CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE
During the normal course of plant development and survival, selective death of specific cells, tissues and organs takes place (Matile et al., 1999). Cell death also constitutes a component of the response of organisms including plants to diverse biotic and abiotic stresses. As the initiation and execution of cell death processes are under strict control of organism, it is referred to as programmed cell death (PCD). PCD is a broad term that refers to a process by which cells promote their own death by the activation of self destructive system. Many plant developmental processes and stress responses are achieved through the operation of PCD. Senescence is an example of PCD; some other examples are xylogenesis, embryogenesis, sex determination in monoecious plants such as maize, abscission zone formation and the hypersensitive reaction (HR) to pathogen infection (Greenberg, 1996; Jones and Dangl, 1996).

Ageing and senescence essentially overlap to a considerable extent. They could be differentiated in that the degenerative changes in the former occur without a reference to death. Senescence is a type of PCD as both its onset and progression take place in a highly regulated manner. The initiation of senescence is particularly regulated by many environmental and autonomous factors. These include pathogen infection, temperature extremes, drought, exposure to ozone, nutrient deficiency, wounding, age, reproductive development and phytohormone levels. The senescence in plant systems could be arrested or even reversed; in contrast, the process in animal systems is altogether irreversible (Buchanan et al., 2000).
Leaf senescence is the ultimate stage of leaf development. It is not simply degenerative but is a recycling process in which nutrients are translocated from senescing leaves to reproductive and developing organs such as young leaves, developing seeds or storage tissues (Quirino et al., 2000). Nitrogen from the leaves of deciduous trees is used for synthesis of storage proteins in stems that will support growth in the following spring (Clausen and Apel, 1991). Through senescence, the water consumption by older, less productive leaves is also eliminated that contributes to the plant water economy. Obviously, leaf senescence serves definite useful purposes in plant's life. The rates of senescence in different plant species/systems differ. They can be very rapid e.g., the flower petals of *Ipomoea tricolor* senesce within a day of flower opening. In contrast, it can take years or even decades to senesce e.g., in case of the leaves of *Pinus longaeva* and *Welwitschia mirabilis*.

Chloroplasts, the organelles containing most of the leaf protein (approximately 70%), are first to lose their integrity. They are known to differentiate into gerontoplasts in senescing leaves. The breakdown of nucleus, in contrast, is relatively a late event (Makino and Osmund, 1991). In fact, the chloroplast senescence is under direct nuclear control as was demonstrated long ago in classical enucleation studies (Yoshida, 1961).

**Biochemical/molecular basis of leaf senescence**

Leaf senescence is a consequence of many biochemical conversions particularly the degradation of chlorophylls and other macromolecules such as proteins, membrane lipids and RNA. Chlorophylls
are first degraded to chlorophyllides by chlorophyllase; the subsequent catabolism relies on Mg dechelatase and phaeoforbide a oxygenase (PaO) (Matile et al., 1992, 1999; Hortensteiner, 2006). Consequent upon chlorophyll degradation, foliar carotenoids become unmasked. The latter together with newly synthesized red anthocyanins and dark coloured oxidation products of phenolics is of great aesthetic value. This defines the polychromatic beauty of autumnal trees (Hortensteiner, 2006). The pigment breakdown is usually accompanied by degradation and mobilization of chloroplastic proteins. Although the enzymes involved in the degradation of chloroplastic proteins are not yet identified precisely, a role of three kinds of proteolytic enzymes namely, (i) cysteine protease (belonging to the family of enzymes involved in seed germination), (ii) an enzyme resembling the papain type and (iii) an enzyme resembling a protein processing one has been suggested (Dangl et al., 2000). Reports are also available about the degradation of pigments and proteins of chloroplasts by vacuolar enzymes (Yoshida et al., 1996). Further, the degradation of SSU of RUBISCO in chloroplasts by a proteolytic system encoded by a nuclear gene has been reported (Schmidt and Mishkind, 1983). Besides cysteine protease, the role of aspartate protease has also been suggested in protein breakdown (Graham et al., 1992; Mclaughlin et al., 1994). The senescence-induced protein breakdown results in the availability of transportable N and S for export from senescing leaves to the other organs.

As a result of the above catabolic changes, photosynthetic activity steadily declines. Inhibition of both PS I and PS II in senescing leaves has
been reported (Biswal et al., 1997). Under the conditions of reduced photosynthesis, the increased energy demand for mobilization and other physiological processes stimulate respiratory and oxidative metabolism. Respiratory burden in the form of senesced leaves with diminished C assimilation is eventually shed through rejection (abscission) by the plant.

