# CHAPTER 2

# REVIEW OF LITERATURE

# 2. Review of Literature

Polyamines are positively charged aliphatic compounds with primary and secondary amines, widely distributed in all the organisms (Galston and Sawhney 1990, Igarashi and Kashiwagi 2000, Tabor and Tabor 1984, Wallace et al., 2003). Most common polyamines among eukaryotes are diamine putrescine, triamine spermidine and tetraamine spermine. Although, prokaryotes does not have spermine synthase activity but some archaebacteria can synthesize an uncommon polyamine named thermospermine, whose presence is also reported in some lower eukaryotes and plants (Kröger et al., 2000, Oshima 1979). Surprisingly, in plants thermospermine synthase was acquired by horizontal gene transfer and has proven to be essential for vascular development in *Arabidopsis* (Minguet et al., 2008). Structure of common and some of the uncommon polyamines are shown in Table 2.1.

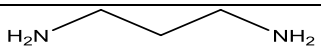
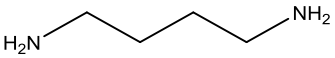
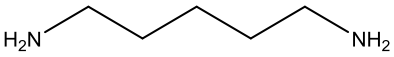
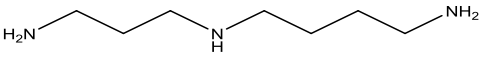
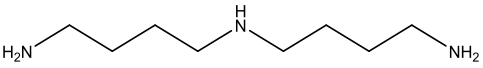
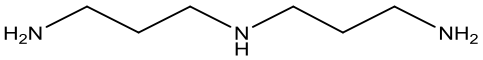
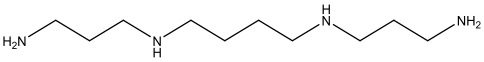
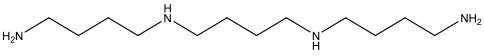
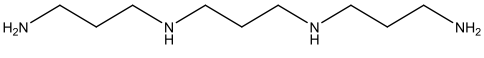
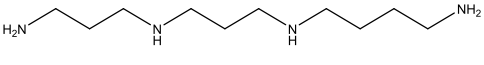
In *Arabidopsis*, thermospermine is synthesized by thermospermine synthase which is encoded by a gene known as ACAULIS5 (ACL5) (Kakehi et al., 2008). It is structural isomer of tetraamine spermine and recently it is proved that thermospermine is widespread in plants and is involved in xylem cell differentiation, cell wall patterning, preventing premature cell death and plant growth (Marina et al., 2013, Muñiz et al., 2008). As several reports demonstrated participation of spermine in plant defense against pathogens (Marini et al., 2001, Urano et al., 2003, Yamakawa et al., 1998), thermospermine defensive role has not been studied so far.

The natural polyamines putrescine, spermidine and spermine are very simple aliphatic amines, essential constituent for all the living organisms. One or more of these compounds are present in each and every single living cell. All prokaryotes and eukaryotes synthesize putrescine and spermidine, while spermine is largely confined to eukaryotic cells. In general, prokaryotes have higher putrescine than spermidine and lack spermine, whereas eukaryotes usually have less putrescine but high concentration of spermidine and spermine (Thomas and Thomas 2001).

Universally, polyamines belong to a broader group of biologically active amines together with biogenic monoamines such as serotonin and tryptamine having important physiological functions (Tabor and Tabor 1984). Although the complete range of biological effects of polyamines are not fully known yet but they influence cellular processes at all stages from gene transcription to protein synthesis, and are important players in regulating cell growth and differentiation. There are reports

which showed direct correlation between proliferative activity of cells and the content of polyamines (Marton and Pegg 1995, Pegg and McCann 1982). The major advantage of polyamine pathway is that cell can control both the production and degradation of polyamines as needed independent of the extracellular environment. It is already known that common and uncommon polyamines are essential for normal growth and development of plants and are characterized in many plants, but the underlying molecular mechanism of their action is still unclear.

**Table 2.1: Structure of some common and uncommon polyamines**

| **Name** | **Molecular Formula** | **Structure** |
|---|---|---|
| **Diamines** | | |
| 1,3-Diamino propane | $NH_2(CH_2)_3NH_2$ |  |
| Putrescine | $NH_2(CH_2)_4NH_2$ |  |
| Cadaverine | $NH_2(CH_2)_5NH_2$ |  |
| **Triamines** | | |
| Spermidine | $NH_2(CH_2)_3\ NH(CH_2)_4NH_2$ |  |
| Homospermidine | $NH_2(CH_2)_4\ NH(CH_2)_4NH_2$ |  |
| Norspermidine | $NH_2(CH_2)_3\ NH(CH_2)_3NH_2$ |  |
| **Tetraamines** | | |
| Spermine | $NH_2(CH_2)_3NH(CH_2)_4\ NH(CH_2)_3NH_2$ |  |
| Homospermine | $NH_2(CH_2)_3NH(CH_2)_4\ NH(CH_2)_4NH_2$ |  |
| Norspermine | $NH_2(CH_2)_3NH(CH_2)_3\ NH(CH_2)_3NH_2$ |  |
| Thermospermine | $NH_2(CH_2)_3NH(CH_2)_3\ NH(CH_2)_4NH_2$ |  |

## 2.1 Distribution and localization

In flowering plants the most abundant polyamines are the diamine putrescine, triamine spermidine and tetraamine spermine (Flores 1990, Smith 1970). Some of the polyamines exist only in the plants of some specific family, like diamine cadaverin and triamine homospermidine are the characteristics of leguminaceae plants (Flores 1990). The uncommon polyamines norspermidine and norspermine are reported in drought tolerant alfalfa under water deficit stress condition and in the cell culture of heat tolerant cotton after high temperature stress (Kuehn et al., 1990). Closely related to these, a tetraamine thermospermine is also reported in extremely thermophiilic bacterium, *Thermus thermophilus,* where it is involved in thermotolerance (Takeda et al., 1983). In eukaryotes, thermospermine biosynthetic gene, ACL5 is also reported from wide range of plants, suggesting that thermospermine is likely to be widely distributed in plant kingdom (Takano et al., 2012).

In plants, polyamines are distributed in all the vegetative and reproductive tissues; roots, stems, flowers, leaves, they are also present in seeds as well as in meristematic tissues (Evans and Malmberg 1989, Felix and Harr 1987). In cells, polyamines are mainly localized in vacuoles, they are also bound to nucleus, mitochondria, chloroplast, ribosomes, cell wall, and membranes and also present in apoplastic fluid (Bagni 1989, Bors et al., 1989, Goldberg and Perdrizet 1984, Slocum 1991).

