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The polyamine "multiverse" and stress mitigation in crops: A case study with seed priming in quinoa \ddagger

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ABSTRACT

The importance of polyamines (PAs) in plant growth and development was recognised several decades ago and, since then, their role in cell proliferation, embryogenesis, organogenesis, flowering, fruit development and ripening, etc. has been investigated to a great extent. In more recent years, most of the attention on PAs has been focussed on their functions in biotic and, especially, abiotic stress responses. Exogenous application, transgenic plants over- or under-expressing PA biosynthetic genes, and mutants have been used to unveil their essential contribution to plant tolerance to salinity, drought, chilling, and heavy metal stresses, among others. In parallel, knowledge on their mechanisms of action has increased greatly and it is today evident that PA functions depend upon their ability to (a) bind electrostatically or covalently to numerous compounds thereby modulating membrane, cell wall, nucleic acid, and protein structure and functions, (b) produce hydrogen peroxide via their catabolic pathways, (c) interact with the biosynthetic and signaling pathways of practically all known phytohormones, and (d) interact with nitric oxide. In this review, a state-of-the-art overview of PA functions in plants and their possible applications in mitigating stress in crop plants is provided. The potential of seed priming with PAs as an economically and environmentally valid approach for enhancing plant tolerance to adverse environmental conditions is discussed and some results from our study on quinoa and salt stress are presented.

1. Introduction

Polyamines (PAs) are organic polycations having two or more primary amino groups. They are present in all living organisms where they are responsible for many fundamental processes, including control of transcription, RNA modification, and protein synthesis as well as modulation of enzyme activities, structural integrity of nucleic acids, and cellular membrane dynamics (Anwar et al., 2015; Takahashi and Kakehi, 2010). Although evidence for the growth regulatory functions of PAs and their involvement in biotic and abiotic stress responses in plants dates back to the late 1960s/early 1970s, it is only in the past two decades that their mechanisms of action have begun to be elucidated. The topic has been dealt with in several recent reviews (Alcázar et al., 2020, Chen et al., 2019, Pál et al., 2021, Pottosin et al., 2021, Sobieszczuk-Nowicka et al., 2019) and the main concepts will be summarized here. The most common PAs in plants are the diamine putrescine (Put), the triamine spermidine (Spd), and the tetraamine spermine (Spm), the latter two sometimes known as "higher" polyamines. Thermospermine is a structural isomer of spermine first discovered in thermophilic bacteria. It is synthesized from Spd in a similar reaction to Spm synthesis. In 2000, the *Arabidopsis* gene encoding thermospermine synthase was identified from the *acaulis5* (*acl5*) mutant, a dwarf phenotype with overproliferation of xylem tissue (Hanzawa et al., 2000). Thermospermine is, therefore, required for normal growth and development (G&D), possibly in relation to the ability of this PA to modulate expression of auxin-related genes (Tong et al., 2014). The homeostasis, physiological functions, and mode of action of thermospermine have been reviewed by Takano et al. (2012).

In plants, PAs are most abundant in actively growing tissues and under stress conditions. Their cellular concentrations are much higher

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than those of other plant growth regulators (PGRs) and, despite strong evidence for their fundamental importance in plant G&D, also corroborated by the fact that PA mutants are often lethal, insight into their mode of action has only recently become available. Current knowledge reveals that PAs exert their functions through many different mechanisms, thus forming a "multiverse" based on catabolism, ionic interactions, covalent binding and interaction with hormonal pathways, and signaling molecules.

1.1. Biosynthesis, degradation, and conjugation of PAs in plants

In plants, Put is synthesized by the decarboxylation of arginine or ornithine catalysed by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), respectively. The addition of two aminopropyl groups to Put in two reactions catalysed by Spd synthase (SPDSYN) and Spm synthase (SPMSYN) leads to the formation of Spd and Spm, respectively. The aminopropyl moieties arise from the decarboxylation of S-adenosylmethionine (SAM); decarboxylated SAM is produced by the enzyme S-adenosylmethionine decarboxylase (SAMDC).

Polyamine catabolic processes are performed by copper-containing diamine oxidases (DAOs) and flavin-containing polyamine oxidases (PAOs). DAOs and PAOs exhibit various subcellular localizations, substrate specificities, and functions (Wang et al., 2019). Putrescine degradation by DAO results in the formation of H₂O₂, ammonia, and 4-aminobutanal; the latter undergoes cyclization to form pyrroline, which is then converted into γ -aminobutyric acid (GABA). PAOs (terminal catabolism reaction type) oxidise Spd and Spm producing H₂O₂, 1, 3-diaminopropane (DAP), and 4-aminobutanal (Spd catabolism) or N-(3-aminopropyl)-4-aminobutanal (Spm catabolism). A second group of PAOs (back-conversion type) catalyse reactions that convert Spm back to Spd and Spd to Put; they also produce 3-aminopropanal and H₂O₂. The importance of PA catabolism in plant development, fruit ripening, and abiotic stress responses has been reviewed by Tavladoraki et al. (2016) and Wang et al. (2019).

