Exogenous Proline-Mediated Abiotic Stress Tolerance in Plants: Possible Mechanisms

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1 Introduction

Plants are constantly exposed to abiotic stresses such as drought, salinity, metal toxicity and extreme temperatures. One of the stress responses in plants is the stimulated production of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) (Hayat et al. 2012; You and Chan 2015). ROS overproduction directly damages cellular biomolecules such as proteins, amino acids, purine nucleotides and nucleic acids and causes the peroxidation of the membrane lipids (Osman 2015; Choudhury et al. 2017). Cells have developed and adapted different mechanisms to maintain low intracellular ROS level. These ROS are scavenged by antioxidative metabolites like glutathione (GSH), ascorbic acid (AsA), α-tocopherol (vitamin E) as well as antioxidative enzymes such as catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) (Gill and Tuteja 2010; de Freitas et al. 2018). In addition to these antioxidants, osmotic regulators like proline also protect plant cell against abiotic stress. They are characterized by low molecular weight and high solubility. Proline accumulation is known to occur under water deficit, salinity, extreme temperature and heavy metal (Ashraf and Foolad 2007; Hayat et al. 2012; Hossain et al. 2014; Aslam et al. 2017, De Freitas et al. 2019). In addition to act as an osmolyte for osmotic adjustment, proline contributes to the stabilization of subcellular structures

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(membranes and proteins), to the scavenging of free radicals, and to buffering of cellular redox potential under stress conditions (Heuer 2010; Hossain et al. 2014). It may also act as protein-compatible hydrotrope, alleviating cytoplasmic acidosis and maintaining appropriate $NADP^+$/NADPH ratios compatible with metabolism (Hare and Cress 1997; Gholami Zali and Ehsanzadeh 2018). In many plant species, proline accumulation under abiotic stress has been correlated with stress tolerance, and its concentration has been shown to be generally higher in tolerant plants than in salt-sensitive plants (Hayat et al. 2012). The level of proline accumulation in plants varies from species to species and can be 100 times greater than in control situation. Thus, the exogenous use of proline is considered as a simple technique to provoke abiotic stress tolerance in plants.

2 Proline and Proline Metabolism in Plants

Proline, abbreviated as Pro or P, is a nonessential proteinogenic amino acid with formula $C_5H_9NO_2$ and a molecular mass of 115.13. Proline is encoded by the codon CCU, CCC, CCA and CCG and is the only proteinogenic amino acid including a secondary amine group called an imine leading to name proline an imino acid (Bhagavan and Ha 2015). The fusion of the three-carbon R-group of proline to the alpha-nitrogen group confers to this compound a rotationally constrained rigid ring structure and thus an exceptional conformational rigidity.

Accumulation of proline under abiotic stress can be mediated by the increase in proline synthesis or a decrease in proline degradation. A diagrammatic representation of proline metabolic pathway and interconnection with polyamine (PA) and gamma-aminobutyric acid (GABA) metabolic pathways is presented in Fig. 1. Proline could be synthesized by two pathways, and, even if glutamate pathway is predominant, ornithine pathway also occurs. The glutamate pathway accounts for major proline accumulation during osmotic stress. Proline is synthesized from glutamic acid via glutamate semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C). The glutamate to GSA reaction is catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS, E.C. 2.7.2.11). GSA is spontaneously converted to P5C, and Δ^1-pyrroline-5-carboxylate reductase (P5CR, E.C. 1.5.1.2) catalyze the transformation of P5C to proline. Proline catabolism occurs in mitochondria in several steps involving proline dehydrogenase (PDH, E.C. 1.5.5.2) producing P5C from proline and P5C dehydrogenase (P5CDH, E.C. 1.2.1.88) converting P5C to glutamate. As previously said, proline can be also synthesized from ornithine in an alternative pathway. Ornithine (Orn) is transaminated to P5C (and GSA) by ornithine delta-aminotransferase (δOAT, E.C. 2.6.1.13), a mitochondrial located enzyme (Hayat et al. 2012; Hossain et al. 2014). P5C is then converted into proline by P5CDH. It has been suggested that the ornithine pathway is important during seedling development and in some plants for stress-induced proline accumulation.

Proline biosynthesis occurs in the cytosol and in the chloroplasts, while proline degradation takes place in mitochondria (de Freitas et al. 2019). Indeed, biosynthetic

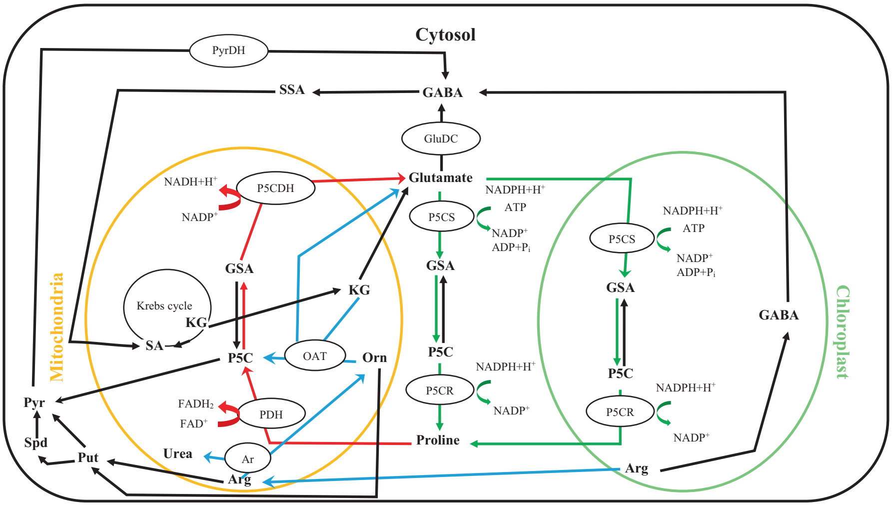

Fig. 1 Proline metabolic pathway in higher plants and possible interconnection with gamma-aminobutyric acid and polyamines pathways. (Adapted from Pál et al. 2018, Huang et al. 2008, Szabados and Savouré 2010, Signorelli et al. 2015). Proline biosynthetic pathways appear with green arrows, catabolic pathway appears with red arrow, and the ornithine pathway is represented with blue arrow. ADP adenosine diphosphate, Ar arginase, Arg arginine, ATP adenosine triphosphate, FAD flavin adenine dinucleotide, FADH2 flavin adenine dinucleotide reduced, GABA gamma-aminobutyric acid, GSA glutamate-semialdehyde, GluDC glutamate decarboxylase, KG apha-ketoglutarate, $NADP^+$ nicotinamide adenine dinucleotide phosphate, NADPH nicotinamide adenine dinucleotide phosphate reduced, OAT ornithine-delta-aminotransferase, Orn ornithine, P5C pyrroline-5-carboxylate, P5CR pyrroline-5-carboxylate reductase, P5CS pyrroline-5-carboxylate synthetase, P5CDH pyrroline-5-carboxylate dehydrogenase, PDH proline dehydrogenase, Put putrescine, Pyr pyrroline, PyrDH pyrolline dehydrogenase, SA succinic acid, Spd spermidine, SSA succinic semiadlehyde

