Osmoprotectants: Types, Action Mechanism & Stressors

Dr. Kuldeep Sharma

Department of Botany

M.L.S. University, Udaipur

Rajasthan, India

Types of Osmoprotectants:

- The three distinct categories on the basis of chemical composition of osmoprotectants are:
  - Amino acids
  - Quaternary ammonium compounds
  - Polyols, sugars, and sugar alcohols

Amino acids:

- Amino acid metabolism in plants under abiotic stress condition play a pivotal role in inducing stress tolerance in plants.
- On the exposure to abiotic stresses and resultant desiccation, the accumulation of different amino acids includes:
  - Proline, alanine, arginine, glycine,
  - Amides such as glutamine and asparagine and
  - Nonprotein amino acids such as gamma-aminobutyric acid (GABA), citrulline, pipecolic acid and ornithine, and
  - Minor amino acids and branched chain amino acids, such as isoleucine, leucine and valine, etc.
  - **Proline (Pro):**
- Among these, proline (Pro), a proteinogenic amino acid, is is one of the abundantly distributed osmoprotectants in plants and widely documented and plays a crucial role in both the metabolism and plant defense as an osmoprotectant.
- As a molecular chaperone, it plays an important role in regulation of enzyme activities and protection of protein integrity.
- It also acts as an antioxidant having singlet oxygen quenching and ROS scavenging abilities.
- On exposure to various stress conditions, including drought, salinity, metal toxicity and high temperature, etc. plants accumulate elevated levels of Pro in the cytoplasm and chloroplast.
- However, it is not yet obvious whether Pro accumulation is an indication of stress, response of stress, or just an adaptive strategy.
- However, Pro can have diverse functions including:
  - Its putative role as an osmoprotectant

    - A stabilizing compound of membranes and proteins
    - Buffer cellular redox potential
    - A scavenger of ROS
    - An inducer of expression of salt stress responsive genes, in particular genes with Pro-responsive elements (e.g., PRE, ACT CAT) in their promoters
    - Proline accumulation helps protect the cell from ROS.
- In the Pro biosynthesis, there are two different precursors i.e. glutamate and ornithin (Orn). Proline synthesis in plant cells can occur through two pathways:
    - Glutamate pathway and
    - Ornithine pathway
- Of these two, **the glutamate pathway** is considered the major source of Pro accumulation, while the ornithine pathway is activated in chloroplasts or cytoplasm, producing Pro from glutamic acid through an intermediate pyrroline-5-carboxylate (P5C) under nitrogen limiting or osmotic stress conditions.
- The two main enzymes in the glutamate pathway are:
    - (1) pyrroline 5-carboxylate synthetase (P5CS), and
    - (2) pyrroline 5-carboxylate reductase (P5CR).
    - In most plants, P5CS and P5CR are encoded by two and one genes, respectively.
- The conversion of glutamate to Pro takes place by two consecutive steps, first is catalyzed by P5CS, which is bifunctional enzyme. It catalyzes activation of glutamate by phosphorylation and second is P5CR activity, which reduces labile intermediate c-glutamyl phosphate into glutamate semialdehyde (GSA), both are involved in Pro biosynthesis and catabolism.
- In a same way, ornithine enzyme occurs in mitochondria can be transmitted to P5C through the action of Orn-d-aminotransferase (OAT).
- Moreover, another pathway of Pro biosynthesis is glutathione via the action of glutamic-g-semialdehyde (GSA) and D1-pyrroline-5-carboxylate (P5C).
- The P5C synthase (P5CS) enzymes catalyses conversion of glutathione to P5C, followed by the action of P5C reductase (P5CR) enzyme, which reduces the P5C to Pro.
- In contrast to this, degradation of Pro is caused by the reverse action of proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH).