Evidence indicating that the conjugated forms of PAs may have essential roles in plant development and stress responses has been accumulating in recent years (Pál et al., 2021). PAs can bind covalently to low-molecular weight compounds, such as hydroxycinnamic acids (HCAs), to form hydroxycinnamic acid amides (HCAAs); these are also known as soluble conjugated PAs or phenylamides. The genes encoding enzymes responsible for the formation of these conjugates (e.g., hydroxycinnamoyl transferases) have been cloned from Arabidopsis, but mutants of these genes show normal growth. However, in some species and under some conditions, HCAAs can accumulate to very high concentrations and even represent the predominant form of the total PA pool. HCCAs crosslink various cell wall polymers via ester and ether linkages, thus modifying cell wall rigidity, which could play a critical role in plant responses to pathogens (e.g., Bartolíc et al., 2020, Muroi et al., 2009), wounding, and attack by insect herbivores (Kaur et al., 2010) as well as in apical growth of pollen tubes (Aloisi et al., 2016). HCAAs have also been indicated as ROS scavengers, particularly with regard to their singlet oxygen (¹O₂) quenching ability (Velikova et al., 2007). In addition to their wall-stiffening and antioxidant capacities, HCCAs have also been shown to have antimicrobial activity, thus they could be classified as phytoalexins (Ube et al., 2019). On the whole, however, few studies have examined the potential role of soluble conjugated PAs in relation to growth, development, and abiotic stress tolerance in crop plants (Pál et al., 2021).

Insoluble bound PAs are those that derive from the linkage of PAs to high molecular weight molecules. In the physiological pH range, PAs are fully protonated and positively charged; they can, therefore, electrostatically combine with negatively charged macromolecules, such as acidic proteins, and nucleic acids. Polyamines readily bind to the negatively charged phospholipid head groups or other anionic sites of cellular membranes; indeed, membrane stabilization, and effects on membrane functions were among the first research topics in the field of PAs (Schuber, 1989). In plant cells, the non-covalent binding between PAs and negatively charged components of the cell wall is thought to be implicated in controlling cell wall structure and properties (Berta et al., 1997). Although few studies have investigated this interaction, a role for cell wall-bound PAs has been proposed under drought stress in triticale (Hura et al., 2015). In most organisms, PAs can be covalently bound to glutamine residues of specific proteins by the action of transglutaminases (TGases). The widespread occurrence of TGase activity in most tissues and organs suggests the importance of inter- and/or intra-molecular cross-linking of proteins mediated by PAs also in plants where TGase has been found associated with growth (cell cycle, pollen tube growth), pollen-pistil interactions, programmed cell death, and stress responses; it is also involved in maintaining the stability of photosynthetic membranes by covalently binding PAs to their protein complexes (Ioannidis et al., 2009; Serafini-Fracassini and Del Duca, 2008). Plastidial TGase was found to be Ca^{2+} -dependent, and light-stimulated and shown to catalyse the incorporation of PAs into thylakoid and stromal proteins (Del Duca et al., 2000). Cross-linking of PAs with cytoskeleton proteins may be associated with gametophytic self-incompatibility in some species (Del Duca et al., 2019).

2. Role of PAs in plant growth and development

Polyamines have a wide range of functions in plant G&D, including embryogenesis, organogenesis, seed germination, flowering, sexual reproduction, senescence, and fruit development and ripening.

Changes in endogenous levels of free and/or conjugated PAs have been observed in relation to these processes. The impact of applying specific inhibitors of Put, Spd and/or Spm biosynthesis (e.g., DFMO, CHA, MGBG) has been investigated to corroborate these findings. Exogenous application of PAs is another approach aimed at establishing their role in G&D, starting with the pioneering work of Bagni (1966) who discovered the ability of PAs to stimulate cell proliferation in explants from dormant tubers of Jerusalem artichoke cultured in the absence of any other exogenous phytohormone to more recent work on the effect of PA application on development, ripening, quality, and shelf-life of climacteric and non-climacteric fruit. Indeed, the latter research topic has received a great deal of attention from the early 1980s and has been reviewed by Fortes and Agudelo-Romero (2018) and Gao et al. (2021). In the last 20 years, molecular and genetic approaches have provided further evidence confirming the importance of PAs in G&D of higher plants. For example, the Arabidopsis genome contains two genes encoding SPDSYN. The spds1spds2 double mutant shows embryonic lethality, indicating the essential role of this PA (Imai et al., 2004).

PAs also have an anti-senescence function in plants. Exogenous application of PAs has been shown to delay flower senescence, possibly as a result of reduced ethylene biosynthesis (Chen et al., 2019 and references therein). PA catabolism also plays a central role in directing senescent leaves towards programmed organ death. Thus, senescence and organ death depend upon which direction PA metabolism takes, toward synthesis/accumulation, or towards the oxidative reactions that generate H_2O_2 . Current understanding of the dynamics involved in the regulation of leaf senescence by PAs has been reviewed recently (Sobieszczuk-Nowicka et al., 2019).

