNP	204	191	198	11.13	10.07	10.60	303	291	297	6.88	5.60	6.24
Mean	248	229		13.60	12.68		358	345		9.33	7.77	
LSD at 5%												
Varieties	V = 3.88(Sig.)			V = 1.44(Sig.)			V = 7.19(Sig.)			V = 0.29(Sig.)		
Treatment	T = 10.64(Sig.)			T = 0.20 (Sig.)			T = 4.37(Sig.)			T = 0.46(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

4.6 EXPERIMENT 6

This experiment was designed to study the combined effect of foliar application of EBL (10^{-8} M) and SNP (10^{-5} M) in countering the Cd toxicity, taking the two same varieties of *B. juncea* (L.) Czern & Coss; Varuna and RH-30. All the agricultural practices, sampling and other parameters studied were same as in the previous experiments. Different levels of Cd (0, 25, 50 or 100 mg Kg^{-1}) were applied to the soil, at the time of sowing while foliar spray of distilled water or SNP and/or EBL was given at 28 and 29 DAS, respectively.

4.6.1 Root and shoot length

Table 61 revealed that the shoot and root length increased from day 30 to 60. Cadmium in different levels (0, 25, 50 or 100 mg Kg^{-1}) significantly decreased the root and shoot length in Varuna and RH-30 at both the stages of growth (30 to 60 DAS), in a concentration dependent manner. Whereas, foliar spray of SNP (10^{-5} M) and/or EBL (10^{-8} M) significantly increased the root and shoot length in stressfree plants, at 60 DAS in both the varieties. The response of Varuna was better, compared to RH-30. The combination of SNP and EBL, increased the length of root and shoot in Varuna by 57% and 47% while in RH-30 by 47% and 41% over that of the respective control (with Cd 0 mg Kg^{-1}, water sprayed) at 60 DAS and also recovered completely the damage caused by 25 or 50 mg Cd Kg^{-1} but partially that of 100 mg Cd Kg^{-1}, at 60 DAS.

4.6.2 Fresh and dry mass of root

With the growth progression from 30 to 60 days the fresh and dry mass of plant root increased (Table 62). Root fresh and dry mass was significantly reduced with the increasing concentration of Cd (0, 25, 50 or 100 mg Kg^{-1}), whereas; significantly increased with the foliar spray of SNP (10^{-5} M) and/or EBL (10^{-8} M). The increase of root fresh and dry mass brought about by SNP plus EBL treatment in Varuna was 42% and 51%, whereas, in RH-30 it was 38% and 44%. Moreover, the combination of SNP and EBL generated complete recovery in the root fresh and dry mass of Varuna and RH-30, against two lower concentrations of Cd whereas, partially that of 100 mg Kg-1 of soil at 60 DAS The values after the complete recovery in the metal stressed plants were more

than that of the control plants. Out of the two varieties, Varuna was more responsive to the treatments than RH-30.

4.6.3 Fresh and dry mass of shoot

Shoot fresh and dry mass of the plants followed a pattern similar to that of the root (Table 63). Increasing level of Cd (0, 25, 50 or 100 mg Kg^{-1}) progressively decreased the fresh and dry mass of shoot in the two mustard varieties (Varuna and RH-30). SNP and EBL increased fresh and dry mass of shoot of non-stressed plants which was 46% and 49% in Varuna and 42% and 46% in RH-30, as compared to control (Cd 0 mg Kg^{-1}, water sprayed) at 60 day stage. Moreover, the treatment with the combination of EBL (10^{-8} M) and SNP (10^{-5} M) to the stressed plants (25 or 50 mg Cd Kg^{-1} of soil) completely neutralized the impact of the meal and the values were more than that of the control and was partially overcome that of 100 mg Cd Kg^{-1} of soil, at 60 DAS. The variety Varuna gained higher values of shoot fresh and dry mass than RH-30.

4.6.4 Leaf area

Table 64 denotes that the leaf area increased with the advancement of plant age (30 to 60 DAS) whereas the presence Cd decreased its values in dose dependent manner (0, 25, 50 or 100 mg Kg^{-1}). Foliar spray of EBL (10^{-6} M) and SNP (10^{-5} M) significantly increased the leaf area, preferably in stress free Varuna followed by RH-30, at 60 day stage. On the other hand, at 60 DAS, Cd at the concentration of 25 or 50 mg Kg^{-1} decreased the values of leaf area in Varuna by 15% and 27% whereas in RH-30 by 27% and 34%, than that of non-stressed control (Cd; 0 mg Kg^{-1} of soil and foliar spray of water) but this damage was completely overcome by EBL+SNP, as a follow up treatment to the stressed plants. Moreover, the treatment also induced partial recovery in the plants exposed to 100 mg Cd Kg^{-1} of soil.

4.6.5 SPAD value of chlorophyll

The leaf chlorophyll content (SPAD value) increased with plant age from 30 to 60 days (Table 64). The foliar application of SNP (10^{-5} M) alone or in combination with EBL (10^{-8} M) significantly increased the leaf chlorophyll content both in Varuna and RH-30. Combination of SNP and EBL improved the values by 29% (Varuna) and 24% (RH-30) at 30 DAS, and at 60 DAS the increase over the respective stress free control was

Table 61. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root and shoot length (cm) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root length						Shoot length					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	14.09	12.03	13.06	25.52	22.69	24.11	29.87	25.02	27.45	74.13	69.64	71.89
SNP	16.07	14.89	15.48	32.93	28.19	30.56	29.91	25.16	27.54	99.81	92.24	96.03
SNP + EBL	16.80	15.34	16.07	40.19	33.41	36.80	30.13	26.01	28.07	109.10	98.50	103.80
$CdCl_2$ (25mg)	09.86	07.57	08.72	19.12	16.39	17.76	25.82	21.63	23.73	61.26	55.68	58.47
$CdCl_2$ (50mg)	08.00	05.01	06.51	16.83	13.01	14.92	22.68	18.36	20.52	56.12	48.73	52.43
$CdCl_2$ (100mg)	06.17	04.16	05.17	14.24	10.98	12.61	19.38	15.25	17.32	51.82	42.81	47.32
$CdCl_2$(25mg)+SNP+EBL	11.47	9.77	10.62	31.68	28.00	29.84	26.18	21.98	24.08	91.09	78.24	84.67
$CdCl_2$(50mg)+SNP+EBL	9.10	6.55	7.83	28.75	25.09	26.92	22.78	18.71	20.75	85.32	73.61	79.47
$CdCl_2$(100mg)+SNP+EBL	7.30	5.26	6.28	22.21	18.72	20.46	19.40	15.39	17.40	67.98	62.37	65.18
Mean	10.98	8.95		25.72	21.83		25.13	20.83		77.40	69.09	
LSD at 5%												
Varieties	V = 0.71(Sig.)			V = 3.88(Sig.)			V = 1.48(Sig.)			V = 5.92(Sig.)		
Treatment	T = 0.83(Sig.)			T = 2.02(Sig.)			T = 2.56(Sig.)			T = 4.25(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 62. Effect of sodium nitroprusside (SNP; 10^{-5} M)and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on root fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root fresh mass						Root dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	2.98	2.79	2.89	5.30	4.62	4.96	0.76	0.61	0.69	1.70	1.27	1.49
SNP	3.02	2.81	2.92	6.83	5.61	6.22	0.78	0.62	0.70	2.35	1.60	1.98
SNP + EBL	3.01	2.83	2.92	7.55	6.40	6.98	0.76	0.61	0.69	2.41	1.77	2.09
$CdCl_2$ (25mg)	1.92	1.66	1.79	3.97	3.03	3.50	0.62	0.47	0.55	1.48	0.92	1.20
$CdCl_2$ (50mg)	1.45	1.29	1.37	3.42	2.72	3.07	0.48	0.34	0.41	1.34	0.77	1.05
$CdCl_2$ (100mg)	1.13	0.92	1.03	3.06	2.14	2.60	0.35	0.19	0.27	1.20	0.63	0.92
$CdCl_2$(25mg)+SNP+EBL	1.85	1.12	1.49	6.31	5.64	5.98	0.69	0.55	0.62	2.18	1.64	1.91
$CdCl_2$(50mg)+SNP+EBL	1.52	0.85	1.19	5.43	4.76	5.10	0.53	0.39	0.46	1.92	1.41	1.67
$CdCl_2$(100mg)+SNP+EBL	1.18	0.54	0.86	4.55	3.87	4.21	0.37	0.23	0.30	1.62	1.17	1.39
Mean	2.01	1.80		5.19	4.26		0.57	0.43		1.85	1.27	
LSD at 5%												
Varieties	V = 0.15(Sig.)			V = 0. 50(Sig.)			V = 0.01(Sig.)			V = 0.31(Sig.)		
Treatment	T = 0.26(Sig.)			T = 0.30(Sig.)			T = 0.02(Sig.)			T = 0.08(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.03(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 63. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on shoot fresh and dry mass (g) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Shoot fresh mass						Shoot dry mass					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	6.09	5.12	5.61	11.16	9.98	10.57	1.79	1.67	1.73	3.22	2.72	2.97
SNP	6.19	5.26	5.73	14.82	12.86	13.84	1.81	1.68	1.75	4.48	3.71	4.10
SNP + EBL	6.25	5.22	5.74	16.28	14.22	15.25	1.84	1.67	1.76	4.59	3.80	4.20
$CdCl_2$ (25mg)	4.70	3.25	3.98	9.34	6.36	7.85	1.25	0.85	1.05	2.77	2.06	2.41
$CdCl_2$ (50mg)	4.20	2.71	3.46	8.70	5.72	7.21	1.11	0.72	0.98	2.41	1.66	2.04
$CdCl_2$ (100mg)	3.65	2.30	2.98	7.86	5.09	6.48	0.97	0.58	0.78	2.06	1.31	1.69
$CdCl_2$(25mg)+SNP+EBL	4.80	3.12	3.96	13.48	11.62	12.55	1.28	0.92	1.10	4.18	3.44	3.81
$CdCl_2$(50mg)+SNP+EBL	4.31	2.67	3.49	12.15	10.09	11.12	1.14	0.79	0.97	3.65	2.90	3.28
$CdCl_2$(100mg)+SNP+EBL	3.88	2.28	3.08	10.25	8.76	9.50	1.00	0.66	0.83	3.11	2.35	2.73
Mean	4.92	3.57		11.61	9.52		1.34	1.05		3.44	2.74	
LSD at 5%												
Varieties	V = 0.22(Sig.)			V = 1.36(Sig.)			V = 0.31(Sig.)			V = 1.06(Sig.)		
Treatment	T = 0.37(Sig.)			T = 0.62(Sig.)			T = 0.10(Sig.)			T = 0.21(Sig.)		
Var. x Treat.	V x T = 0.53(Sig.)			V x T = (NS)			V x T = 0.12(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 64. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on leaf area (cm^2) and SPAD value of chlorophyll of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Leaf area						SPAD value of chlorophyll					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	24.58	22.83	23.71	36.39	29.67	33.03	43.23	32.13	37.68	58.61	49.08	53.85
SNP	24.65	22.87	23.76	43.73	34.25	38.99	51.39	37.10	44.25	69.01	56.63	62.82
SNP + EBL	24.48	22.27	23.38	47.22	37.17	42.20	55.82	39.85	47.84	72.24	58.84	65.54
$CdCl_2$ (25mg)	18.94	15.51	17.23	30.88	21.73	26.30	34.21	20.99	27.60	50.35	35.57	42.96
$CdCl_2$ (50mg)	15.82	13.15	14.49	26.67	19.71	23.19	27.22	13.91	20.57	43.96	28.52	36.24
$CdCl_2$ (100mg)	12.77	10.40	11.59	23.72	15.31	19.52	21.01	09.04	15.03	38.19	22.14	30.17
$CdCl_2$(25mg)+SNP+EBL	19.21	15.78	17.50	42.91	33.81	38.36	47.52	34.94	41.23	68.05	57.03	62.54
$CdCl_2$(50mg)+SNP+EBL	15.76	12.90	14.33	39.63	31.80	35.72	45.87	33.61	39.74	64.65	52.43	58.54
$CdCl_2$(100mg)+SNP+EBL	13.29	9.72	11.51	35.31	27.47	31.39	38.02	27.04	32.53	57.96	47.27	52.62
Mean	18.83	16.16		36.27	27.88		40.48	27.62		58.11	45.23	
LSD at 5%												
Varieties	V = 0.18(Sig.)			V = 1.33(Sig.)			V = 1.71(Sig.)			V = 1.27(Sig.)		
Treatment	T = 0.31(Sig.)			T = 2.00(Sig.)			T = 1.96(Sig.)			T = 2.19(Sig.)		
Var. x Treat.	V x T = 1.60(Sig.)			V x T = (NS)			V x T = (NS)			V x T = 3.10 (Sig.)		

Sig = Significant; NS = Non-significant

23% and 20% at the two growth stages. In contrary to the above, soil fed with Cd (25, 50 or 100 mg Cd Kg^{-1}) significantly reduced the of chlorophyll level at both the stages of growth. However, the plants raised with 25 or 50 mg Cd Kg^{-1} and supplemented with SNP and EBL exhibited the SPAD values above the non-stressed control plants at 30 and 60 DAS, in Varuna and RH-30. Moreover, the treatment was more effective with Varuna than RH-30.

4.6.6 Photosynthetic parameters

Photosynthetic characteristics (net photosynthetic rate; P_N, stomatal conductance; g_s, internal CO_2 concentration; C_i, transpiration rate; E and maximum quantum yield of PSII; Fv/Fm) of the two varieties increased as the growth progressed from 30 to 60 day stage (Tables 65-67). The foliage of the plants that received the treatment of SNP (10^{-5} M) alone or in combination with EBL (10^{-8} M) exhibited significant improvement in all the aforesaid traits, compared with the control. The per cent increase of P_N, g_s, C_i, E and Fv/Fm was more in SNP plus EBL treated plants which at 30 day stage in Varuna was 13%, 36%, 23%, 34% and 6%, whereas, in RH-30 was12%, 32%, 20%, 27% and 5% higher as compared to control (Cd; 0 mg Kg^{-1} of soil, sprayed with water). The plants raised in the presence of Cd exhibited a loss in the values of all the above parameters. However, the foliar spray of stressed plants with SNP and EBL completely regained the normal rate (that of control) of photosynthetic and associated attributes in the two varieties against 25 and 50 mg Cd Kg^{-1}, whereas, it was partial against 100 mg Cd Kg^{-1} of soil, at the two stages of growth. Varuna showed better photosynthetic responses than RH-30.

4.6.7 Leaf water potential (LWP)

Table 67 revealed that the LWP was relatively higher at 60 DAS than at 30 DAS in both the varieties. The soil amended with Cd (0, 25,. 50 or 100 mg Kg^{-1}) led to a significant loss of LWP in concentration dependent manner at the two stages of growth. However, the LWP values improved as the foliage of the stressed plants was sprayed with SNP and/or EBL. At 60 DAS, the per cent increase of LWP under SNP and EBL treatment was 49% in Varuna and 44% in RH-30, respectively, as compared to control

plants (Cd; 0 mg Kg^{-1} of soil, water sprayed). Moreover, the values of LWP lowered by 25 and 50 mg Cd Kg^{-1} of soil was completely recovered through foliar treatment of SNP and EBL combination, whereas, that of 100 mg Cd Kg^{-1} of soil was only partially neutralized at 60 DAS, in the two cultivars. Out of the two varieties, Varuna exhibited higher values of LWP than RH-30.

4.6.8 Nitrate reductase (NR) and carbonic anhydrase (CA) activity

The activity of NR and CA was more at 60 DAS than at 30 DAS i.e. increased with plant age (Table 68). In Varuna and RH-30, Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) decreased the activity of both the enzymes in a manner dependent on Cd concentration. Against lowest concentration (25 mg Cd Kg^{-1} of soil), the recorded decline of NR and CA activity in the leaves of Varuna and RH-30 was 14% and 29% (NR) and 24% and 27% (CA), at 30 DAS. On the other hand, the treatment with SNP (10^{-5} M) and/or EBL (10^{-8} M) to the non-stressed plants significantly increased the NR and CA activity over the control. Moreover the combination of SNP plus EBL applied to the stressed (25 or 50 mg Cd $Kg^{-1}$of soil) plants improved the values of NR and CA which were comparable with those of the control (Cd; 0 mg Kg^{-1} of soil, water sprayed). However, the recovery was partial in plants growth with 100 mg Cd kg^{-1} of soil. The activity of the two enzymes was higher in Varuna over RH-30, irrespective of the treatments.