The reduced photosynthesis during senescence often results in sugar starvation, thus activating the conversion of lipids to sugars. Lipids released from thylakoid breakdown are known to be converted to sugars through the glyoxylate pathway (Kim and Smith, 1994 a,b). The simple sugars produced from lipids are transported to other growing and demanding parts of the plant in appropriate transportable form(s). Apparently, the expression of genes for enzymes participating in the process of glucogenesis for production of sucrose plays an important role in senescence. For example, the mRNA encoding pyruvate orthophosphate dikinase is reported to be abundant in senescing maize leaves (DeLong et al., 1993; Gray et al., 1997). The activation of glucogenesis in senescing leaves is associated with functional transformation of peroxisomes to glyoxysomes (Pastori et al., 1997; Del Rio et al., 1998).

Although molecular degradation is one of the major events that occur during leaf senescence, the process nevertheless involves synthesis of some RNAs and proteins de novo (Smart, 1994; Buchanan-Wollastan and Ainsworth, 1997). The genes participating in degradation of macromolecules and mobilization of breakdown products may not be senescence-specific; they might be common with other processes/systems.
In fact, the expression of these genes has also been observed in young and mature leaves; however, their expression enhanced during senescence (Nooden et al., 1997). The proteases called Clp and ClpC are the plastid homologs of the ATP-dependent protease and associated ATPase of E.coli Clp gene family, respectively.

In addition to the expression of genes for proteases, senescence induced enhancement of expression of the genes for synthesis of nucleases has been reported (Taylor et al., 1993). Nucleic acids serve as the source of inorganic phosphates on hydrolysis during senescence (Herman and Feller, 1998). Phosphatases are active during senescence. The nucleoside products of nuclease and phosphatase action presumably are cleaved into sugars, purines and pyrimidines. Further catabolism of guanosine results in the production of CO2 and NH3. Often, an increased expression of RNase occurs rather late in the course of senescence which might be connected to the point of irreversibility that is when senescence leads to necrosis and death (Taylor et al., 1993; Oh et al., 1997; Lers et al., 1998).

A polypeptide of a cDNA clone that has similarity with plant ATP sulphurylase has been examined in Brassica during senescence (Buchanan-Wollastan and Ainsworth, 1997). Its enhanced expression during senescence might modulate the levels of cysteine and its conversion to glutathione. The latter not only plays a key role in minimizing the levels of toxic oxygen free radicals but may also be involved in storage and transport of S from senescing leaves to growing plant parts.
Altered oxidative metabolism is a central feature of senescence at the cellular level. The senescing leaves exhibit elevated levels of reactive oxygen species (ROS) e.g., superoxide anions ($O_2^-$), hydroxyl radicals (OH), perhydroxyl radicals ($HO_2^-$) and hydrogen peroxide ($H_2O_2$). This is accompanied by enhanced levels of an array of low molecular weight antioxidants e.g., glutathione (GSH), ascorbic acid (Asc) etc. and concomitantly altered activities of antioxidant enzymes e.g., catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) etc. (Strother, 1988). The changes in oxidative metabolism of senescing leaves are commonly studied in terms of lipid peroxidation (MDA levels) and osmolyte (e.g., K$^+$) leakage.

Regulation of leaf senescence by exogenous ascorbic acid (Asc) and H$_2$O$_2$ has been studied. Exogenous application of Asc to excised Triticum aestivum leaf segments increased the activities of ascorbate peroxidase (APX) and catalase (CAT) while reducing the lipid peroxidation (MDA contents). In contrast, H$_2$O$_2$ application increased the proteolytic activities decreasing protein contents and decreased catalase activity leading to the promotion of senescence (Shin et al., 1997). The role of ascorbate-glutathione cycle in mitochondria and peroxisomes showed differential action during senescence; mitochondria were much more susceptible to oxidative damage than peroxisomes. Consequently, the latter participated in the cellular oxidative mechanism longer than mitochondria (Jimenez et al., 1998). With respect to the altered redox metabolism, redifferentiation of peroxisomes into glyoxysomes could be of special significance in
senescence. This transition was clearly associated with an altered profile of superoxide producing- and antioxidant enzymes (Biswal, 1995). Senescence-induced enhanced expression of the catalase gene might help in reducing ROS pool (Thomas and de Villiers, 1996; Buchanan-Wollastan and Ainsworth, 1997).