enzymes are preferentially located in cytosol (PCS and PCR), whereas enzymes of proline catabolism are preferentially located in the mitochondria (PDH, PCDH, and OAT) (Szbados and Savouré 2010). This compartmentalization of proline metabolism suggests the occurrence of intracellular proline transport between the cytosol, the chloroplast and the mitochondria. If some proline carriers have been identified like mitochondrial proline uniporter and proline/glutamate antiporter, the involvement of basic amino acid transporters is also needed to transfer arginine and ornithine through mitochondrial membrane. Moreover, the preferential localization of proline catabolic enzyme in the mitochondria and the involvement of glutamate and alpha-ketoglutarate (KG) in the ornithine pathway suggest the interconnection with Krebs cycle (or tricarboxylic acid cycle) (Rana et al. 2017). Through glutamate and pyrroline, proline pathway could also be connected to GABA. Indeed, Δ^1-pyrroline is converted to GABA thanks to pyrroline dehydrogenase (PyrDH, E.C. 1.2.1.19) even if GABA is mainly produced from glutamate by glutamate decarboxylase (GluDC, E.C. 4.1.1.15). GABA accumulation occurs during several stresses leading to attri-

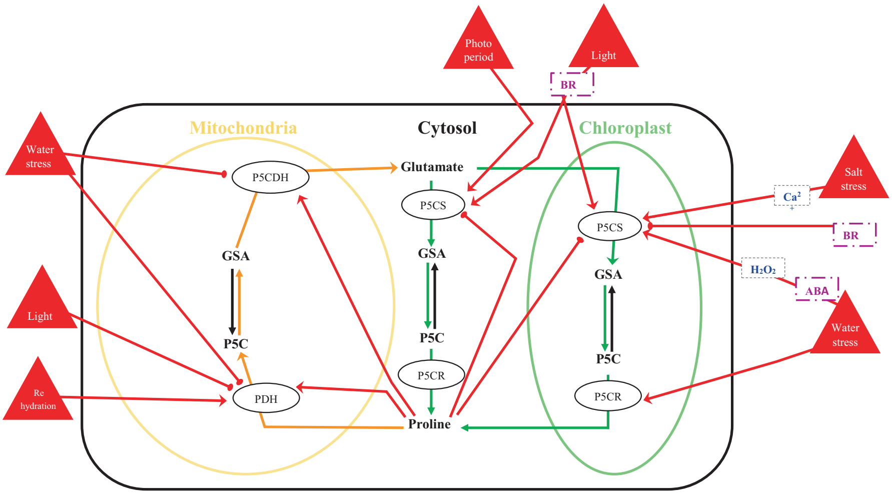

Fig. 2 Possible regulation ways of proline metabolic pathway in higher plants by abiotic factors. (Adapted from Szabados and Savouré 2010). ABA abscisic acid, BR brassinolides, GSA glutamate-semialdehyde, KG alpha-ketoglutarate, P5C pyrroline-5-carboxylate, P5CR pyrroline-5-carboxylate reductase, P5CS pyrroline-5-carboxylate synthetase, P5CDH pyrroline-5-carboxylate dehydrogenase, PDH proline dehydrogenase

bute to this molecule several protective roles (like proline, GABA could be involved in osmoregulation, cell signaling, and protection against oxidative stress, cytosolic pH regulation). Recently, Signorelli et al. (2015) proposed an alternative pathway to connect proline to GABA via pyrroline through nonenzymatic reactions that would explain the simultaneous accumulation of GABA and proline under oxidative stress. Moreover, Pál et al. (2018) suggested the existence of an interconnection between proline pathways and polyamine (putrescine, spermidine) pathways. Indeed, putrescine is synthetized by ornithine decarboxylase (E.C. 4.1.1.17) from ornithine or indirectly by arginine decarboxylase (E.C. 4.1.1.19) from arginine via agmatine. Thus, complex interactions between proline, polyamines, GABA synthesis pathways, and ROS balance exist, and new connections must be probably deciphered in the future as abscisic acid plays also an important role in these stress responses.

Proline metabolism is regulated by multiple factors (Fig. 2), and the regulation processes are still poorly known. Proline biosynthesis is stimulated during dehydration while its catabolism is reduced. At the contrary, the process is reversed during rehydration. Proline biosynthesis is stimulated by light and osmotic stress, whereas proline catabolism is stimulated in dark and during stress relief. Proline accumulation is also reported to be repressed by brassinosteroïds, whereas it was stimulated during salt stress.