4.6.9 Activity of antioxidant system

The data depicted in tables 69 and 70 showed that the activity of antioxidant enzymes (peroxidase; POX, catalase; CAT, superoxide dismutase; SOD) and leaf proline content increased as the growth progressed from 30 to 60 DAS. Moreover, the soil amendment with Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) and/or the foliage applied with SNP and/or EBL further increased the antioxidant enzymes activity and proline level in the two varieties (Varuna and RH-30) at both the growth stages. The values were higher at 60 DAS than that of 30 DAS. Maximum increase in enzymes activity was recorded in plants that received the foliar spray of SNP with EBL in concentration with 100 mg Cd Kg^{-1} of soil. The increase in the activity of POX, CAT, SOD and proline level in RH-30 was 77%, 37% 86% and 109%, whereas, in Varuna it was 102%, 52% 106% and 113%,

Table 65. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on net photosynthetic rate (P_N; μ mol CO_2 $m^{-2}sec^{-1}$) and stomatal conductance (g_s; mol H_2O m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Net photosynthetic rate						Stomatal conductance					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	14.09	12.02	13.06	19.54	16.26	17.90	0.081	0.062	0.072	0.093	0.078	0.086
SNP	14.83	12.59	13.71	20.18	16.63	18.41	0.091	0.069	0.080	0.119	0.093	0.106
SNP + EBL	15.86	13.48	14.67	21.29	17.28	19.29	0.110	0.082	0.096	0.131	0.104	0.118
$CdCl_2$ (25mg)	12.43	09.72	11.08	15.29	10.79	13.04	0.048	0.041	0.045	0.077	0.060	0.068
$CdCl_2$ (50mg)	10.49	07.28	08.89	12.62	07.85	10.24	0.037	0.030	0.033	0.059	0.042	0.050
$CdCl_2$ (100mg)	09.29	06.01	07.65	10.95	05.63	08.29	0.024	0.019	0.022	0.042	0.026	0.034
$CdCl_2$(25mg)+SNP+EBL	15.12	12.82	13.97	20.23	16.78	18.51	0.119	0.088	0.104	0.133	0.109	0.121
$CdCl_2$(50mg)+SNP+EBL	14.19	12.06	13.13	19.64	16.30	17.97	0.096	0.072	0.084	0.108	0.089	0.099
$CdCl_2$(100mg)+SNP+EBL	12.67	10.38	11.53	17.95	14.88	16.42	0.083	0.063	0.073	0.091	0.075	0.083
Mean	13.22	10.71		17.52	13.62		0.076	0.059		0.095	0.075	
LSD at 5%												
Varieties	V = 1.10(Sig.)			V = 1.55(Sig.)			V = 0.014(Sig.)			V = 0.001(Sig.)		
Treatment	T = 0.25(Sig.)			T = 0.31(Sig.)			T = 0.003 (Sig.)			T = 0.002(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.005(Sig.)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 66. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on internal CO_2 concentration (Ci; ppm) and transpiration rate (E; m mol m^{-2} sec^{-1}) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Internal CO_2 concentration						Transpiration rate					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	312	289	301	350	304	327	3.05	2.97	3.01	4.56	4.28	4.42
SNP	357	323	340	376	323	350	3.85	3.33	3.59	5.49	4.90	5.20
SNP + EBL	385	346	366	398	338	368	4.09	3.77	3.93	5.81	5.14	5.48
$CdCl_2$ (25mg)	280	229	255	322	253	288	2.79	2.50	2.64	4.32	3.51	3.92
$CdCl_2$ (50mg)	258	208	233	308	231	269	2.55	1.99	2.27	4.03	3.20	3.62
$CdCl_2$ (100mg)	238	188	213	300	211	255	2.28	1.76	2.02	3.73	2.96	3.35
$CdCl_2$(25mg)+SNP+EBL	371	341	356	395	337	366	3.42	3.25	3.33	5.38	4.90	5.14
$CdCl_2$(50mg)+SNP+EBL	346	312	329	377	320	349	3.24	3.12	3.18	5.10	4.62	4.86
$CdCl_2$(100mg)+SNP+EBL	294	254	274	333	271	302	2.87	2.60	2.74	4.65	4.31	4.48
Mean	316	277		348	289		3.13	2.81		4.79	4.20	
LSD at 5%												
Varieties	V = 9.32(Sig.)			V = 7.74(Sig.)			V = 0.07(Sig.)			V = 0.31(Sig.)		
Treatment	T = 6.14(Sig.)			T = 7.40 (Sig.)			T = 0.12(Sig.)			T = 0.23(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = 0.17(Sig.)			V x T = 0.76(Sig.)		

Sig = Significant; NS – Non-significant

Table 67. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on maximum quantum yield of PSII and leaf water potential (MPa) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Maximum quantum Yield of PS II						Leaf water potential					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.773	0.763	0.768	0.807	0.778	0.793	-0.61	-0.72	-0.67	-0.43	-0.59	-0.51
SNP	0.793	0.781	0.787	0.816	0.788	0.802	-0.42	-0.50	-0.46	-0.34	-0.45	-0.39
SNP + EBL	0.817	0.805	0.811	0.824	0.808	0.816	-0.26	-0.28	-0.27	-0.22	-0.33	-0.28
$CdCl_2$ (25mg)	0.727	0.698	0.713	0.773	0.733	0.753	-0.71	-0.86	-0.78	-0.50	-0.66	-0.58
$CdCl_2$ (50mg)	0.660	0.620	0.640	0.727	0.680	0.704	-0.77	-0.94	-0.86	-0.55	-0.73	-0.64
$CdCl_2$ (100mg)	0.582	0.535	0.559	0.660	0.623	0.642	-0.84	-1.03	-0.93	-0.62	-0.81	-0.71
$CdCl_2$(25mg)+SNP+EBL	0.801	0.784	0.793	0.831	0.795	0.813	-0.47	-0.58	-0.53	-0.29	-0.47	-0.38
$CdCl_2$(50mg)+SNP+EBL	0.793	0.777	0.785	0.824	0.789	0.807	-0.56	-0.68	-0.62	-0.36	-0.53	-0.44
$CdCl_2$(100mg)+SNP+EBL	0.786	0.765	0.776	0.818	0.776	0.797	-0.65	-0.79	-0.72	-0.44	-0.60	-0.528
Mean	0.748	0.725		0.787	0.752		-0.59	-0.71		-0.42	-0.57	
LSD at 5%												
Varieties	V = 0.006(Sig.)			V = 0.004(Sig.)			V = 0.05(Sig.)			V = 0.03(Sig.)		
Treatment	T = 0.003(Sig.)			T = 0.004(Sig.)			T = 0.06(Sig.)			T = 0.05(Sig.)		
Var. x Treat.	V x T = 0.04(Sig.)			V x T = 0.10(Sig.)			V x T = 0.11(Sig.)			V x T = 0.07(Sig.)		

Sig = Significant; NS = Non-significant

Table 68. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on the activity of nitrate reductase [NR; n mole NO_2 $g^{-1}s^{-1}$ (FM)] and carbonic anhydrase [CA; mole (CO_2) g^{-1} (FM) s^{-1}] in the leaves of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Nitrate reductase activity						Carbonic anhydrase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	418	393	406	434	421	428	2.07	1.95	2.01	2.37	2.22	2.30
SNP	478	429	554	498	462	480	2.66	2.40	2.53	2.89	2.56	2.73
SNP + EBL	552	497	525	579	542	561	3.03	2.63	2.83	3.32	2.84	3.08
$CdCl_2$ (25mg)	358	279	319	393	306	350	1.58	1.43	1.50	2.11	1.85	1.98
$CdCl_2$ (50mg)	324	218	271	367	257	312	1.46	1.29	1.37	1.81	1.31	1.56
$CdCl_2$ (100mg)	284	184	234	337	207	272	1.29	1.07	1.18	1.58	0.97	1.27
$CdCl_2$(25mg)+SNP+EBL	522	462	492	580	524	552	2.47	2.19	2.33	2.79	2.57	2.68
$CdCl_2$(50mg)+SNP+EBL	481	427	454	530	489	509	2.22	2.03	2.12	2.66	2.43	2.54
$CdCl_2$(100mg)+SNP+EBL	388	339	364	443	422	433	1.97	1.66	1.81	2.52	2.15	2.34
Mean	423	359		462	403		2.07	1.84		2.44	2.09	
LSD at 5%												
Varieties	V = 30.53(Sig.)			V = 17.40(Sig.)			V = 1.39(Sig.)			V = 0.95(Sig.)		
Treatment	T = 33.44(Sig.)			T = 20.10(Sig.)			T = 0. 15(Sig.)			T = 0.13(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = (NS)		

Sig – Significant; NS – Non-significant

with respect to respective control plants (Cd, 0 mg Kg^{-1} of soil, water sprayed) at 60 DAS. RH-30 recorded lower values as compared to Varuna.

4.6.10 Cd accumulation in root and shoot

Table 71 indicates that Cd progressively accumulated in the root and shoot of two varieties (Varuna and RH-30) with the plant growth (30 to 60 DAS). Cd is preferably accumulated in root than shoot with the increasing soil Cd level (i.e. 25 < 50 < 100 mg Kg^{-1} of soil). Combination of SNP (10^{-5} M) and EBL (10^{-8} M) significantly suppressed the Cd accumulation in both the tissues at 60 DAS. RH-30 accumulated higher Cd as compared to Varuna at the two stages of growth. Cd accumulation in root and shoot of RH-30 treated with 25 mg Cd Kg^{-1} of soil and SNP (10^{-5} M) plus EBL (10^{-8} M) was 63μg and 11μg Cd g^{-1} of tissue dry mass at 60 DAS. At the same stage of growth the control plants (received Cd 0 mg Kg^{-1} and foliar spray of water) accumulated 0.86μg and 0.54μg Cd g^{-1} of tissue dry mass, at 30 and 60 DAS, respectively.

4.6.11 Yield characteristics

The data presented in table 72 represents that Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) induced stress significantly reduced the yield characteristics (number of pods per plant, seeds per pod, mass of 100 seeds and seed yield per plant). In contrary to this, SNP (10^{-5} M) and/or EBL (10^{-8} M) as foliar application, significantly increased the number of pods per plant and seed yield per plant in two varieties, with stress free conditions. The loss of seed yield per plant under 25 mg Cd Kg^{-1} of soil, was completely neutralized by the foliar treatment of stressed plants with SNP and EBL in both the varieties whereas; the recovery was partial with 50 or 100 mg Cd Kg^{-1} of soil. The foliar spray of SNP plus EBL alone or in combination had a positive impact on the number of pods per plant. Varuna clearly excelled the yield performance over RH-30.

Table 69. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on peroxidase [POX; units (g FM)$^{-1}$] and catalase [CAT; μ mol H_2O_2 decomposed (g FM)$^{-1}$] activity of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Peroxidase activity						Catalase activity					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	12.90	7.21	10.06	17.35	9.30	13.33	417	376	397	438	385	412
SNP	16.71	9.12	12.92	21.49	11.12	16.31	486	419	453	478	413	446
SNP + EBL	17.87	9.66	13.77	24.29	12.93	18.61	538	478	508	525	447	486
$CdCl_2$ (25mg)	16.78	8.52	12.65	21.41	10.63	16.02	509	415	462	486	423	455
$CdCl_2$ (50mg)	18.39	9.84	14.12	22.97	11.98	17.48	534	440	487	508	448	478
$CdCl_2$ (100mg)	20.01	11.19	15.70	24.73	13.39	19.06	566	464	515	536	467	502
$CdCl_2$(25mg)+SNP+EBL	21.79	11.55	16.67	26.68	13.58	20.13	626	495	560	577	473	525
$CdCl_2$(50mg)+SNP+EBL	26.90	13.04	19.97	32.30	14.98	23.64	705	524	614	610	501	556
$CdCl_2$(100mg)+SNP+EBL	28.39	15.08	21.74	35.09	16.49	25.79	738	573	656	667	526	597
Mean	19.99	10.58		24.97	12.57		569	465		536	454	
LSD at 5%												
Varieties	V = 0.97(Sig.)			V = 2.19 (Sig.)			V = 13.55(Sig.)			V = 15.94(Sig.)		
Treatment	T = 1.24(Sig.)			T = 1.30 (Sig.)			T = 23.47 (Sig.)			T = 27.60(Sig.)		
Var. x Treat.	V x T = 2.39 (Sig.)			V x T = 2.36(Sig.)			V x T = (NS)			V x T = (NS)		

Sig = Significant; NS = Non-significant

Table 70. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on superoxide dismutase [SOD; units g^{-1} (FM)] activity and proline content [μ mol (g FM)$^{-1}$] of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Superoxide dismutase activity						Proline content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	125	110	118	149	128	139	14.98	12.00	13.49	19.01	14.83	16.92
SNP	144	128	136	170	150	160	18.28	14.40	16.34	21.90	16.76	19.33
SNP + EBL	160	138	149	187	158	173	20.08	15.51	17.80	24.82	18.69	21.76
$CdCl_2$ (25mg)	177	129	153	197	148	172	21.22	15.02	18.12	25.06	17.93	21.50
$CdCl_2$ (50mg)	193	152	173	225	168	196	24.20	17.83	21.02	28.29	20.99	24.64
$CdCl_2$ (100mg)	226	189	207	267	190	229	27.30	20.97	24.14	31.66	24.12	27.89
$CdCl_2$(25mg)+SNP+EBL	233	178	205	246	190	218	26.71	21.12	23.92	31.95	24.30	28.13
$CdCl_2$(50mg)+SNP+EBL	239	204	221	267	213	240	30.42	24.01	27.22	35.54	27.38	31.46
$CdCl_2$(100mg)+SNP+EBL	268	225	246	307	239	273	33.37	27.09	30.23	40.42	30.95	35.69
Mean	197	162		224	177		24.18	18.63		28.74	21.78	
LSD at 5%												
Varieties	V = 15.88(Sig.)			V = 28.68(Sig.)			V = 1.32(Sig.)			V = 1.09(Sig.)		
Treatment	T = 13.50(Sig.)			T = 12.68(Sig.)			T = 2.38 (Sig.)			T = 1.89(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 2.67(Sig.)		

Sig = Significant; NS = Non-significant

Table 71. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8}M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} soil) on Cd content (µg g^{-1} DW) in root and shoot (µg g^{-1} tissue DW) of *Brassica juncea* cv. Varuna and RH-30 at 30 and 60 DAS

Treatment	Root Cd content						Shoot Cd content					
	30 DAS			60 DAS			30 DAS			60 DAS		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	0.52	0.71	0.62	0.80	0.86	0.83	0.37	0.50	0.44	0.47	0.54	0.50
SNP	0.52	0.71	0.61	0.60	0.66	0.63	0.35	0.45	0.40	0.36	0.45	0.41
SNP + EBL	0.45	0.50	0.47	0.38	0.46	0.42	0.33	0.47	0.40	0.32	0.42	0.37
$CdCl_2$ (25mg)	50.82	72.60	61.71	66.29	73.14	69.72	10.23	14.82	12.53	12.11	20.26	16.19
$CdCl_2$ (50mg)	105.40	148.20	126.80	137.84	173.03	155.44	25.94	37.54	31.74	30.23	48.29	39.26
$CdCl_2$ (100mg)	159.90	212.10	186.00	195.07	153.87	224.47	59.80	83.46	71.63	66.64	105.41	86.03
$CdCl_2$(25mg)+SNP+EBL	31.27	55.51	43.39	58.58	63.12	60.85	8.20	11.40	9.80	7.93	10.74	9.33
$CdCl_2$(50mg)+SNP+EBL	53.50	101.28	77.39	124.50	149.64	137.07	14.41	27.42	20.92	18.91	30.02	24.47
$CdCl_2$(100mg)+SNP+EBL	77.98	156.37	117.18	184.12	242.91	213.51	41.16	65.46	53.31	47.68	75.60	61.42
Mean	53.37	83.11		85.35	106.41		17.87	26.84		20.53	32.35	
LSD at 5%												
Varieties	V = 0.21(Sig.)			V = 0.11(Sig.)			V = 0.05(Sig.)			V = 0.05(Sig.)		
Treatment	T = 0.36(Sig.)			T = 0.20(Sig.)			T = 0.19(Sig.)			T = 0.08(Sig.)		
Var. x Treat.	V x T = 0.51(Sig.)			V x T = 0.28(Sig.)			V x T = 0.13(Sig.)			V x T = 0.12 (Sig.)		

Sig = Significant; NS = Non-significant

Table 72. Effect of sodium nitroprusside (SNP; 10^{-5} M) and 24-epibrassinolide (EBL; 10^{-8} M) against soil amended $CdCl_2$ (0, 25, 50 or 100 mg Kg^{-1} of soil) on yield characteristics of *Brassica juncea* cv. Varuna and RH-30 at harvest

Treatment	Pods plant^{-1}			Seeds pod^{-1}			100 seed mass (mg)			Seeds yield plant^{-1} (g)		
	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean	Varuna	RH-30	Mean
Control	215	208	212	12.58	11.93	12.26	307	303	305	8.30	7.52	7.91
SNP	256	229	243	12.77	12.08	12.43	320	314	317	10.46	8.69	9.57
SNP + EBL	282	265	274	12.87	12.18	12.53	328	322	325	11.90	10.39	11.15
$CdCl_2$(25mg)	189	172	181	12.01	11.12	11.57	291	283	287	6.61	5.42	6.01
$CdCl_2$(50mg)	176	157	167	11.68	10.82	11.25	282	269	276	5.80	4.57	5.19
$CdCl_2$(100mg)	165	146	156	11.06	09.96	10.51	269	255	262	4.90	3.72	4.31
$CdCl_2$(25mg)+SNP+EBL	258	246	252	12.26	11.36	11.81	297	288	293	9.39	8.03	8.71
$CdCl_2$(50mg)+SNP+EBL	237	226	232	11.91	11.01	11.46	287	273	280	8.10	6.79	7.45
$CdCl_2$(100mg)+SNP+EBL	217	198	207	11.22	10.16	10.69	273	258	266	6.63	5.18	5.91
Mean	222	205		12.04	11.18		295	285		8.01	6.70	
LSD at 5%												
Varieties	V = 6.10(Sig.)			V = 2.09(Sig.)			V = 2.48(Sig.)			V = 0.25(Sig.)		
Treatment	T = 10.47(Sig.)			T = 0.29(Sig.)			T = 6.45(Sig.)			T = 0.18(Sig.)		
Var. x Treat.	V x T = (NS)			V x T = (NS)			V x T = (NS)			V x T = 0.61 (Sig.)		