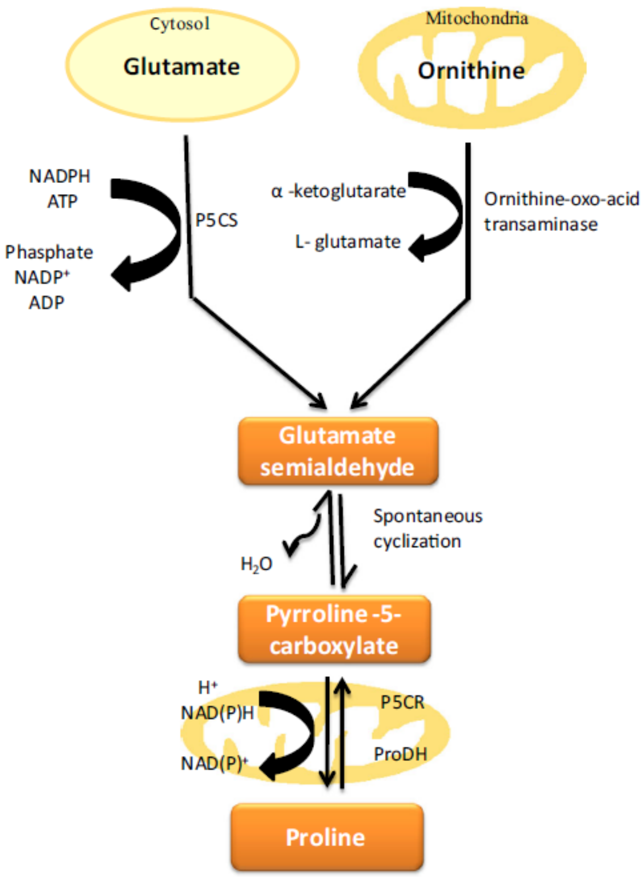

Fig.: Biosynthetic pathway of proline through glulamate and ornathine in plants.
P5CS pyrroline-5-carboxilate synthetase, P5CR pyrroline-5- carboxilate synthetase, ProDH proline dehydrogenase

- After plant exposure to stress conditions, catabolism of Pro in the mitochondria contributes to oxidative respiration and produces energy for resuming plant growth.
- It acts as a metabolic signal for stabilizing metabolite pools and, hence, causes a beneficial impact on growth and development.
- Moreover, Pro synthesis in the chloroplast under stress conditions:
  - Balances the low NADPH: $NADP^+$ ratio,
  - Sustains the electron flow between photosynthetic excitation centers
  - Regulates the redox balance
  - Alleviates cytoplasmic acidosis
  - Protects from photoinhibition and damage of photosynthetic apparatus.
- However, there are species-specific differences in Pro accumulation in plants under stress conditions.

- **Quaternary amines: (Glycine Betaines (Gb), B-Alaninebetaine, Prolinebetaine, Choline-O-Sulphate, Dimethyl Sulphoniopropionate, Hydroxyproline Betaine, And Pipecolate Betaine)**

- **Glycine betaine (GB):**

- Glycine betaine (GB) [$(CH3)3N^{+}CH2COO^{-}$] is a major and efficient putative osmoprotectant that accumulates in different plant species.
- It is widely believed that GB can protect the plants against exposure to harsh environmental conditions such as drought, high temperature and salinity without causing cellular toxicity.
- Glycine betaine is a zwitterionic, dipolar and electrically neutral (at physiological pH) quaternary ammonium compound which is a N-methylated derivative of glycine and accumulates abundantly in many plant species, under a range of environmental stresses.
- Owing to its unique structure, GB tends to interact with both the hydrophilic and hydrophobic domains of plant cellular macromolecules such as enzymes and proteins.
- The intrinsic levels of GB are believed to be ontogenetically regulated because it is found in young tissues during continued stress, while its degradation does not significantly occur in plants.
- The ability of plants to synthesize/ accumulate excess levels of GB in young tissues under stressful environments does not depend on N availability. This supports the viewpoint that plant N allocation is required to safeguard the developing tissues, even under N- deficit regimes.
- Since GB is not actively degraded/ metabolized in plant tissues, therefore its concentration depends on synthesis, transport and dilution in plants. The extent of accumulation depends on the plant species and degree of their stress.
- It has been reported that GB accumulation in plants in response to abiotic stresses protects the reproductive organs resulting in high yields. It increases the cell osmolality in plants under abiotic stress conditions.
- In angiosperms, GB is synthesized widely in chloroplasts and protects membranes, enzymes, and proteins of the photosynthetic machinery (e.g., Rubisco and PSII) under harsh environmental conditions.
- Generally, GB biosynthesis in higher plants is via two substrates, choline and glycine. GB synthesis from choline in the chloroplast is a two-step process.
- Biosynthesis of GB occurs in the chloroplast from serine through ethanolamine, choline, and betaine aldehyde.

- Choline in plants is transformed to betaine aldehyde by the choline monooxygenase enzyme, the betaine aldehyde being subsequently converted to GB by the action betaine aldehyde dehydrogenase (BADH).