Under stress, proline metabolism is regulated by multiple and complex pathways that can drastically influence cell death and survival of the organism. Indeed, the coupling of proline metabolic pathways with the mitochondrial and chloroplastic electron transport chain (through NADPH/NADP$^+$, NADH/NAD$^+$, FADH$_2$/FAD$^+$)

Table 1 Summary of proline functions in plant at different organization levels from cell to whole plant

Organization level	Proline compounds and pathways	Function
Cell wall	PRPs	Wall component
		Drought stress
Plasma membrane	RPRPs	Roots: sensitivity to ABA
	HRGPs	Links between plasma membrane and cytoskeleton
Cell	PRPs	Cell elongation
		Root hair development
	HRGPs	Cell wall assembly Cell wall remodeling
		Intercellular communications
Callus	HRGPs	Somatic embryogenesis Germination of somatic embryos
	HyPRPs	Cell elongation Size increase
Phloem	PRPs	Expression during drought stress
Embryo	Proline biosynthesis impairment	Embryo death Impaired seed development
Leaf	Normal proline level	Flavor compounds
Floral buds	PRPs	Style structural integrity
	Proline accumulation	Bud break
Pollen and style	HRGPs	Pollen tube growth Style growth
Floral nectars	Proline accumulation	Pollinators attraction
Flower	Normal proline level	Flavor compounds
	PRPs	Flower development Cotton fiber development
Fruit	Proline accumulation	Enhanced fermentability (grapevine)
Seed/grain	Normal proline levels	Seed germination
		Flavor compounds
Plant	PRPs	Abscission, senescence
		Development, abiotic stress tolerance
	Proline transporters	Xylogenesis
	Proline biosynthesis impairment	Reduced protein synthesis Cyclin genes downregulated

Adapted from Kavi Kishor et al. (2015)

ABA abscisic acid, *HRGPs* hydroxyproline-rich glycoproteins, *HyPRP* hybrid proline-rich proteins, *PRPs* proline-rich proteins

induces an opportunity to balance the redox state by regulating the generation of ROS. For example, Zhang and Becker (2015) indicated that proline metabolism may influence ROS signaling pathways to delay the senescence.

Proline functions in plants are complex, are not entirely deciphered, and depend on the organization level (Table 1). Proline is a main element of the cell wall matrix

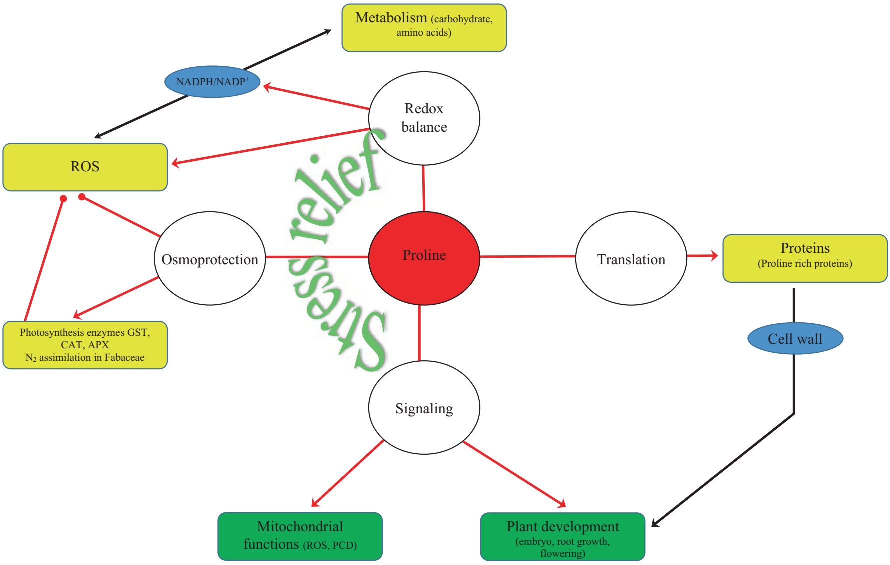

Fig. 3 Proline role in plant functioning. (Adapted from Szabados and Savouré 2010, Verdoy et al. 2006)

through proteins like hydroxyproline-rich glycoproteins (HRGP) or proline-rich proteins (PRPs), thus giving to proline a key role in the plant development (Fig. 3). For example, proline is vital for proper seed development and for producing viable seeds. In in vitro culture, proline via HRGPs is necessary to embryo for their regeneration and their germination during somatic embryogenesis. HRGPs are also necessary to pollen tube and style development. Proline is a key actor in root elongation and in flower initiation but also in the further reproductive tissue development (Kavi Kishior et al. 2015). Thus, proline is not only involved in protein synthesis but regulates also key functions like osmotic adjustment or protein protection. It should be noted that a positive correlation probably exists between proline and glycine betaine, another molecule playing a highly beneficial role in plants exposed to stress (Murmu et al. 2017). Through its involvement in cell wall synthesis, root growth, embryo formation, and germination, proline becomes therefore a major stakeholder during all the plant life cycle.

3 Genetic Features of Proline Metabolism and Regulation

As described above, the proline metabolism occurs through two pathways interconnecting various organelles in plants (Fig. 1). This metabolism appears conserved between prokaryote and eukaryote organisms, and various genes are involved in the

Table 2 Genetics features of genes involved in the main network of proline biosynthesis

Gene name	Plant	E.C number	Chrom.	Gene/protein length (bp/aa)	Exons (number)	Response to abiotic stress in plants
P5CS1	*A. thaliana*	2.7.2.11	2	2154/717	20	Salinity/drought /oxidative stress /Light/phosphate starvation/cold/heat
P5CS2			3	2181/726	20	NaCl (weak)/cold/H_2O_2
P5CS3	*L. regale*		?	2139/712	?	Salinity/drought
P5CR	*A. thaliana*	1.5.1.2	5	831/276	7	Salinity/drought/heat/cold
P5CDH	*A. thaliana*	1.2.1.88	5	1671/556	16	Salinity/cold/drought/dark
PDH1	*A. thaliana*	1.5.5.2	3	1500/499	4	Salinity/drought/ hypo-osmolarity/ phosphate starvation/ABA
PDH2			5	1431/476	4	Salinity/sucrose
δOAT	*A. thaliana*	2.6.1.13	5	1428/475	10	Salinity/drought/H_2O_2