Sig = Significant; NS = Non-significant

Chapter 5

DISCUSSION

CHAPTER - 5

DISCUSSION

Plants face various abiotic stress signals that normally restrict their growth and productivity. One of the most important is the soil heavy metal stress caused by various anthropogenic activities. Among these metals, cadmium (Cd) is the most abundant soil contaminant with high enrichment factor in plants that generates an oxidative stress, inactivates metabolic enzymes directly or indirectly and alters membrane potential and its permeability. Various strategies are adopted to minimize/neutralize the toxic effects, generated by heavy metals in plants. Phytohormones are the natural protectors and plant growth regulator to enable them to withstand heavy metal stress and other unfavourable conditions. Here a new class of recognized hormones, the brassinosteroids (BRs) plays a pivotal role in cell division, cell elongation and expansion, seedling growth, xylem differentiation, pollen fertility, fruit setting and finally yield output (Fariduddin et al., 2013b). Out of the various analogues of BRs, 28-homobrassinolide (HBL) and 24-epibrassinolide (EBL) are very active, under field conditions (Khripach et al., 2003). Nitric oxide (NO), on the other hand is gaseous plant growth regulator plays an essential role in seed germination, hypocotyl growth, root organogenesis and stomatal closure (Beligni and Lamittina, 2000; Hayat et al., 20103b; Chaki et al., 2009). It also modulates redox signals, under normal and abiotic stress conditions in a dose dependent manner (Steber and McCourt, 2001; Neill et al., 2003; Divi et al., 2010). Therefore, an attempt has been made to investigate the efficacy of the two analogues of BRs in association with NO to improve plant growth under Cd stress. Two mustard varieties viz. Varuna and RH-30 have been selected for the study, based on our earlier experience.

An increase in soil Cd level additively favors its accumulation in the plants of Brassica (heavy metal accumulator; Szczygłowska et al., 2011). Present study also indicated that the Cd content increased in the root and shoot tissues with increasing soil Cd concentration (0-100 mg Cd Kg^{-1} of soil) in both the varieties i.e. Varuna and RH-30 (Table 11). However, RH-30 accumulated more Cd than Varuna this could be due to increased rate of root detoxification in Varuna which checked the excess Cd uptake

(Dong et al., 2007). Such varietal differences have also been reported by Song et al. (2003), Metwally et al. (2005), Sharma et al. (2010) and Akhtar (2012). Moreover, plant roots of both the varieties accumulated more Cd than the aerial parts. The main factors determining the Cd accumulation in root and shoot include Cd binding to extracellular matrix, cellular detoxification and complexation and Cd transport efficiency (Horst, 1995, Marchiol et al., 1996, Cobbett et al., 1998; Zhu et al., 1999). The retention of Cd at the root level is regarded as an important preventive mechanism of several plants (Rauser and Meuwly, 1995; Akhtar, 2012) which sometimes may reach above 75% of total metal uptake of the plant (Wojcik and Tukiendorf, 2005). Comparable observations regarding the increased tissue Cd level have already been noticed in *Brassica juncea* in response to Cd feeding (Mobin and Khan, 2007; Gill et al., 2011).

Metal toxicity is known to induce high permeability of the membrane to organic acids in young root cells and activation of anion channels, located in the cell membrane which are proposed to mediate the transport of organic acids to outside the cell (Mariano et al., 2005; Yang et al., 2013). This detoxifies the metal in the soil, by slowing down its further import. In the present experiment, Varuna has shown a lower level of Cd and better defense as compared to RH-30 possibly due to aforesaid reasons. Application of 10^{-8} M BRs (HBL/EBL) further reduced the accumulation of Cd in root and shoot of both the varieties (Table 47). BRs, possibly improved the membrane stability of root hairs that could have restricted the redial uptake of Cd. Moreover, NO (as SNP) also lowered the heavy metal uptake in the two varieties of mustard (Table 59). A combined application of 10^{-8} M of EBL and 10^{-5} M of SNP generated the best response in preventing the Cd uptake at all its levels (25, 50 or 100 mg Kg^{-1} soil) (Table 71). This observation indicated that BR and/or NO might have caused the exudation of organic acids to detoxify Cd in the soil. BRs have been implicated to be involved in the regulation of the level of sulfur-rich molecules (Mussig et al., 2002; Ahammed et al., 2013) and the activity of antioxidant enzymes (Cao et al., 2005). Up-regulation of ATP sulfurylase and increased level of cysteine rich pool viz. glutathione, ascorbate and phytochelatins (Anjum et al., 2007; Khan et al., 2009; Hossain et al., 2012; Mohamed et al., 2012; Manara, 2012) under the regulation of BRs could have possibly checked the uptake and accumulation of

metal in the aerial biomass of the plants (Khripach et al., 1999; Bajguz, 2000a; Kroutil et al., 2010). Moreover, NO is known to positively regulate the development of lateral roots and root hairs in plants (Correa-Aragunde et al., 2004; Lombardo et al., 2006), and hence the root absorption of soil metals. Consistent with the current study, the protective role of SNP (NO) was reported by Jhanji et al. (2012) in *B. napus* under Cd stress. Recently, beneficial interaction of BRs and NO is also suggested by Hayat et al. (2010b) in tomato plants. Root signals are diversely modulated by the interactions of BRs and NO with auxins (Nemhauser et al., 2004; Mouchel et al., 2006; Chen et al., 2010; Lanza et al., 2012) and ABA (Zhang et al., 2009, 2011) to regulate the mineral absorption, root elongation, root hairs induction, transport and exudation of organic acids (Lombardo et al., 2006; Rubio et al., 2009; Yi-Kai et al., 2010; Yang et al., 2012). In mustard varieties, BRs and NO induced protective role could have been exerted due to a reduction in Cd uptake and its further transport to the shoot tissues (Hayat et al., 2010b).

Carbonic anhydrase (CA) is a ubiquitous Zn metallo-enzyme which in green plants reversibly catalyzes the conversion of HCO_3 and CO_2 in plants (Xin Bin et al. 2001; Khan, 2004) and participates in a range of biological functions viz. carboxylation, acid-base buffering, ion exchange and also participates in respiration and photosynthesis (Tashian, 1992). The activity of CA is determined by the availability of Zn, CO_2, light intensity, hormonal signaling and regulation of genetic expression of the transcripts (Reed and Graham, 1981, Barcelo and Poschenrieder, 1990, Kim et al., 1994, Tiwari et al., 2005). Mustard plants grown in the soil, fed with Cd, exhibited a lower level of CA activity in a concentration dependent manner (Tables 8, 44, 56 and 68). This loss in the activity of CA, under Cd stress is also reported by Hayat et al. (2007a) and Hasan et al. (2008) which could be due to metal-induced degradation of mRNA associated with CA (Park et al., 2012), Cd-binding to -SH group of CA, restricted stomatal gaseous (CO_2) exchange (Barcelo et al., 1986a, b) or an impact on Zn availability (Aravind and Prasad, 2005). BRs, on the other hand, stabilize the membrane potential; increase stomatal conductance (Hasan et al., 2011; Hayat et al., 2012b) and CO_2 availability (Yu et al., 2004b), the substrate for the activity of CA (Okabe et al., 1980). BRs mediated increase in CA activity was reported in *L. esculentum* under Cd (Hayat et al., 2012b) and Co stress

(Hasan et al., 2011) and under Ni stress in the cultivars of *Triticum aestivum* and *Vigna radiata* (Yusuf et al., 2011; 2012). These earlier findings are in amenity with the present report on *B. juncea*, under Cd stress (Tables 20 and 44). Nitric oxide also regulates the stomatal movement by modulating the activity of ion channels and Ca^{2+} level in stomatal guard cells (Lamattina et al., 2003, Neill et al., 2008), which in turn determines the level of CA activity through the internal concentration of CO_2. The activity of CA is improved with the application of SNP both in (Cd) stressed as well as in (stressfree) control plants (Tables 32 and 56). Similar observations have also been reported Hayat et al. (2010b) in tomato cultivars, Khan et al. (2012) in mustard plants and Singh et al. (2008) in wheat roots. Therefore, the cumulative effect of BR and NO is suggested to regulate stomatal conductance in a concentration dependent manner by interacting with Ca^{2+} to regulate the substrate (CO_2) availability for CA, and hence, its activity (Garcia-Mata and Lamattina, 2001; Hayat et al., 2010b; Hayat and Ahmad, 2003b). In the present studies, a follow-up spray of SNP (10^{-5} M) and EBL (10^{-8} M) had an additive effect in promoting the CA activity, under Cd stressed and non-stressed plants (Table 68).

The other enzyme, nitrate reductase (NR) catalyzes the conversion of nitrate to nitrite (Larcher, 1995) to ensure the adequate supply of nitrogen to plants for proper growth and productivity (Srivastava, 1995). This process of nitrate reduction depends on three main factors (a) substrate (NO_3^{2-}) level in the cytoplasm (b) the level of functional NR and/or (c) the activity level of functional NR. In a cell, each of these processes is, directly or indirectly dependent on the metabolic sensors and/or signal transducers (Campbell, 1999) and transporters (Loque et al, 2003). However, the major rate limiting step in this process of nitrate reduction is the conversion of nitrate to nitrite (Salisbury and Ross, 1992) which is inhibited by the presence of the Cd in the soil (Tables 8, 44, 56 and 68 and Rai et al., 1998; Chaffei et al., 2004; Siddhu and Khan, 2012). This loss in NR activity could partially be due to the Cd mediated effect on plasma membrane fluidity (Meharg, 1993); activity of plasma membrane proton pump (Obata et al., 1996) and/or uptake of nitrate, the substrate (Hernandez et al., 1996; Campbell, 1999, Rhizzardo et al. 2012). However, BRs had a positive effect on the activity of NR both in the stressed and non-stressed plants (Tables 20 and 44) that could be due to its impact on the integrity of

cell membranes (Dalio et al., 2013), partially overcoming the Cd-induced damage of cell membranes (Hayat et al., 2007a) and/or favoring the transcription and/or translation (Khripach et al., 2003). Similar observations have also been reported in maize (Shen et al., 1990), rice (Mai et al., 1989) and wheat (Sairam, 1994) when treated with BRs. Moreover, NO alternatively, regulates the redox status of the root cells with the mediation of hemoglobin-NO cycle maintaining oxygen tension at very low level (Stohr and Stremlau, 2006). Nitric oxide also regulates the stability of root plasma membrane and induction of root hairs (Lombardo et al., 2006, Clark et al., 2010) which could have facilitated the uptake of a larger quantity of NO_3^- (inducer of NR). Besides increased substrate (NO_3^-) availability, NR activity could have also been stimulated by NO through post translational regulatory pathway (Kaiser and Huber, 2001; Jin et al., 2009). BRs, on the other hand mediate plasma membrane transport of ions that may have favored the uptake of substrate through root hair formation and regulated activity of transporters in association with auxins (Zhang and Forde, 2000; Stohr and Stremlau, 2006; Chen and Kao, 2012). Increased NR activity could also be due to phosphorylation and de-phosphorylation mediated post-translational regulation (Beevers et al., 1965; Sawhney and Naik, 1990), *de novo* synthesis of mRNA and enzymes induced through the interaction of BRs regulated NO (Zhang et al., 2011) which seems to be the another possible explanation of increased NR activity through BR and NO treatment (Table 68).

The presence of Cd in the soil led to the loss of leaf chlorophyll in the two mustard varieties in a concentration dependent manner (Tables 4, 40, 52 and 64). This observation seems to be due to Cd-mediated inactivation of chlorophyll molecules and of biosynthetic enzymes (Jain et al., 2007). Besides mimicking the divalent cations (Chmielowska-Bak et al., 2013), Cd potentially substitutes the central metal of chlorophyll molecule to inactivate its function (Kupper et al., 1996). Cadmium also checks the biosynthesis of chlorophyll molecules by interfering with the functional sulfhydryl-group of several enzymes such as ALA synthetase, ALA dehydratase and protochlorophyllide reductase, responsible for its synthesis (Mysliwa-Kurdziel et al., 2004; Stobart et al., 1985). Cadmium stress enhanced the activity of enzyme, chlorophyllase which could have led to the degradation of chlorophyll molecules (Abdel

Basset et al., 1995) or related regulatory proteins e.g. *Cab1* (Chlorophyll a/b-Binding Protein1) to repress chlorophyll content (Jhanji et al., 2012; Park et al., 2012). Cadmium is known to induce lipoxygenase activity that may have also contributed to the chlorophyll oxidation (Klein et al., 1984; Somashekaraiah et al., 1992). Cadmium challenges the uptake of several essential nutrients e.g. Mg, Fe, Ca, K (Greger and Ogrer, 1991, Ouzounidou et al., 1997) that could have adversely affected the level of cellular Mg and Fe, required for chlorophyll biosynthesis. Therefore, all the above factors might have contributed to the decrease in the values of leaf chlorophyll content which is in conformity with Usha and Mukherji (1992) and Gadallah (1995). On the other hand, the application of BRs partially relieved the plants from Cd toxicity that is expressed as an improvement in the leaf chlorophyll level (Table 47). This impact of BRs is possibly mediated by the increased activity of enzymes of pigments biosynthesis (Bajguz and Asami, 2005) and suppressed activity of chlorophyll catabolic enzymes. An increase in the pigment contents, under the impact of BRs has been reported earlier by Hayat et al. (2000; 2001b), Fariduddin et al. (2003), Yu et al. (2004b), Ali et al. (2006, 2007) and Bajguz and Hayat (2009). Application of BRs induces a recovery in photosynthesis under stress (Hola, 2011) which could be due a shift in the oxidative state and chlorophyll metabolism (Deitz et al., 2011). In addition to this, BRs are known to regulate oxidative stress through regulating enzymes and molecules of antioxidant system (Goda et al., 2002; Ahammed et al., 2013). This study and that of Qayyum et al. (2007), Anuradha and Rao (2009), Hola (2011) indicated that BRs nullified the damaging effect of metal on chlorophyll in a manner dependent upon the structure/activity of BR analogue and its concentration or plant genotype.

The increase in chlorophyll content (Tables 28 and 52), under the impact of NO may be due to the uptake of iron, in additional quantities and detoxification of Cd mediated generation of ROS. Since deficiency of iron also impairs the chlorophyll biosynthesis and chloroplast development (Zhang et al., 2012). Similarly, NO declined iron stress-induced chlorosis symptoms by increasing chlorophyll a and b concentration in wheat cultivars (Graziano et al., 2002) by activating the conversion of Mg-protoporphyrin to protochlorophyllide and subsequently to chlorophyll (El-Abdin Abdel-

Kader, 2007). Nitric oxide mediated increase of chlorophyll level has also been reported by Fan et al. (2007) in cucumber and recently by Tewari et al. (2013) in *Arabidopsis*. Moreover, BRs have positive impact on core transcription module to regulate the biosynthesis of chlorophyll and chloroplast development (Cheminant et al., 2011; Bai et al., 2012) and BRs interaction with endogenous NO could have up-regulated the absorption of nitrate and Mg-Fe ions to favour chlorophyll biosynthesis. The metal stressed plants applied with BRs and NO showed higher chlorophyll value over the metal stressed plants alone (Table 64) might be due to the prevention of chlorophyll degradation under metal stress (Dietz et al., 1999).