Since they are positively charged molecules at physiological pH, they can interact with phospholipid membranes and affect membrane rigidity as well as influence DNA conformation and stability (Edreva 1996). In plants, polyamines may interact with negatively charged cell wall components and affect various cellular processes such as control of cell division, root formation, flowering, senescence, thylakoid membrane stabilization, including response to various stresses (Alcázar et al., 2010, Edreva 1996, Evans and Malmberg 1989, Galston et al., 1997, Galston and Sawhney 1990). Recent studies on *Arabidopsis* also revealed its involvement in vascular tissues development and prevention from premature cell death (Hanzawa et al., 2000, Kakehi et al., 2008, Muñiz et al., 2008)

## 2.2 Polyamines interaction with cellular molecules and subcellular structure

### 2.2.1 Interaction with nucleic acids

Polyamines interact with macromolecules and subcellular structures due to their cationic nature. They can bind with DNA as the electrostatic interaction is established between the amino and imino groups of polyamines with the phosphate groups of DNA. This interaction leads to important conformational changes, complexation and stabilization of DNA structure and chromosome condensation resulting in less denaturation of DNA by enzymes and X-ray radiations. Polyamines also modulate mRNA, tRNA and rRNA conformation thus involved in regulating posttranscriptional events (Mehta et al., 1991, Slocum et al., 1984, Tofilon et al., 1982).

### 2.2.2 Interaction with proteins and enzymes

Polyamines primarily modulate protein synthesis *in vivo* by controlling the formation of polysomes and binding of aminoacyl tRNA to ribosomes, negatively charged loci of ribosomes is the active site for polyamines binding. Polyamines also enhance the rate of peptide chain initiation and elongation thus increasing the yield of full length translational product. Polyamines also regulate various enzymes activity through modulation of phosphorylation and dephosphorylation pattern, through various types of ionic interactions and covalent binding (Datta et al., 1987, Veluthambi and Poovaiah 1984, Xiang et al., 1994).

### 2.2.3 Interaction with membranes and cell wall components

Polyamines influence the membrane structure and function by interacting with the negatively charged phospholipid heads of membrane bilayer and also with the membrane bound proteins. Polyamines can lay parallel to the membrane surface and interacts with more than one negative charge thus forming bridge between lipid-lipid and lipid-protein components, stabilizing and rigidifying the membrane structure. Exogenously applied polyamines also stabilizes membrane structure and functions (Schuber 1989, Tadolini 1988, Tiburcio et al., 1994). Polyamines also regulate structure and function of plant photosynthetic membranes. Exogenously supplied polyamines prevent the loss of chlorophyll and preserve the integrity of chloroplast membrane (Cohen et al., 1979, Tiburcio et al., 1994). The negatively charged pectin and lignin components of the cell wall also bind electrostatically with the polyamines and thus stabilizes the cell wall structure (Varner and Lin 1989).

## 2.3 Metabolism and regulation

Polyamine metabolism includes their synthesis, conjugation, transport and degradation according to the cellular needs. Complete metabolism of polyamines is carried out by several enzymes which are very tightly regulated and in addition to this their transport is also maintained both inside and outside the cell as per the cellular function. Cellular polyamines concentration has to be tightly regulated because excessive polyamine accumulation is harmful for normal cellular function and viability. Polyamine can be supplemented exogenously in case of cell is deprived of intracellular polyamines which has the same effect as of intracellular polyamines. All these three processes of polyamines metabolism are summarized below.

### 2.3.1 Biosynthesis

Polyamine biosynthesis is an ancient metabolic pathway present in all organisms. Biosynthetic pathway of these common polyamines remain conserved from bacteria to animals and plants (Tabor and Tabor 1984) (Figure 2.1). In animals and most of the fungi it is a single step process which is initiated by decarboxylation of ornithine to form putrescine by ornithine decarboxylase (ODC) (EC 4.1.1.17), while in plants the dominant pathway for putrescine synthesis is three step process where decarboxylation of arginine by arginine decarboxylase (ADC) (EC 4.1.1.19) to form agmantine which is then deaminated by agmantineiminohydrolase to form N-carbamoylputrescine which is further catalyzed by N-carbamoylamidohydrolase to finally form putrescine (Edreva 1996). Interestingly, the model plant *A. thaliana* does not contain the gene for ornithine decarboxylase so it synthesizes putrescine via arginine decarboxylase (Hanfrey et al., 2001). Further, putrescine is converted to triamine spermidine and then to tetraamines spermine or thermospermine by the addition of aminopropyl group from decarboxylated S-adenosylmethionine (dcSAM). Putrescine is converted to spermidine, spermine and thermospermine by successive activities of spermidine synthase (SPDS) (EC 2.5.1.16), spermine synthase (SPMS) (EC 2.5.1.16) and thermospermine synthase (ACL5) (EC 2.5.1.79), respectively. The dcSAM is produced by S-adenosylmethionine decarboxylase (SAMDC) (EC 4.1.1.50) from S-adenosyl methionine (SAM) (Edreva 1996). SAMDC is considered to be a major regulatory enzyme in the synthesis of spermidine and spermine and is also known to influence the rate of ethylene

production in plants which is known to be senescence hormone in plants. Ethylene and polyamines play antagonistic role to each other in the development of plants, thus the balance between these two pathways is necessary for the proper development of plants (Evans and Malmberg, 1989). However, all the mammals and many eukaryotic organisms lack arginine decarboxylase (ADC), so they don't have any other option except ornithine decarboxylase pathway to produce putrescine (Yatin 2002).

Thus, the key enzymes making up the polyamine biosynthetic pathway in eukaryotic cells are ornithine decarboxylase (ODC) that forms putrescine from L-ornithine; s-adenosylmethionine decarboxylase (SAMDC) that forms decarboxylated S-adenosylmethonine, which act as an aminopropyl donor; spermidine synthase (SPDS) that transfers the aminopropyl group from dcSAM to putrescine and spermine synthase (SPMS) or thermospermine synthase (ACL5) that transfers the aminopropyl group from dcSAM to spermidine, whereas in some plants and bacteria, arginine decarboxylase (ADC) initiates an alternative pathway to putrescine. All the aminopropyltransferases SPDS, SPMS and ACL5 share a high degree of sequence similarity including the active putative centers, although they have different substrate specificity (Hashimoto et al., 1998, Teuber et al., 2007).

A number of studies have used the polyamines inhibitors to modulate the cellular level of polyamines in order to understand their role in plant developmental processes. Four commonly used inhibitors of polyamines synthesis are diflouromethyl ornithine (DFMO), an irreversible inhibitor of ODC (Bey et al., 1987), diflouromethylarginine (DFMA), an irreversble inhibitor of ADC (Bitonti et al., 1987), methyl-glyoxyl- bisguanylhydrazine (MGBG), a competitive inhibitor of SAMDC (Williams-Ashman and Schenone 1972), cyclohexylamine (CHA), competitive inhibitor of SPDS (Hibasami et al., 1980).