3. PAs in biotic and abiotic stress responses

3.1. Biotic stress

In early studies, enhanced biosynthetic activity and accumulation of both free and soluble conjugated PAs were reported in response to pathogen attack; using the hypersensitive response (HR) in the *Nicotiana tabacum*-tobacco mosaic virus (TMV) model system, Torrigiani et al. (1997) observed increasing concentrations of free and conjugated Put and Spd towards the centre of the necrotic lesion. Programmed cell death (PCD) plays a critical role during this and other HRs in plant defence from some pathogens. One of the components that triggers PCD is the oxidative burst, which is generated, among other pathways, via PA oxidation (Yoda et al., 2006). Thus, catabolism is an important aspect of the biological activities of PAs, especially with regard to defence from pathogens, due mainly to their involvement in PCD, cell wall reinforcement and, possibly, to direct toxicity (Bassard et al., 2010).

3.2. Abiotic stress

Climate change, urbanization, deforestation, and agricultural practices leading to soil impoverishment are having, and will increasingly have, a huge impact on agriculture worldwide. Drought, salinity, and temperature extremes are among the main abiotic stresses that negatively affect crop productivity today. Indeed, one of the research areas on PAs in plants receiving most attention in recent years has been their implication with abiotic stresses, in particular water deficit, high salinity, high temperature, UV radiation, heavy metals, nutrient deficiency, chilling, ozone, and hypoxia. Many studies have provided evidence for enhanced biosynthesis and accumulation of PAs in response to such adverse conditions. This raises the question: are increased PA levels markers of stress or indicators of stress tolerance (Pál et al., 2015)? Like H₂O₂, PAs may actually have a dual role as pro-oxidative molecules and as signaling molecules essential for the activation of developmental and stress tolerance processes (Gupta et al., 2016). Another open question is: do Put, Spd, Spm, and (more recently) thermospermine have different functions? For example, in rice, salt tolerance was positively correlated with increased Spd, but negatively correlated with Spm levels (Do et al., 2014). However, "correlation is not cauzation" and thus further studies are needed to corroborate these results. Partial answers to these questions come from experimental data on the effects of exogenous application of a PA on the stress tolerance of a plant. Overall, studies have revealed that in a many crop species exogenous PAs have a protective effect and improve tolerance to drought, salinity, and temperature stresses (Alcázar et al., 2020 and references therein; Chen et al., 2019). In particular, there are many examples of studies reporting the protective effects of pre-treatment with either Spd or Spm against drought and salinity stresses in different plant species, including citrus (Shi et al., 2010), bermudagrass (Shi et al., 2013), tomato (Hu et al., 2014), wheat (Ebeed et al., 2017), finger millet (Satish et al., 2018), and rose (Hassan et al., 2018). In parallel, molecular investigations have been undertaken to provide more compelling evidence for the protective role of PAs under environmental stress (Marco et al., 2011). Over- or under-expression of PA biosynthetic genes has been shown to modulate tolerance to several types of abiotic stress in pear (Wen et al., 2008, 2011), and this was associated with the degree of PA accumulation (Alcázar et al., 2020).

While the generally protective role of PAs in acclimation to stressful conditions is, therefore, well established, further research is still needed to identify the action mechanisms underlying these effects. In several cases, the protective effect has been attributed to increased activities of antioxidant enzymes/lower levels of H2O2 and to enhanced osmolyte content (Ebeed et al., 2017; Hassan et al., 2018; Li et al., 2016; Satish et al., 2018; Shi et al., 2010). Another example is the ability of PAs to regulate ion channels, either by direct binding or, indirectly, by inducing membrane depolarization or modulating the abundance of signaling molecules, such as ROS and NO (see below). Hyperpolarization-activated Ca²⁺ influx and NO-induced release of intracellular Ca²⁺ causing increases in cytoplasmic Ca²⁺ concentration are essential components of stress responses, including stomatal closure. The direct and indirect effects of PAs and the products of their catabolism (i.e., H₂O₂ and GABA) on the activity of plasma membrane or to noplast ion-transporting proteins, such as H^+ and Ca^{2+} pumps as well as K⁺-selective and cation channels, and their importance in stress tolerance have been reviewed recently (Pottosin et al., 2021). Polyamines also alleviate stress-induced reduction of photosynthesis by stabilizing thylakoid ultrastructure via regulation of the expression, structure, and function of photosynthetic membrane proteins, and

promotion of chlorophyll biosynthesis (Hassan et al., 2020; Pottosin et al., 2021 and references therein).

In fact, current knowledge on how PAs exert their multifaceted role in plant G&D and stress responses indicates that these can be divided into five main categories:

- · Ionic interactions with positively charged biomolecules.
- Conjugation with phenolic compounds.
- TGase-mediated covalent binding with proteins.
- Catabolic production of H₂O₂.
- Crosstalk with hormonal signaling cascades and signaling molecules.