Multiple factors regulate the net photosynthetic rate (P_N) of plants which includes stomatal conductance (g_s), intercellular CO_2 concentration (Ci), transpiration rate (E), maximum quantum yield of PSII (Fv/fm), CA activity and leaf chlorophyll level. The plants raised in Cd fed soil exhibited loss in photosynthetic attributes (Tables 5-7). Cadmium brings about the closure of stomata by decreasing partial pressure of CO_2 in the stroma (Barcelo and Poschenrieder, 1990) and affects the activity of Rubisco (Siedlecka et al., 1997) which would naturally have a negative impact on CO_2 reduction. Cadmium induced decrease in P_N might also be due to its impact on the enzymes of Calvin cycle, glycolysis and Krebs cycle (Van Assche and Clijsters, 1990) as Cd is known to interact with the active site -SH groups of different enzymes. The lower level of chlorophyll and Cd-interference in electron transport chain at thylakoid membrane could have also affected chlorophyll fluorescence or Fv/Fm (Krupa and Baszynski, 1995; Lopez-Millan et al., 2009; Vanova et al., 2009). Cadmium induced alteration in one or more of the above mentioned processes seemingly led to decline in the rate of photosynthesis (Figures 1-6). These results are also in conformity with Lopez-Millan et al. (2009) and Hasan et al. (2011). However, in several crop plants, BRs have been reported to improve P_N and related attributes, under different abiotic stresses (Ali et al., 2008a, b; Fariduddin et al., 2009a, b; 2013a, b) as they protect photosynthetic apparatus (Yu et al., 2004b; Hasan et al., 2011), regulate membrane anion channels (Zhang et al., 2005), facilitate stomatal conductance (Gudesblat et al., 2012), CO_2 assimilation (Jiang et al., 2012) and also improve the activity of Rubisco (Braun and Wild, 1984; Yu et al., 2004b). In the

present study also BRs improved photosynthetic parameters, under Cd stress (Tables 41-43). A positive correlation of P_N with CA activity (Figures 1-3) and chlorophyll level (Figures 4-6) further proved the regulation of photosynthesis by multiple factors. The application of SNP (10^{-5} M) to Cd-stressed and non-stressed plants of *Brassica juncea* improved the values of photosynthetic attributes (Tables 28-31 and 53-55). SNP generated nitric oxide should have the control on stomatal opening (García-Mata and Lamattina, 2001), promotes ROS scavenging, balances mineral nutrition, counteracts the Cd-induced damage of photosynthetic apparatus (Wenhai et al., 2006) and also maintains the photosynthetic efficiency (Jhanji et al., 2012). It is suggested that higher level of NO along with EBL regulates the Cd-induced inhibition of photosynthetic electron transport chain (Takahashi and Yamasaki, 2001; Wodala et al., 2008; Thapar et al., 2008). The response of BRs and NO interaction seems to be the result of independent actions in root and shoot domains to favor photosynthetic attributes in response to ABA signals (Zhang et al., 2009; 2011). Therefore, the interactive effect of NO and EBL provided best photosynthetic values as compared to their individual application, against Cd toxicity (Tables 64-67). These findings are in conformity with Hayat et al. (2011) who reported positive effects of BR and NO combination on photosynthesis of tomato plants.

Abiotic stresses generate a large quantity of reactive intermediates in plants that oxidize cellular components that results into cellular abnormality (Gill and Tuteja, 2010). However, plants are endowed with antioxidant metabolites (ascorbate, glutathione, tocopherol and proline) and enzymes (superoxide dismutase, catalase, peroxidase and glutathione reductase) that could counter these noxious species, depending on the dose and length of stress regime, and plant genotype (Schutzendubel and Polle, 2002; Gill and Tuteja, 2010). Here also the plants fed with increasing concentrations of Cd, exhibited more and more activity of antioxidant enzymes (Tables 9-10, 45-46, 57-58 and 69-70). Similar findings were also reported in chickpea 2001; Wodala et al., 2008; Thapar et al., 2008). Moreover, BRs and NO additive effects appeats due to their individual actions favoring photosynthetic attributes, signaled through ABA (Yu et al., 2004; Garcia-Mata and Lamattina, 2007; Hayat et al., 2010b; Zhang et al., 2011), tomato (Hayat et al. 2010b;

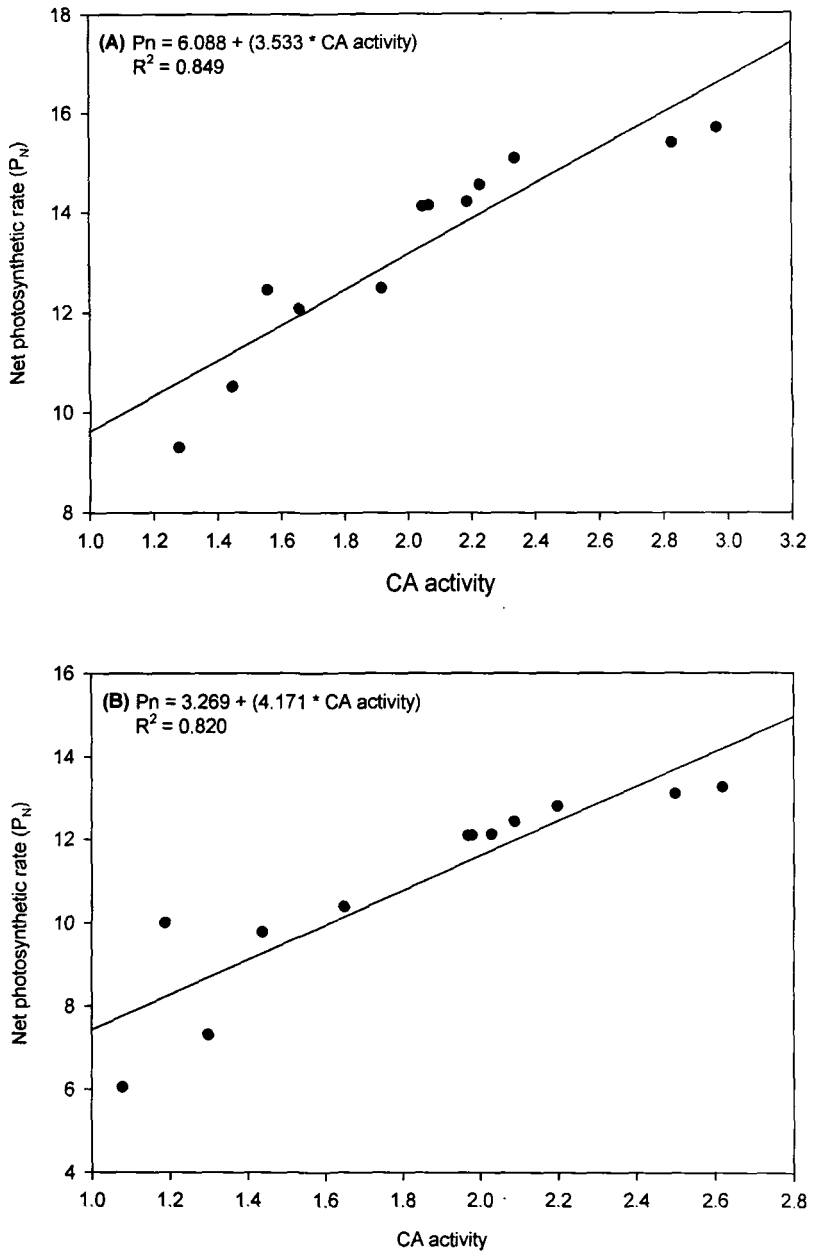


Figure 1 Correlation coefficient values between net photosynthetic rate (P_N) and CA activity in (A) Varuna (B) RH-30 (Experiment 4).

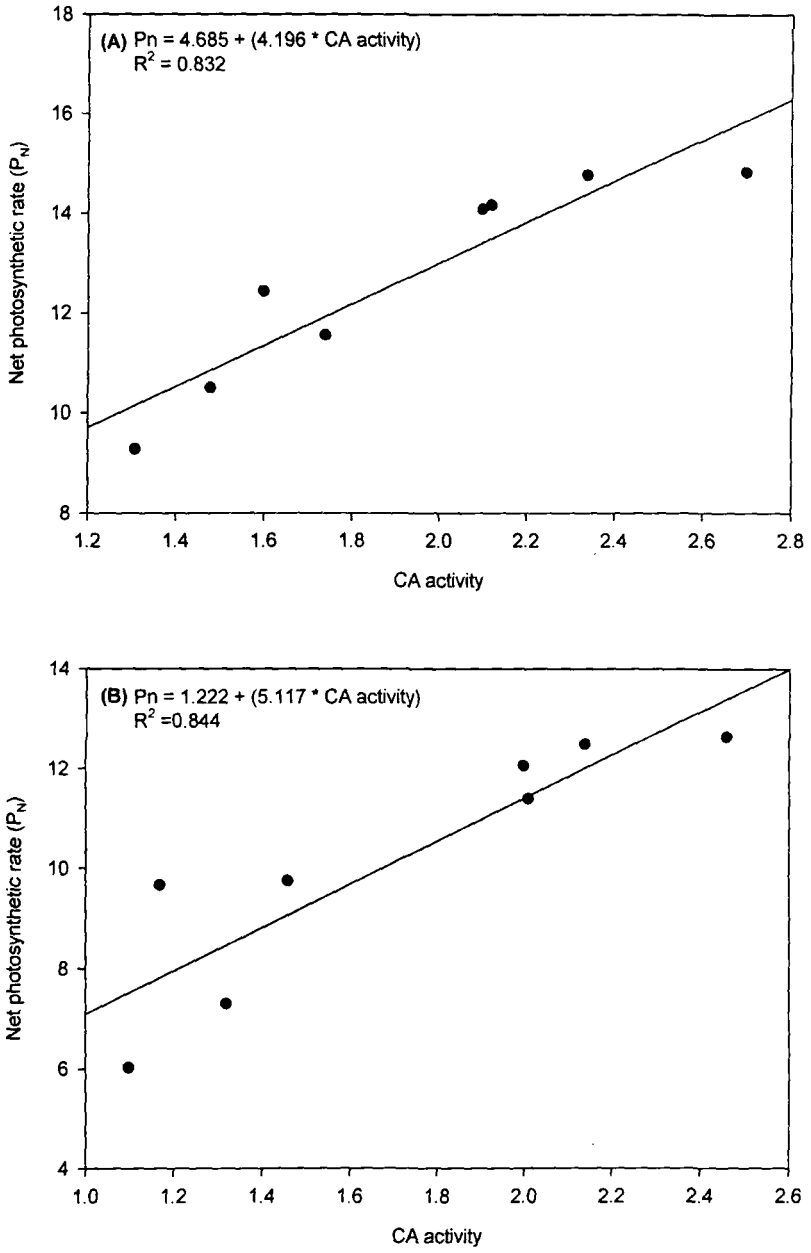


Figure 2 Correlation coefficient values between net photosynthetic rate (P_N) and CA activity in (A) Varuna (B) RH-30 (Experiment 5).

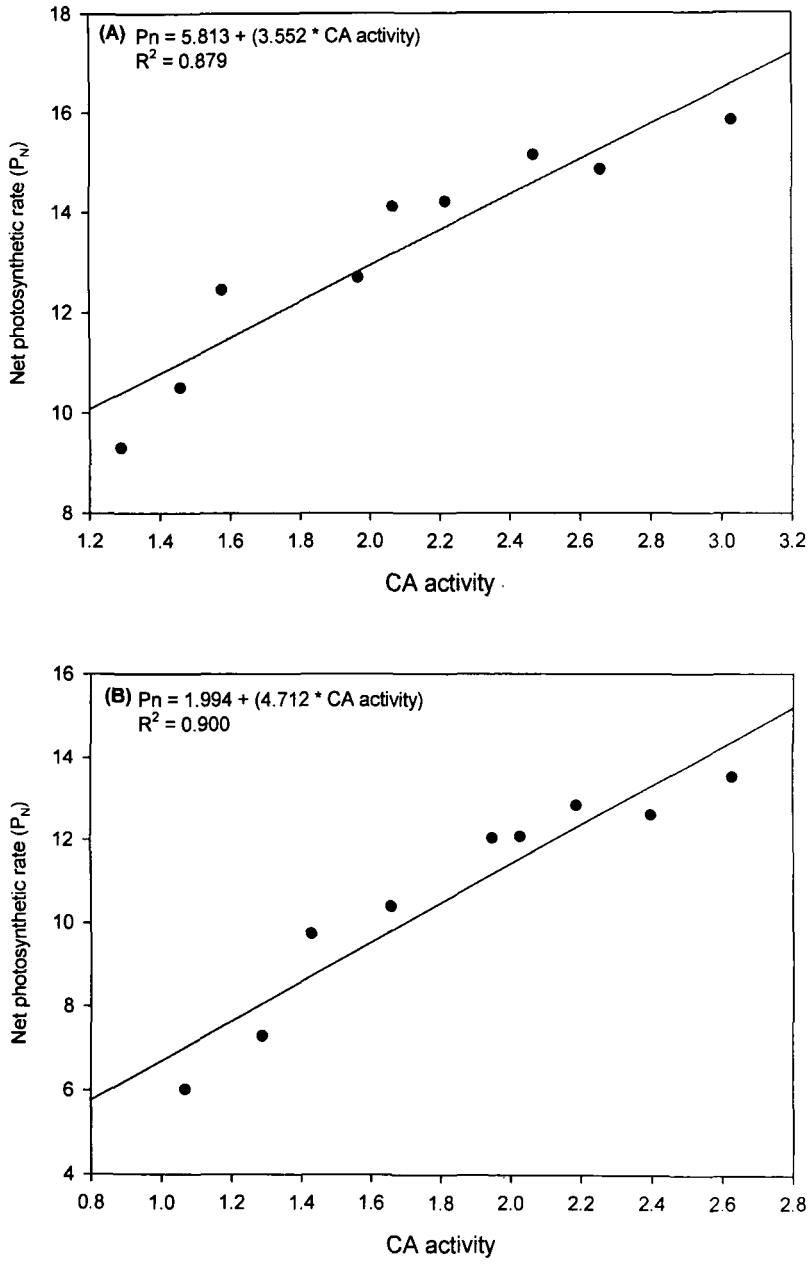


Figure 3 Correlation coefficient values between net photosynthetic rate (P_N) and CA activity in (A) Varuna (B) RH-30 (Experiment 6).

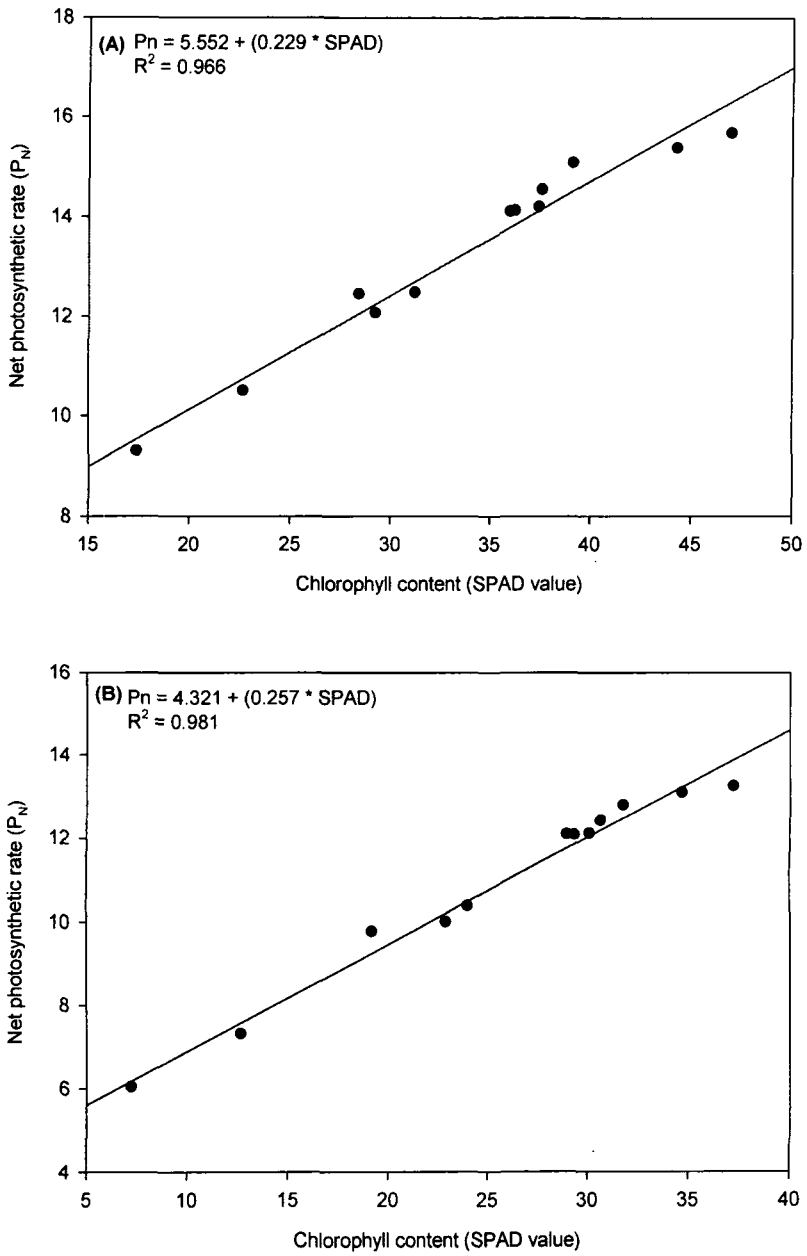


Figure 4 Correlation coefficient values between net photosynthetic rate (P_N) and chlorophyll content (SPAD level) in (A) Varuna (B) RH-30 (Experiment 4).