### 2.3.2 Degradation

Catabolism of polyamines is more important process than synthesis in regulating the equilibrium of cellular polyamines concentrations in the entire living organism (Wuddineh et al., 2018). Basically there occurs oxidative deamination of polyamines during their degradation which gives rise to different compounds which are biochemically active during different sets of reactions. Mostly two enzymes play role in the catabolism of polyamines, these are Diamine oxidases (DAO) (EC 1.4.3.22)

and Polyamine oxidases (PAO) (EC 1.5.3.14) (Edreva 1996) (Figure 1). Besides these two enzymes, the key regulatory enzyme of degradation pathway is spermidine/spermine N1 –acetyltransferase (SSAT). Spermidine and spermine can be converted back to putrescine via this SSAT which is present in cytosol. SSAT acetylates both spermidine and spermine by using acetyl co-A and then this acetylated spermidine and spermine are degraded by PAO to produce $H_2O_2$ and other byproducts (Yatin 2002). In addition to PAO, there is another enzyme which uses spermine as its substrate namely spermine oxidase (SMO). It catabolizes spermine to spermidine without being acetylated. For spermidine back conversion to putrescine only SSAT is responsible (Minois et al., 2011).

Polyamines oxidation leads to generation of hydrogen peroxide ($H_2O_2$) which is a signaling molecule involved in polyamine induced regulation of various biological processes (Gupta et al., 2016). Another important molecule, GABA, also produced from polyamines degradation is implicated in numerous physiological functions in all organisms (Bouche and Fromm 2004, Bown and Shelp 2016).

DAO has broad range of substrate specificity. It acts on the primary amino group of polyamines to release ammonia, $H_2O_2$ as well as aminoaldehydes. It can act on aliphatic as well as aromatic mono-, di- and polyamines. It is generally found to be loosely attached with cell wall so that it can be easily released in apoplastic fluid. On the other hand PAO has very confined substrate specificity which is limited to spermidine and spermine only. PAO acts on imino group of spermidine and spermine to release $H_2O_2$, aminoaldehydes and other byproducts which are sequentially converted to other compounds and excreted out from the cells (Edreva 1996).

So, it has become clear now that catabolism of polyamines serves a vital role in regulation of plant growth and development (Tisi et al., 2011).

### 2.3.3 Conjugation

In plants, polyamines may found in free form as well as in conjugation with phenolic compounds, nucleic acids and proteins. Polyamines mainly conjugates with hydroxycinnamic acid amides (HCAA), also known as phenolamides, produced by hydroxycinnamoyl acylation of the mono-, di- or tri- phenolic acid substitution of polyamines by enzymes namely hydroxycinnamoyltransferases (Peng et al., 2016). On the other hand conjugation of polyamines with proteins occurs via covalent

linkage to the glutamyl residues to form either mono-γ-glutamyl-polyamines or bis-γ-glutamyl-polyamines. These reactions are catalyzed by transglutaminases (Del Duca et al., 2014). However, concentration of free versus conjugated polyamines depends on growth and developmental stages of different organs and their interconversion has also been reported (Luo et al., 2009). These polyamines conjugates are shown to involve in various biological processes such as pollen development, plant defense responses to pathogens as well as abiotic stresses (Aloisi et al 2016, Tiburcio et al 2014, Walters et al 2001).

### 2.3.4 Transport

Transport of polyamines across the cell is very imperative to maintain the cellular polyamine level as excessive accumulation as well excessive degradation together has severe effects on the normal cellular function. Polyamines transport system is well studied in bacteria and yeast but relatively less in plants and animals.

In *Escherichia coli,* mainly there are two types of polyamine transport systems which include ABC transporters family. These systems are polyamines specific as one transport only spermidine and other one is specific to putrescine (Igarashi et al., 2001). Different transporters of ABC transporter family play role in their transport if any transporter is missing then the polyamine transport will not be effective. Besides these, there are some exporters and proteins which excrete out polyamines outside the cells. In yeast *Saccharomyces cerevisiae,* polyamines transport system requires energy which includes phosphorylation and dephosphorylation (Igarashi et al., 2010). In addition to these, there are some other proteins which take part in their uptake, transport and excretion (Minois et al., 2011).

In plants and animals, polyamine transport systems have been proposed but very little work has been done to understand the regulatory mechanism. Polyamine transport systems are reported in protoplasts, vacuoles and mitochondria in plants. The uptake of polyamines is driven by a transmembrane electrical gradient i.e., difference between ionic potential across the cell membrane (Pistocchi et al., 1987). Other than this there may be some protein carriers present across the plasma membrane which helps in polyamine transport. Thus, in plants polyamine transport is carrier mediated and carried out by antiport transport mechanism between external and internal polyamines (Pistocchi et al., 1988). Recently in rice and *Arabidopsis* some specific importers have been identified and characterized (Fujita et al., 2012,

Mulangi et al., 2012a). The first protein OsPUT1 (Polyamine uptake transporter) was reported from rice and it mainly functions as a spermidine importer and is expressed in all tissues except mature roots and seeds (Mulangi et al., 2012b). Also, a LAT (L-type amino acid transporter) protein namely RMV1 (Resistant to Methyl Viologen 1) is reported which is involved in proton dependent incorporation of polyamines with high affinity for spermine in *Arabidopsis* (Fujita et al., 2012). Recently, it has been reported that three out of five LATs in *Arabidopsis* displayed different cellular localization indicating that they may be involved in inter or intracellular transport or some different cellular activities. For example, AtLAT1 is located in plasma membrane and hence involved in intercellular transport of polyamines and paraquat, while AtLAT3 and AtLAT4 were shown to localize in endoplasmic reticulum and golgi apparatus, respectively (Li et al., 2013). In mammals, the polyamines are transported in the cells via some carrier or transporters driven by a membrane potential i.e., pH gradient across the cell membrane (Casero and Marton 2007, Soulet et al., 2004). There is another model system which has been proposed in animals i.e., Glypican 1 (GPC1) recycling helps in polyamine transport (Belting et al., 2003).