4. Crosstalk with hormonal signaling pathways and with other signaling molecules

The notion of crosstalk between PAs and other phytohormones stems from studies showing that some physiological or developmental processes regulated by a phytohormone can be synergistically or antagonistically modulated by PAs. There are also reports suggesting a role for PAs in signal transduction pathways, possibly mediated by interactions with other signaling molecules, such as NO and H₂O₂, that are essential in G&D and stress responses. Over the years, evidence has accumulated for crosstalk between PAs and all major classes of phytohormones (auxins, cytokinins, ABA, ethylene, brassinosteroids, etc). The topic was reviewed by Anwar et al. (2015). A few examples will be described in the following paragraphs.

4.1. PAs and ethylene

While ethylene promotes leaf or flower senescence and abscission as well as climacteric fruit ripening, PAs are generally abundant in actively growing tissues (e.g., young developing fruit) and seem to have an antisenescence effect (Sobieszczuk-Nowicka et al., 2019). In the 1990s, evidence for an inverse metabolic relationship between ethylene and PAs came from studies showing that ethylene inhibited PA biosynthetic activities (Roustan et al., 1994), that exogenous application of PAs inhibited ethylene production (Li et al., 1992) and, conversely, that an inhibitor of PA biosynthesis stimulated ethylene production (Scaramagli et al., 1999). Based on these observations, crosstalk between ethylene and the higher PAs was assumed to derive from competition for SAM, the common precursor in their respective biosynthetic pathways. However, in SAMDC overexpressing transgenic tomato fruits that accumulated two- to threefold higher Spd/Spm, the rate of ethylene production was much higher than in control fruit (Mehta et al., 2002), suggesting that availability of SAM was not rate limiting. Moreover, on-vine ripening was delayed in the transgenic fruit in spite of enhanced ethylene production, providing evidence for the preponderant role of Spd/Spm over ethylene during the ripening process. In a similar study, Nambeesan et al. (2010) showed that tomato fruits overexpressing the spermidine synthase gene (SPDSYN) also exhibited increased Spd as well as ethylene production and yet had extended shelf-life compared to wild-type fruits. Today, microarray data demonstrate that PAs interact with ethylene not only at the biosynthetic level, but also by modulating expression of genes encoding components of the ethylene signaling pathway (Anwar et al., 2015). For example, exogenous application of Spd to peach fruit mesocarp lowered ethylene production and led to higher transcript levels of the ethylene receptors ETR1 (ethylene receptor1) and ERS1 (ethylene sensor1) (Ziosi et al., 2006); peach fruits treated with Spd also showed enhanced expression of subfamily II receptors and CTRs (serine/threonine kinases known as Constitutive Triple Response factors), components of the ethylene signaling cascade (Torrigiani et al., 2012). Transcriptome data also indicate that PAs may increase ethylene production by upregulating expression of its key biosynthetic enzyme, 1-aminocyclopropane-1-carboxylate synthase (ACS). Evidence obtained using these molecular approaches have revealed the complexity of the PAs-ethylene crosstalk, especially due to

the many gene families involved, both at the level of biosynthesis and action.

4.2. PAs and jasmonates

Jasmonates (JAs) are mainly involved in tolerance to pathogens and insect herbivores; they also induce wounding responses (Koo and Howe, 2009). These effects may derive from their ability to enhance production of secondary metabolites, including phenylamides (HCCAs), i.e., soluble conjugated PAs. Application of methyljasmonate (MeJA) was reported to upregulate expression of PA biosynthetic genes and to induce accumulation of free and soluble conjugated PAs (Biondi et al., 2001; Walters et al., 2002; Ziosi et al., 2009). Indeed, MeJA application or mutants having constitutive JA signaling exhibited increased foliar levels of caffeoyl-Put, whereas mutants defective in JA perception showed decreased production of caffeoyl-Put in tomato leaves (Chen et al., 2006). MeJA-induced overproduction of PAs involves R2R3-MYB8, a JA-responsive transcription factor (Kaur et al., 2010). Conversely, the effects of PAs on biosynthesis, conjugation, perception, and signal transduction of JAs have also been investigated, mainly using Arabidopsis and tomato plants overexpressing PA biosynthetic genes, such as those encoding ADC, SAMDC, and SPMSYN (Anwar et al., 2015 and references therein).