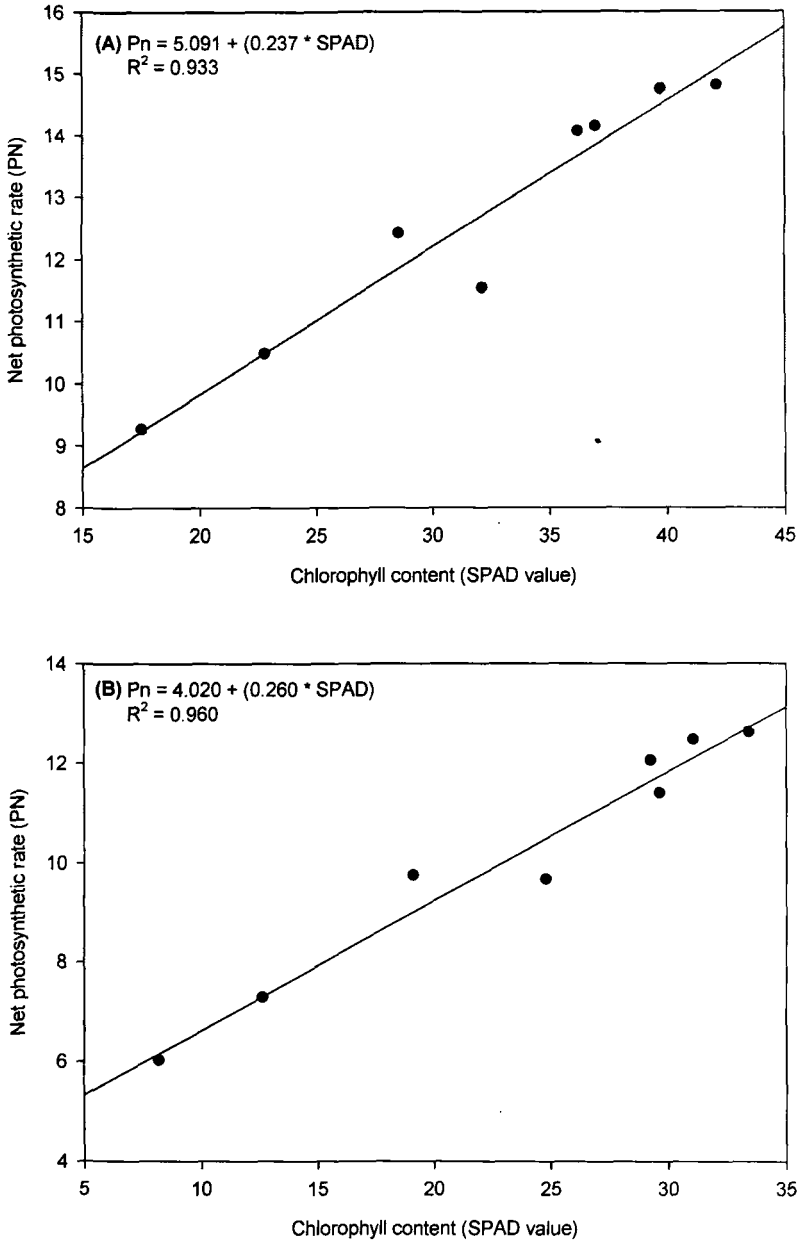


Figure 5 Correlation coefficient values between net photosynthetic rate (P_N) and chlorophyll content (SPAD level) in (A) Varuna (B) RH-30 (Experiment 5).

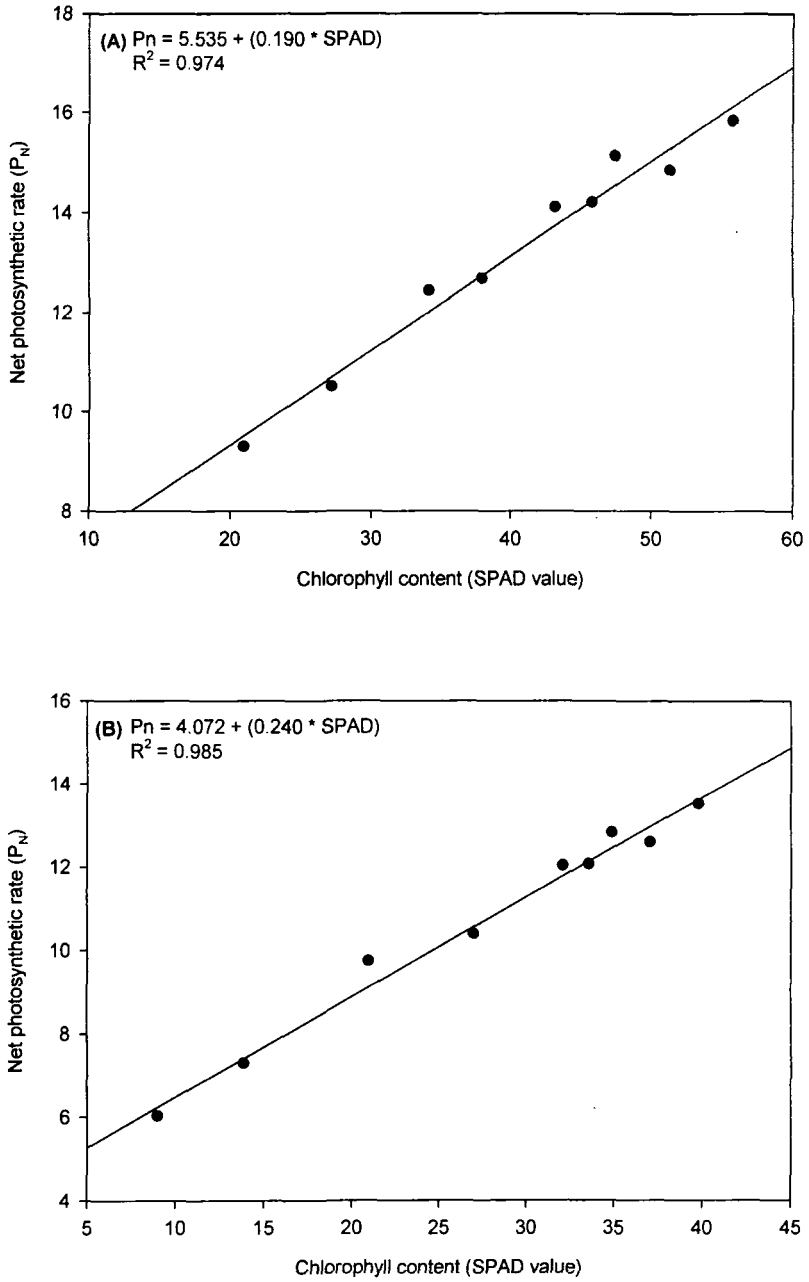


Figure 6 Correlation coefficient values between net photosynthetic rate (P_N) and chlorophyll content (SPAD level) in (A) Varuna (B) RH-30 (Experiment 6).

2011; 2012b, Hasan et al. 2009; 2011), mustard (Hayat et al., 2007a; Markovska et al., 2009) and pea plants (Metwally et al., 2005). However, phytohormones not only help to detoxify the active free radicals but also regulate the antioxidant system activity. BRs regulate this cellular redox status through several ways, like change in transcription and translation machinery (Khripach et al., 2003; Choudhary et al., 2012). BRs modified antioxidant enzyme activities in *Cicer arietinum* (Hasan et al., 2008) and *Vigna radiata* (Ali et al., 2008a) under heavy metal stress. It has been suggested that Cd and BRs induced signaling share the pathways that alter final gene responses in sensitive and tolerant varieties which not only protected plants from metal-induced oxidation but also optimized antioxidant system activity (Villiers et al., 2012 and Table 45). BRs, in association with lower concentration of NO also enhanced the antioxidant enzymes activity in metal-stressed plants (Tables 33-34 and 57-58). NO mediates, BR-induced ABA biosynthesis involved in oxidative stress tolerance is reported in maize leaves (Zhang et al., 2010; 2011) and a combination of BRs and SNP, under Cd stress activated SOD, POX and CAT thereby counteracting ROS (Tables 69-70). These findings are in conformity with Hayat et al. (2010b) and Verma et al. (2013).

Stress induced consequences in plants undergo protection by multifunctional metabolite, the proline (Szabados and Savoure, 2010) which is also recognized as the regulator of important enzymes (Shah and Dubey, 1998). Its level in our study, under Cd stress, increased and improved further, if the plants were given a follow up treatment with BRs (Tables 10, 46, 58 and 70). Here, specific genes are said to be involved in the regulation of proline level, under stress in *Arabidopsis* (Rentsch et al., 1996) and tomato (Schwacke et al., 1999). Recently, Hayat et al. (2013) reported positive impact of proline on the antioxidant system and photosynthesis to secure plant growth, under stressed conditions. This proline aided insurgence, at the cellular level, caused the loss of leaf water potential (Tables 7, 43, 55 and 67). Therefore, proline level was found to be negatively correlated with LWP (Venekamp et al., 1987; Jianchang et al., 1995). Similar observations have also reported in cucumber (Fariduddin et al, 2013b), tomato (Hasan et al., 2011; Hayat et al., 2012b), mung bean (Yusuf et al., 2012), and wheat (Yusuf et al., 2011). Moreover, root hairs may have lost water/capability to uptake water, under the

impact of the metal as it causes the damage to the cellular membrane (Hall, 2002). The knowledge of the direct mechanism of BR and/or osmolyte induced metal stress alleviation and its homeostasis in plant is scare. However, BRs in combination with polyamine (another compatible osmolyte) enhanced the Cu stress tolerance in *Raphanus sativus* (Choudhary et al., 2012). The level of proline, induced by BRs could have checked the cellular uptake of metal ions in the cytoplasm thereby working as compatible osmolyte (Sharma and Bhardwaj, 2007; Bhardwaj et al. 2011).

The exogenously applied SNP along with BRs enhanced the proline content in non-stressed and Cd-stressed plants (Tables 34 and 58). Similarly, SNP applied under osmotic stress favored proline accumulation and higher relative water content (RWC) with lower loss of leaf water (Tan et al., 2008). It has therefore been suggested that SNP (-the NO donor) regulates the polyamine and proline metabolism in the leaves (Filippou et al., 2013) and NO mediates the BR induced ABA signals under oxidative stress (Zhang et al., 2010; 2011). It may be proposed that the application of BRs alone or with SNP under stressed/non-stressed conditions could have enabled plants to sustain its higher leaf water content (Tables 19, 43 and 67), by further improving the proline level.

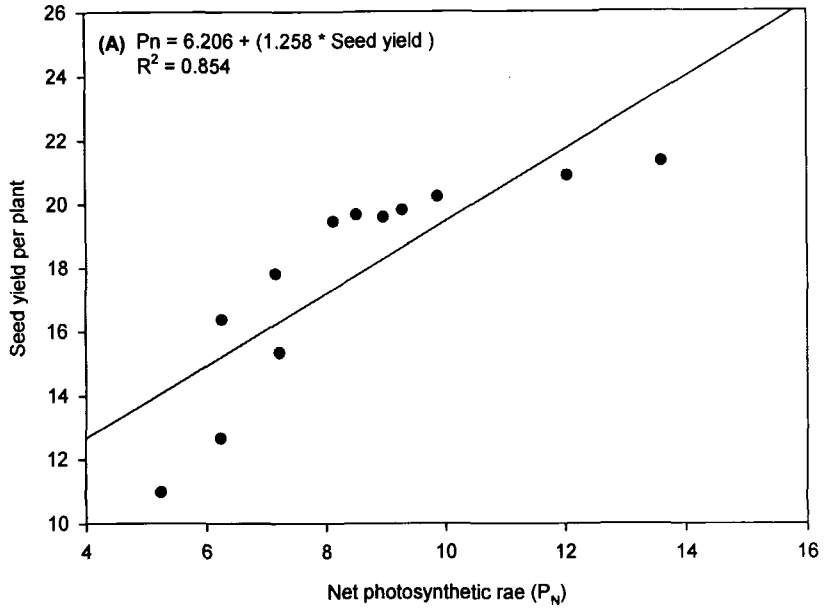
Heavy metals presence induces the loss of cellular turgor, inhibition of cellular division and cell enlargement (Gabbrielli et al., 1990, Gajewska et al., 2006). In particular, Cd causes nutrient deficiency, competes with the uptake of other micronutrients (e.g. Ca, Zn, Mg, Mn, Fe, S and P) and disrupts the physiological functions (Marshner, 1995; Irfan et al., 2012). Cadmium may even change the concentration of basic macronutrients such as that of nitrogen and phosphorous in plant tissues (Siedlecka, 1995; Chen et al., 2009). Moreover, Cd-induced retardation of photosynthetic efficiency limits the dry mass accumulation (Vassilev et al., 2004). These damaging factors in a cumulative action had a negative impact on the growth of plants in terms of their length, fresh and dry mass and leaf area (Tables 1-4, 37-40). These observations are in conformity with the earlier observations of Hayat and Hayat (2011) and Hasan et al. (2008) in chickpea, and Hayat et al. (2007a) in mustard. However, application of BRs to Cd-stressed or non-stressed plants improved all the growth characteristics (Tables 13-16 and 37-40) as it is involvement in cell elongation (Azpiroz

et al., 1998, Catterou et al., 2001), via regulation of correlated genes i.e. XETH (xyloglucan endotransglucosylase/hydrolase), expansins, cellulose synthase and sucrose synthase (Cosgrove, 1997; Ashraf et al., 2010; Xie et al., 2011), that regulates membrane permeability and proton pump activity, under stress conditions (Hamada, 1986). Moreover, enhanced expression and activity of XETH caused cell wall loosening and growth in Azuki bean epicotyls (Kaku et al., 2004) and *Arabidopsis* and tomato hypocotyls (Miedes et al., 2011; 2013) where ATPase regulated localized changes in apoplastic and cytoplasmic pH are associated with growth initiation as shown in *Arabidopsis thaliana* root hair development (Bibikova et al., 1998). In addition to this, EBL is also involved in the improvement of transcript level of cyclin-D3 proteins, a key regulator of cell cycle in *Arabidopsis* (Ashraf et al., 2010), which could be assigned as the direct role in BR activated cell division and cellular enlargement (Clouse and Sasse, 1998; Bajguz and Tretyn, 2003). These findings are further corroborated by other studies where BR improved the leaf area in *Vigna radiata* under Al stress (Ali et al. 2008a), *Brassica juncea* (Alam et al., 2007; Fariduddin et al., 2009b) under Ni/Cu stress and in tomato under Cd stress (Hayat et al., 2012b). The increased growth of mustard plants under Cd-stressed conditions as in our results also gets support from the findings of Zeng et al. (2010), Hasan et al. (2011), Rady and Osman (2012) and Hayat et al. (2012b).

Application of SNP (the source of NO) improved the growth characteristics (length, fresh and dry mass of root and shoot, and leaf area) of metal-stressed and non-stressed plants of mustard (Tables 25-28 and 49-52). Similarly, NO-induced improvement in plant growth, under stress has been reported earlier (Hayat et al., 2009; Chaki et al, 2009; Jhanji et al., 2012; Irfan et al., 2013). The stimulative effect of NO (released from SNP) on growth in *Lonicera japonica* is assigned to its favourable action on the activity of exo- and endo-β-D-glucanase in the cell wall (Terasaki et al., 2001). The same has been verified by using NO deficient mutants, where the decreased activity of this enzyme led to restricted plant growth (Guo et al., 2003). The glycosidic linkages of cell wall on being broken by these enzymes facilitate wall loosening and extension (Zhang et al., 2003) to drive plant growth, under internal turgor pressure. The increase of leaf water potential with NO (Table 31) gets support from the study of Hayat et al.

(2010b) in tomato. NO also controlled root organogenesis in cucumber (Pagnussat et al., 2002) and indeterminate nodule formation in *Mililotus truncatula* (Pii et al., 2007). In *Arabidopsis*, NO promoted root sequestration of essential micronutrients (e.g. iron), up-regulated the associated genes (Besson-Bard et al., 2009) and modulated Cd influx (Ma et al., 2010). However, combined application of BR and NO, against the Cd stress resulted in a much better growth performance (Tables 61-64). BR and NO together have improved the growth of tomato plant under normal conditions (Hayat et al., 2010b). Nitric oxide mediated promotion of root hairs and BRs mediated selective absorption of mineral acquisition (Nafie and El-Khallal, 2000) seems to be the one of the reason for better plant growth in a combined application (Lombardo et al., 2006; Ali et al., 2006).

