CHAPTER I

INTRODUCTION
The amino acids, i.e., arginine (Arg) and ornithine (Orn), are the precursors of polyamine formation in plants and animals. In plants, the physiological functions of polyamines, putrescine (Put), spermidine (Spd), spermine (Spr), etc., is still not well understood (Walden et al., 1997). PAs occur in free form as cations highly protonated at physiological pH or are often conjugated with small phenolic acids as well as various macromolecules. Due to their cationic nature, they are known to bind strongly to phospholipids of cell membranes, nucleic acids, and proteins to exert their biological functions (Tiburcio et al., 1993; Slocum et al., 1984). PAs are low molecular weight, polycationic, aliphatic, nitrogenous, non-protein forming molecules, with functionally active positively charged amino groups at their ends and or in the middle of the molecules.

Polyamines are of ubiquitous occurrence in plants, animals, and human beings. In general, prokaryotes have a higher concentration of Put than Spd and lack Spm (Flores et al., 1989). Though Spm has been reported recently in few thermophilic bacteria (Oshima, 1983). The plant cells in general contain large quantities of Spd followed by Put and little quantities of Spm. There are three forms of PAs occurring naturally in plants, viz., a) Free cationic form, b) Conjugated form, c) Covalently bound form. The earlier studies have generally reported free PAs, whereas, the studies including the conjugated and covalently bound forms of PAs appeared lately (see Rajam, 1997). The conjugated forms of PAs are conjugated to low molecular weight secondary metabolites in plants by formation of amide linkages. This linkage is made to provide an activated carboxyl group by utilizing esters of coenzyme A by the PAs. The phenolic acids are the main conjugants of PAs, namely, cinnamic acid, ferulic acid, coumaric acid, and caffeic acid (Martin - Tanguy, 1985; Smith et al., 1983). A third form of PAs is covalently bound to cellular proteins which may account for the significant portion of the metabolic pool (see Rajam, 1997). Bagni (1966) was first to demonstrate the growth regulatory action of PAs in dormant tubers explants of Helianthus tuberosus. Altman (1989) termed PA as bioregulators and proposed their role as 'secondary messenger' in mediating hormonal effects.
Since then many investigators have demonstrated that the endogenous titres of aliphatic amines change considerably with the development of tissues or organs, and are correlated with several physiological processes, viz., cell growth and differentiation, physical and chemical properties of the membranes, modulation of the enzyme activities, protein synthesis, regulation of synthesis and function of nucleic acids and hormones, root induction, floral development and inhibition, fruit set and development, pollen development and senescence, etc. (Walden et al., 1997; Walters, 1995; Sharma and Rajam, 1995; Tiburcio et al., 1994; Kakkar and Rai, 1993). Cells undergoing division contain high levels of free PAs, whereas, low levels are found in the cells undergoing expansion (Galston and Kaur-Sawhney, 1995).

Intercellular PAs are attached to ribosomes in vacuoles, mitochondria, chloroplast and various RNA species (Smith, 1981). Spermine is associated with nucleus (Smith, 1985). The Spd and Spm synthases have been demonstrated to be present in chloroplast by Cohen et al. (1982). The PA catabolizing enzymes (diamine oxidase and polyamine oxidase) have been suggested to be located in the cell wall (Federicio and Angelini, 1986). Alteration of Spm contents of DNA can apparently result in transformation from the functional B form to the nonfunctional Z-form of DNA by attachment of PAs to the later, especially at G and C-residues. (Rich et al., 1984). They occur in plant tissue varying from 0.05 - 10 mM (Solcum et al., 1984; Galston and Sawhney, 1990). Exogenously supplied PAs have been reported to delay senescence and Put and Cad levels are regulated in oxidation rather than biosynthesis during seed and seedling germination in chichpea (Torrigniani and Scoccianti, 1995). The decline in PA titre along with arginine decarboxylase activity seems to parallel the pattern of appearance of senescence in many plants. Ethylene and PA show opposite effect in relation to fruit ripening (Kakkar and Rai, 1993).

Most of the genes encoding PA’s biosynthetic enzymes, viz., arginine and ornithine decarboxylase (ADC and ODC), have been isolated from plants and compared with
those of other eukaryotic genes encoding for the same enzymes suggesting a similar
catalytic mechanism of the both, plants and animals (Walden et al., 1997; Michael et
al. 1996). The ADC m-RNA levels increase from immature green to breaker stage
in tomato (Rastogi et al., 1993). The expression patterns for ADC genes support
the idea that it is related directly to Put synthesis during cell elongation (Rastogi et
al., 1993). A changed PA metabolism has been observed in mutants of tobacco
cell lines (Fritze et al., 1995) and transgenic plants in tobacco from oat gene (Masgrau
et al., 1996; DeScenzo and Minocha, 1993).