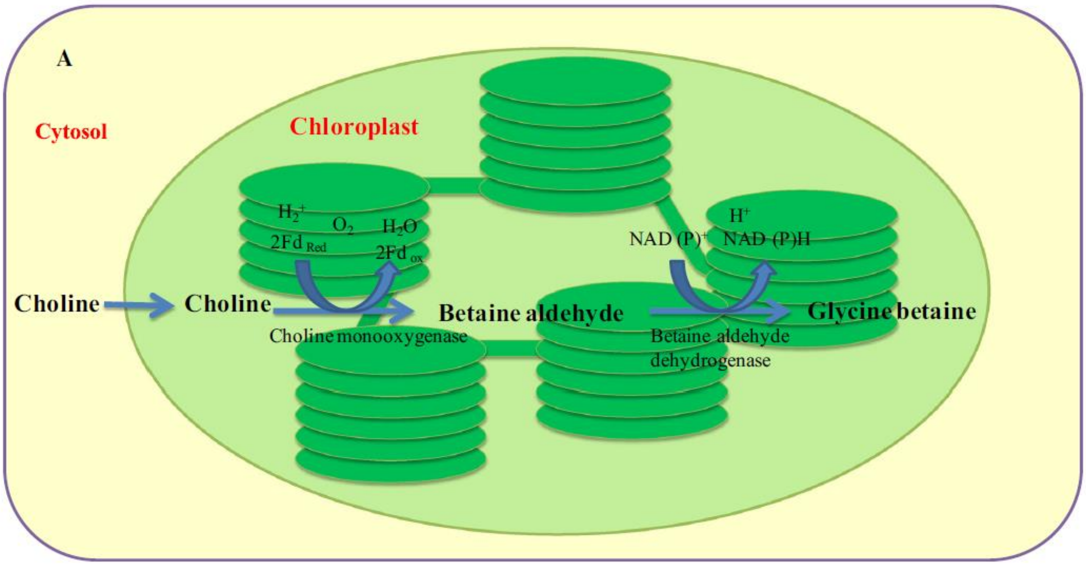

Fig.: Biosynthetic pathway of glycine betaine in higher plants. Choline is converted to betaine aldehyde, by choline monooxygenase, which is then converted to glycine betaine by betaine aldehyde dehydrogenase.

- Although other pathways including direct N-methylation of glycine also exist, the choline to GB pathway has been detected in all GB-accumulating plant species.
- Under normal conditions, plants accumulate low levels of GB which increase at the onset of abiotic stress.
- If GB is supplied exogenously to older parts, it is rapidly re-translocated to younger expanding tissues.
- As a matter of fact, it is synthesized/accumulated in young tissues/organs of plants exposed to stressful cues as well as at N deficit regimes.
- Thus, it can be inferred that GB plays a critical role in safeguarding young expanding tissues.
- However, there are some species that are naturally non accumulators of GB under both normal and stress conditions. For instance, major cereals like maize, wheat or barley partially lack the natural ability of adequate GB accumulation under harsh conditions.
- Additionally, rice, tomato, and tobacco are among those crops that completely lack the GB accumulation ability under normal or stress conditions.

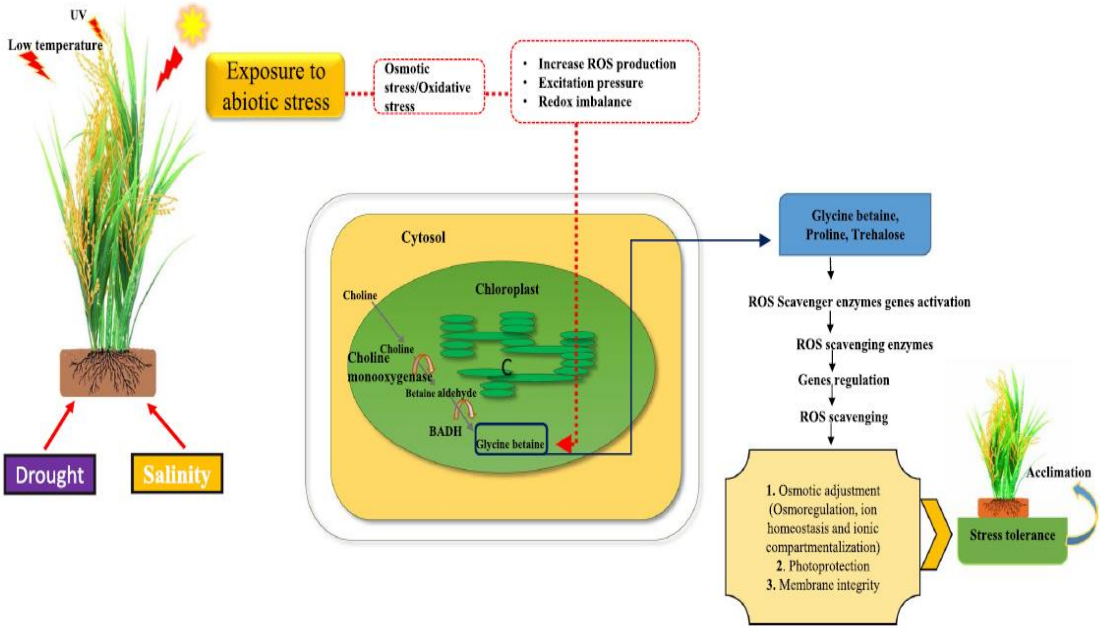

Fig. Schematic example showing involvement of an osmoprotectant (glycine betaine) in abiotic stress tolerance.