A decrease in the number of seeds per pods and seed yield per plant in metal stressed plants is possibly due to their slower rate of photosynthesis and subsequent restriction on growth and leaf area (Tables 12, 48, 60 and 72). It is further strengthened by the establishment of a significantly positive correlation between net photosynthetic rate and seed yield, at harvest (Figures 7, 8 and 9). However, the Cd-stressed and stress-free plants supplemented with BRs exhibited healthy growth (Tables 37-40) with an improvement in the characteristics of seed yield (Table 48). In a natural course, the fruit bearing capacity of the plants is primarily determined by the density of the flowers retained to set fruits and the metabolic state of the plants (Carrari and Fernie, 2006; Yong-Ling et al., 2012). BRs are characterized to slow down the process of senescence of the flowers before and/or after pollination (Zhao et al., 1987, Sugiyama and Kuraishi, 1989, Iwahori et al., 1990). Similarly, the fruit setting in tomato was improved by a synthetic brassinolide analogue; TS-303 (Kamuro and Takatsuto, 1999). Moreover, the delayed rate of senescence allowed the attachment of leaves to the mother body for a longer duration with improved rate of photosynthesis (Hayat et al., 2000, 2001b, Yu et al., 2004a; Liu and Guo, 2013) and speeded up translocation of photosynthates to the sink (Fujii et al., 1991, Petzold et al., 1992, Fujii and Saka, 2001) that enhanced the seed bearing capacity of the plants (Table 24 and 48). Ali et al. (2006) have also pointed out the similar reasons in tomato for an increase in yield under the treatment of BRs.



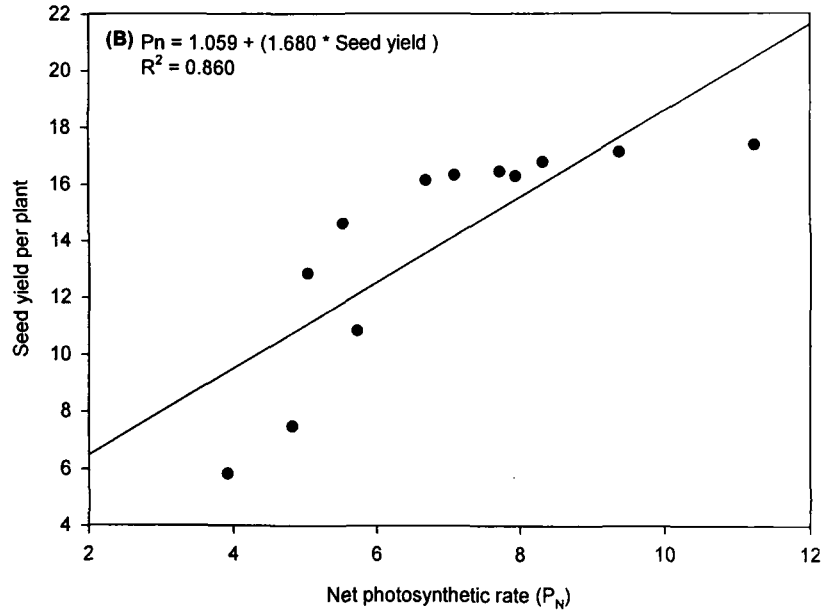


Figure 7 Correlation coefficient values between net photosynthetic rate (P_N) and seed yield per plant in (A) Varuna (B) RH-30 (Experiment 4).

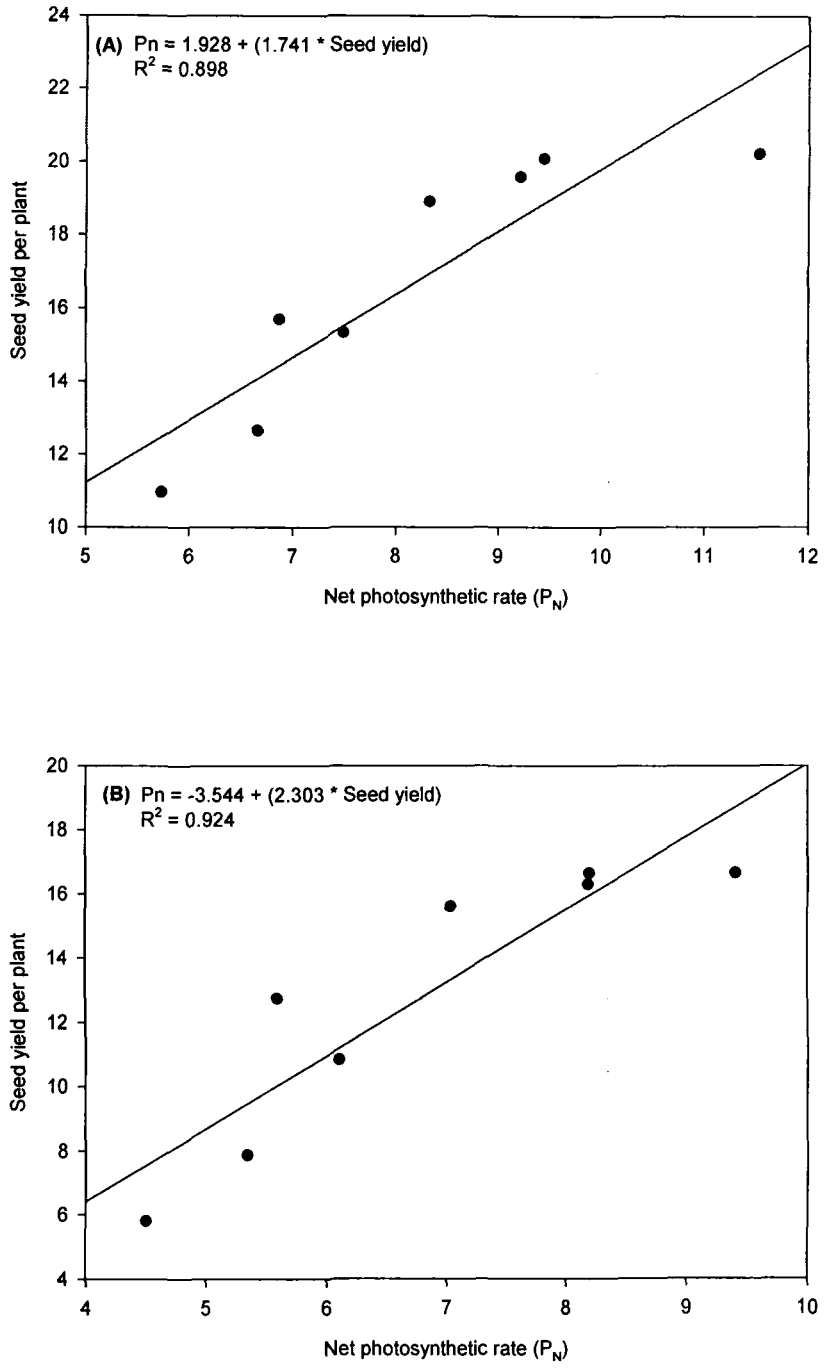
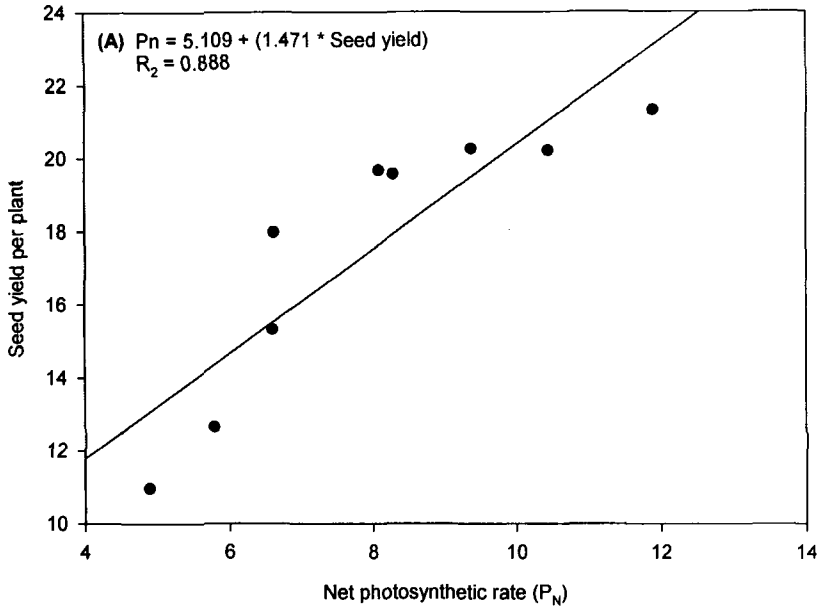


Figure 8 Correlation coefficient values between net photosynthetic rate (P_N) and seed yield per plant in (A) Varuna (B) RH-30 (Experiment 5).



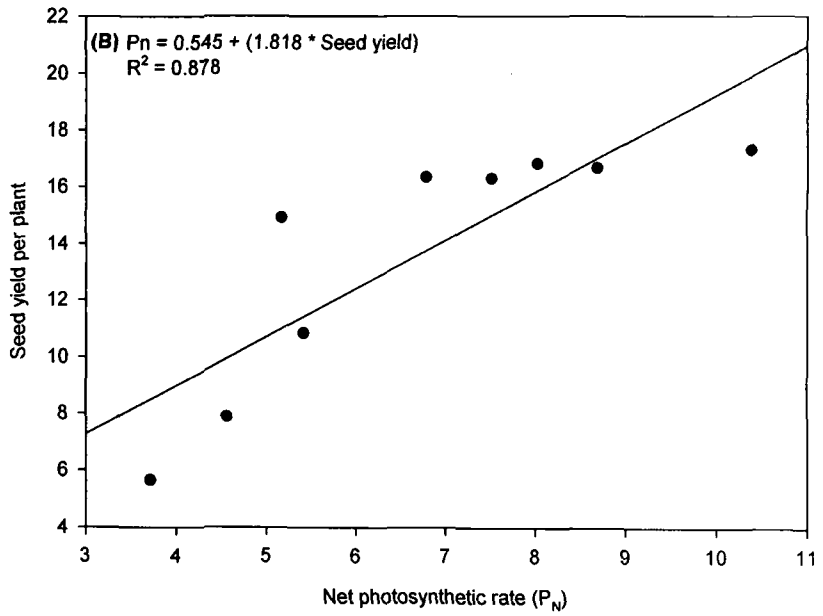


Figure 9 Correlation coefficient values between net photosynthetic rate (P_N) and seed yield per plant in (A) Varuna (B) RH-30 (Experiment 6).

Leaf applied SNP also improved the yield parameters both in metal-stressed and non-stressed mustard plants (Tables 60 and 72). However, there is no direct evidence of the role of NO in seed production but it is often elucidated to be involved in flowering.NO application induced flowering in duckweed, *Lemna aequinoctialis* Welw. under non-inductive conditions (Khurana et al., 2011). The findings of Seligman et al. (2008) revealed that synthesis of NO occurs only in differentiated stigmatic papillae of the floral bud, and in the stamen, only anthers that are producing pollen grains synthesize NO. In addition to this, NR-deficient plants emits less NO that results into mature blossom when compared with wild-type plants which reflects its importance in flowering and ultimately result into the yield enhancement. Nitric oxide with BRs, rather than alone, generated better yield output both in non-stressed and Cd-stressed mustard plants (Table 72) possibly because of BR mediated induction of ABA synthesis (Zhang et al., 2011) which has key role during plant maturation and seed production. BRs signals are also involved in the floral-induction of *Arabidopsis* (Li et al., 2010; Kutschera and Wang, 2012) and sex determination in maize (Hartwig et al., 2011) which might be directed by NO to regulate plant yield attributes.

Comparing the response of selected concentrations/analogues of plant hormones, the foliar spray of 10^{-5}M SNP, as compared to its other doses (10^{-4} and 10^{-6} M SNP), outperformed against Cd-induced toxicity in mustard plants. However, higher dose of SNP/NO (10^{-4} M) possibly generated reactive radicles which resulted into a lower state of growth. Among the two BR analogues, EBL more effectively improved most of the physiological and biochemical characteristics in the presence as well as in the absence of Cd. This functional superiority of EBL over HBL may be corroborated to differences in their structure and stability (Khripach et al., 2003). Most of the BRs carry an S-oriented alkyl (methyl or ethyl) group at C-24 of side chain, EBL is among the exceptions, along-with castesterone (another analogue of BR) which carry R-oriented alkyl group on the side chain of the steroid nucleus. It is speculated that the conformational difference of BR-receptor (BRI-1) binding at the plasma membrane could be the factor for the varied responses of HBL and EBL. EBL-receptor complex might have more active conformation in triggering favorable signaling cascades than HBL. However, further studies regarding the role of structure-function of BR-receptor complex in signaling by taking different analogues would

possibly disclose the differences in BRs activity. However, the activated transcription factors regulate BKI-1 after dissociation with BAK-1 facilitates the BR-BRI-1 binding at membrane. A series of reactions are also elicited for the internalization/inactivation of BRI-1 to switch-off the BR response once it has been achieved (Russinova et al., 2004; Swaczynova et al., 2007; Hategan et al., 2010; Codreanu and Russinova, 2010). The applied steroids are more effectively absorbed at the young rapidly growing leaves surface and could be transported through phloem to other actively growing tissues or synthesized *in-vivo*. The optimal sensitivity and biosynthesis of BRs is reported in rapidly growing meristems (apical meristems, axillary buds, differentiating vasculature, flower buds their pollens, and root apical meristem *etc.*). Furthermore, BR sensitivity is directly correlated with the density of BR-receptors, expressed on the plasma membrane of meristematic cells. After BR-BRI1 binding, the receptors are hetero-dimerized to transduce downstream signals and carry on its effects (Plate 1 and 2A).

Metabolically, a significant difference in proline level was recorded in the two tested varieties of mustard (RH-30 and Varuna) which could be one of the important determinants to express sensitive or resistant responses. Higher proline contents in BRs and/or SNP sprayed plants (Table 46, 58 and 70) is in coherence with favorable growth characteristics further support the above hypothesis.

Conclusions

The present study revealed that:

1. A significant decline in morphological, physiological and biochemical parameters was noted within the plants increasing level of Cd the in soil.
2. The soil amended with Cd (25, 50 or 100 mg Kg^{-1}) generated toxicity in the mustard plants, in a concentration dependent manner, where, 100 mg Cd Kg^{-1} soil developed maximum toxicity and damage to the plants.
3. Out of the two varieties (RH-30 and Varuna) the later was more tolerant to Cd.
4. Out of the two BR analogues (HBL or EBL), the performance of EBL excelled over HBL in terms of favourable responses both under Cd stress and stress-free conditions.

5. Out of the various concentrations of NO (10^{-4} M, 10^{-5} M or 10^{-6} M) of SNP, 10^{-5} M generated most favorable response.
6. The combination of SNP (10^{-5} M) and EBL (10^{-8} M) was most effective in overcoming the toxicity generated by Cd.

Chapter 6

SUMMARY

SUMMARY

This thesis comprises of the following five chapters.

Chapter 1 introduces the significance of the problem entitled, "Effects of brassinosteroids and nitric oxide against cadmium stress in *Brassica juncea*".

Chapter 2 reviews the available literature related with the above problem, in terms of growth, metabolism, and yield characteristics of the plants.

Chapter 3 elaborates the details of the materials and methods employed in conducting the experiments and chemical analysis of the biological material.

Chapter 4 comprises of tabulated data, recorded during the aforesaid study, and a brief description of the results.

Chapter 5 deals with the possible explanations for the observations, in the light of the earlier findings.

The summary of the observations, recorded in each of the five experiments is given below:

Experiment 1

This experiment was carried out to compare the effect of different concentrations of Cd on two varieties of *Brassica juncea* L. Czern & Coss, Varuna and RH-30. The soil of each set of pots was supplemented with different doses of Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) in the form of $CdCl_2$ at the time of seed sowing. Surface sterilized seeds of two varieties were sown in earthen pots (25 × 25 cm) filled with sandy loam soil and farmyard manure, in a ratio of 6:1. Thinning was done 7 days after sowing (DAS); leaving three plants per pot and five pots were maintained per treatment, as replicates. The pots were arranged in a completely randomized block design, in the net house of Department of Botany, Aligarh Muslim University, Aligarh. The plant samples were collected at 30 and 60 DAS to assess various growth characteristics, enzymes activitiy (NR, CA, CAT, POX and SOD), photosynthetic attributes, leaf water potential, contents of proline and Cd. Rests of the plants were allowed to grow up to maturity and were harvested to study the yield characteristics, at harvest. All the above parameters except antioxidant enzymes, proline content and Cd content, showed a significant decrease in response to Cd treatment where

maximum damage was caused at a Cd concentration of 100 mg $Kg^{-1}$of soil, more prominently in RH-30 as compared to Varuna. However, Cd treatment resulted in a significant increase in the activity of antioxidant enzymes (CAT, POX and SOD) and content of proline along with the accumulation of Cd in root and shoot tissues. The activity of antioxidant enzymes and proline level was higher in Varuna which accumulated a lower level of Cd in its root and shoot tissues and was found to be more tolerant to the metal, compared to RH-30.