biosynthesis of the different enzymes (Table 2). P5CS, the eukaryotic key fusion enzyme exhibiting the two conserved domains glutamate 5-kinase (GK, EC: 2.7.2.11; N-terminal) and γ-glutamyl phosphate reductase (GPR, EC: 1.2.1.41; C-terminal) (Pérez-Arellano et al. 2010; Fichman et al. 2015), is synthetized by two duplicated *P5CS* genes in most plants (*P5CS1* and P*5CS2*). From several studies, *P5CS* revealed to play distinct roles according to the stress, in an organ-specific manner and following cell spatiotemporal expression patterns (thoroughly reviewed by Rai and Penna 2013, Amini et al. 2015, and Rana et al. 2017). For example, *P5CS1* is mediated by hyperosmotic stress and regulated by abscisic acid, while *P5CS2* appears as a constitutive and ubiquitous gene in plants (Savouré et al. 1997; Székely et al. 2008; Verslues and Sharma 2010). Recently, a third *P5CS* gene (*P5CS3*) was found in the dicot *Medicago truncatula* (Kim and Nam 2013) and in the monocot *Lilium regale* (Wei et al. 2016). These genes contribute also to proline accumulation and abiotic stress tolerance. At the contrary, the second reduction step leading to proline from P5C is managed by only one gene of the plant genome. However, two P5CR isoforms were identified from pea and spinach allowing a lingering doubt on the exact number of *P5CR* genes in these plants (Murahama et al. 2001; Lehmann et al. 2010). In addition, although P5CS represent a rate-limiting step, the absence of a functional P5CR prevents both routes for proline biosynthesis what raise the *P5CR* gene to a paramount converging point of the two anabolic pathways. Therefore, the unique P5CR supposed fine transcriptional regulation although a post-translational regulation is highly suggested even more evident (Forlani et al. 2015; Anwar et al. 2018). As mentioned above, *δOAT* and *P5CDH* constitute another pathway for proline metabolism although *δOAT* is involved in the anabolism route contrary to *P5CDH* that corresponds more precisely to the proline catabolism pathway (cf. Fig. 1). Whatever, both genes are described for having only one copy in the nuclear plant genomes, and *AtP5CDH* exhibits a ubiquitous low basal level but can be upregulated by proline as shown in Fig. 2 (Deuschle et al. 2001). Concurrently, catabolism of proline to Glu is performed through *PDH* and *P5CDH* gene transcription. *PDH* is represented by two copies in the *Arabidopsis thaliana* genome (forming two isoforms, PDH1/PDH2), and their suppression leads to Pro accumulation. Indeed, as for *P5CDH*, proline cellular level insures the post-transcriptional regulation on *PDH* (Verbruggen and Hermans 2008). However, the two protein isoforms were shown to be differentially expressed (Funck et al. 2010).

Overall, many transcription factors (TFs) revealed to be involved in the regulation of the proline metabolism genes. Several TFs gene families like *MYC/MYB*, *bZIP*, *AP2/EREBP*, *RAV*, *PHR1*, *PHL*, etc. participate to the abiotic stress tolerance in plants as already demonstrated or reported (Aleksza et al. 2017; Fichman et al. 2015; Roychoudhury et al. 2015; Anwar et al. 2018). In addition, various binding sites were predicted or demonstrated like the ACTCAT cis-acting element of the *PDH1* promoter (Satoh et al. 2004; Weltmeier et al. 2006) or the HD-HOX, bZIP-DOF, AP2/EREBP, and P1BS-binding sites of *AtP5CS1*, *AtP5CS2*, *AtP5CR*, and *AtOAT* promoters (Fichman et al. 2015).

Owing to the loss of crop productivity and the role of proline in the plant tolerance to abiotic stresses, engineering strategies using plant mutants for proline anabolism/catabolism allow to improve the knowledge on the molecular factors modulating these biological pathways (cf. reviews of Kavi Kishor et al. 2015, Singh et al. 2017, and Hasanuzzaman et al. 2019). Biotechnologies could therefore give substantial advantage for developing new food crop cultivars tolerant to multiple abiotic stresses. Moreover, parallel to plant molecular enhancements, researchers proposed another tool for a higher crop productivity as described hereinafter.

4 Application of Exogenous Proline on Plants Grown Under Abiotic Stresses

4.1 Effect of Exogenous Proline Application on Plant Water Status

In an analysis of the beneficial effect of exogenous proline in plants exposed to abiotic stress (Table 3 and Fig. 4), it is important to consider the role of proline supplementation in plant water status.

Drought stress is known to induce a decline in water content in plants tissues. For example, in cowpea (*Vigna unguiculata* L.) grown under three levels of water deficit (60, 40, and 20% of soil water holding capacity), Merwad et al. (2018) reported that water stress induced a significant decrease of leaf relative water content (LRWC). In

Table 3 Improvement in growth and regulation of various physiological and biochemical processes in different plant species by exogenous application of proline under abiotic stress

Mode of application	Concentration	Type and level of stress	Species	Response	Reference
Foliar spray	5 µM	Salt stress (15 mM)	Lettuce	Increased photosynthetic rate and total yield	Orsini et al. (2018)
Foliar spray	5 mM	Salt stress (150 and 300 mM NaCl)	Rice	Alleviated oxidative damages by enhancing the antioxidant and glyoxalase systems	Hasanuzzaman et al. (2014)
Foliar spray	(0.4, 0.6, 0.8, 1.0, and 1.2 mM)	Salt stress (50 mM)	Chili	0.8 mM was the best concentration Improved growth, physiological, ionic, and biochemical attributes (biomass, photosynthetic rate, transpiration rate, and antioxidant enzyme activities)	Butt et al. (2016)
Foliar spray	30 mM	Salt stress (75 mM)	Sorghum	Decreased membrane damage and regulated proline levels	De Freitas et al. (2019)
Foliar spray	5 mM	Salt stress (saline soil with EC = 1.84, 6.03, and 8.97 dS m^{-1})	Common bean	Increased the activities of antioxidant enzymes and concentrations of carotenoids, ascorbic acid, and endogenous proline, increased the concentrations of P and K+, decreased Na+ ion concentrations, enhanced the growth	Abdelhamid et al. (2013)
Foliar spray	30 mm	Drought stress	Maize	Increased the content of seed sugar, oil, protein, moisture, fiber, and ash, increased the oil oleic and linoleic acid contents, increased the concentrations of antioxidant compounds in the seed oil, enhanced oil DPPH· free radical scavenging activity	Ali et al. (2013)
Foliar spray	4 mM	Drought stress	Pea	Increased the yield and soluble protein concentration, increase nonenzymatic antioxidant defense system, enhanced the production and translocation of assimilates from source to sink	Osman (2015)

(continued)

Table 3 (continued)