- **Sugars:**
- **Trehalose (Tre):**
- Trehalose (Tre), a naturally occurring non-reducing disaccharide sugar, plays a critical role in plant metabolic processes as a reserve carbohydrate and an osmoprotectant (stress protector) in several living organisms including plants.
- Trehalose is composed of two glucose residues (D-glucopyranose units) joined by a very stable α-α- ($1 \rightarrow 1$) linkage.
- Trehalose is highly soluble in nature and chemically unreactive because of its non-reducing property that makes it a compatible solute or osmoprotectant even at its high concentrations.
- Trehalose possesses high hydrophilicity because of the absence of internal hydrogen bonding.
- Owing to this unique property, Tre acts as a protective molecule for cellular, membranous and proteinaceous structures.
- It has been suggested that Tre has the ability to function as scavenging ROS, thus providing protection to protein synthesis machinery of plants.
- Tre is responsible for expression of genes and signaling pathways associated with stress response and detoxification.

- Under stress conditions, Tre protects the structures of membranes and proteins by making an amorphous glass structure and by acting with surrounding polar phospholipids head groups or with amino acids via hydrogen bonding.
- This amorphous glassy structure formation saves the biomolecules from the adverse effects of abiotic stresses especially dehydration and helps in recovery of their specific functions on the onset of normal non-stress environmental conditions.
- Trehalose 6-phosphate (Tre6P), an intermediate of trehalose biosynthesis, is of paramount importance as a signal metabolite in plants, connecting growth and development to carbon status.
- Trehalose biosynthesis in plants follows the OtsA–OtsB pathway which depends on two key molecules:
    - Uridinediphospho- glucose (UDP-Glc) and
    - Glucose-6-phosphate (Glc-6-P) in a two-reaction process
- Initially, the enzyme trehalose phosphate synthetase (TPS) catalyzes UDP-Glc and Glc-6-P into trehalose- 6-phosphate (T-6-P) and uridine diphosphate (UDP).
- Subsequently, the T-6-P is dephosphorylated into Tre by trehalose-6-phosphate phosphatase (TPP).
- Additionally, the trehalase enzyme catalyzes the hydrolysis of Tre.

Sugar alcohols or polyols:

Polyols originate via the reduction of aldoses or their phosphate esters and are generally water soluble in nature.

- Sugar alcohols are categorized into two groups:
    - Cyclic polyols (e.g., pinitol, galactinol, ononitol and inositol) and
    - Acyclic or linear polyols (e.g., orbitol, mannitol, xylitol and ribitol)
- Their accumulation in plants is believed to perform multiple functions including facilitation of osmotic adjustment, and regulation of redox system (ROS scavengers) and molecular chaperons.
    - **Cyclic polyols (e.g., pinitol, galactinol, ononitol and inositol)**

- **Inositol:**
- Myo-inositol (cyclohexane hexol) is a sugar-like unique carbohydrate, which is critically important for a myriad of plant cellular processes and also found to contribute as an osmoprotectant for plant protection against abiotic stress.

- Of the seven isomers of inositol, myo-inositol is the most abundant.
- Besides inositol (myo-inositol), its derivates such as pinitol, galactinol, and ononitol also accumulate in plants and perform diverse functions including osmoprotection.
- The glycosidic linkage present in inositol and its derivates (pinitol, galactinol, and ononitol) is not hydrolysis-labile and owing to this property, inositol derivatives are among the most stable compounds in the plant cell.
- Moreover, this compound is reported to control:
    - Transport of plant hormones such as auxins,
    - Membrane biogenesis,
    - Phytic acid biosynthesis,
    - Signal transduction,
    - Plant immunity and
    - Programed cell death.
- In addition, myo-inositol also contributes to the synthesis of phosphoinositide, which aids in a well-defined signalling pathway (P1), especially under osmotic stress signalling.
- It has been observed that under salinity stress, inositol functions in two ways:
    - As a protectant against ROS and
    - As a controller of cell water potential.
- The biosynthesis of inositol is a two-step biochemical pathway that involves:
    - Enzymatic conversion of d-glucose- 6-P into myo-inositol-1-P mediated by myo-inositol-1-P synthase and
    - Dephosphorylation of myo-inositol-1-P resulting in myo-inositol that further produces different inositol containing compounds such as phospholipids.

    - **Acyclic polyols (e.g., mannitol)**

- **Mannitol:**
- Of the acyclic sugar alcohols, mannitol is the six-carbon liquor polyol, that is considered as the most important and common osmoprotectant widely accumulated in various plant species except halophytes to counteract ROS, a repository of reducing power, and as a carbon stockpiling compound thus, has a critical role in photosynthesis and abiotic stress tolerance.