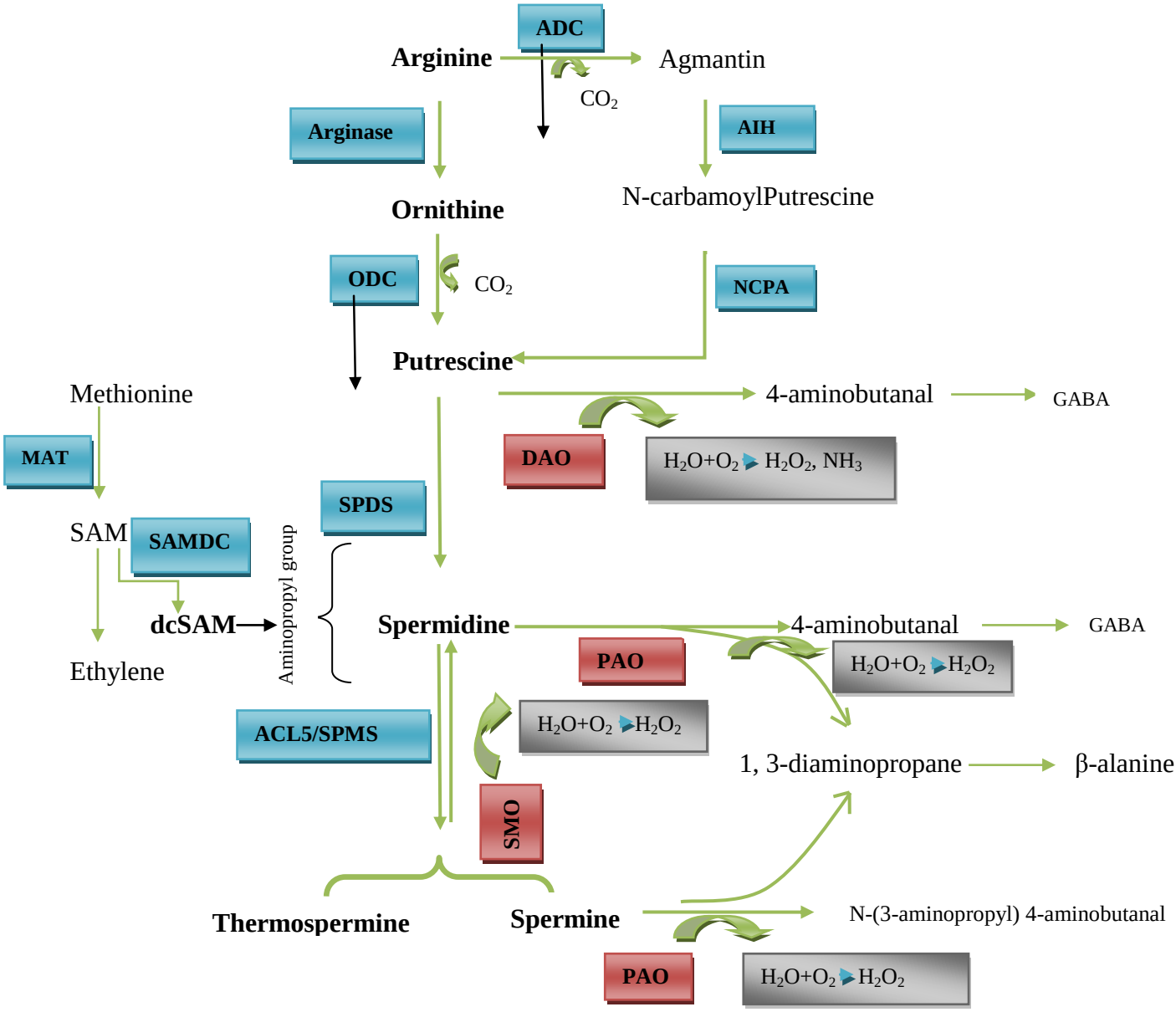



**Figure 2.1**: **Metabolic pathway of polyamines in plants**. Blue boxes represent biosynthetic enzymes, red boxes representing catabolic enzymes and Grey boxes showing byproduct of polyamines catabolism. ADC, Arginine decarboxylase; ODC, Ornithine decarboxylase; AIH, Agmantineiminohydrolase; NCPA,N-carbamoylputrescineamidohydrolase; SPDS, Spermidine synthase; SPMS, Spermine synthase; ACL5, Thermospermine synthase; MAT, Methionine adenosyltransferase; SAMDC, S-adenosyl methionine decarboxylase; DAO, Diamine oxidase; PAO, Polyamine oxidase; SMO, Spermine oxidase

## 2.4 Functions of polyamines in plants

In plants, polyamines have been implicated to function in wide range of cellular and biological processes including cell division, embryo formation, root development, flowering, senescence, thylakoid membrane stabilization, and in response to various stresses (Alcázar et al., 2010, Cohen 1998, Edreva 1996, Evans and Malmberg 1989, Galston et al., 1997, Galston and Sawhney 1990)

### 2.4.1 Polyamines in growth and development

Polyamines are known to be involved in various plant developmental processes such as cell division, embryogenesis, root growth, pollen development, floral initiation and development, fruit development and ripening as well as senescence (Bais and Ravishankar 2002, Evans and Malmberg 1989, Galston et al., 1997) (Table 2.2). During these developmental processes there is change in the activity of polyamines biosynthetic enzymes namely ADC, ODC and SAMDC resulting into changes in polyamines level. Earlier work had shown that increase in the polyamines are associated with the rapid cell division in many plants, for example, carrot embryogenesis (Feirer et al., 1984, Montague et al., 1979), tomato ovaries (Heimer and Mizrahi 1982) and fruit development (Kakkar and Rai 1993). In contrast, some other studies have shown that correlation between polyamines and plant growth processes especially somatic embryogenesis is not universal and may be specific to some plant species (Bais and Ravishankar 2002, Evans and Malmberg 1989, Galston et al., 1997). Generally, cells undergoing division, apical meristems and apical shoots prior to flowering and also flower parts of many plants contain high level of free and conjugated polyamines (Cabanne et al., 1981, Galston and Kaur-Sawhney 1995, Martin-Tanguy 1985). Polyamine conjugates especially HCAA are abundant in the seeds and reproductive organs of some plants (Bassard et al., 2010). Furthermore, spermidine synthase is suggested to essential for embryo development in *Arabidopsis* (Imai et al., 2004b). It is also reported that in *Arabidopsis* ADC gene is required for normal seed development (Urano et al., 2005). Also enhanced expression of yeast SAMDC in tomato resulted in increased spermidine and spermine content leading to an increase in lycopene content, prolonged wine life, and enhanced fruit juice quality (Mehta et al., 2002).

In pea plant, it is observed that SPDS1 is highly expressed in actively growing tissues, while SPDS2 had high expression in fully elongated stem (Alabadí and

Carbonell 1999). In *Citrus clementina*, polyamines are involved in parthenocarpic fruit development (Trénor et al., 2010). In poplar plant, it is observed that polyamine biosynthetic gene ACL5 is involved in vascular development (Milhinhos et al., 2011). It is also shown that the occurrence of polyamines and the enzymes involved in their biosynthesis at every stage of plastid development play roles such as in their differentiation, structure, function and senescence (Sobieszczuk-Nowicka and Legocka 2014). It has also been reported that polyamines participate in pollen growth and development (Rodriguez-Enriquez et al., 2013).