Senescence-induced increase in the expression of homologues of ferritin gene in *Brassica* has been reported. The possible role of ferritin could be to complex and transport Fe from senescing leaves to growing parts of the plant. Likewise, an enhanced expression of homologues of metallothionein (MT) genes in *Arabidopsis* (Thomas and de Villiers, 1996) and in *Brassica* (Buchanan-Wollastan and Ainsworth, 1997) seems to be associated with binding by MTs of free metal ions released from protein breakdown; MT-metal ion complex being suitable for storage and transport. In this manner, the production of free radicals catalysed by free metal ions would be minimized.

Altered gene expression during leaf senescence was demonstrated using *in vitro* translation followed by gel electrophoresis to detect changes in the translatable mRNA populations (Watanabe and Imaseki, 1982). Most leaf mRNAs significantly diminished during the progression of senescence whereas some translatable mRNAs increased (Watanabe and Imaseki, 1982; Davies and Grierson, 1989; Becker and Apel, 1993; Smart *et al.*, 1995). Differential screening of cDNA libraries made from mRNA of senescent leaf tissues also demonstrated that the expression of a vast majority of genes is down regulated. These are senescence-down regulated
genes (SDGs); in contrast, the expression of other genes is upregulated
during senescence, the upregulated genes are senescence-associated
genes (SAGs). More than 30 SAGs have been isolated, cloned and
characterized in different plant systems e.g., *Arabidopsis* (Hensel *et al*.,
1993; Taylor *et al*., 1993; Lohman *et al*., 1994; Oh *et al*., 1996), asparagus
(King *et al*., 1995), barley (Becker and Apel, 1993), *Brassica napus*
(Buchanan-Wollastan, 1994), maize (Smart *et al*., 1995), radish (Azumi and
Watanabe, 1991) and tomato (Davies and Grierson, 1989; Drake *et al*.,
1996).

Although leaf senescence is characterized by both activation and
inactivation of distinct sets of genes, gene inactivation per se is not
sufficient for causing senescence; rather gene expression within leaf cells is
required for senescence to proceed. The senescence process can be
blocked by inhibitors of RNA and protein synthesis and by enucleation.

**Hormonal regulation of leaf senescence**

The onset and progression of leaf senescence is regulated by an
interplay of diverse internal and external factors including hormones. The
five major classes of plant hormones namely, auxins, gibberellins,
cytokinins, ABA and ethylene and other plant growth regulators such as
jasmonates either delay or promote senescence (Smart, 1994; Gan and
Amasino, 1997). Ethylene is by far the strongest promoter of leaf
senescence. A rapid rise in ethylene production prior to senescence is
commonly observed (Abeles *et al*., 1992; Reid and Wu, 1992). Ethylene
enhanced leaf senescence even in the evergreen plants like *Hedera helix*
Data from various experimental approaches show the involvement of ethylene in the regulation of leaf senescence. Thus, the promotion of senescence by precursors of ethylene biosynthesis like 1-amino-cyclopropane-1-carboxylic acid (ACC) and inhibition by inhibitors like aminooxyacetic acid (AOA), amino iso- butyric acid (AIB), Co$^{2+}$ and Ag$^{2+}$ are consistent with ethylene regulation of senescence (Abeles et al., 1992; Podwyzynska and Goszcynska, 1998; Moeder et al., 2002). Furthermore, the ethylene insensitive mutant (etrI) of Arabidopsis (Grbic and Blecker, 1995) and transgenic tomato with reduced ethylene production (John et al., 1995) showed retarded leaf senescence. The importance of ethylene in regulation of senescence is also implied by ore 3, an allele of ein 2 (ethylene-insensitive 2 mutation) (Oh et al., 1997).

The ABA-induced acceleration of leaf senescence is well established and documented. A substantial increase in endogenous ABA levels in excised senescing leaves and acceleration of senescence by exogenous ABA are reported (Zeevaart and Creelman, 1988). Despite the exogenous ABA-induced acceleration of leaf senescence, there is no consistent correlation between the endogenous ABA concentrations and stages of senescence e.g., in case of Phaseolus vulgaris leaves (Colquhown and Hillman, 1975). It is likely that ABA serves as a trigger in early stages of senescence with no necessary correlation at later stages.