- In plants, biosynthesis of mannitol starts from fructose-6-P by the actions of different enzymes including mannose-6-P isomerase (phosphomannose isomerase), mannose-6-phosphate reductase and mannose-1-phosphate phosphatase.
- In this way, mixture of glucose/fructose is got interchanged into gluconic acid and mannitol via simultaneous activities of two enzymes.
- The enzyme mannitol dehydrogenase which then controls the catabolism of mannitol, produces mannose which on phosphorylation is converted into mannose 6-P. Further, the enzyme mannose 6-P isomerase converts mannose 6-P into fructose 6-P.
- Mannitol is not accumulated naturally in all plant species and thus introduction of mannitol into non mannitol accumulators can improve their tolerance of harsh environmental conditions.

Non-Proteinogenic Amino Acid

- **γ-amino butyric acid (GABA)**

- A four carbon non-proteinogenic amino acid, γ-amino butyric acid (GABA), widely existing in uni- and multicellular organisms including plants, performs diverse functions in plant life cycles.
- GABA was first discovered during the 1949s in potato tubers by Steward et al. 1949. Since then it has received increased attention by plant physiologists investigating its role in plant metabolism.
- GABA is produced endogenously in plant cells and acts as a signalling molecule which rapidly accumulates under biotic stress conditions and provides osmoprotection to plants.
- Additionally, under harsh environmental conditions, GABA contributes to:
    - Regulation of redox status
    - Osmotic pressure
    - Maintenance of cytosolic pH
    - Maintenance of N fluxes and C–N metabolism
- It can have a scavenging activity against ROS exceeding those of proline and glycine betaine.
- The synthesis of GABA by glutamate decarboxylation catalyzed by GAD could be conducive to the dissipation of excess energy and release of CO2, enabling the Calvin cycle to function while employing a lower influence on photosynthetic electron chain along with decreasing both ROS and photo-damage.

- GABA is metabolized in plants via a pathway known as the GABA shunt, which bypasses two steps of the tricarboxylic acid cycle (TCAC).
- In plant cells, GABA biosynthesis primarily occurs in the presence of the enzyme α-glutamate decarboxylase (GAD) via the decarboxylation of glutamate in the cytosol from where GABA is transported to mitochondria.

Ammonium compound group:

- **Polyamines [PAs (Putrescine, Spermidine and Spermine, etc.)]**

- Polyamines are low molecular weight small aliphatic nitrogenous compounds with hydrocarbon chains and amino groups which are positively charged at physiological level and regulate the pH of cellular components.
- By virtue of their endogenous protonation (cationic nature) ability at cell physiological pH, polyamines belonging to biogenic amines group are widely distributed in plants and regulate diverse biological activities such as:
    - Cell division,
    - Cell differentiation
    - Organogenesis
    - floral induction
    - Root formation
    - Pollination
    - Tuber development
    - Fruit ripening and
    - Programmed cell death
- PAs have great affinity to bind with DNA, RNA and proteins through electrostatic linkages.
- These ubiquitous low molecular weight compounds also function as anti-stress agents in plants.
- Polyamines are important for counterbalancing the excess ROS levels from the plant cell under abiotic stress conditions.
- These positively charged molecules also protect plant cells from oxidative damage via a direct or an indirect route.
- Directly, polyamines act as antioxidants and indirectly they regulate the enzymatic and non-enzymatic oxidants in the cell environment correlating with the level of stress tolerance in plants.

- Among the various osmoprotectants, polyamines are contemplated as the most important for alleviating the negative effects of environmental stresses.
- In higher plants, the most abundant and studied polyamines are putrescine, spermidine, and spermine.
- Besides these major polyamines, others including cadaverine and homospermine also occur in living organisms including plants.
- In addition to their role as osmoprotectants, polyamines are known as nitrogen sinks in plants.
- In plants, the biosynthesis pathway involves decarboxylation of arginine or ornithine, catalyzed by arginine decarboxylase (ADC) or arginase to give rise putrescine.
- The agmatine resulting from arginine is then transformed to putrescine, by agmatine iminohydrolase (AIH) and N-carbamoylputrescine amidohydrolase (CPA).
- Spermine and spermidine are derived by the consecutive addition of aminopropyl groups to putrescine and spermidine from decarboxylated S-adenosylmethionine (SAM) by the action of SAM decarboxylase.