Plant hormones auxin, cytokinin, gibberellin and ethylene are also correlated with change in polyamines metabolism during plant growth and development. The changes occur in both at endogenous level of polyamines and the activity of their biosynthetic enzymes. Thus, polyamines which may or may not be mobile in plants, acts as intracellular mediators of hormone actions (Galston and Kaur-Sawhney 1995, Young and Galston 1983). Both ethylene and polyamines have antagonistic roles in plants as polyamines inhibits senescence of leaves and fruit ripening, while ethylene promotes (Kakkar and Rai 1993, Kaur-Sawhney et al., 1982). However, polyamines and ethylene regulates each other synthesis directly or indirectly via metabolic competition for SAM, a common precursor for their biosynthesis (Even-Chen et al., 1982, Fuhrer et al., 1982). Polyamines inhibit ethylene biosynthesis by blocking the conversion of SAM to ACC and further to ethylene, whereas ethylene is an effective inhibitor of ADC and SAMDC which are the key enzymes of polyamines biosynthetic pathway (Apelbaum et al., 1981, Apelbaum et al., 1985, Icekson et al., 1985). Thus polyamines may affect senescence and ripening by modulating polyamine and ethylene biosynthesis.

Polyamines such as putrescine, spermidine and spermine are found to play important roles in various developmental processes. External application of these polyamines also shows effects on plant growth as well as response against stress. It has been reported that plants do not require spermine for normal growth since knockout plants of SPMS gene that encodes spermine synthase are viable (Imai et al., 2004a). There is a second gene in plants, ACL5 that was originally also identified to encode a spermine synthase. Mutations in ACL5 gene lead to severe developmental defects. However, it has now been elucidated to actually encodes thermospermine rather than spermine that rescues the dwarf phenotype of the mutants (Knott et al., 2007).

Thermospermine which is also a tetraamine is structural isomer of spermine, produced from spermidine by thermospermine synthase. Spermine is reported to present only in angiosperms, whereas thermospermine is indicated to present in all plants. Recent evidences suggest that thermospermine synthase was acquired by an algal ancestor of plants through horizontal gene transfer from archaea (Minguet et al., 2008). In *Arabidopsis*, it is shown to be essential for stem development (Kakehi et al., 2008). Loss of function of thermospermine synthase in *Arabidopsis* affected xylem specification and involved in prevention of premature cell death (Muñiz et al., 2008). Also, *Arabidopsis* samdc4 mutant exhibits semi dwarfism and improper vascular development, partially resembling the acl5 mutant phenotype (Cui et al., 2010, Ge et al., 2006).

*Arabidopsis* ADC1/ADC2, SPDS1/SPDS2 and SAMDC1/ SAMDC4 double loss of function mutants which are impaired in the biosynthesis of putrescine, spermidine or spermine are found to be embryo lethal in plants (Ge et al., 2006, Imai et al., 2004b).

**Table 2.2**: **Developmental roles of polyamines in various plant species**

| **Polyamine biosynthetic gene** | **Plant species** | **Developmental role** | **References** |
|---|---|---|---|
| ADC | *Daucus carota* | Embryogenesis | Feirer et al., 1984; Montague et al., 1979 |
| | *Arabidopsis thaliana* | Seed development | Urano et al., 2005 |
| | | Embryo development | Ge et al., 2006, Imai et al., 2004b |
| ODC | *Solanum lycopersicum* | Ovary development | Heimer and Mizrahi 1982 |
| | | Fruit ripening | Kakkar and Rai 1993 |
| SAMDC | *Solanum lycopersicum* | Fruit shelf life and quality improvement | Mehta et al ., 2002 |
| | *Arabidopsis thaliana* | Embryogenesis, Shoot and root architecture | Ge et al., 2006 |
| SPDS | *Arabidopsis thaliana* | Embryo development | Imai et al., 2004a |
| | *Pisum sativum* | Increased level in actively growing tissues and elongating stem | Alabadí and Carbonell 1999 |
| | *Arabidopsis thaliana* | Pollen development | Rodriguez-Enriquez et al., 2013 |
| | | Embryo development | Imai et al., 2004b |
| ACL5 | *Arabidopsis thaliana* | Vascular development and prevention of premature cell death | Muñiz et al., 2008 |
| | *Populus trichocarpa* | Vascular development | Milhinhos et al., 2011 |

### 2.4.2 Polyamines in stress

The complex relationship between stress and polyamines are well studied as increased polyamines protect plants from damage as they have capability to deal with oxidative radicals or cause damage to them as polyamines catabolism leads to $H_2O_2$ production. But the observed increase in tolerance of plants against stress when the cellular content of polyamines gets elevated by either exogenous application or through genetic engineering approach with genes encoding polyamines biosynthetic enzymes is indicator of protective role of polyamines (Minocha et al., 2014).

Polyamines are known for protecting the plants directly as they are cationic in nature so they can bind to various negatively charged molecule and stabilize the cellular structures under stress conditions (Pottosin et al., 2014). They also act as signaling molecule during stress responses (Jiménez Bremont et al., 2014). Various reports suggested that polyamines are involved in stress adaptive responses as there is fluctuation in their level at the time of stress condition (Pal et al., 2015). Variation in level of polyamines during different stress condition has been reviewed in many plant species (Alcázar et al., 2010, Hussain et al., 2011, Kusano et al., 2008). This accumulated size of cellular polyamines can also be correlated with the stress tolerance ability of the plants, further explaining the effectiveness of polyamines during stress conditions (Hatmi et al., 2015).This accumulation of polyamines at the time of stress condition is due to the *de novo* synthesis of free polyamines as it is mainly regulated at transcriptional level (Liu et al., 2015). This is further supported from the fact that most of the polyamines biosynthetic genes including ADC, SPDS, SPMS and SAMDC have also shown to be upregulated during stress conditions, also there are reports which shown that the other polyamines like norspermine and thermospermine also plays role in stress tolerance (Cvikrová et al., 2012, Liu et al., 2006, Liu et al., 2011).

#### 2.4.2.1 Abiotic stress

Change in polyamine levels have been generally observed in various plant species when affected by range of abiotic stresses such as drought, cold, salt, high temperature and others (Liu et al., 2007) (Table 2.3). In some cases, it has been reported that three polyamines namely, putrescine, spermidine and spermine level increases to very high following abiotic stress (Yang et al., 2007). For instance, when apple callus treated with salt, putrescine level increased (Liu et al., 2006).

Similarly, when sweet orange exposed to salt and cold treatment its callus showed increased spermidine content (Wang and Liu 2009). Grape (*Vitis vinifera*) plants also showed drastic accumulation of spermidine and spermine content after salt stress (Ikbal et al., 2014). In *Arabidopsis*, putrescine is the main polyamine involved in salt tolerance (Urano et al., 2004). Effect of salt stress on plant growth and polyamines level in *Lycopersicum esculentum, Brassica oleracia* and *Beta vulgaris* have revealed that polyamines level changed with the salinity stress and mostly increased level of spermidine and spermine observed during salinity tolerance (Zapata et al., 2004).