Altered concentrations of PAs have been found under stress conditions
( Basu and Ghosh, 1991; Flores and Galston, 1984a) and in response to growth
regulators (auxins and gibberellins) and certain external stimuli (light, dark, etc)
(Lin, 1984; Dai et al., 1981a). PAs especially Put accumulates considerably in various
plants species in response to the various stresses, viz. water deprivation due to
drought (Flores et al. 1989), osmotic shocks (Tiburcio et al. 1993b), high
concentration of ammonium (Smith, 1983), K\(^+\), Mg\(^+\), Ca\(^+\) deficiencies (Young and
Galston, 1984) and anaerobiosis (Reggiani and Bertani, 1990). The physiological
significance of this increase in endogenous Put levels is not yet clear although the
recent work suggests an adaptive and protective role of PAs (see Rajam, 1997;
Galston and Sawheny, 1990). One of the possible mechanism of PAs involvement
for the antisenescence effects is possibly mediated through inhibition of lipid
peroxidation (Borrell et al., 1997).

In response to the biotic stresses, e.g., viral and fungal infections, the plant cells
accumulate free and bound PAs which are conjugated with hydroxycinnamic acids
(HCAs) (Martin- Tinguy, 1993). In one of the earliest studies an antifungal factor,
p-coumaroyl agmatine (a PA), was suggested to be responsible to create resistance
in young barley seedlings from Helimenthosporium sativum (Stoessl and Unwin,
1978). ‘Hordatines’, the dimers of p-coumaryl agmatine, were found to show
streptomycin like response to the fungus.
Cu²⁺-diamine oxidase (DAO. E.C. 1.4.1.6) and Polyamine oxidases (PAO. E.C. 1.4.3.4) are the two major classes of enzymes which catalyzes oxidative deamination of PAs. The DAOs are copper containing and PAOs are flavin containing (FAD) -amine oxidases. PAs are catabolized by various amine oxidases, of which DAO and PAO are most active (Smith, 1985a). The oxidases serve to regulate intracellular PA levels in plants and animals. The DAO has broad substrate specificity in leguminoseae (see Sharma, 1997). It converts (1) Put. to NH₃, H₂O₂ and \( \Delta¹ \)-pyrroline. The \( \Delta¹ \)-pyrroline is further converted \( \gamma \) -aminobutyric acid (GABA), and / or (2) oxidizes Spd into aminoproply pyrroline. On the other hand PAO oxidises (1) Spd. to 1,3-diaminopropane, pyrroline and \( \text{H}_2\text{O}_2 \). Diaminopropane in turn may get converted to L-alanine. Werle and Raub (1948) first demonstrated the presence of amine oxidases activity in plants. All DAOs contains Cu (II ) as the metallic cofactor. The second prosthetic group, an organic cofactor, was reported to be pyridoxal phosphate for the first time. Later, in 1987, Glatz et al. suggested pyrroroloquinoline quinone (PQQ) to the carbonyl group of DAO. In Janes et al. (1990) suggested 6-DOPA (6-dihydroxydopa quinone) as organic cofactor and it was confirmed to be 3,4,6- trithydoxy TOPA quinone (TOPA) in E. coli K-12 by Copper et al. (1992). Diamine oxidase have been localized in the association with membranes and cell walls of the leaves. In roots, the enzyme shows an active presence in the well differentiating zone and, in differentiating tissues it is associated with cell walls and nuclei (Federico and Angelini, 1985). In cotyledons the amine oxidases are found on plasma membranes, on the membranes of protien bodies and on the vacuolar surfaces (Federico and Angelini, 1985).

Diamine oxidase may have a role in seed germination and growth of plants by regulating the auxin synthesis (Srivastava et al., 1981). The DAO has been reported to be inactive in the ungerminated seeds whereas in the germinating seedling the enzyme is highly active (Choudhary and Singh, 1997; Torrigiani and Scoccianti, 1995; Di Tomaso et al. 1992; Federico and Angelini, 1988). The DAO activity decreases generally with increase in age of seedling. This decrease may be due to higher...
levels of accumulation of secondary metabolites like phenols and flavonoids. (Angelini et al., 1998b; Srivastava et al., 1981).