In last some years the molecular mechanism of ABA action has been understood to a reasonable extent. In several plant systems, ABA has been demonstrated to rely essentially on the availability of Ca$^{2+}$ ions. This was
first demonstrated in mid 1980s by the group of Mansfield for ABA-induced stomatal closure in epidermal peels of *Commelina communis* on the basis of following experimental evidence: (i) exogenous ABA and Ca\(^{2+}\), applied individually, caused stomatal closure; the magnitude of closure was synergistic when the two effectors were applied in combination and (ii) in the presence of EGTA, a Ca\(^{2+}\) chelator/or Ca\(^{2+}\) channel blockers namely, nifedipine and verapamil, the extent of ABA-induced stomatal closure was much reduced (De Silva *et al.*, 1985 a,b). Dependence of ABA for its action on Ca\(^{2+}\) was also shown in other systems such as respiration of isolated *Avena* protoplasts (Owen and Wellburn, 1987) and heterocyst differentiation in *Nostoc* (Huddart *et al.*, 1986). Consequently, a second messenger hypothesis, wherein Ca\(^{2+}\) acts as a second messenger via its elevated cytosolic concentrations was proposed. Role of Ca\(^{2+}\) in ABA-induced inhibition of PCD in aleurone layer was also shown (Wang *et al.*, 1996; Bethke *et al.*, 1999). In our laboratory, Ca\(^{2+}\) requirement for ABA-induced acceleration of excised leaf senescence of *T. majus* was observed (Tyagi, 2001). However, a similar Ca\(^{2+}\) requirement for seed germination inhibition in *Brassica juncea* induced by ABA was not indicated (Sharma *et al.*, 1992). The function of Ca\(^{2+}\) as a second messenger is not restricted to ABA only, but has also been shown for other phytohormones e.g., auxin- (Mc Anish *et al.*, 1990; Irving *et al.*, 1992) and kinetin (Hare and Van Staden, 1997)-dependent processes. Furthermore, Ca\(^{2+}\) regulates an array of cellular processes ranging from adaptive stress responses to survival and programmed cell death. The specificity of Ca\(^{2+}\) signal generation is
dependent on influx and efflux of Ca\(^{2+}\) from the extracellular sites, cytosol and intracellular organelles. A major aspect of plant Ca\(^{2+}\) signalling relies on specific spatio-temporal changes in cytosolic-free Ca\(^{2+}\) which encode for information that brings about the physiological responses (Berridge et al. 1988, 2000; Sanders et al., 1999, 2002; Schroeder et al., 2001). Elevation of cytosolic Ca\(^{2+}\) concentrations through stimulation of Ca\(^{2+}\) channels may be a central step in many ROS-mediated processes such as stress, hormone signalling, polar growth development and possibly mechano-transduction. The activation of Ca\(^{2+}\) permeable cation channels is one of the important ROS-signalling components that is emerging in several plant transduction pathways (Mori and Schroeder, 2004).

Cytokinins retard the senescence process. Accordingly, endogenous cytokinin levels decrease in senescing leaves and exogenous application of kinetin causes a delay in senescence (Wingler et al., 1998). Cytokinin-mediated retardation of senescence is mainly due to the enhancement of sink activity in plants, tissues with largest cytokinin contents being the strongest metabolic sinks. It is evident that cytokinins retard leaf senescence by enhancing photosynthetic activity per unit area. The contents of chlorophylls, carotenoids, proteins and rubisco are known to enhance after the application of certain herbicides (benzyladenine and metribuzin) having cytokinin activity (Chernyad, 2000). The enhanced production of cytokinins in senescing tissue delayed the age-dependent decline in NADH-dependent hydroxy-pyruvate reductase (HPR), ribulose-1,5 biphosphate carboxylase/oxygenase and other enzymes involved in
photosynthetic metabolism. The transgenic plants with overexpression of ipt, a gene encoding isopentenyltransferase and hence enhanced cytokinin levels showed delayed senescence (Gan and Amasino, 1997). Cytokinins seem to influence the senescence by being a part of signalling pathway that represses the expression of SAGs.

Cytokinin effects in relation to leaf senescence and oxidative mechanisms on creeping bentgrass (Agrostis palusteris) in relation to heat stress were studied. Exogenous ZR (zeatin riboside) significantly suppressed lipid peroxidation and electrolyte leakage, increased superoxide dismutase (SOD) and catalase (CAT) activities while retaining the chlorophyll contents. Thus, ZR alleviated the stress-induced lipid peroxidation of bio-membranes (Liu and Huang, 2002).