4.3. PAs and ABA

Evidence for crosstalk between PAs and ABA initially came from studies in which ABA and PA levels were monitored during seed maturation and germination, fruit growth and ripening, as well as under several abiotic stress conditions, such as low temperature, water deficit, and excess UV-B radiation (Anwar et al., 2015). Supplying exogenous PAs has been shown to influence ABA biosynthesis and accumulation (Marco et al., 2019). Moreover, Arabidopsis mutants and transgenic plants have been used to demonstrate that, under stress conditions, expression of PA biosynthetic genes increases in response to ABA and is impaired in ABA-insensitive, and ABA-deficient mutants (Alcázar et al., 2006). In fact, the presence of ABA-responsive elements (ABREs) in promoter regions of ADC2, SAMDC1, SAMDC2, SPDS1, and SPMSYN genes is likely to regulate ABA-induced PA biosynthesis (Minocha et al., 2014). The first committed step in ABA biosynthesis is catalysed by 9-cis-epoxycarotenoid dioxygenase (NCED). Experiments using Put-deficient adc mutants, ADC over-expressing transgenic plants, or exogenous Put application under chilling or drought conditions have shown that Put and ABA positively regulate each other's biosynthesis under abiotic stress (Cuevas et al., 2008; Diao et al., 2017; Espasandin et al., 2014).

4.4. PAs and signaling molecules

(a) Hydrogen peroxide

It has been proposed that the role of PAs in developmental processes and biotic and abiotic stress responses could be, at least in part, mediated by their capacity to regulate, via oxidation and/or scavenging, the abundance of H_2O_2 involved in signal transduction (Pál et al., 2015; Wimalasekera et al., 2011). H_2O_2 is an important signaling molecule mediating many abiotic stress-related responses, including ABA-induced stomatal closure under drought and salinity stress. It also activates other key signaling pathways, such as those of calcium, JAs, ethylene, salicylic acid, and NO (Gupta et al., 2016). Transduction of H_2O_2 signals into biologically relevant information is coordinated by a complex network of sensors/receptors (e.g., MAP kinases) and transcription factors. Miller et al. (2010) reported 27 genes encoding transcription factors and other regulatory genes whose abundance was modulated by H_2O_2 in roots and shoots of *Arabidopsis* under osmotic or salt stresses (H_2O_2 -responsive transcripts). H_2O_2 can also post-translationally modify proteins via oxidation of thiol groups (e.g., Cys). *Arabidopsis* has about 400 H_2O_2 -responsive protein families.

As mentioned above, H₂O₂ is one of the products of PA catabolism through the activities of DAOs and PAOs. Polyamines, especially Spd, also induce superoxide anion (O2-) production by the activation of NADPH-oxidase; O₂⁻⁻ dismutates spontaneously or enzymatically to form H₂O₂ (Pál et al., 2015). On the other hand, the radical scavenging properties of PAs have been indicated by some authors (Velikova et al., 2007) and their capacity, when supplied exogenously, to activate antioxidant enzymes has also been reported (Choudhary et al., 2011; Zhang et al., 2009). Thus, PAs are both sources of ROS and potential ROS scavengers and may, therefore, play a critical role as redox homeostasis regulators (Saha et al., 2015). H₂O₂ produced by PA catabolism can also be used by cell wall-localised peroxidases involved in stress-induced cell wall stiffening and maturation through cross-linking of its components (Angelini et al., 2010). With regard to G&D, a relationship between PAs, H₂O₂, and NO during development of somatic embryos in cotton cell cultures was described in a study by Cheng et al. (2015).

(a) Nitric oxide

Since its discovery in plants, evidence has accumulated to show that NO is a gaseous signaling molecule involved in various developmental processes (e.g., rooting, seed germination, flowering, and senescence), as well as abiotic and biotic stress responses, all of which are also regulated by PAs. This functional overlap may derive from a metabolic interrelationship. In fact, they share common precursors (L-arginine, and L-methionine) in their biosynthetic pathways; moreover, PAs can induce formation of NO (Tun et al., 2006) and, conversely, exogenous NO modifies PA metabolism (Pál et al., 2015; Sobieszczuk-Nowicka et al., 2019 and references therein). It has been suggested that NO may be a potential mediator of PA action (Wimalasekera et al., 2011).

Exposing secondary amines to NO results in the formation of (poly)amine/NO conjugates known as NONOates. They are a unique group of compounds with important biological functions as NO donors having great potential in biomedical treatments (e.g., cancer therapy) that require rapid or prolonged release of NO *in vivo* (Sobieszczuk-Nowicka et al., 2019 and references therein). Spermine NONOate was used as an NO-releasing compound in gibberellin-treated wheat aleurone layers (Wu et al., 2013), and in *Azolla* where it caused dose-dependent root abscission (Cohen et al., 2015), but further investigation on the use of NONOates in plant biology is still needed.

5. PA functions in crop plants

Given the well-established involvement of PAs in plant G&D as well as in biotic and abiotic stress responses, it is not surprising that there is a plethora of information concerning the metabolism and functions of PAs in crop plants and their beneficial effects when applied exogenously. The processes whose control by PAs are most frequently investigated are briefly discussed below.