Experiment 2

This experiment was set up with an aim to study the effect of two analogues of brassinosteroid (BRs; EBL or HBL), administered at the concentration of 10^{-8} M. Surfactant tween-20 was mixed with hormone solution just before the spray on the foliage of 29 days old plants. Control plants were sprayed with distilled water, 0.5% solution of tween-20 or 5% ethanol. All the agricultural practices remained the same as in Experiment 1. Plant samples were collected at 60 and 90 DAS to assess various growth characteristics, enzymes activity (NR, CA, CAT, POX and SOD), photosynthetic attributes, leaf water potential, contents of proline and Cd and the yield characteristics, at harvest. The values for the aforesaid parameters increased significantly, except Cd accumulation, at 60 DAS. Moreover, pod number and seed yield also increased by hormone application. No significant effect of tween-20 or ethanol appeared as compared to water treated, control. EBL excelled over HBL and generated more favorable effect in Varuna than RH-30.

Experiment 3

This experiment was laid with an objective to study the effect of different concentrations of sodium nitroprusside (10^{-4}, 10^{-5} or 10^{-6} M SNP, the donor of nitric oxide) on two varieties of *Brassica juncea*; Varuna and RH-30. All the agricultural practices were same as in Experiment 1. The plants were sprayed with DDW (control), tween-20 (0.5%), potassium ferricyanide (10^{-4} M) and 10^{-4}, 10^{-5} or 10^{-6} M of SNP. The plant samples were collected at 30 and 60 DAS to assess various growth characteristics, enzymes activity (NR, CA, CAT, POX and SOD), photosynthetic attributes, leaf water potential, contents of proline and Cd and yield characteristics, at harvest. Out of the various concentrations of SNP, 10^{-5} M proved best and generated a significant increase in the values of the aforesaid parameters,

except Cd level in root and shoot. Potassium ferricyanide (10^{-4}M) did not induce a significant impact on the plants. Varuna responded more favorably to NO than RH-30.

Experiment 4

This experiment was performed with an objective to elucidate the effect of exogenous BRs (10^{-8}M; HBL/EBL) on Cd-induced changes in two mustard cultivars (Varuna and RH-30). All the agricultural practices were same as in Experiment 1. The different doses of Cd (0, 25, 50 or 100 mg Kg^{-1} of soil) were supplemented in the form of $CdCl_2$ to the soil, at the time of seed sowing. The foliage of 29 day old plants was sprayed with DDW/10^{-8} M aqueous solution of BR (HBL or EBL). The plant samples were collected at 30 and 60 DAS to assess various growth characteristics, enzymes activity (NR, CA, CAT, POX and SOD), photosynthetic attributes, leaf water potential, contents of proline and Cd. The plants raised in Cd fed soil had lower values for all the aforesaid parameters, except Cd level, proline content and antioxidant enzymes activity. The exogenous application of BRs (HBL<EBL) alleviated the adverse effects generated by Cd therefore improved the values of the aforesaid parameters, and of yield charactersitics except the Cd level in root and shoot tissues. BR (HBL/EBL) completely restored the values of the above parameters, against Cd stress more promisingly against its lower concentrations (25 or 50 mg Kg^{-1} soil) than the higher concentration (100 mg Kg^{-1} soil). EBL proved more effective than HBL and Varuna responded better to BRs than RH-30.

Experiment 5

This experiment was conducted with an objective to elucidate the effect of exogenous SNP on the Cd-induced changes in two mustard varieties (Varuna and RH-30). Cadmium (0, 25, 50 or 100 mg Kg^{-1} of soil) was administered to the soil at the time of seed sowing in the form of $CdCl_2$. All the agricultural practices were same as in Experiment 1. At the stage 29 DAS, the plants were sprayed with DDW (control) or 10^{-5} M SNP. The plant samples were collected at 30 and 60 DAS to assess various growth characteristics, enzymes activity (NR, CA, CAT, POX and SOD), photosynthetic attributes, leaf water potential, contents of proline and Cd. The presence of Cd decreased the values for most of the parameters in a concentration dependent manner, except tissue Cd level, proline content and antioxidant enzymes

activity. The exogenous application of 10^{-5} M SNP alleviated the adverse effects generated by Cd and improved the aforesaid parameters, except the Cd level in root and shoot tissues. The SNP treatment significantly improved the yield characteristics, against Cd stress of 25 mg Kg^{-1} soil. Varuna possessed higher values in most of the above parameters than RH-30 and showed better response to 10^{-5} M SNP.

Experiment 6

This experiment was laid with an objective to elucidate the interactive effect of exogenous application of EBL (10^{-8} M) and SNP (10^{-5} M) on the Cd-induced changes in two mustard varieties i.e. Varuna and RH-30. Cadmium (0, 25, 50 or 100 mg Kg^{-1} soil) was administered to the soil at the time of seed sowing in the form of $CdCl_2$. All the agricultural practices were same as in Experiment 1. Plants were sprayed with DDW (control), 10^{-5} M SNP (28 DAS) and 10^{-8} M EBL (29 DAS), as follow up spray. The plant samples were collected at 30 and 60 DAS to assess various growth characteristics, enzymes activity (NR, CA, CAT, POX and SOD), photosynthetic attributes, leaf water potential, contents of proline and cadmium. The presence of Cd decreased the values for most of the parameters in a concentration dependent manner, except tissue Cd level, proline content and antioxidant enzymes activity. The combined application of 10^{-5} M SNP and 10^{-8} M HBL completely alleviated the adverse effects generated by Cd; 25 or 50 mg $Kg^{-1}$of soil but partially against 100 mg Kg^{-1} of soil. The treatment also proved beneficial in improving the yield characteristics of the plants, exposed to Cd stress (25 or 50 mg Kg^{-1} soil). Varuna possessed higher values than RH-30, expressing better response to the application of SNP and BRs.

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APPENDIX

APPENDIX

1. Preparation of reagents for nitrate reductase activity

1.1 0.1M Phosphate buffer (pH 7.4)

27.2 g of KH_2PO_4 and 45.63 g of $K_2HPO_4.7H_2O$ was dissolved, separately in 1000 cm^3 of DDW. The above solutions of KH_2PO_4 and $K_2HPO_4.7H_2O$ were mixed in the ratio of 16:84.

1.2 0.2M KNO_3

20.2 g of KNO_3 was dissolved in sufficient DDW and final volume was made up to 1000 cm^3, using DDW.

1.3 5% Isopropanol

5 cm^3 of isopropanol was pipetted into sufficient DDW and final volume was made up to 100 cm^3, using DDW.

1.4 1% Sulphanilamide

1 g of sulphanilamide was dissolved in 100 cm^3 of 3N HCl which was prepared by dissolving 25.86 cm^3 of HCl in sufficient DDW and final volume was maintained to 100 cm^3, by using DDW.

1.5 0.02% N-1-Naphthyl-ethylenediamine dihydrochloride (NED-HCl)

20 mg of NED-HCl was dissolved in sufficient DDW and final volume was made up to 100 cm^3, by using DDW.

2. Preparation of reagents for the estimation of carbonic anhydrase activity

2.1 Cystein hydrochloride solution (0.2M)

48 g cystein hydrochloride was dissolved in sufficient DDW and final volume was made up to 1000 cm^3, by using DDW.

2.2 Sodium Phosphate buffer (pH 6.8)

27.8 g NaH_2PO_4 and 53.65 g Na_2HPO_4 was dissolved, separately in sufficient DDW and final volume was made up to 1000 cm^3. 51 cm^3 of NaH_2PO_4 and 49 cm^3 of Na_2HPO_4 were then mixed to get the required solution.

2.3 Alkaline sodium bicarbonate solution

16.8 g sodium bicarbonate ($NaHCO_3$) was dissolved in aqueous 0.2M NaOH solution [0.8 g NaOH (1000 cm^3)$^{-1}$] and final volume was made up to 1000 cm^3, by using DDW.

2.4 0.002% bromothymol blue

0.002 g of bromothymol blue was dissolved in sufficient DDW and final volume was made up to 1000 cm^3, by using DDW.

2.5 0.5N HCl

4.3 cm^3 of pure HCl was pipetted in sufficient DDW and final volume was made up to 1000 cm^3, by using DDW.

2.6 Methyl red indicator

5 mg of methyl red was dissolved in sufficient ethanol and final volume was made up to 100 cm^3, by using ethanol.

3. Reagent for peroxidase estimation

3.1 Pyrogallol phosphate buffer

It was prepared by mixing 25 cm^3 of pyrogallol in 75 cm^3 phosphate buffer (pH 6).

4. Reagents for catalase estimation

4.1 Phosphate buffer (0.1M) for pH 6.8

3.54 g of Na_2HPO_4 was dissolved in 100 cm^3 of DDW and 3.72 g of NaH_2PO_4 was dissolved to 100 cm^3 of DDW separately. 12.3 cm^3 of Na_2HPO_4 was then added to 87.7 cm^3 of NaH_2PO_4 to get the buffer.

4.2 H_2O_2 (0.1M)

0.34 cm^3 of H_2O_2 was added to 100 cm^3 of distilled water.

4.3 Sulphuric acid (2%)

2 cm^3 of H_2SO_4 was added to 98 cm^3 of DDW.

4.4 0.1N Potassium permanganate

This was made by dissolving 0.162 g of $KMnO_4$ in 500 cm^3 of DDW.

5. Reagents for superoxide dismutase

5.1 Phosphate buffer (50mM) for pH 7.8

It was prepared by dissolving 1.78 g Na_2HPO_4 and 1.56 g of NaH_2PO_4 in 100 cm^3 of DDW separately. 91.5 cm^3 of Na_2HPO_4 was mixed with 8.5 cm^3 of NaH_2PO_4 to get pH 7.8.

5.2 Methionine (13mM)

It was prepared by dissolving 0.193 g of methionine in 100 cm^3 of DDW.

5.3 Nitrobluetetrazolium (NBT) (75µM)

6.13 mg of NBT was dissolved in 100 cm^3 of DDW.

5.4 Riboflavin (2mM)

0.732 mg of riboflavin was dissolved in 100 cm^3 of DDW.

5.5 EDTA (0.1M)

2.92 g EDTA was dissolved in 100 cm^3 of DDW.

6. Preparation of reagents for proline estimation

6.1 Sulphosalicylic acid (3%)

3 g of sulphosalicylic acid was dissolved in sufficient DDW and final volume was maintained to100 cm^3, by using DDW.

6.2 Acid ninhydrin solution

1.25 g of ninhydrin was dissolved in a mixture of warm, 30 cm^3 of glacial acetic acid and 6 M phosphoric acid (pH 1.0) with agitation till it got dissolved. It was stored at 4°C and used within 24 h.

The 6M phosphoric acid was prepared by mixing 11.8 cm^3 of phosphoric acid with 8.2 cm^3 of DDW.

7. Preparation of reagents for cadmium accumulation

7.1 Calcium chloride (5mM)

0.06 g of $CaCl_2$ (anhydrous) is dissolved in sufficient DDW and final volume was maintained to 100 cm^2.

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ORIGINAL ARTICLE

Effect of cadmium on the growth and antioxidant enzymes in two varieties of *Brassica juncea*

Mohd Irfan [a], Aqil Ahmad [a], Shamsul Hayat [a,b,*]

[a] *Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh 202002, India*
[b] *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia*

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Abstract Increasing contamination and higher enrichment ratio of non-essential heavy metal cadmium (Cd) induce various toxic responses in plants when accumulated above the threshold level. These effects and growth responses are genotype and Cd level dependent. An experiment was conducted to analyze the effect of Cd toxicity in *Brassica juncea* [L] Czern and Coss by selecting its two varieties Varuna and RH-30. Cadmium (0, 25, 50 or 100 mg $CdCl_2$ kg^{-1} of soil) fed to soil decreased the values of growth characteristics, activity of nitrate reductase and leaf water potential, whereas activities of antioxidant enzymes and proline content increased with the increasing concentration of Cd, observed at 30 and 60 day stages of growth, in both the varieties. Moreover, Cd uptake by the roots was higher in RH-30 than Varuna. Also the activity of antioxidant enzymes and proline accumulation were higher in Varuna with increasing soil level of Cd. Out of the two varieties, Varuna was more tolerant than RH-30 to Cd stress.

1. Introduction

Untreated sewage sludge, industrial waste water or inappropriate use of phosphate fertilizers to agricultural fields are progressively increasing the soil Cd level. Therefore, metal accumulator crop species take up and store Cd in their tissues at high concentration which not only poses threat to plant survival but also induces several toxic responses and raises the danger of food adulteration in crop plants. Cd competes with the uptake of other essential minerals and causes desiccation stress (Hernandez et al., 1996). When taken up in the cellular environment it binds with membranes and enzymes interfering with their functions and stability (Karcz and Kurtyka, 2007). Plant species generate a range of defense mechanisms to resist Cd induced toxicity and to recover the subsequent damages (Meharg, 1993; Mohamed et al., 2012) eliciting their genotype based biochemical responses. However, the resistance response relies on the interaction of genotype with dose of toxicity to show comparative resistance. The excessive uptake of nonessential bivalent cations to the aerial plant parts shifts its cellular phosphorylation state, eliciting oxidative stress and a range

* Corresponding author at: Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia.
E-mail addresses: hayat_68@yahoo.co.in, shayat@lycos.com (S. Hayat).

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of physiological disturbances (Meharg, 1993; Akhtar and Macfie, 2012).

To counter this oxidative stress plants have an efficient system of stress enzymes and antioxidant non-enzyme molecules, that is termed as antioxidant system. Among these enzymes, superoxide dismutase (SOD) is the first line of defense against ROS, dismutating O_2^- to oxygen molecule and H_2O_2. Another enzyme is catalase (CAT), that breaks H_2O_2 to water and oxygen while peroxidase (POX) scavenges H_2O_2 in chloroplast and cytosol of plant cells (Gill and Tuteja, 2010; Gill et al., 2011). The metabolite proline serves multiple functions in plant stress adaptions. It works as protein-compatible hydrotope, osmo-protectant, ROS scavenger and regulator of cellular redox status. Proline regulates the redox signal governing the metabolite pool and expression of several genes that affect plant growth and development (Kavi Kishor et al., 2005; Szabados and Savoure, 2010; Hayat et al., 2012).

Species of mustard are good accumulators of sufficient quantities of Cd in their tissues. The brown mustard or *Brassica juncea* [L] Czern and Coss is economically very important crop, primarily used to harvest edible oil and also as a vegetable. However, Cd toxicity responses of different varieties vary greatly and are dependent on the interaction of the genotype with the type of metal and its concentration. The varieties of *B. juncea* could be classified as sensitive or resistant based on their responses to Cd toxicity. The objective of the study is to assess the level of oxidative stress, internal Cd level and the efficiency of antioxidant enzymes which might play a regulatory role against Cd induced metabolic shift.

2. Materials and methods

Seeds of *B. juncea*; Varuna and RH-30, procured from the National Seed Corporation, New Delhi, India, were surface sterilized (with 0.01% $HgCl_2$) followed by repeated washings with double distilled water (DDW). A completely randomized block design experiment was arranged in the net house of Department of Botany of Aligarh Muslim University, Aligarh, India during September-February 2009–2010 under the ambient environmental conditions with optimum temperature that varied from 10 to 30 °C.

Seeds were sown in earthen pots (25 × 25 cm) filled with 5 kg of soil containing sandy loam soil and farmyard manure (6:1 v/v), urea, single superphosphate and muriate of potash were added at 40, 138 and 26 mg kg^{-1} of soil, respectively. Soil in the selected pots was mixed with Cd (0, 25, 50 or 100 mg $CdCl_2$ kg^{-1} of soil) and watered on alternate days. Both the varieties were sampled at two growth stages (30 and 60 DAS). The plants were removed from the pots along with the soil and were dipped in a bucket filled with tap water. The plants were gently moved to remove the adhering soil particles.

2.1. Growth analysis

The length and fresh mass of roots and shoots were measured using a meter scale and an electronic balance, respectively. The leaf area was measured manually using a graph sheet, where the squares covered by the leaf were counted. The plants were then placed in an oven at 80 °C for 72 h. The dried plants were then weighed to record plant dry mass.

2.2. Nitrate reductase activity

Nitrate reductase (NR) activity was measured by the method of Jaworski (1971) in fresh leaf samples that were cut into small pieces. The absorbance was read at 540 nm and the activity of NR [*n* mole NO_2 g^{-1} (FM) s^{-1}] was calculated.

2.3. Antioxidant enzyme activities

The activity of peroxidase (POX) and catalase (CAT) were assayed following the procedure described by Chance and Maehly (1955). The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The amount of enzyme which causes 50% inhibition in photochemical reduction of NBT was considered as one enzyme unit.

2.4. Leaf water potential and proline content

Leaf water potential, was measured in fresh, detached leaves of the sampled plants by using PSYPRO, leaf water potential system (WESCOR, Inc. Longman, USA). The proline content in fresh leaf samples was determined by the method of Bates et al. (1973). The absorbance of the toluene layer was read at 528 nm, on a spectrophotometer (Milton & Roy, USA).

2.5. Cd accumulation in root and shoot

The root and shoot samples were placed for 10 min in ice cold 5 mM $CaCl_2$ solution to displace extracellular Cd, rinsed with DDW and then oven dried (Meuwly and Rauser, 1992). Cd concentration in tissues was estimated after digesting the samples in nitric acid:perchloric acid (3:1, v/v). Cd concentration was determined by an atomic absorption spectrophotometer (Perkin-Elmer A, Analyst, 300).