Diamine oxidases have been purified to homogeneity from various sources using standard purification methods involving homogenisation, salting out, and various chromatographic procedures (see Medda et al., 1995). The SDS-PAGE and native PAGE show that DAO from various sources is a dimer of 2 similar units of molecular weight ranging from 54-81 KDa. The native and SDS-PAGE and gel chromatographic techniques have shown the Mr ranging from 118-162 KDa. Cu-contents vary from 0.082-0.10% and carbohydrate contents vary from 12-14% (w/w) (see Medda et al., 1995). The DAOs are reported to have best specificity which Put and Cad with optimum pH of 7.0. The DAOs have been suggested to be substrate inducible enzymes (Srivastava et al., 1981, 1977). The DAO has been purified to homogeneity in *Hordeum vulgare* (Cogoni et al., 1990), *Oryza sativa* (Chaudhury and Ghosh, 1984), *Zea mays* (Suzuki and Hagiwara, 1993), *Trigonella foenum-graecum* (Luhova et al., 1995), *Onobrychis viciifolia* (Zajonocova et al., 1997), *Pisum sativum* (Srivastava and Prakash, 1977; Deveci and Guvenilir, 1995), *Lathyrus sativus* (Padiglia et al., 1991), *Lens esculenta* (Padiglia et al., 1991) etc.

The day light has been reported to depress DAO activity in plants whereas etiolated seedling and leaves show the higher enzyme activity (Angelini et al., 1988; Macholan and Minar, 1974). The enzyme level is increased in plants upon exposure of red light and reversed by far red light (Joseph and Srivastava, 1995; Joseph et al., 1995b). An increase in free calcium concentration in the cells as a result of light stimulus may lead to increased enzyme synthesis through, (1) gene activation, (2) Ca-dependent protein Kinases or, (3) Ca-dependent enzyme activation (Joseph et al., 1996). Similar phytochrome mediated enzyme synthesis and activation through phytochrome mediation via signal transduction have been reported for nitrate reductase (Raghurom and Sopory, 1996).

Some of the other factors that effect DAO are plant growth regulators e.g., IAA and 2,4-D showed a reduced DAO activity in pea seedlings (Srivastava et al., 1977).
whereas, e.g., gibberllins, Put, Spd, and ornithine, treatments increased enzyme activity in pea seedlings (Srivastava et al., 1977). Upon exposure to ozone the plants show a decreased DAO activity (Rajam, 1997). Sodium chloride caused salinity increased the enzyme activity upon long term exposure (Das et al., 1995).

Industrial release of pesticides and fertilizers used in agriculture practices, automobile exhaust, acids, alkali, carbohydrates, coal, dyes, paints, fats, soaps, toxic metals, oils, resins, alkaloids, rubber, detergents, etc., are the major contributors to water, air and soil pollution. The heavy use of the fertilizers is one of the major reasons of soil salinity and sodicity. Dumping of the municipal and industrial waste without detoxification, results in leaching of toxic chemicals including heavy metals, which seep into soil and groundwater. More than twenty human diseases are associated directly with groundwater contamination with the toxic substances.

The metals that are considered to be toxic to the human beings, animals and plants are, Hg$^{2+}$, Pb$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, As$^{3+}$, Cr$^{3+}$, Ni$^{2+}$, Si$^{2+}$, etc. The metals are mainly heavy metals and they are called so due to their heavy nucleus containing more neutrons. The heavy metal toxicity at cellular and molecular levels causes disturbances in reproduction, differentiation, and maturation. They effect cell membrane permeability and disturb the energy metabolism, decreases the ability of lysosomal membrane to disrupt cell functioning and release of various hydrolases. At molecular level the metals interact with proteins leading to denaturation and precipitation (see Singh et al., 1997 and references therein). It also causes allosteric effects for the enzyme inhibition (Assche and Clijstr, 1991). In the fluctuating environment the plants may experience shifting internal concentration of the bioreactive metals as stated above. Therefore, it is beneficial for plants to have mechanisms that maintain the internal concentration of essential metals between deficient and toxic limits and to keep non-essential metals below the toxic threshold. The proteins and peptides known as metallothionins (MTs) sequester metals and thereby may accomplish the cellular metal homeostasis and detoxification. These
molecules are rich in cysteins that provide thiols for binding the metals mentioned so far (Rauser, 1995). The legumes produce the homologous tripeptide homoglutathione or $\gamma$-Glu-Cys-$\beta$-Ala in response to the metal stress and the Cd-binding complex from such plants contained the peptides $(\gamma$-Glu-Cys)$_n$$\beta$-Ala (Meuwly et al., 1995). These stress-induced proteins are commonly called heat-shock proteins (HSPs) also (Vierling, 1991; Nover, 1990; Schlesinger, 1982). The amino acid sequences of many of the low molecular weight (LMW)-HSPs have been determined through gene sequencing (Waters et al., 1996). In contrast to the detailed knowledge about the amino acid sequences of the LMW-HSPs, there is relatively little known about their functions (Wu and Laidman, 1997).