5.1. Fruit ripening, shelf-life, and preservation of quality traits

In agricultural studies, one of the best documented topics is that of PAs and fruit development, ripening, and shelf-life. Fortes et al. (2019) provided an overview on the transcriptional control of PA metabolism in fruit as related to pre- and post-harvest stresses. They reviewed evidence based on the use of mutants, priming, and treatment with exogenous PAs or PA inhibitors. More recently, Gao et al. (2021) provided a state-of-the art review on the physiological and molecular mechanisms underlying PA metabolism and action during fruit ripening where they compared climacteric and non-climacteric fruits. Many research articles dealing with Put, Spd, or Spm application aimed at improving abiotic stress tolerance (e.g., chilling injury; Saba et al., 2012) and quality attributes of post-harvest fruit have also appeared in the last decade (e.g.,

Koushesh Saba et al., 2012; Phornvillay et al., 2019; Serrano and Valero, 2018; Wannabussapawich and Seraypheap, 2018). It has been pointed out, however, that post-harvest treatments are not always the optimal solution for maintaining fruit quality and pre-harvest treatments may be more suitable (Mirdehghan and Rahimi, 2016).

5.2. Biotic and abiotic stress tolerance

Using PA applications to enhance abiotic stress tolerance in crops is another topic of major interest (Alcázar et al., 2020 and references therein). Numerous studies have been conducted with respect to drought (e.g., Ebeed et al., 2017), salinity (e.g., Chen et al., 2019 and references therein; Li et al., 2016; Wu et al., 2018), chilling (e.g., Shen et al., 2000), high temperature (Sang et al., 2017) and heavy metal (Naz et al., 2021) stresses in various species (cucumber, rice, wheat, tomato, maize, etc.).

Much work is aimed at establishing the biochemical, physiological, and molecular processes that are affected by treatment with PAs. For example, Ahanger et al. (2019) reported that application of exogenous Spm alleviated salt stress-induced effects on growth and photosynthesis in tomato plants by modulating osmolyte and secondary metabolite accumulation. Spermidine has been reported to alleviate the harmful effects of drought stress in maize and white clover by enhancing the antioxidant defence system and by modulating endogenous phytohormone and PA levels (Li et al., 2018; 2016). Cucumber seedlings treated with 0.8 mM Put exhibited enhanced tolerance to 75 mM NaCl due to improved ion homeostasis, since they had increased K⁺ and decreased Na⁺ concentrations in xylem sap; this was associated with enhanced expression of ion transporter genes, *SOS1* and *NHX*, involved in regulating Na⁺ flux in the plant (Yuan et al., 2019).

Despite the large amount of data regarding the involvement of PA biosynthetic and catabolic genes in controlling plant G&D and stress tolerance levels, transgenic approaches with individual candidate genes have, so far, had little impact on breeding programmes (Alcázar and Tiburcio, 2016). Likewise, the numerous positive results obtained with exogenous PAs to mitigate the effects of adverse environmental conditions on crops have not yet found a wide application in agriculture, mainly due to costs and unknown effects on the environment. They have, however, opened the way for exploring the potential of PAs in seed priming.

5.3. Seed germination and seedling establishment

Seed germination is a critical stage in a plant's life cycle and the first step toward successful plant establishment, especially under adverse conditions. Seed priming, a non-invasive and cost-effective technique, is a pre-sowing treatment that allows partial rehydration, without leading to radicle emergence. Priming can hasten and synchronize germination rate and improve productivity of horticultural crops in a sustainable manner (Zulfiqar, 2021). The most commonly used priming techniques are hydropriming, priming with phytohormones (e.g., ABA), and osmopriming (with PEG). Priming activates processes associated with the early phases of germination, but there is still a great lack of information on what these processes are, both at the physiological and transcriptional levels.

Priming (also known as sensitization) is also a procedure by which plants can be prepared to better tolerate future stresses, either of the same ("stress memory") or of a different ("cross-adaptation") type (Sorrentino et al., 2021). Priming can be achieved by exposure of plants or seeds to a mild environmental stress that acts as a cue. Cold priming, halopriming, exposure to low doses of UV-B (Thomas and Puthur, 2019), or chemical priming with stress-related compounds, such as H_2O_2 , H_2S , and PAs (Hussain et al., 2016; Savvides et al., 2016) have been tested.

Improved stress tolerance of seedlings from primed seeds as compared with non-primed ones has been reported for several species. It has been suggested that seed priming, such as hydropriming, could trigger germination-related processes at the physiological (e.g., respiration, starch metabolism) and molecular (gene transcription and translation) levels. On the other hand, chemical priming with stressrelated compounds may impose a mild stress that represses radicle protrusion but stimulates responses, such as the production of protective molecules (e.g., LEAs, osmolytes, antioxidants, etc.), potentially inducing cross-tolerance (Chen and Arora, 2013). In particular, seed priming with PAs, especially Spd and Spm, has been receiving increasing attention and has been tested on several crop species (rice, maize, wheat) under various types of abiotic stresses, including water deficit (Li et al., 2014), chilling stress (Fu et al., 2020; Sheteiwy et al., 2017) and, especially, salinity stress (Feghhenabi et al., 2020; Hongna et al., 2021; Ibrahim, 2016; Paul and Roychoudhury, 2017; Stassinos et al., 2021).