Among other hormones, the senescence delaying effects of auxins e.g., in Prunus (Osborne, 1967) and gibberellins e.g., in Taraxacum officinale (Whyte and Luckmill, 1966) had become clear in 1960s itself. GA3 retarded senescence has been reported in several other species (Jiang et al., 1999; Maity et al., 1999; Ranwala and Miller, 2000).

Jasmonates (JA) are known to promote leaf senescence in Arabidopsis (He et al., 2001); the JA-promoted senescence could be counteracted by cytokinins (Parthier, 1991). The JA-induced down-regulation of photosynthetic genes seems to be a strong determinant in the onset of senescence (Creelman and Mullet, 1997). Also, the SAGs exhibit inducibility by jasmonates (Park et al., 1998). Genetic evidence for the involvement of brassinosteroids in leaf senescence has been reported.
Chory et al. (1991) demonstrated delayed senescence in *Arabidopsis det 2* (de etiolated 2) mutant that had a defect in brassinosteroid biosynthesis. Besides imposing the individual influences, various phytohormones interact among themselves in affecting the regulation of senescence.

**Relationship between stomatal movements and leaf senescence**

The status of stomatal movements seems to determine the onset, progression and eventually the rate of leaf senescence; stomatal closure induced by appropriate treatments generally promotes leaf senescence and *vice versa*. Thimann and Satler (1979a) showed the treatment of *Avena* leaves with phenyl mercuric nitrate or 1 M mannitol solutions to cause rapid senescence that was accompanied by stomatal closure in white light. A similar result was obtained with ABA treatment of *Tropaeolum* leaves (Thimann, 1985). On the other hand, factors inducing stomatal opening in dark such as certain antibiotics in *Tropaeolum* and kinetin in *Avena* leaves (Thimann and Satler 1979b; Park and Thimann, 1990) delayed the senescence. Further, the reversal of ABA-induced acceleration of *T. majus* leaf senescence by t-cinnamic acid (tCA) was found to correspond with reversal of ABA-dependent stomatal closure (increased SDR) by tCA (Sharma et al., 1995).

**Plant stress(es) and leaf senescence**

Plants experience a variety of abiotic stresses during their life cycle e.g., water deficit, salinity, low and high temperatures, toxic metal ions, UV radiations etc. (Aspinall and Paleg, 1981; Munns, 2005; Sharma and Dietz, 2006). All these stressful growth conditions adversely affect the plant
growth and developmental processes including senescence. Examples of stresses known to accelerate leaf senescence include water deficit (Irigoyen et al., 1997), high or low temperature, salinity (Sahoo et al., 2001), heavy metals, UV radiations (Biswal et al., 1997; Donan and Gallois, 1998; Lewandowski and Skorska, 2003), ozone (Mikkelsen and Heide-Jorgensen, 1996), SO$_2$ (Kargiolaki et al., 1991) etc. There is evidently a considerable overlap between the plant responses to various abiotic stresses and senescence. It is implied that genes/gene products involved in stress responses may also affect the longevity and senescence. For example, Chen et al. (2002) monitored the expression profiles of 402 potential stress related genes encoding known or putative transcription factors from Arabidopsis in various organs at different developmental stages and under various biotic and abiotic stresses. Among the 43 transcription factor genes induced during senescence, 28 were induced by stress treatment strengthening the extensive overlap between stress responses and senescence. The stress related genes known to be induced during senescence include metallothionein-like genes from rice (Wang et al., 2004) and Arabidopsis (Hensel et al., 1993; Hsieh et al., 1995) and those encoding ascorbate oxidase (Collard et al., 1996).

There are several commonalities at the level of hormonal regulation as well as with regard to the metabolites synthesized by plant tissues during stress and senescence. For example, ABA, that is involved in adaptation and coordination of plant responses to abiotic stresses, is a strong
determinant in the regulation of onset and progression of leaf senescence (Zeevaart and Creelman, 1998; details described in an earlier section).