2.6. Statistical analysis

The experiment was conducted according to simple randomized block design. Each treatment was replicated five times and three plants were maintained in each pot, representing a replicate. Treatment means were compared by the analysis of variance (ANOVA) using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Least Significant Difference (LSD) between treatment means was calculated at the 5% level of probability.

3. Results

3.1. Growth parameters

Cd (0, 25, 50 or 100 mg kg^{-1}) administered through the soil significantly declined the growth (length, fresh mass, dry mass of root and shoot and leaf area) parameters in both the varieties in a concentration dependent manner both at 30 and 60 DAS (Fig. 1A–G). The highest concentration of Cd (100 mg kg^{-1}) caused maximum damage and decreased the root and shoot length by 65% and 39%, fresh mass 67% and 55%, dry mass 69% and 65%, and leaf area 54%, respectively, as compared to control plants of RH-30, at 30 DAS.

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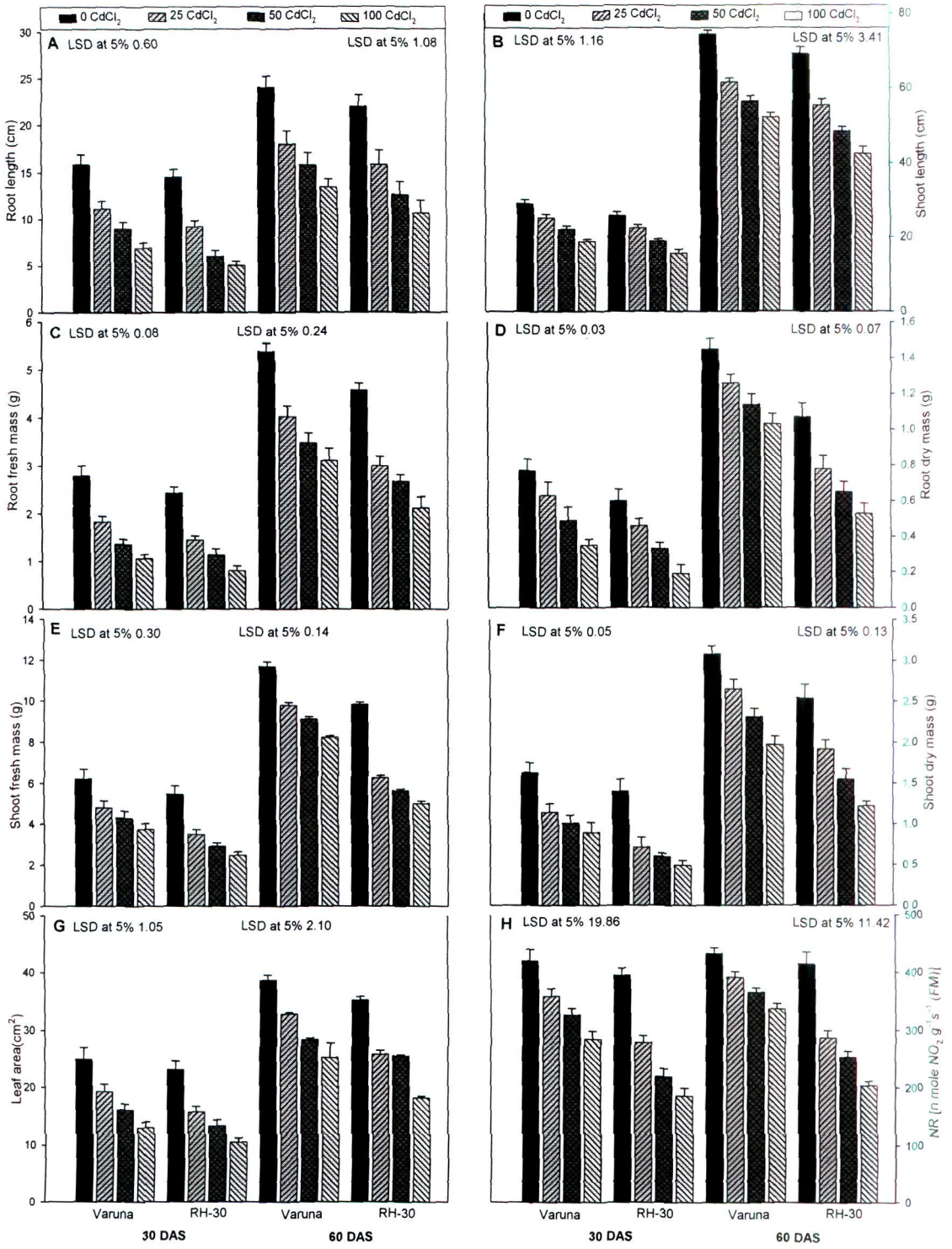

Figure 1 Effects of soil amended cadmium ($CdCl_2$; 0, 25, 50 or 100 mg Kg^{-1} of soil) induced changes in the (A) root length, (B) shoot length, (C) root fresh mass, (D) root dry mass, (E) shoot fresh mass, (F) shoot dry mass, (G) leaf area, (NR activity of Varuna and RH-30 varieties of *Brassica juncea* L. plants at 30 and 60 DAS.

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The reduction was higher in RH-30 than Varuna at both the growth stages (30 and 60 DAS). However, per cent loss was more at 30 DAS.

3.2. Nitrate reductase activity

As depicted in Fig. 1H, nitrate reductase (NR) activity decreased significantly as the concentration of soil Cd increased in both the varieties at 30 and 60 DAS. The maximum decline was observed at a concentration of 100 mg kg^{-1} of Cd which reduced the activity by 32% and 22% in Varuna and 53% and 51% in RH-30 at 30 and 60 DAS, respectively, as compared to their control plants. The loss in the activity was more prominent in RH-30 than Varuna. The per cent activity increased with the age of plants from 30 to 60 DAS.

3.3. Antioxidant enzyme activity

The activity of antioxidant enzymes; [peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD)] in the leaves of plants increased significantly in response to Cd in a concentration dependent manner at both the growth stages (Fig. 2A–C). The highest Cd level (100 mg kg^{-1}) caused maximum increase in enzyme activity that was 57% and 55% (POX), 36% and 23% (CAT) and 81% and 71% (SOD) in Varuna and RH-30, respectively, as compared to the control, at 30 DAS. Per cent enzyme activity decreased with the growth advancement from 30 to 60 DAS. Varuna possessed more enzyme activity than RH-30 at both the growth stages.

3.4. Proline content

It is evident from Fig. 2D that soil amended with Cd caused a significant increase in the proline content in a concentration (0, 25, 50 or 100 mg $CdCl_2$ kg^{-1}) dependent manner in Varuna and RH-30 at both the growth stages. The highest concentration (100 mg kg^{-1}) of Cd caused maximum accumulation of proline that was 82% and 66% in Varuna and 75% and 63% in RH-30, as compared to their control plants at 30 and 60 DAS, respectively. Varuna accumulated more proline as compared to RH-30 in response to all the treatments.

3.5. Leaf water potential (ψ)

The leaf water potential (LWP) decreased with an increase in the Cd level in the soil in both the varieties at 30 and 60 DAS (Fig. 2E). The highest level of Cd caused maximum reduction that was 37% and 43% in Varuna and RH-30 at 60 DAS, respectively, compared to the control plants. RH-30 was more vulnerable to Cd stress than Varuna at both the growth stages. The degree of Cd toxicity was more at early stage (30 DAS) of the growth than at latter stage (60 DAS).

3.6. Cd accumulation in root and shoot

An increasing trend of Cd accumulation was recorded both in root and shoot tissues with the increase of $CdCl_2$ in the soil (Fig. 2F and G). Shoot comparatively accumulated lesser quantities of Cd than root in both the mustard varieties. The percent increase in the Cd accumulation was higher in RH-30 than Varuna at 30 DAS. At the highest concentration of Cd (100 mg kg^{-1}) Varuna accumulated 148 μg and 60 μg Cd and RH-30 170 μg and 83 μg Cd g^{-1} of root and shoot dry mass, respectively, at 30 DAS.

4. Discussion

Plant genotypes differ in their ability to take up and translocate soil-amended Cd from roots to shoots (Metwally et al., 2005). The ability to check root uptake and aerial distribution of Cd depends on its binding to extracellular matrix, root efflux, intracellular detoxification and its transport efficiency (Marchiol et al., 1996; Akhtar and Macfie, 2012; Meng et al., 2012). In this study Varuna, compared with RH-30, accumulated lesser quantity of Cd both in root and shoot tissues (Fig. 2F and G) because of the reasons mentioned earlier. The absorbed Cd accumulates preferably in plant roots followed by shoots, which often restricts the uptake and distribution of other nutrients (Gomes et al., 2013 and Fig. 2F and G). This study indicates, the level of the metal increased with a progressive increase in the soil Cd content (0, 25, 50 or 100 mg kg^{-1}) both in root and shoot.

Cadmium uptake at toxic level causes mineral deficiency, desiccation and cellular metabolic disturbances (Marshner, 2012; Gomes et al., 2013) in plants. Cadmium alters the membrane permeability and hence cellular LWP (Fig. 2D). Cd affected membrane potential and proton pump activity could restrict the growth of maize plants (Karcz and Kurtyka, 2007). Moreover, Cd brought about aquaporin mediated reduction in maize root hydraulic conductivity that reduced the cellular turgor and leaf elongation even without changing transpiration (Ehlert et al., 2009). Therefore, an increase in Cd concentration both in root and shoot (Fig. 2F and G) partially damaged the membrane which resulted in decreased LWP (Fig. 2D). However, proline accumulation is an adaptive mechanism to counter osmotic stress caused by decreased LWP; therefore, it reestablishes the LWP and augments the loss of cellular osmoticum (Alia and Saradhi, 1991; Albert et al., 2012). Varuna accumulated more proline than RH-30 which could have favored the maintenance of LWP in this cultivar (Fig. 2D and E).

The Cd binding to root epidermal membrane affects the functioning of transporter proteins either through direct binding to the ion transporters or via membrane assisted ROS production. The competitive exclusion of the substrate (NO_3^{2-}) potentially impeded the NR activity (Hernandez et al., 1996; Campbell; 1999; Fig. 1H) or, alternatively, the metal could have bound with the –SH group, directly affecting the enzyme structure and its functions (Choudhary and Singh, 2000). Cadmium induced supra-optimal generation of ROS could interfere with the active state of NR rendering it inactive. The NR activity exhibited a progressive decline in response to increasing dose of Cd (Fig. 1H). However, proline protects membranes and subcellular structures, hydrates the enzymes to restore their activity and neutralizes reactive oxygen/nitrogen species (Hare and Cress, 1997; Kavi Kishor et al., 2005), its increased detoxification capacity (Fig. 2A–C) may have potentially protected the NR activity more effectively in Varuna as compared to RH-30.

Besides proline, antioxidant enzymes are also the key players in maintaining cellular redox status and stress induced

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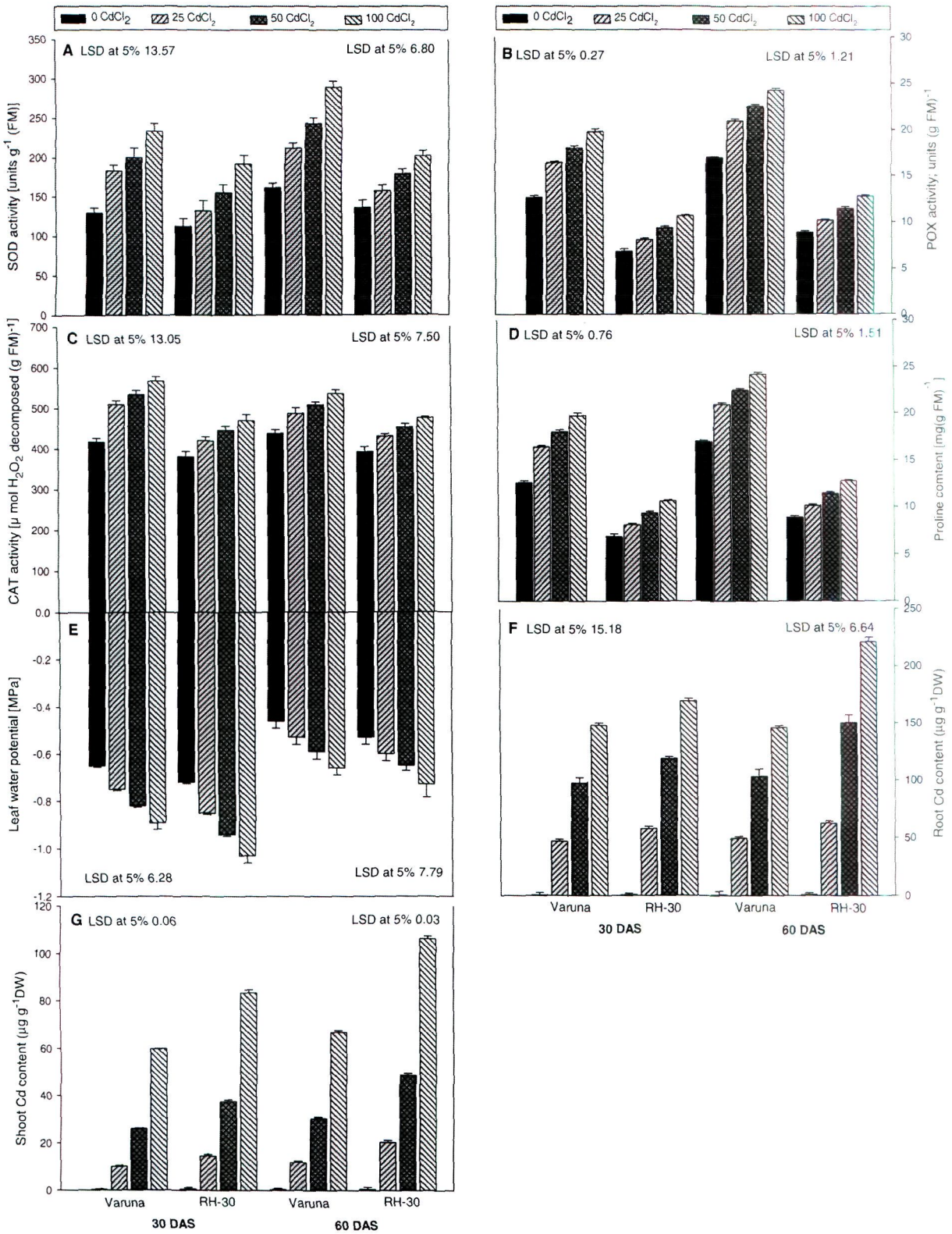

Figure 2 Effect of soil amended cadmium ($CdCl_2$; 0, 25, 50 or 100 mg Kg^{-1} soil) induced changes in the (A) SOD activity, (B) POX activity, (C) CAT activity, (D) proline content, (E) leaf water potential, (F) root Cd content, (G) shoot Cd content of Varuna and RH-30 varieties of *Brassica juncea* L. plants at 30 and 60 DAS.

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plant tolerance (Kavi Kishor et al., 2005; Gill and Tuteja, 2010; Gill et al., 2011; Hayat et al., 2012). The higher activity of antioxidant enzymes (i.e. POX, CAT and SOD) was in proportion to the progressive increase in the concentration of Cd ($CdCl_2$; 0, 25, 50 or 100 mg Kg^{-1} of soil; Fig. 2A–C). Moreover, the per cent increase in antioxidant enzymes was more in Varuna as compared to RH-30 (Figs. 2A–C). Mohamed et al. (2012) has shown in *B. juncea* that the higher activity of antioxidative enzymes offers a greater detoxification efficiency which provides better resistance to a plant variety against heavy metal induced oxidative stress.

The increased uptake and accumulation of heavy metal in plants cause osmotic shift, metabolic alterations and also ROS induced damages (Gill and Tuteja, 2010; Gill et al., 2011) while Cd induced mineral stress could reduce plant dry weight accumulation (Marshner, 2012). Cadmium induced restricted water uptake hampers turgor mediated wall extensibility (Marchiol et al., 1996) which could decrease cell division (Marshner, 2012). Cadmium mediated cumulative effect of these factors caused a decrease in leaf area, fresh and dry mass, and length of root and shoot (Fig. 1A–G). The values for all these growth characteristics decreased in a dose dependent manner of Cd level in the two mustard varieties (Varuna and RH-30). However, the increased activity of antioxidant enzymes and that of proline level presumably protected the metabolic machinery, stabilized the membranes to prevent water loss and supported nutrient uptake to augment growth performance more in Varuna than RH-30. The present findings get additional support from the work of Sharma et al. (2010) and Hasan et al. (2011) in tomato and Hayat et al. (2011) in brassica, respectively, under heavy metal stress.

5. Conclusion

The two mustard varieties (Varuna and RH-30) responded differentially against Cd induced oxidative stress. Varuna was more resistant than RH-30. The increased activity of antioxidant enzymes and leaf proline level protected the plant growth in a genotype dependent manner besides the restricted uptake and transport of Cd.

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