Such treatments presumably increase stress tolerance in the developing plant by reprogramming metabolic mechanisms during the germination stages (Mahmoudi et al., 2020). Indeed, data obtained from molecular approaches applied to some cultivated plant species (e.g., rice, rapeseed, tomato) have begun to shed light on the molecular cues involved in priming efficiency. Thus, gene expression and transcriptomic analyses have shown that transcript levels of numerous genes are affected by stress priming (Ding et al., 2013; Fan et al., 2019 and references therein). In Medicago truncatula, more than 1300 genes were differentially regulated during priming with PEG; some transcription factors and genes involved in the synthesis of osmoprotectants, such as proline, may also be regulated in seedlings obtained from primed seeds, leading to increased amount of proline, and the expression of stress-related genes is often more rapid (Lutts et al., 2016 and references therein). Huang et al. (2017) reported that exogenous Spd improved seed germination of sweet corn via changes in phytohormone levels (such as gibberellins, ABA, and ethylene) and related gene expression. Seed priming with Spd and Spm of a salt-sensitive vs a salt-tolerant indica rice variety was studied with respect to expression of genes involved in metabolic pathways controlling salt tolerance; expression profiling of genes encoding non-enzymatic and enzymatic antioxidants, osmolyte biosynthesis, the key ABA biosynthetic enzyme (NCED), transcription factors, the vacuolar Na⁺antiporter NHX1, and PA metabolic enzymes (SAMDC, SPDS, SPMS, DAO, PAO) showed that both PAs differentially modulated gene expression levels in the two varieties (Paul and Roychoudhury, 2017).

6. Case study: Priming quinoa seeds with PAs under salinity stress

Salt stress severely reduces seed germination, plant G&D, and productivity of most high-value agricultural crops as these are sensitive to salinity (i.e., glycophytes). Thus, soil salinity, currently affecting approximately 6.5% of the total land area worldwide, is one of the major abiotic stresses impacting agriculture, especially in arid and semi-arid regions of the planet. In addition to naturally saline soils, global warming and irrigation with brackish water is expected to lead to the loss of half of all arable land by 2050 (Rozema and Flowers, 2008).

Quinoa (*Chenopodium quinoa* Willd.) is a halophytic crop with strong resistance to several other abiotic stress factors (drought, chilling, and frost) in addition to salinity and whose seeds have excellent nutritional properties (Ruiz et al., 2016). It can complete its life cycle at seawater levels of salinity (corresponding to ~400 mM NaCl) and grow under even higher salinity levels (600–700 mM NaCl; Orsini et al., 2011). However, like most halophytes, quinoa can be sensitive to salt at the seed germination and seedling emergence stages (Ungar, 1978). In commercial agriculture, successful seed germination and rapid and uniform seedling emergence are important. Consequently, treatments that can support these early stages of plant establishment are useful for boosting quinoa as a crop for salinized soils in a climate change scenario and for an increasing world population (Bazile et al., 2016).

In this case study, we investigated the potential of improving quinoa

seed germination under strongly saline conditions by priming them with Spd or Spm and analyzed some stress-related responses at the biochemical and gene expression levels.

7. Materials and methods

7.1. Plant material

Quinoa seeds of a landrace known as Pandela (belonging to the salares ecotype) were obtained from Colchane, northern Chile. They were selected by seed diameter ($\geq 2 \text{ mm}$) and subsequently sterilised with chlorine gas for 2 h followed by ventilation for 6 h under sterile air flow. They were then primed by soaking in distilled water (water-primed, WP), 1 mM Spd (P-SPD), or 0.5 mM Spm (P-SPM) solutions (5 mL/g seeds) for 30 min; preliminary experiments were performed with 0.5, 1, and 5 mM Spd and Spm to choose the best concentration, i.e., improved rate of germination and percentage germination at 24 h on saline medium. An aliquot of primed seeds was washed, collected and immediately frozen in liquid N₂ for further analyses. The remaining primed seeds were transferred to plates containing solid half-strength MS (Murashige and Skoog, 1962) medium containing 0, 450, or 600 mM NaCl and kept under a 16/8 h light/dark photoperiod at 21 °C. Seedlings were collected after 4, 24, or 168 °Ch, flash frozen in liquid N₂ and then freeze-dried. Non-primed (NP) seeds were collected at the end of sterilization.

7.2. Proline determination

Proline concentration was estimated following the method of Bates et al. (1973) with slight modifications (Scoccianti et al., 2016).

7.2.1. HPLC determination of polyamines

Free Put, Spd, and Spm concentrations were determined after tissue extraction in 4% (v/v) cold perchloric acid and dansylation of the supernatants as previously described (Scoccianti et al., 2016).