The heavy metals are non-degradeable and, therefore, readily enter the food chain subsequently endanger animal and human health (Parsad et al., 1997; Singh et al., 1997a, b; Panda and Patra, 1997). Contamination of the environment by heavy metals further jeopardise human welfare by reducing the productivity of the agricultural crops and their yield (Singh et al., 1997a, b; Panda and Patra, 1997; Singh and Sheoran, 1993). The uptake and the accumulation of these heavy metals in plants occur either directly through adsorption from aerial parts, or through mineral water uptake system via the roots. The metal contamination of the soil, and air is increasing very rapidly due to industrialisation. The uptake and accumulation of heavy metals by plants depend upon several nutritional and endogenous factors (Panda and Patra, 1997; Singh et al., 1997a, b). The metal absorbed and accumulated in the cells cause cellular and morhological changes as a result of alteration / inhibition of the metabolic processes (Van Bruwaene et al., 1984). Heavy metal contamination in the soil is known to inhibit / delay seed germination, seedling growth, biomass production, productivity and yield (Panda and Patra, 1997; Singh et al., 1997a, b; Kumar et al., 1993; Singh and Sheoran, 1993; Singh et al., 1988). The inhibitory effect of heavy metals on growth may also arise from interference of the metals with auxin regulated cell elongation (Lane et al., 1978). The uptake of certain ions is inhibited by heavy metals, possibly either by blocking the entry of ion or by binding with the respective ion carriers (Kannan and Koeppa, 1976).
Lead and cadmium have been reported to inhibit chlorophyll and carotenoid biosynthesis in many plants and photosynthesis has been found highly sensitive to these metals (see Singh et al., 1997 a,b; Singh and Sheoran, 1993; Muthuchelian et al., 1988). Enzyme of reductive pentose phosphate pathway is also inhibited by these metals (Hamppe et al., 1974). The reduction in photosynthesis is also attributed to increased stomatal resistance and decreased PS-II activity (Bazzaz and Govindjee, 1974; Hamppe et al., 1973) and also due to disorganisation of chloroplast membrane (Kumar, 1990). Pb\textsuperscript{2+} is found to inhibit the mitochondrial electron transport in dark (see Singh et al., 1997; Miller et al., 1973). The rate of nitrogen fixation and assimilation has been reported to decline in mungbean seedlings during Cd\textsuperscript{2+} (Sawhney et al., 1990) and Pb\textsuperscript{2+} contamination (Dabas et al., 1995). The adverse effects of the heavy metal, especially Pb\textsuperscript{2+} and Cd\textsuperscript{2+} on symbiotic nitrogen fixation in legumes is well documented (Lakshman et al., 1991, Wicliff and Evans, 1980). The reports of Mancuso (1951) have shown that these heavy metals above 40 mg/100g blood causes brain damage in children.

The responses of the plants to salinity have been reported by various workers (Singh et al., 1997b; Bharti and Singh, 1994; Lasher 1983; Salim and Pitmaru, 1983). Salinity in soil the water is an ever increasing problem in arid and semi-arid regions of the world today. The total area affected by excess of salts is about 25% of the world's potentially arable land (Singh et al., 1997b). In India, 7 million ha of land is salt affected and out of which about 40% is found in Indo-Gangetic plains of Punjab, Haryana and U.P. (Abrol and Gill, 1984). Haryana accounts to 5.2 Lakhs Hac which is about 14% of the total cultivated land in the state (Rana, 1977; Abrol and Bhumbla, 1971). Salinity is caused due to a high concentrations of soluble salts in the soil profile which get deposited as thick crusts on the soil surface during dry seasons. The sodic soils have a characteristic high exchangeable sodium percentage (ESP > 15%) and a high pH (> 8.0). The saline soils have high electrical conductivity exceeding 4 mhos cm but the pH of the soil is less than 8.0. Soil salinity may effect growth productivity, physiology and metabolism of plants by decreasing water
potential (osmotic potential), distributing the ionic balance of the soil solution and by increasing electrical conductivity which leads to the so called Shunting effect. The sodium chloride caused salinity is reported to effect growth and development of the plants through osmotic as well as specific ion effects. It has been reported to impair the uptake of nitrogen in barley plants (Dabas et al., 1993). The NaCl mediated decrease in nitrate reductase activity due to lowering of water potential has been reported by Plaut (1974).