**Stress metabolites: proline, polyamines**

The 'stress metabolites' produced often by plants in response to the diverse abiotic stresses include free proline, glycine-betaine, polyamines etc. Accumulation of proline is one of the common responses to various abiotic stresses (Hare and Cress, 1997; Schat et al., 1997; Sharma and Dietz, 2006). Proline, a proteinogenic amino acid, is predominantly synthesized from glutamate; alternatively, it could be generated from ornithine (Delauney and Verma 1993; Sharma and Dietz, 2006). Proline, considered to be a compatible solute, has been ascribed several adaptive functions under stress conditions such as that of osmoregulation (Kavikishor et al., 1995), N reserve, metal ion chelator (Sharma et al., 1998) etc. The osmoregulatory function of Pro is revealed in studies involving transgenic approaches. Thus, Kavikishor et al. (1995) demonstrated transgenic tobacco containing elevated constitutive proline contents to be less susceptible to the inhibitory effects of severe osmotic stress imposed by water deficit or enhanced salinity. Besides contributing to the osmotic adjustment at relatively high concentrations, proline at low concentrations affected the expression of certain genes associated with osmotolerance (Kiyosue et al., 1996). Experimental evidence for the antioxidative properties of Pro, both from *in vitro* and *in vivo* studies, has been presented. Thus, Smirnoff and Cumbes (1989) demonstrated the hydroxyl radical (OH \(^{-}\)) scavenging by proline. Recently, Kaul et al. (2006) demonstrated the
free radical scavenging properties of proline in a graft co-polymerization assay system wherein methyl acrylate (a monomer) is grafted onto the cellulose backbone in the presence of a redox initiator (Ce⁴⁺ or γ-radiation). Grafting is essentially mediated by free radical generation which was shown to be suppressed by Pro. The antioxidative function of proline in vivo was also reported in a transgenic high proline producing microalga *Chlamydomonas reinhardtii* (Siripornadulsil et al., 2002).

Although the protective/adaptive role of proline against stress-induced growth repression is well known from studies including transgenics (e.g., Kavikishor et al., 1995; Siripornadulsil et al., 2002), in recent years, some reports have appeared showing the growth inhibitory effects of proline. Chen and Kao (1995) demonstrated the inhibition of rice seedling growth due to the application of exogenous proline. Similarly, external supply of proline was found to be toxic in *Nicotiana* (Bonner et al., 1996). Proline toxicity has also been reported in *Arabidopsis thaliana* (Hellman et al., 2000) and in animal cells (Maxwell and Davies, 2000). In the light of the evidence reported recently, proline toxicity seems to be linked to the metabolism of applied Pro. Proline degradation is catalyzed by sequential action of two enzymes namely, Pro-dehydrogenase (ProDH) and P5C-dehydrogenase (P5CDH). Mani et al. (2002) demonstrated that antisense suppression of AtProDH in *Arabidopsis* resulted in hypersensitivity to Pro. This finding shows the toxicity to be due to Pro per se. Besides, the proline toxicity has also been proposed to be mediated by P5C, the degradation product of proline, which is not metabolized rapidly (by P5CDH).
Accordingly, yeast strains deficient in P5CDH were found to be hypersensitive to Pro (Deuschle et al., 2001; Nomura and Takagi, 2004). Apparently, the proline toxicity is not restricted to plants only. In humans, defects in HsP5CDH result in type 2 hyperprolinemia with variable degrees of mental retardation (Geraghty et al., 1998; Morita et al., 2002). Deuschle et al. (2004) established the proline toxicity to be due to P5C accumulation. They demonstrated the overexpression of AtP5CDH to cause a decreased sensitivity to externally supplied proline.

The foregoing account clearly reveals the toxic effects of exogenous proline application in certain plant and animal systems. Apparently, this newer dimension does not fit into the known properties of proline being a compatible solute which could accumulate in high concentrations without interfering with the cellular metabolic activities. The integration of the two diagonally opposite influences of proline is awaited. Since proline levels increase in senescing leaves (Sahoo et al., 2001; Rabiza-Swider et al., 2004), it was of interest to monitor the effects of proline on leaf senescence.