Exogenous application of polyamines at different concentration has also been shown to increase stress tolerance to certain plant species (Duan et al., 2008). It has been observed that exogenously applied putrescine improved thermotolerance to wheat (Kumar et al., 2014). Moreover, exogenous application of putrescine have successfully been used to increase plant tolerance against salinity (Chattopadhayay et al., 2002, Ndayiragije and Lutts 2006), cold (Nayyar 2005, Nayyar and Chander 2004), drought (Zeid and Shedeed 2006), high temperature (Murkowski 2001), osmotic stress (Liu et al., 2004) and flooding (Yiu et al., 2009). Also, exogenously applied spermidine to *Cucumis sativus* enhanced salt tolerance as there was accumulation of spermidine, spermine and putrescine conjugates in the roots during salt stress (Duan et al., 2008). In a recent study it was demonstrated that exogenous application of spermine reduced chilling injury during low temperature storage of grape berries increasing shelf life of fruits (Champa et al., 2015).

Recent evidence suggested that metabolic pathway associated with polyamines metabolism also play a key role in providing drought stress tolerance (Hatmi et al., 2014). It has been shown that overexpression of polyamines biosynthetic genes provide salt tolerance to rice, tobacco, tomato and *Arabidopsis* (Kasukabe et al., 2004, Roy and Wu 2001, Wi et al., 2006). In *Arabidopsis* overexpressser lines of SAMDC showed enhanced drought tolerance, also there was significant accumulation of main polyamines (Wi et al., 2014). Similarly in rice, overexpression of *Datura* ADC showed increased tolerance to drought and also enhanced level of spermidine and spermine (Capell et al., 2004).

A recent study revealed that elevated polyamines level activates defense mechanism under the condition of heat stress (Cvikrová et al., 2012). Tomato plants

overexpressing SAMDC showed higher level of spermidine and spermine and were more heat tolerant than the control plants, through increasing the activities of antioxidant enzymes and by protecting lipid peroxidation (Cheng et al., 2009). Heat stress triggers induced activity of both ADC and ODC and also an increase in the level of spermidine and spermine was observed due to their *de novo* synthesis in the leaves and root of tobacco. Uncommon polyamines were also studied and shown to provide thermotolerance to plants during heat stress (Roy and Ghosh 1996).

Evidences also suggested that polyamines protects the plants under flood stress by stabilizing the plasma membrane integrity (Yiu et al., 2009). Polyamines content also increases when plants are challenged by chilling stress. It has been reported that polyamine biosynthetic enzyme ADC is mainly induced in *Arabidopsis* during cold stress (Hummel et al., 2004). Evidences suggested that polyamine provide chilling tolerance to cucumber by acting as antioxidative compound (Zhang et al., 2009).

As observed in other stresses, polyamines titer also shown to be increased in plants after oxidative stress caused by UV radiations (Kramer et al 1991) and polyamines protected the plants by stabilizing the membrane structure and inhibit lipid peroxidation (Slocum et al 1984, Tadolini 1988).

Furthermore, generation of transgenic plants with polyamines biosynthetic genes such as ADC, ODC SAMDC and SPDS enhanced their tolerance to varieties of environmental stresses (Liu et al., 2007). Such multiple stress tolerance is of practical importance as plants face all these stresses concurrently during their life cycle.

**Table 2.3: Polyamine roles during various abiotic stress conditions**

| Plant species | Stress | Polyamine biosynthetic enzyme involved/ level | References |
|---|---|---|---|
| *Malus sylvestris* | Salt | ADC/Putrescine increased | Liu et al., 2006 |
| *Citrus sinensis* | Salt, Cold and Heat | ADC and SAMDC/ Putrescine,spermidine and spermine increased | Wang and Liu 2009 |
| *Vitis vinifera* | Salt | SAMDC/Spermidine and spermine increased | Ikbal et al., 2014 |
| *Arabidopsis thaliana* | Salt | ADC/ putrescine accumulation | Urano et al., 2004 |
| *Arabidopsis thaliana* | Cold | ADC activity increased | Hummel et al., 2004 |
| *Arabidopsis thaliana* | Drought | SAMDC activity increased | Wi et al., 2014 |
| *Arabidopsis thaliana* | Drought | ADC | Alcazar 2010 |
| *Cucumis sativus* | Salt | Spermine, spermidine and putrescine conjugates | Zapata et al., 2004 |
| *Solanum lycopersicum* | Heat | SAMDC/spermidine and spermine | Cheng et al., 2009 |
| *Oryza sativa* | Drought | ADC/putrescine increased | Capell et al., 2004 |
| | Salt | ADC | Roy and Wu 2001 |
| *Triticum aestivum* | Heat | Putrescine increased | Kumar et al., 2014 |
| *Solanum melongena* | Salt, Drought, Cold | ADC,ODC/putrescine, spermidine and spermine increased | Prabhavathi and Rajam 2007 |
| *Nicotiana tabacum* | Salt and Drought | SAMDC/putrescine, spermidine and spermine accumulation | Waie and Rajam 2003 |

#### 2.4.2.2 Biotic stress

Plants are frequently challenged by a range of microbial pathogens throughout their lifecycle and to prevent themselves they have developed a complex defense system (Takahashi et al., 2003). One of the defense reaction associated with localized cell death is known as hypersensitive response (HR) that prevents further spreading of infection from invasion site. Several reports have suggested that polyamines are also involved in plant defense responses against biotic stresses (Sagor et al., 2012) (Table 2.4). Role of polyamines during plant pathogen interactions has been proposed earlier and it has been investigated that cellular level of polyamines gets altered at the time of pathogen attack (Walters 2003). The most studies have observed change in polyamines level or activities of polyamines biosynthetic enzymes during pathogen attack (Walters 2000) (Table 2.4). The ODC, ADC and SAMDC are considered as major regulatory enzymes of polyamines biosynthetic pathway and it has been observed that during biotic stress, activity of these biosynthetic enzymes increases and result in accumulation of polyamines in plants (Jiménez-Bremont et al., 2014). Moreover, an increase in accumulation of free polyamines also leads to accumulation of polyamine conjugates in plants (Martin-Tanguy 2001). These polyamine conjugates are observed mainly during HR towards the plant pathogens (Flores and Martin-Tanguy 1991, Martin-Tanguy 2001). Interestingly, accumulated polyamine conjugates hydroxycinnamic acid amides (HCAAs), which are conjugated form of hydroxycinnamic acids with putrescine, spermidine or spermine, have been shown to have antifungal effect (Walters et al., 2001).

In plant cells polyamine content increases due to viral infections. It has been observed that there is a drastic increase in the level of spermine and activity of ODC at the time of *Tobacco mosaic virus* (TMV) infection to tobacco especially in the necrotic cells (Negrel et al., 1984). Also, ODC activity increases accumulation of HCAAs in tobacco cells which further prevents the TMV infection (Martin-Tanguy 1985). Besides this, in *Arabidopsis*, spermine prevents the infection of *Cauliflower mosaic virus* (CaMV) by activating signaling cascade leading to activation of defense related genes. Similarly, the structural isomer of spermine, thermospermine prevents the viral infections to the same extent by activating defense related genes, mitochondrial alternative oxidase (AOX), mitogen activated protein kinase 3

(MAPK3), and transcription factors, such as WRKY40 and bZIP60 etc. (Sagor et al., 2012).