Mode of application	Concentration	Type and level of stress	Species	Response	Reference
Foliar spray	80 mM	Heavy metal stress (400 μM, $CuSO_4.5H_2O$)	Wheat	Reduced the generation of reactive oxygen species and enhanced accumulation of proline and protein contents, enhanced the plant height and the shoot and root fresh and dry weight, enhanced the photosynthetic capacity	Noreen et al. (2018)
Foliar spray	10 mM	Heat stress (45 °C)	Barley	Increased the tolerance of photosystem II through protection of the oxygen evolving complex (OEC)	Oukarroum et al. (2012)
Foliar spray	60 mM	Salt stress (100 mM)/nickel stress (100 μM)	Pea and ray grass	Improved growth, total chlorophyll content, photosynthetic attributes, RWC, and membrane stability index (MSI), increased activity of enzymatic antioxidants, enhanced osmolytes, lipid peroxidation, and polyamine metabolism	Shahid et al. (2014)
Foliar spray	2, 4, and 6 mM	Water deficit	Cowpea	Improved growth criteria, yield characteristics, contents of chlorophylls a and b, total carotenoids, shoot and seed nutrients, RWC, membrane stability index, activity of leaf antioxidant enzymes, and content of leaf proline	Merwad et al. (2018)
Rooting medium	25 and 50 mM	Salt stress (100 and 200 mM)	Olive	50 mM was the best concentration Increased photosynthetic activity, LRWC, chlorophyll and carotenoid, and starch contents, reduced the Na^+ content in leaves and roots of stressed plants	Ben Ahmed et al. (2011)
Rooting medium	1, 10, and 100 mM	Cold stress (4 °C)	Larch, sitka spruce, and oak	Increased growth rate and reduced K^+ leakage	Gleeson et al. (2004)

Rooting medium	10 µM	Heat stress (30/25 °C, 35/30 °C, 40/35 °C, and 45/40 °C)	Chickpea	Reduced oxidative injury by elevating enzymatic and nonenzymatic antioxidants, improved chlorophyll content and LRWC, enhanced the activities of enzymes of carbon metabolism	Kaushal et al. (2011)
Rooting medium	25, 50, and 100 µM	Heavy metal stress (1, 2, 4, and 6 ppm selenium in hydroponic medium)	Mung bean	50 µM was the best concentration Increased the endogenous proline content and enhanced the growth, increased the enzymatic and nonenzymatic antioxidants, stimulated components of the ascorbate-glutathione cycle, reduced lipid peroxidation and H_2O_2 content	Aggarwal et al. (2011)
Rooting medium	20 mM	Salt stress (200 mM)	Tobacco	Increased fresh mass and the activities of enzymatic antioxidant	Hoque et al. (2007)
Rooting medium	10 and 20 mM	Cadmium stress (10 and 30 mg Cd kg^{-1} soil)	Date palm	Alleviated the oxidative damage, increased the activity of antioxidant enzymes in roots and leaves	Zouari et al. (2016a)
Rooting medium	20 mM	Cadmium stress (10 and 30 mg Cd kg^{-1} soil)	Olive	Enhanced photosynthetic activity, nutritional status, plant growth, and oil content of olive fruit	Zouari et al. (2016b)

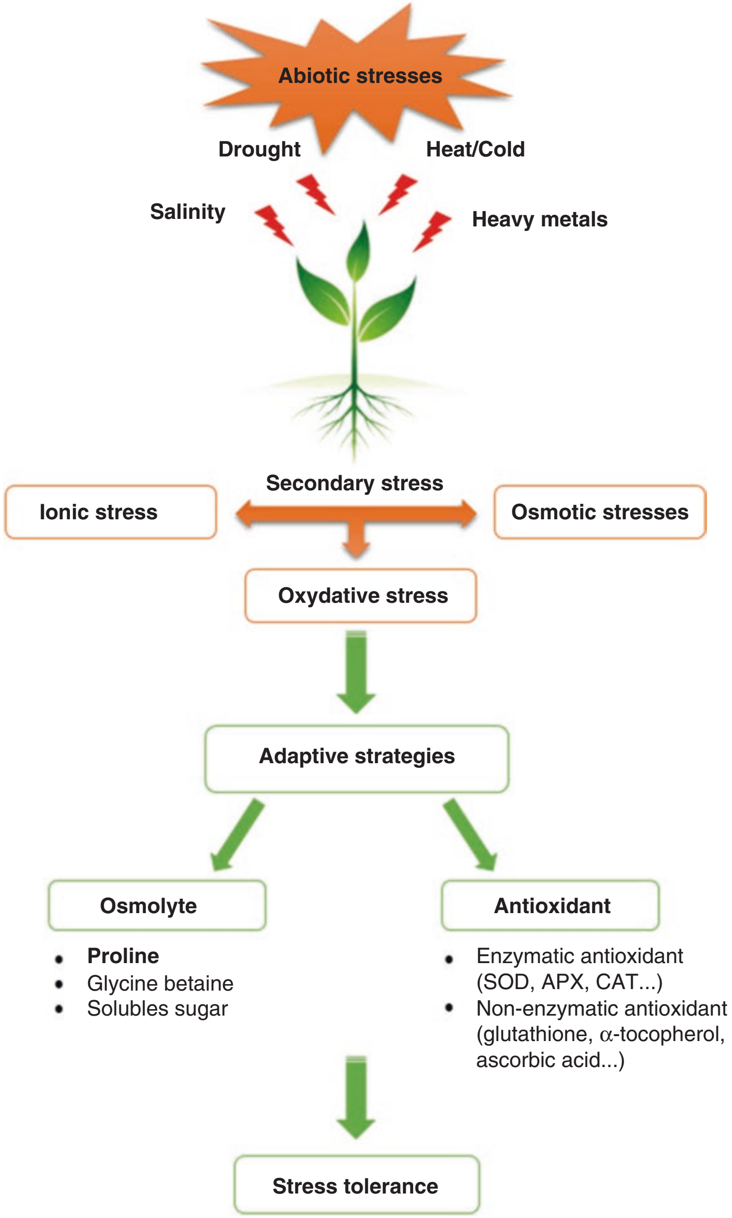

Fig. 4 Plant responses to abiotic stresses

this study, the decline in LRWC due to water deficit stress can be explained by the decrease in the ability of osmotic adjustment due to the reduced absorption of nutrients, especially K^+. The same authors reported that, when proline (6 mM) was applied as foliar spray treatments, LRWC increased. This enhancement was attributed to the significant accumulation of proline in cowpea that proves the important adjusting role of this osmolyte under unfavorable conditions. Its contribution to osmotic adjustment is considered as a mechanism to maintain water relations and postpone dehydration under osmotic stress. In addition, Iqbal (2018) observed that under drought conditions, the exogenous application of proline increased its endogenous level that decreased the water potential in cells to a level lower to the one in soil. This may facilitate the uptake of water by roots and therefore maintain the turgor pressure within cells.