Polyamines (PAs) are low molecular weight, aliphatic amines that are ubiquitous to all organisms. PAs are organic cations due to protonation at cytoplasmic pH i.e. putrescine$^{2+}$, spermidine$^{3+}$ and spermine$^{4+}$. In higher plants, these are the most prevalent polyamines. Different PAs are produced by plants under abiotic stresses (Evans and Malmberg, 1989). Senescing leaves also exhibit elevated concentrations of PAs (Moriguchi, 2004).
PAs are synthesized from ornithine, citrulline or arginine. Putrescine (Put) is directly synthesized from Orn by Orn decarboxylase (ODC) and indirectly from Arg by arginine decarboxylase (ADC) and two other enzymes. The activities of ODC and ADC are regulated in a developmental and tissue-specific manner (Minocha and Minocha, 1995; Walden et al., 1997). Spermidine (Spd) is formed from Put by the addition of an aminopropyl group derived from decarboxylated S-adenosyl-methionine (SAM) by the enzyme Spd synthase. The addition of another aminopropyl group to Spd gives rise to spermine (Spm). Decarboxylated SAM is produced from SAM via SAM decarboxylase. These three decarboxylases have short lives indicating that they are important metabolic control points in the cell (Cohen, 1998).

A variety of roles have been proposed for polyamines in the growth, development and stress responses of plants (Minocha and Minocha, 1995; Cohen, 1998; Bouchereau et al., 1999). Polyamines also serve as precursors for secondary metabolites such as nicotine and can be conjugated with phenolic acids to produce plant defence-related compounds (Martin-Tanguay, 1997). The biological activity of PAs is attributed to their cationic nature. PAs can associate with the surface of membranes through their interactions with anionic components of biomembranes such as phospholipids (Roberts et al., 1986) rendering them stabilized and thus retard membrane deterioration. PAs have been shown to be effective against ozone damage (Bors et al., 1989) and to show free radical scavenging properties in certain in vitro assay systems (Drolet et al.,
1986). Due to these attributes, PAs retard lipid peroxidation and hence show antioxidant activity. PAs also regulate DNA replication, transcription and translation, cell division, differentiation and other morphogenetic processes (Smith, 1990).

Polyamines have been reported to retard leaf senescence (Apelbaum et al., 1981; Kaur-Sawhney and Galston, 1991). Convincing evidence suggests the involvement of polyamines (PAs) and ethylene in the process of leaf senescence and fruit ripening. Although the biosynthetic pathways of both PAs and ethylene are interrelated, S-adenosyl-methionine (SAM) being the common precursor, their physiological functions are distinct and often antagonistic. Their biosynthesis and the balance play a crucial role in the process of leaf senescence and fruit ripening (Pandey et al., 2000). PAs have been shown to retard leaf senescence by inhibiting ACC synthesis (Hong and Lee, 1996; Lee et al., 1997). The ethylene biosynthesis could also be modulated by the in vivo synthesis of PAs since both biosynthetic pathways share SAM as a common precursor/intermediate and could compete for the available SAM during senescence (Katoh et al., 1987).

It was of interest to examine the influence of exogenous PAs (Put, Spd, Spm) on the senescence of excised *T. majus* leaves particularly with regard to the aspects of membrane integrity. Furthermore, both free proline and PAs are known to increase in concentration in senescing leaves, a comparison of the co-accumulating metabolites in a model system like
Tropaeolum majus leaves could provide a comparative view as to the role of 'stress-metabolites' in regulation of leaf senescence.

**Flower senescence**

Floral senescence, representing the ultimate stage of flower development, culminates in the PCD of the petals. Senescing symptoms of flowers, in general, include wilting of flowers, fading and abscission of flower or floral parts (Stead and Van Doorn, 1994). The floral senescence is regulated by highly coordinated changes in gene expression and is under hormonal regulation. Flowers provide an excellent model system for the study of senescence as they generally have a defined life span.

Many flowers exhibit age-related colour change (e.g., bright blue flowers of chicory fade over the course of time of their opening) whereas some others (particularly those in leguminosae and verbenaceae) change colour in response to pollination (Van Doorn, 1997). Wilting of corolla commonly marks the end of life of flowers. Solomos and Gross (1977) demonstrated the petal wilting to be accompanied by a decrease in water uptake. The metabolic changes associated with floral senescence include the change in the rate and/or pathways of respiration, glyoxylate cycle, lipid metabolism, protein turnover and cell wall metabolism. During senescence flowers lose upto 95% of their sugars. Removal of this carbohydrate source by removal of mature flowers is known to cause a substantial reduction in the opening of subsequent flowers (Waithaka et al., 2001). Increased membrane permeability has been attributed to a reduction in membrane fluidity resulting from changes in the composition of membrane. Borochov
et al. (1994) studied the specific activities and products of a number of kinases and lipases involved in the synthesis and catabolism of membrane lipids using plasma membranes isolated from senescing petunia petals. Activities of acid phosphatases, ribonuclease, ATPase have been shown to increase in senescing carnation petals (Hobson and Nicholas, 1977). Panavas et al. (1998) showed senescence-associated increase in specific activities of cellulase, polygalacturonase and β-galactosidase in daylily flowers.