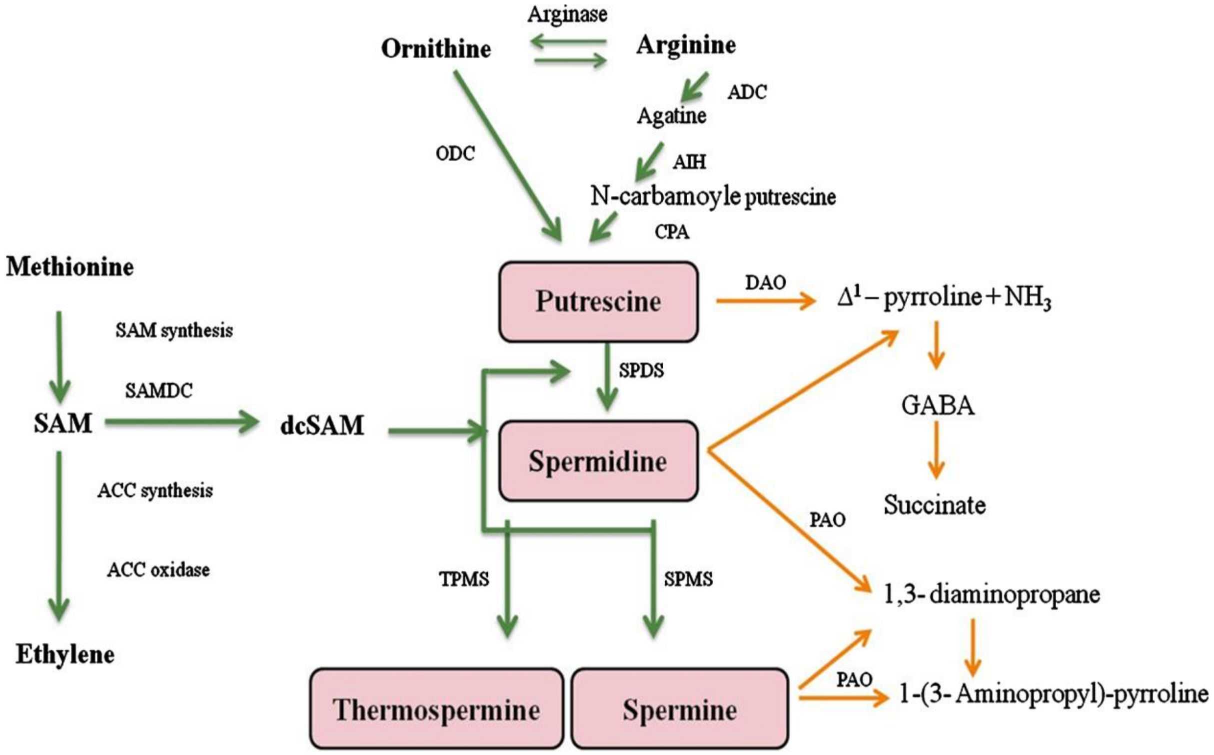

Fig. Polyamine biosynthetic, breakdown pathways and related metabolites in higher plants. ACC 1-amino-cyclopropane- 1-carboxylic-acid, ADC arginine decarboxylase, AIH agmatine iminohydrolase, CPA N-carbamoylputrescine amidohydrolase, DAO diamine oxidase, dcSAM decarboxylated Sadenosylmethionine, GABA c-aminobutyric acid, ODC ornithine decarboxylase, PAO polyamine oxidase, SAM sadenosylmethionine, SAMDC S-adenosylmethionine decarboxylase, SPDS spermidine synthase, SPMS spermine synthase, TPMS thermospermine synthase.

- In a similar way, degradation of PAs are also take place via oxidative deamination process catalyzed by amine oxidases, specifically diamine oxidases (DAO) and polyamine oxidases (PAO) enzymes.
- Diamine oxidases enzymes (DAOs) have a great affinity to diamines, whereas polyamine oxidases enzymes (PAOs) cause oxidation of secondary amine group of Spd and Spm.
- The Put catabolization takes place via the action of diamine oxidases (DAOs) enzymes.
- In this process, Put converts into D1-pyrroline and generates ammonia and H_2O_2 as a by product.
- Further, in oxidation of Put, D1-pyrroline is catabolized into c- aminobutyric acid (GABA) which is then converted into succinic acid, a component of the Krebs' cycle.

Tolerance mechanism of osmoprotectants in response to salinity & drought stress in plants:

- Against different abiotic stresses plants produce a wide range of organic solutes or osmoprotectants belonging to distinguished groups such as ammonium compounds, sugar and sugar alcohols, and amino acids group (Fig.).
- The elevated concentrations of these osmoprotectants in plant system provide:
  - Protection to cellular organs by increasing water uptake potential and
  - Facilitating the enzyme activity
- By two underlying mechanisms:
  - Improving the antioxidant defence system and another
  - Ion homeostasis