Polyamine metabolism also gets altered in response to the bacterial attack. It has been shown that accumulation of thermospermine reduced *Pseudomonas viridiflava* and *Xanthomonas compestris* multiplication in *Arabidopsis* indicating the role of thermospermine against bacterial pathogens (Marina et al., 2013). In addition, spermidine and putrescine were also shown to provide tolerance to tobacco and *Arabidopsis* against *Pseudomonas chichorii* and *Pseudomonas syringae,* respectively (Marina et al., 2013, Yoda et al., 2009). It has also been observed that accumulation of spermine provides tolerance in toabcco during infection of *P. syringae* and *P. viridiflava* (Marina et al., 2008, Moschou et al., 2009). In *Arabidopsis*, overexpression of SAMDC1 has been reported to elevate the level of spermine which resulted in increased tolerance of plants against *P. syringae* (Marco et al., 2014). Similarly, sweet orange overexpressing SPDS have been reported to tolerant against *P. syringae* (Marco et al., 2014).

Similar to both viral and bacterial attack, polyamines level also gets altered at the time of fungal infection. In barley infected with powdery mildew, induced activity of ODC, ADC and SAMDC was observed which lead to increased level of putrescine, spermidine and spermine in plant cells (Walters et al., 2001). Also, *Blumeria graminis* infection to barley lead to hypersensitive reaction which caused rigorous increase in free as well as conjugated putrescine and spermidine in plants (Cowley and Walters 2002a). In case of flax also, increase in polyamines content during the infection of *Fusarium* was reported (Wojtasik et al., 2015). These findings suggest that polyamines play some precise roles during fungal pathogen attack in plants.

As a part of the defense system in plants, polyamine catabolism also plays an important role as its catabolism leads to production of reactive oxygen species (ROS) molecules. Some reports have shown that in plants DAO and PAO contributes in providing resistance, mainly through the production of $H_2O_2$ molecule which is known to mediate cross linking of cell wall components which leads to cell wall stiffening in response to damage and during defense action against plant pathogens (Apel and Hirt 2004, Torres and Dangl 2005). It is shown that the incompatible reaction between barley and powdery mildew results in an increase in the activity of catabolic enzymes, DAO and PAO which leads to production of $H_2O_2$ that is

responsible for lignification and rigidity of cell wall (Cowley and Walters 2002b, Walters 2003). Similar DAO activity in the cell wall of chickpea also reported in response to wounding (Cona et al., 2006). Besides these, the cell death driven developmental alterations were also reported in *Arabidopsis* due to increased activity of DAO (Moller and McPherson 1998).

Generation of $H_2O_2$ and other ROS such as $OH^-$, $O_2^-$ are one of the early responses induced during plant pathogen interaction (Bolwell 1999). These ROS act directly as antimicrobial molecules or may behave as signaling molecule in the regulation of defense responses. $H_2O_2$ also induces phenylalanine ammonia lyase (PAL) which is a phenylpropanoid pathway enzyme responsible for providing resistance to plants against diseases (Dorey et al., 1997). Phenylpropanoid pathway provides intermediates for the synthesis of isoflavanoids, phytoalexins, various phenolic derivatives and signaling molecule such as salicylic acid (Dixon and Paiva 1995). So, it can be speculated that $H_2O_2$ is a part of signal transduction pathway which leads to activation of defense related genes in response to pathogens (Sudha and Ravishankar 2002). $H_2O_2$ also shown to activate $Ca^{2+}$ ion channel that results in induction of signal transduction cascade related to defense processes. $Ca^{2+}$ signaling plays an important role in plant defense responses which includes hypersensitive response (Lecourieux et al., 2006). After pathogen attack to a plant ROS generated in the apoplast due to catabolism of polyamines induces $Ca^{2+}$ influx and amplifies defense related signals (Garcia-Brugger et al., 2006). So, this can be suggested that polyamine catabolism and ROS generated due to this causes increase in $Ca^{2+}$ which is involved in the progression of HR cell death. In addition, it is also predicted that elevated $Ca^{2+}$ can also activates metacaspases which are known as PCD executioner proteins in plants (Watanabe and Lam 2011), thus increased ROS directly or indirectly involved in plant defense responses and protect them during pathogen attack.

Recently, it has been revealed that the catabolism of spermine provides resistance to *Arabidopsis* and tobacco plants against bacterial infections (Gonzalez et al., 2011). Moreover, unusual polyamine, thermospermine catabolism also shown to protects *Arabidopsis* from *P.viridiflava* although its catabolic products are not yet known (Marina et al., 2013). So all these studies provide further evidence that catabolism of

polyamines plays a very important role in protecting plants against various infections.

**Table 2.4: Polyamine roles during biotic stress conditions**

| Pathogen | Host Plant | Polyamine enzyme involved | Polyamine Level | References |
|---|---|---|---|---|
| **Bacteria** | | | | |
| *Pseudomonas viridiflava* | *Arabidopsis thaliana* | ACL5 | Thermospermine increased | Marina et al., 2013 |
| *Xanthomonas compestris* | *Arabidopsis thaliana* | ACL5 | Thermospermine increased | Marina et al., 2013 |
| *Pseudomonas cichorii* | *Nicotiana tabacum* | DAO & PAO | Putrescine and spermidine oxidation increased | Yoda et al., 2009 |
| *Pseudomonas syringae* | *Arabidopsis thaliana* | DAO & PAO | Putrescine and spermidine oxidation increased | Yoda et al., 2009 |
| **Fungus** | | | | |
| *Blumeria graminis (powdery mildew)* | *Hordeum vulgare* | ODC & SPMS | Free and conjugated forms of Putrescine and Spermine increased | Cowley and Walters 2002a |
| *Peronospora tabacina, Erysiphe cichoracearum & Alternaria tenuis* | *Nicotiana tabacum* | NA | Putrescine and spermidine decreased | Edreva 1997 |
| *Ascochyta rabiei* | *Cicer arietinum* | DAO | Putrescine decreased | Cona et al., 2006 |
| *Phoma exigua* | *Solanum tuberosum* | NA | Feruloyl putrescine accumulation | Malmberg 1984 |
| **Virus** | | | | |
| *Tobacco mosaic virus (TMV)* | *Nicotiana tabacum* | ODC | Putrescine increased | Negrel et al., 1984 |
| *Tobacco mosaic virus (TMV)* | *Nicotiana tabacum* | NA | HCAA including Feruloyltyramine accumulation | Martin-Tanguy et al., 1976 |
| *Tobacco mosaic virus (TMV)* | *Nicotiana tabacum* | DAO | Putrescine decreased | Marini et al., 2001 |
| *Tobacco mosaic virus (TMV)* | *Nicotiana tabacum* | SPMS | Spermine increased | Yamakawa et al., 1998 |
| *Cauliflower mosaic virus (CMV)* | *Arabidopsis thaliana* | SPMS | Spermine increased | Mitsuya et al., 2009 |
| *Citrus exocortis viroid (CEVd)* | *Solanum lycopersicum* | ODC | Putrescine decreased | Belles et al., 1993 |
| **Nematodes** | | | | |
| *Heterodera Schachtii* | *Arabidopsis thaliana* | SPDS | Spermidine increased, SPDS activity increased | Hewezi et al., 2010 |