Salinity stress affects also plant-water relations. De Freitas et al. (2019) studied the impact of proline supply to sorghum (*Sorghum bicolor* L.) exposed to salt stress (75 mM NaCl) and observed a significant increase in LRWC of stressed plants sprayed with 30 mM proline solution. The beneficial role of exogenous proline was also obtained in salt-stressed plants such as rice (*Oryza sativa* L.) (Hasanuzzaman et al. 2014) and searocket (*Cakile maritima* L.) (Messedi et al. 2016). Referring to these authors, lowering of leaf osmotic potential by proline supplementation might be the result of higher accumulation of endogenous proline, which enhances the osmoregulation ability of plants under salt stress conditions. The same authors suggest that exogenous proline supplementation can restore water use efficiency, leaf water status, production of free proline, and membrane damage during salinity stress. Proline can enhance water influx and decrease water efflux to restore water content in plant exposed to stress.

Other environmental stress conditions like extreme temperatures similarly account for a significant reduction in plant water status (Kaushal et al. 2011; Oukarroum et al. 2012). In these studies, exogenous proline application maintained the leaf water status, whereas it was reduced in non-treated plants. According to these authors, the maintained leaf water status in proline-treated plants may be attributed to higher accumulation of compatible solutes like proline that possibly improved the turgor content.

Plant-water relations are also affected by heavy metal stress. Zouari et al. (2016a) showed that LRWC and water potential (WP) were decreased in the leaves of date palm (*Phoenix dactylifera* L.) exposed to cadmium stress. The same authors reported that exogenous supply of proline improved the water status of Cd-stressed plants and attributed this enhancement to the interactive effect of proline on osmotic adjustment. Similarly, Shahid et al. (2014) demonstrated that proline application significantly mitigated the alteration of water status of pea (*Pisum sativum* L.) induced by the phytotoxic effect of nickel. According to Aggarwal et al. (2011), exogenous application of proline (50 μM) increased its endogenous levels that antagonized the toxic effects of selenium by improving water status of bean (*Phaseolus vulgaris* L.) seedlings.

4.2 Effect of Exogenous Proline Application on Nutrient Status

Absorption of mineral elements is a key process for plants to survive and grow. However, it is well known that several abiotic stresses result in decreased nutrient uptake and consequently reduced mineral nutrients content in plant tissues. Several studies reported that the exogenous supplementation of proline can ameliorate the uptake and accumulation of inorganic nutrients in stressed plants.

Ali et al. (2008) reported that maize plants (*Zea mays* L.) subjected to drought stress by maintaining moisture content at 60% field capacity presented a decrease in N, P, K^+, Ca^{2+}, and Mg^{2+} contents in the shoots and roots. In the same study, exogenously proline (applied at 30 and 60 mM) increased endogenous proline and promoted the uptake of all the macronutrients under water stress conditions. According to these authors, 30 mM proline concentration was more beneficial than 60 mM as this concentration appeared more effective to increase the transpiration rate. Leaf transpiration creates the water tension necessary to the root absorption of essential nutrients from the soil solution. Similar findings were reported by Merwad et al. (2018) who noticed lower nutrient contents (shoots and seed N, P, and K^+ contents) in cowpea plant submitted to drought stress than in control ones. These authors reported that exogenous proline has maintained nutrient status by promoting the uptake of N, P, and K^+ under water stress.

Salt stress causes also ion imbalance. Abdelhamid et al. (2013) reported that highly saline soil ($EC = 8.97\ dS\ m^{-1}$) resulted in an increase of Na^+ and in a decrease of P and K^+ content in bean plants. In the same study, spraying bean plants with 5 mM proline significantly increased the content of P and K^+ and the K^+/Na^+ ratio and decreased Na^+ levels in salt-affected plants. Butt et al. (2016) also found similar results. These authors grown two chili genotypes under 50 mM NaCl saline condition with and without various concentrations of proline (0.4, 0.6, 0.8, 1 and 1.2 mM) applied as a foliar spray and concluded that proline supply had increased the K^+ concentration in leaves of stressed plants. In this study, authors reported that K^+ efflux was significantly reduced by the application of proline and ionic homeostasis was maintained by enhancing the H^+ATPase activity. In the same way, Sobahan et al. (2009) reported that exogenous proline application reduces the Na^+-enhanced apoplastic flow to reduce Na^+ uptake and transport by plants, suggesting that proline interact with macromolecules in the Na^+ diffusion pathways.

It has been demonstrated that heavy metal stress may result in disturbance of ionic homeostasis. According to Noreen et al. (2018), the uptake of Ca^{2+}, Mg^{2+}, and K^+ ions by root, shoot, and leaf organs of wheat (*Triticum aestivum* L.) was reduced by copper stress. On the other hand, copper content substantially increased in root organs compared to shoot and leaf organs under copper stress environment. The foliar spray of proline increased the uptake of Ca^{2+}, Mg^{2+}, Na^+, and K^+ by root, shoot, and leaf organs, while the copper uptake was reduced in all parts. Ashraf and Foolad (2007) reported that ion uptake by plants was regulated by proline spray under stress. In young olive plants (*Olea europaea* L.) treated with 30 mg $CdCl_2$

kg^{-1} soil, Zouari et al. (2016b) demonstrated that the content of Ca^{2+}, Mg^{2+}, and K^+ was strongly reduced, while Cd^{2+} content was increased in leaf and root tissues. According to these authors, this perturbation of mineral nutrient status could be due to competitions between Cd^{2+} and essential elements via common transporters. In the same study, exogenous addition of proline to growth medium resulted in increased Ca^{2+}, Mg^{2+}, and K^+ contents and in reduced Cd^{2+} content.

4.3 Effect of Exogenous Proline on Photosynthetic Performance

Abiotic stresses generally affect the plant performance and development by altering the photosynthetic machinery (Hayat et al. 2012).