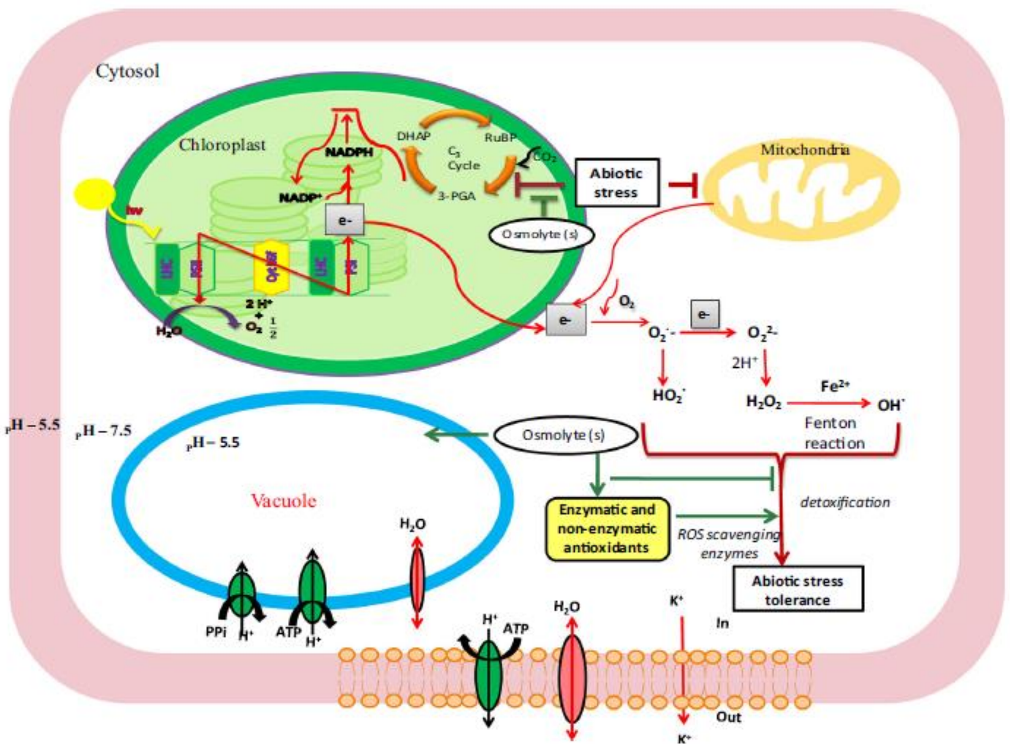

Fig.: During abiotic stress conditions changes in the photosynthetic electron transport lead to the formation of superoxide radicals because the molecular oxygen competes with NADPH for reduction at the acceptor side of photosystem I. Also, damages in proteins of photosystem II (PS II) are associated with the decrease of PSII chemistry caused by ROS. At the same time, different osmolytes accumulate under salt and drought stress conditions and they detoxify the adverse effects of stress by increasing enzymatic and non-enzymatic antioxidants and also they maintain water and ion movement

Antioxidant defence systems:

- In response to severe oxidative stress caused by different abiotic stresses, like salinity and drought, etc., plants trigger PAs, GB, sugar and alchohal sugar and Pro, etc that up-regulate antioxidant enzymes activities that includes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) to reduce adverse effect of oxidative stress.
- And also some other nonenzymatic low molecular weight antioxidants like glutathione (GSH), ascorbate (ASC) and carotenoids are also induced which provide protection via quenching of toxic ROS.

- In a series of detoxifying mechanisms, plants enhanced the production of metalloenzyme SOD which is responsible for the conversion of $O_2^{\cdot -}$ to H_2O_2 and further CAT and PODs catalyze breakdown of H_2O_2.
- Although CAT is apparently absent in the chloroplast, however, H_2O_2 can be detoxified in a reaction catalyzed by an ascorbate specific peroxidase often present in high levels in this organelle through the ascorbate–glutathione cycle.
- Ascorbate can also be oxidized via direct reaction with O2 - or by serving as a reductant of a-chromoxyl radical of oxidized atocopherol.
- The osmoprotectants avoid participation in any biochemical reaction and stored in the cytosol, may enhance the potential of defence enzymes and resolve water crisis during osmotic stress caused by salinity and drought, etc.
- **Ion homeostasis:**
- In response to various abiotic stresses such as drought and salinity plants accumulate diverse groups of osmoprotectants, which provide osmotic adjustment and ion homeostasis via the ion exchange activity.

  - **Osmoprotectants & Salinity:**

- The most common effect of salinity is the reduction in growth and productivity due to direct specific ion toxicity like Na^+ and Cl^- toxicity that decrease the uptake of essential nutrients like phosphorus (P), potassium (K^+), nitrogen (N), and calcium (Ca^{++}).
- These toxic ions exert negative impacts on intracellular K^+ influx, attenuating acquisition of this essential nutrient by cells.
- Moreover, Na^+ toxicity is associated with its competitive nature with K^+ for binding essential sites, which carry normal cellular function.
- It is believed that decrease in K^+/Na^+ ratio may result in K^+ deficiency in the cell therefore, K^+ homeostasis is an important factor in salinity (Fig.).
- In order to tolerate salinity, plants tend to store Na^+ in vacuoles, which defend the cytosolic enzymes from toxic action.
- However, excess concentration of Na^+ in the vacuolar system may enhance the osmotic pressure of other cellular compartments leading to damage of cellular organelles.
- At the same time, ion homeostasis depends on transportation potential of transmembrane proteins, which regulate ion fluxes (influx and efflux) including H^+ translocating ATPases, Ca^{++} ATPases and pyrophosphatases.