### 2.4.3 Polyamines as antioxidants

Reactive oxygen species are generated within a cell as a result of normal metabolic processes but when the cells are under various stress there is an increase in the production of these molecules (Halliwell and Gutteridge 2015).When ROS are produced in such a high concentration to overcome the antioxidant defense, oxidation of DNA, protein and fatty acids occurs, that resulting in lipid peroxidation and loss of membrane function. Such damage is referred as oxidative stress and is considered a very sensitive biomarker of many important environmental stresses (Burritt 2008, Burritt and Mackenzie 2003, Lesser 2006). Numerous studies have revealed that cells with reduced concentrations of polyamines are more sensitive to oxidative damage (Chattopadhayay et al., 2002, Rider et al., 2007) suggesting that polyamines may play role in protecting the cells from oxidative damage caused by elevated ROS levels. Hence one of the modes of action of polyamines is to act as antioxidants. It is proposed that polyamines can act as antioxidants as they have ion binding properties and they have been shown to inhibit both lipid peroxidation and metal catalyzed induction of oxidative stress (Tadolini 1988).

### 2.4.4 Polyamines as anti-senescence molecules

Polyamines are implicated as anti-senescence molecules as they are involved in prolonged survival of excised organs or senescing organs *in vivo* namely, leaves, flowers and fruits (Cai et al., 2015). Many studies have focused on the involvement of polyamines in plant senescence by exogenous application of polyamines or by genetically overproducing polyamines (Mattoo et al., 2010, Nambeesan et al., 2010). However, there are some contradictory report, whether polyamines level increases or decreases during senescence (Cai et al., 2015). It is now identified that during senescence polyamines metabolism is connected with many intracellular metabolic pathways, including signaling molecules and other metabolites associated with stress response. Processes interlinked with the increase or decrease in polyamines level during senescence and ability of plants to control senescence in relation to their ability to metabolize polyamines are slowly becoming clear (Sequera-Mutiozabal et al., 2016, Sobieszczuk-Nowicka et al., 2015).

Polyamines may play important role in controlling leaf senescence process. In barley, it became apparent when they observed a decrease in the endogenous level of free polyamines in senescing chloroplasts (Sobieszczuk-Nowicka and Legocka

2014). Previously polyamines were shown to inhibit senescence in oat and *Petunia* leaves, and polyamines were found to be strongly bounded with high molecular weight proteins (Mizrahi et al., 1989). In oat leaves exposed to osmotic stress, exogenous application of spermidine and spermine inhibited protein degradation, chlorophyll loss and stabilized thyllakoid proteins (Besford et al., 1993, Legocka and Zajchert 1999). Exogenous application of spermidine to excised leaves of barley senescing in darkness led to inhibition of RNase activity and chlorophyll degradation (Legocka and Zajchert 1999). In development related and dark induced oat senescing leaves, the best single indicator of leaf senescence was spermine, which decreased during senescence of leaves kept in dark (Kaur-Sawhney et al., 1982). It was suggested that this influence of polyamines on senescence related process is may be due to their cationic nature, which enables direct interaction with nucleic acid, phospholipids and many proteins (Cohen et al., 1979). It is also observed that polyamines delay leaf senescence in oats and petunias, where they have noticed a strong association of polyamines and proteins, suggesting this interaction may be responsible for the observed phenomenon (Mizrahi et al., 1989).

### 2.4.5 Polyamines in cell death

In plants, the growth and development process requires selective elimination of single or group of cells via a highly controllable process known as programmed cell death (PCD). PCD is a process that occurs throughout the lifespan of every plant life starting from embryo development to senescence of plants. It is a normal genetically encoded physiological process occurs during the plant development which can also be induced by abiotic and biotic stresses. Till date, comparatively little is known about the regulators of PCD in plants, various factors among which polyamines and growth regulators have been shown to play role in regulating PCD. The role of polyamines in plant PCD appears to be complex as on one side they act as pro-survival molecules and on the other hand they are known to accelerate PCD (Cai et al., 2015).

Polyamines are involved directly as well as indirectly in regulating PCD process. Polyamines and their analogues may directly induce PCD through their regulatory effect on ion channels (Wu et al., 2010, Zepeda-Jazo et al., 2011). In plants, low $K^+$ activates metacaspase and nucleases promoting PCD (Saha et al., 2015). Polyamines influence $K^+$ level by sensitizing ROS-induced channel conductance and $K^+$ efflux.

Also, stress induced PCD in plants requires activity of plasma membrane and vacuolar ion channels, and it is known that polyamines effect many of these (Saha et al., 2015). Indirectly polyamines regulate PCD through their metabolic derivatives, such as catabolic and interconversion products, like $H_2O_2$ and aminoaldehydes (Moschou and Roubelakis-Angelakis 2013).

Polyamines also known to have effect on gene transcription delaying cell death (Moschou and Roubelakis-Angelakis 2013). In tomato, an increase in spermidine or spermine level by overexpression of SAMDC orthologue gene from yeast resulted in increase in life span of fruits (Mehta et al., 2002). Polyamines also delays PCD by covalently interacting with the molecules involved in cell death. Recently, it is reported that an enzyme transglutaminase (TGase), is involved in such type of interactions. After studying various plant models for cell death, TGase appears to be involved by similar molecular mechanism as they did during apoptosis in animal cells. Thus, TGase acts as cross linker of polyamines and protein and involved in delaying PCD (Del Duca et al., 2014).

### 2.4.6 Polyamines as DNA protectant

It has been shown that polyamines can enhance the stability of DNA, and protect the DNA from damage caused by oxidative stress, ionizing radiations and from endonuclease digestion. Polyamines could protect the DNA in two ways. It has been suggested that polyamines can directly scavenge ROS specially hydroxyl radicals that readily targets DNA (Nayvelt et al., 2009). On the other hand because of their positive charge they can bind electrostatically to negatively charged DNA. Some spectroscopic evidence has shown that polyamine analogues can bind to guanine bases and the phosphate group of DNA as well spermidine and spermine bind to the major and minor grooves of DNA and also to the phosphate groups. DNA compaction is induced when 90% of the charges associated with DNA have been neutralized by binding to the polyamines, hence protecting the DNA against oxidative damage (Vijayanathan et al., 2002). In light of the above, the present work has focused on characterizing the possible roles of some of the identified polyamine biosynthetic genes during plant development and stresses in tomato using molecular genetics and biochemical approaches.