Salinity stress is one of the most common abiotic factors that inhibit crop growth and productivity by reducing the photosynthetic capacity of plants. De Freitas et al. (2019) reported that under NaCl stress, photosynthesis rate, stomatal conductance, transpiration rate, and internal CO_2 concentration of sorghum were significantly decreased as compared to the control. Salt toxic effects on photosynthesis can be generated by stomatal factors, including restrictions for CO_2 diffusion, and by non-stomatal limitations such as decreased Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) activity or damage on photosynthetic apparatus by photosystem II performance impairment. The same authors indicated that stressed plants treated with proline presented higher CO_2 assimilation in comparison to proline-untreated stressed plants, a response closely related to increases in stomatal conductance and transpiration rate. These responses indicate that proline supplementation might play a key role for CO_2 assimilation and photosynthesis recovery in plants against salt stress.

Kaushal et al. (2011) studied the comportment of chickpea (*Cicer arietinum* L.) grown under heat stress and investigated the effects of exogenous proline on total chlorophyll content. Proline-treated plants improved their chlorophyll content by 18% at 40/35 °C and by 44% at 45/40 °C in comparison to untreated plants. According to these authors, proline application significantly reduced the decrease in chlorophyll contents due to heat stress, and such physiological enhancement could result from leaf water status improvement and in possibly reduced photooxidation. The same authors suggested that proline may play an important role in maintaining respiratory metabolism and membrane structure of cells and organelles like chloroplast.

Hayat et al. (2012) and Hossain et al. (2014) reported that proline application under drought conditions may maintain the photosynthetic capacity not only through increasing stomatal conductance but also by protecting the subcellular structures such as the chloroplast ultrastructure, the electron transport complex II in mitochondria, as well as the activity of many enzymes like Rubisco which thereby improved the photosynthetic capacity. Referring to Hare and Cress (1997) and Gholami Zali and Ehsanzadeh (2018), proline biosynthesis is a reductive pathway that require

NADPH (for the reduction of glutamate to P5C and P5C to proline) and generate $NADP^+$ which can be used further as electron acceptor and dissipate electron pressure in thylakoid electron transport chain thus avoiding the photoinhibition and thereby the alteration of photosynthetic machinery.

4.4 *Effect of Exogenous Proline on Antioxidant Defense System*

Plants naturally synthesize ROS as byproducts of cellular oxidative metabolism. The role of proline as ROS scavenger was firstly observed in vivo by Smirnoff and Cumbes (1989) on *Arabidopsis* P5CS insertion mutants. Then, Ashraf and Foolad (2007) confirmed that proline was an effective scavenger of hydroxyl ($OH^\cdot$) and peroxide ion. Hong et al. (2000) concluded that the role of proline as a free radical scavenger is more important in alleviating stress than its role as a simple osmolyte. Reduced lipid peroxidation and H_2O_2 contents, along with the upregulation of the antioxidant defense system, were reported in rice seedlings under salt stress conditions when treated with proline (Hasanuzzaman et al. 2014; Wutipraditkul et al. 2015). Similar patterns were observed also by Butt et al. (2016) in chili genotypes subjected to salt stress and treated with various concentrations of proline. The results proved that both genotypes can cope with salt stress conditions by reducing lipid peroxidation and through the modulation of antioxidant enzymes (SOD and CAT) with exogenous application of proline. It has been reported that proline activates defense mechanisms in response to salt stress, such as activation of antioxidant enzymes. Proline also plays an important role in stress-induced phenolic synthesis, which exhibit antioxidant activities. It has been suggested that proline synthesis stimulates biosynthesis of phenolics via shikimate and phenylpropanoid pathways (Shetty 1997).

Drought stress induces a severe oxidative stress in pea leading to oxidative damages as the antioxidant defense system was unable to cope with this stress (Osman 2015). In this study foliar applied proline (4 mM) enhanced the tolerance of peas to oxidative damage by enhancing ROS detoxification systems. These findings suggest that proline has protective effects against drought-induced oxidative stress by reducing H_2O_2 content and by increasing the enzymatic antioxidant defense system (SOD, CAT, and APX). Ghaffari et al. (2019) noticed in sugar beet (*Beta vulgaris* L.) exposed to drought stress (50% water requirement of plant) that foliar proline applications (low, 5 mM; high, 10 mM) increased enzymatic antioxidant activities and then reduced levels of MDA (malondialdehyde) and H_2O_2. Referring to these authors, proline foliar application might induce the drought tolerance in plants by up-regulating the antioxidant enzymatic activities, quenching the ROS and improving cellular membrane stability.

In regard to heat stress, Kaushal et al. (2011) reported that elevated temperature causes significant reduction in proline content and antioxidant enzymes and resulted in severe membrane lipid peroxidation in chickpea plants. In this respect, a synchronic increase in some components of the antioxidative system would be necessary in order to obtain an improvement in heat stress tolerance. In this connection, exogenous application of proline increased enzymatic (SOD and APX) and nonenzymatic antioxidants (AsA and GSH) to a significant level comparing with control. According to these authors, proline has been shown to function as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes.

4.5 Effect of Exogenous Proline on Growth and Yield Quantity and Quality

Several studies reported that different abiotic stresses reduced cell division and cell expansion, resulting in substantial growth reduction. Inhibition of stem and leaf development negatively affects plant height and leaf area and consequently reduces photosynthesis and crop productivity (Dawood et al. 2014; Osman 2015; Zouari et al. 2016a).

Proline regulates many aspects of growth and development, particularly under abiotic stresses. Transgenic rice overexpressing P5C genes presented increased root and shoot growth and increased biomass production under drought conditions. Transgenic plant accumulated more proline than the control (Su and Wu 2004). Therefore, it has been postulated that exogenous application of proline can effectively stimulate growth and yield attributes. Ali et al. (2013) reported that foliar applied proline significantly increased the seed oil content of maize under well irrigated and water-deficit conditions. Furthermore, exogenous application of proline increased the oil oleic and linoleic acid contents. In a similar study, Teh et al. (2016) reported that proline supplementation significantly increased the plant height and the number of roots of rice under salt stress. More recently, Merwad et al. (2018) reported that foliar application of proline ameliorated growth criteria (shoot dry weight, plant height, leaf area, and number of branches per plant) and yield characteristics (dry seed weight, biological yield per plant, and 100-seed weight) of cowpea submitted to water stress.