- The extrinsically bound Na^+/H^+ antiporters at the plasma membrane and tonoplast acquired energy from electrochemical H^+ gradients, and couple passive movement of H^+ to active movement of Na^+ out of the cell and into the vacuole, respectively (Fig.).

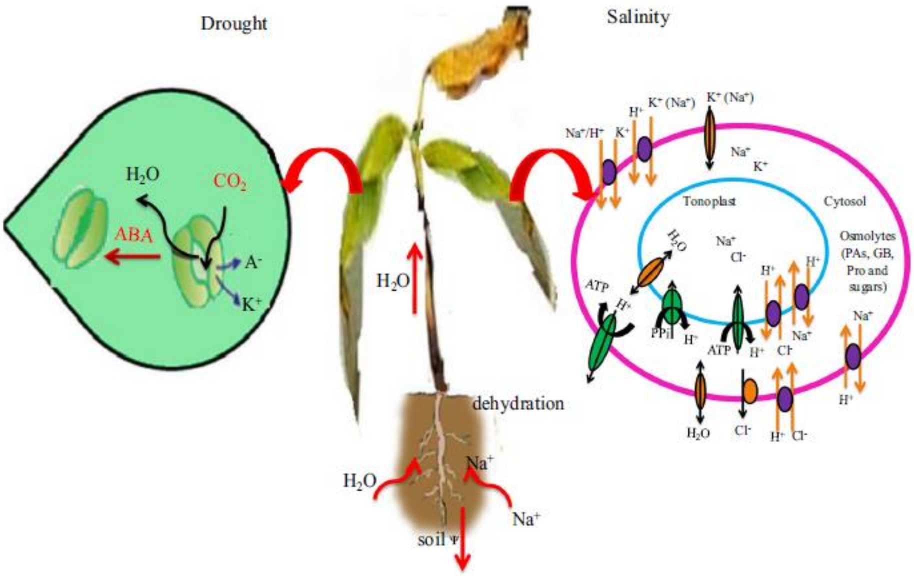

Fig. The schematic arrangement of a plant cell includes three compartments that are defined by the extracellular space; cytosolic space and vacoular space. Indicated are the osmolytes and ions compartmentalized in the cytoplasm and vacuole, and transport proteins responsible for Na? and Cl- homeostasis across the plasma membrane and tonoplast. Similarly, under drought stress ABA plays important role in ion homeostasis and water balance through stomata closing and decreases in water potential (W).

- Accumulation of K^+ into the vacuole against electrochemical gradient is essential to generate efficient turgor for stomatal opening, and this uphill K^+ transport has to be mediated by secondary active carriers.
- Some compatible osmolytes such as PAs, Pro, sucrose, soluble carbohydrates, glycine betaine, and other solutes accumulate in the cytosol and organelles to balance the osmotic pressure of ions in the vacuole thus maintaining the cell turgor and improving water uptake from drying soil.
- The accumulation of these compounds under salinity was linked to the maintenance of a high K^+ concentration and thus a lower Na^+/K^+ ratio, with a better performance of the cell water status.
- Even the exogenous application of Pro and trehalose suppressed Na^+ uptake and accumulation, reduced Na^+/K^+ ratio as well as enhanced activities of antioxidants enzymes in rice plants under salinity.

Osmoprotectants & Drought Stress:

- Similar to salinity, drought stress also causes an impairment of ionic balance in plants. During drought stress, anions and cations, such as Cl^- and K^+, water transport systems in the plasma membrane and tonoplast induce turgor pressure changes in guard cells, which result into stomatal closure.
- Under water deficit, ABA inhibits H^+ATPase activity by reducing H^+ATPase phosphorylation level, and this is important to maintain membrane depolarization.
- H^+ATPase induces negative electric potential gradient inside the plasma membrane which causes a hyper polarization of the plasma membrane and subsequent opening of voltage-regulated inward K^+ channels, resulting in stomatal opening.
- Here, the vacuolar K^+/H^+ exchange is a critical component involved in vacuolar remodeling and the vacuolar pH regulation during stomatal movements. T
- The ABA mainly produced in leaf vascular tissues in response to drought stress is transported to guard cells where it induces stomatal closure.
- The ABA, which is produced rapidly in response to drought and salinity, plays a critical role in the regulation of stress responses and induces a series of signalling.
- Specifically, the accumulation of ABA promotes stomatal closure to minimize water loss, accelerates leaf senescence, down regulates plant growth, and induces the biosynthesis of protective substances under drought and salinity.
- The ABA promotes an efflux of K+ ions from the guard cells, which results in loss of turgor pressure leading to stomata closure (Fig. 6).
- Nayyar and Walia (2003) have reported that accumulation and mobilization of Pro was associated with enhanced tolerance to drought stress.
- Exogenous GB application causes increased accumulation of endogenous osmolytes such as free Pro and GB; they maintain ion homeostasis under stress.