Floral senescence is accompanied by changes in endogenous levels of ethylene, ABA and cytokinins. These changes are mediated through signal transduction mechanisms that control the process. Once the flower is pollinated or is no longer receptive to pollination (which otherwise becomes costly to be maintained in terms of respiration), breakdown of the macromolecules and organelles occurs to mobilize their constituents to other developing tissues i.e. developing seeds. A number of genes encoding hydrolytic enzymes, upregulated during senescence, have been identified (Jones, 2005). Flower senescence in many plant species is stimulated by ethylene. Transgenic Petunia (35 S : etr 1-1) that are insensitive to ethylene exhibit delayed flower senescence. As such, the transgenic plants with delayed senescence provide a suitable model to study the interaction of plant hormones (ethylene, ABA, cytokinin) and the signal transduction mechanisms involved. Expression of ipt, a cytokinin biosynthetic gene from Agrobacterium tumefaciens, under the control of promoter from
senescence-associated gene (SAG\textsubscript{12}) delayed senescence and reduced sensitivity to ethylene in transgenic with SAG\textsubscript{12}-IPT Petunia corolla (Chang et al., 2003). Endogenous ethylene production is induced upon pollination in both WT (wild type) and ipt corollas, but the senescence is delayed in IPT flowers accompanied by increased cytokinin accumulation. Pollination induced increase in ethylene is through increased expression of ACC synthase.

Pollination induced ethylene production may be associated with pollen as there is a report of a pollen-specific novel ACC synthase gene expressed only in pollen (Lindstrom et al., 1999). Induction of upregulation of genes involved in production of ACC and ethylene provide the inter-organ communication of senescence within the flower. It is reported that after compatible pollination, the signal that coordinates ethylene production is translocated from the style to the ovary and petal within 12 to 16 h of pollination (Jones and Woodson, 1999; Wagstaff et al., 2005; Stead et al., 2006). In contrast, the flowers that are insensitive to ethylene, due to treatment with ethylene action inhibitors or transgenics (etr 1-1), do not exhibit pollen-induced corolla-senescence. These facts support the inter-organ signalling of ethylene.

ABA, a known inducer of senescence, upregulates the parameters of senescence such as loss of membrane permeability, increase in lipid peroxidation and induction of proteinase and RNase activities. Endogenous ABA levels increase before flower opening and continue to increase during petal senescence (Panavas et al., 1998). In daylily (Hemerocallis) flowers,
which die within 24 h after opening, exogenous ABA prematurely upregulates the same parameters of senescence as those occurring during natural senescence. ABA seems to be a part of signal transduction chain leading to programmed cell death. On the other hand, cytokinins are reported to delay flower senescence when applied exogenously in carnations (Upfold and Van Staden, 1990), roses (Mayak and Halevy, 1974) and petunia (Taverne et al., 1999).

The establishment of proline-induced acceleration of leaf senescence of excised *T. majus* leaves in this study, prompted us to examine whether exogenous proline could exert similar influences on flower senescence also. This interest was further strengthened by the fact that floral tissues have been reported to contain high proline contents (Verbruggen et al., 1996; Deuschle et al., 2001).

**Objectives**

On the basis of available literature and work done in our laboratory (Sharma et al., 1995; Tyagi, 2001), following plan of investigations was proposed for the doctoral programme.

1. To monitor the changes in endogenous free proline contents in excised senescing leaves of *Tropaeolum majus* L. and to determine the influence of exogenous proline on senescence of excised leaves
2. To examine whether the responses of senescing leaves to proline are affected by Ca$^{2+}$ availability
3. To examine the influence of exogenous polyamines (Put, Spm, Spd) on senescence of excised leaves
4. To examine the relationship, if any, between stomatal movements and senescence of excised leaves as affected by proline

5. To monitor the influence of exogenous proline on floral senescence

The studies have been carried out with *Tropaeolum majus* leaves. However, in case of floral senescence, besides *T. majus* flowers, flower buds those of two more species namely, *Vinca major* and *Ipomoea hederacea* were also employed with a view to generalize the findings.