Amelioration of plant growth and yield attributes due to proline application might be due to (i) the improved synthesis of compatible solutes leading to better osmotic adjustment (Dawood et al. 2014); (ii) the enhanced accumulation of total soluble phenolics, thus protecting the tridimensional structure of proteins and enzymes (Ashraf and Foolad 2007; Rasheed et al. 2014); (iii) the improvement in chlorophyll contents (Zouari et al. 2016b); (iv) the reduced oxidative damages (Shahid et al. 2014); (v) the increased antioxidant system activities (Osman 2015); (vi) the stabilization of biological membranes (lipids, protein, plasma membrane) (Hayat et al. 2012); (vii) the enhancement of Rubisco activity (Kaushal et al. (2011); and (viii) the improved photosynthesis (De Freitas et al. 2019). The growth-promoting effect of proline application could be also attributed to its role in protein synthesis.

5 Effective Concentrations of Exogenous Proline

Exogenous application of proline to abiotic-stressed plants generally provides a stress preventing or recovering effect. Despite the beneficial effects of exogenous proline application, proline has toxic effects if over-accumulated and/or applied at excessive concentrations (Ashraf and Foolad 2007). Therefore, it is essential to determine optimal concentrations of proline that provide beneficial effects for each plant species. In maize plants, for example, it was determined that foliar applied proline at 30 mM mitigated the adverse effects of NaCl stress, but, at 60 mM, proline inhibited the growth of salt-stressed and non-stressed plants (Ali et al. 2008). In lettuce (*Lactuca sativa* L.), exogenous proline spraying at 10 μM was very effective in alleviating the effects of salt stress, while higher concentrations (15 μM) were not beneficial (Orsini et al. 2018). Butt et al. (2016) applied various concentrations of proline (0.4, 0.6, 0.8, 1.0, and 1.2 mM) as a foliar spray on chili seedlings submitted to salt stress. Among all proline concentrations, 0.8 mM proved to be the best concentration regarding growth, physiological, ionic, and biochemical attributes. Proline application in high concentrations has shown to present harmful effects, such as an inhibition of growth and cellular metabolism (Ashraf and Foolad 2007). Thus, in spite of its protective role, the toxicity effect of proline at high concentrations may be a problem. This toxicity could be due to the repression of genes involved in key functions of the plant metabolism like photosynthesis or synthesis of cell wall-associated proteins (Verbruggen and Hermans 2008). The available information from different studies suggest that optimal concentrations of proline may be species- or genotype-dependent, which need to be determined a priori before commercial application of exogenous proline to improve crop stress tolerance.

6 Conclusion and Future Perspectives

Abiotic environmental stresses remain the major obstacle in plant growth, development, and global crop productivity. However, understanding the physiological and biochemical responses of plants to stress remains necessary to plant science researchers around the world. Scientists are constantly developing new strategies to improve plant stress physiology. In this regard, many studies have provided the notion that the exogenous application of proline provided better protection against different abiotic stresses such as salinity, drought, metal toxicity and extreme temperatures, etc. Under these stressful environmental conditions, exogenous applications of proline have been shown to:

(i) Increase the endogenous levels of proline and compatible solute which provide protection to cells through osmotic adjustment.
(ii) Help to maintain cellular ionic homeostasis.

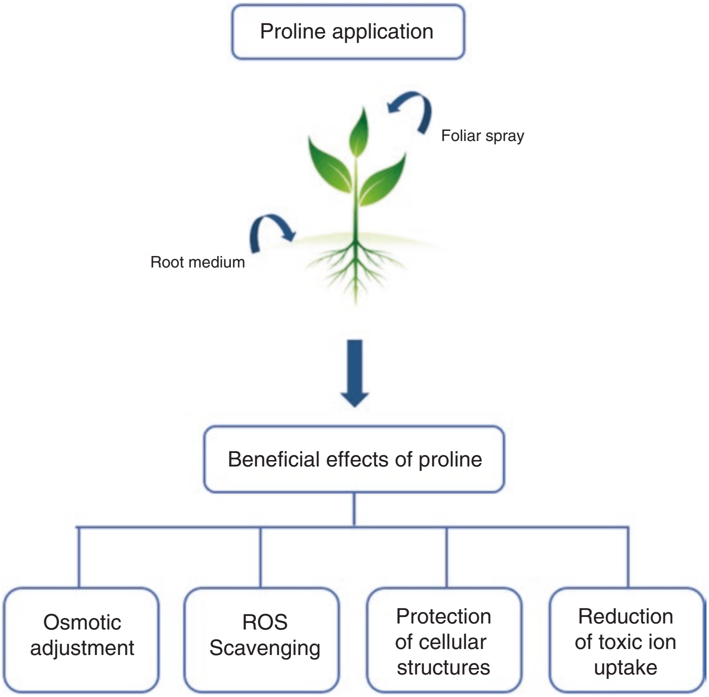

Fig. 5 Beneficial effects of exogenous proline application on plants under abiotic stresses

(iii) Act as an antioxidative defense which efficiently scavenge toxic ROS, confer detoxification processes, and reduce oxidative damages through stabilizing antioxidant enzymes.
(iv) Affect plant-water relations by maintaining turgidity of cells under stress and increase the photosynthesis rate.
(v) Enhance plant growth and final crop yield (Fig. 5).

Deciphering proline metabolic pathways and their interconnections with TCA cycle, GABA, polyamine pathway, etc. is of major interest to develop future applications of proline-mediated stress abiotic tolerance. GMO crop fully benefiting from these future breakthroughs are probably not ready before several decades and will be probably not accepted by the public, as they are not biological and environmental friendly. In that sense, combination of proline, glycine betaine, and polyamine exogenous application could constitute a main key to help plant coping with many stresses induced through climate change and global warming even if the exact effects of these applications must be elucidated and their effect on soil microbiota clarified. For plants, climate change leads to increased drought stress, salinity, and heavy metal stresses linked with the fast growing of water reuse techniques occurring currently. Assisting plants in their adaptation to this changing environment is probably the key to maintain crop production at an acceptable level to insure human survival and “proline engineering” is certainly one of the possible solutions.

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