The more toxic effect of the metal is noticed in the environment when the plants grow in saline conditions. The Cu^{2+}, Pb^{2+} and Cd^{2+} mediated decrease in biomass accumulation and nitrate assimilation in sesame had been reported under NaCl caused salinity was nullified to a great extent (Bharti and Singh, 1994). The other salts e.g., CaCl_{2}, K_2HPO_4, KN0_3 have also been reported to recover the above said inhibitory effects of the metals in seseme (Bharti et al., 1996). The Pb^{2+} mediated inhibition of the biomass accumulation and chlorophyll biosynthesis could recover by the amendment of potassium salts in Vigna radiata seedlings (Singh et al., 1994, 1996).

Vigna radiata (L) Wilczek, commonly called as the mungbean, is native to India and is an important grain legume of sub-tropical countries (see Jaiwal and Gulati, 1995). It is an important grain legume due to its high protein contents (24 %). The protein is rich in lysine, as compared to other proteinaceous cereal grains. Mungbean protein is, however, defecient in methionine, cystine and cysteine, sulfur containing amino acids which are abundantly present in the cereal crops (Tsou et al., 1979). Sprouted mungbean seeds provides a succulent and nutrientous vegetable, rich in proteins, minerals and vitamins. It fixes nitrogen through its symbiotic root nodules which susiquently gets assimilated into amino acids and other nitrogenous forms (see Jaiwal and Gulati, 1995).

The global mungbean growing area has increased during the past 20 years at the rate of 2.5 % upto 3.4 × 10^6 hac with an average yield of 384 Kg / hac (Jaiwal and
India accounts for about 2/3 of the total world production of the mungbean with the national average yield of 338 Kg/hac. (Jeswani and Blaldev, 1990).

Despite the efforts of plant breeders to improve the yield of mungbean, the production has not been increased with much success due to insufficient level of genetic variability within the germplasm (Tiyagi and Alam, 1992). Some of the factors responsible for the limitation to improve the production of this plant are, Susceptibility to fungal, viral, bacterial pathogens and insect pests, Salinity, drought, heavy metal pollution, Sensitivity to photoperiod, temperature and shattering of pods, etc. (Kim, 1994).

The genes responsible for attaining resistance in mungbean to biotic and abiotic stresses have been found in many wild and related species which are sexually incompatible with the cultivated Vigna sp. These genes are to be transferred to cultivated Vigna in order to increase and stabilize its production.

Inhibition of PA biosynthetic enzymes (ADC and ODC) in mungbean resulted in a decrease in Put contents in leaves and root whereas the same parameters increased in shoot tissue during salt and osmotic stress (Friedman et al., 1989; Tiburco et al., 1986). In V.mungo the PA concentration was found to be highest during flowering, and the Put and its biosynthetic enzyme decreased with senescencing while DAO levels increased. (Lahiri et al., 1992). Goldberg and Perdrizet (1984) showed that in young cells, most PAs were located in protoplasm whereas in older cells they are bound to the cell walls.

We could not get any report on DAO in V. radiata. The studies of the enzyme under heavy stress in V. radiata, are also not available in the literature as per our knowledge.

Therefore, the understanding of the underlying physiological and biochemical alterations in mungbean under various stress condition is important to manipulate
the crop for the desired stress tolerant traits. Role of PAs in the stress is also an ill
defined phenomenon and the studies related to PAs metabolism in this plant under
the heavy metal stress is very relevant to the currently identified gap areas in the
knowledge.

The present study was planned with these perspectives and the following specific
objectives.

(i) Screening DAO activity in five V. radiata cultivars in light and dark growth
conditions.

(ii) Regulation of DAO and PA under heavy metals (Pb^{2+} and Cd^{2+}) contamination.

(iii) Regulation of DAO under salt stress and in combination to Pb^{2+} and Cd^{2+}.

(iv) Purification of DAO from mungbean seedlings.

(v) Characterization of mungbean Cu^{2+} -diamine oxidase for their specific
properties.

(vi) Immobilization of mungbean DAO and characterization of the immobilized
enzyme.