7.2.2. Gene expression analysis

Total RNA extraction and real-time quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) analysis of gene transcript levels were performed essentially as described by Ruiz et al. (2017). Primers were designed by consulting the ChenopodiumDB database (http://www.cbrc.kaust.edu.sa/chenopodiumdb/). Primer details are provided in Table S1 (Supplementary Material). The housekeeping gene, Elongation Factor1 α (EF1 α) was used to calculate relative gene expression. Total RNA was extracted from roots and hypocotyls by performing three separate extractions of 0.1 g FW for each replicate. RNA yield and purity were checked by means of UV absorption spectra, whereas RNA integrity was determined by electrophoresis on agarose gel. DNA was removed using the TURBO DNA-free™ (Applied Biosystems, California, USA) from 10 to 20 µg aliquots of total RNA. First-strand cDNA was synthesized from 6 µg of the DNase-treated RNA by means of the High-Capacity cDNA Kit (Applied Biosystems) using random primers. The reaction mixture for the qRT-PCR analysis was made in a final volume of 25 μ L containing 5 ng of cDNA, 5 pmol of each primer, and 12.5 µL of the PowerUp SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. PCRs were carried out with StepOnePlusRM 7500 Fast (Applied Biosystems) for 2 min at 50 °C, 2 min at 95 °C and then for 40 cycles as follows: 95 °C for 3 s, 60 °C for 30 s. Expression levels of genes in all samples are the mean values of three replicates. Fold changes in RNA expression compared with controls were estimated using threshold cycles and analyzed by the comparative threshold cycle method, also known as the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

7.2.3. Statistical analysis

Germination and dry weight measurements were performed on 300

seedlings for each NaCl treatment, while for the biochemical determinations and RT-qPCR analyses, three to four samples of roots and hypocotyls were pooled for the extractions. Three technical replicates were used for each sample. Data were analyzed using one-way ANOVA and means were compared using Fisher's LSD post hoc test, at 5% significance level. For transcript data, a one-way ANOVA was applied followed by the DGC test, at 5% significance level. Genes were considered up- or down-regulated when fold change was ≥ 2 relative to the control.

8. Results and discussion

8.1. Seed germination and seedling growth

As shown in Fig. 1, in WP seeds 10 h after sowing, germination on NS medium was 49% and already more than 90% at 24 h, while with 450 mM NaCl it was less than 15% at 10 h and 42% at 24 h; by 168 h, germination reached~80% and~50% with 450 and 600 mM NaCl, respectively. P-SPM slightly but significantly (P < 0.05) enhanced germination both with 450 and 600 mM NaCl starting from 24 h and maintained higher germination percentages up to 168 h even at 600 mM NaCl (29% higher than in WP seeds). P-SPD also significantly (P < 0.05) improved percentage germination with 450 and, especially, 600 mM NaCl starting from 24 h (+74%, +39%, and + 25% at 24, 48, and 168 h, respectively). Seedling dry weight (DW) at 168 h was not significantly different in WP seedlings as compared to P-SPD and P-SPM ones under NS conditions, while under NaCl treatment DW was significantly improved by priming with 0.5 mM Spm both with 450 and 600 mM NaCl, but only with 600 mM by priming with 1 mM Spd (Fig. 2).

8.2. Polyamines

To check if the exogenously supplied PA was taken up, Spd and Spm contents were measured at the end of the 30 min priming period. Results showed that Spd concentration in P-SPD seeds was ca. 13-fold higher than in WP seeds and Spm concentration in P-SPM seeds was almost 56-fold higher (Table 1). Thus, although Spm was supplied at a lower dose than Spd, it was more readily absorbed.

Changes in PA concentrations were then measured in seedlings at 4, 24, and 168 h after transfer to non-saline (NS) medium or to medium supplemented with 450 or 600 mM NaCl. After 4 h, Put, Spd, and Spm contents were not significantly affected by salt in WP seedlings (Fig. 3A-C). The strong accumulation of Spd in P-SPD and of Spm in P-SPM seedlings was still evident on NS medium, but declined dramatically on saline media, though levels still remained well above those of WP seedlings: on 450 mM NaCl, Spd was 2.7-fold higher in P-SPD seedlings than in WP ones (Fig.3B), and Spm was ca. 6-fold higher in P-SPM seedlings (Fig. 3C). The salt-induced decline could be explained by PAO-catalysed degradation that, by producing H₂O₂, may contribute to the signaling process that activates tolerance mechanisms. After 24 h, Put content declined to the same extent in the presence of both NaCl concentrations as compared with NS medium in WP seedlings (Fig. 3D); this was partially consistent with the upregulation of DAO1/2 genes (see below Table 2). Instead, they accumulated Spd and Spm ca. three-fold (Fig. 3E, F). This is in accord with previous data showing that salinity decreased Put concentrations and enhanced the (Spd+Spm)/Put ratio in quinoa seedlings (Ruiz et al., 2011) and indica rice (Paul and Roychoudhary, 2017). A reduction in Put levels has been reported in other species exposed to salinity stress (e.g., Liu et al., 2008). On the other hand, the protective role of Spd and Spm in plants exposed to abiotic stresses is well established (Wen et al., 2008; Roychoudhury et al., 2011) and may, among other factors, be related to ion channel regulation by PAs discussed earlier (Pottosin et al., 2021). This is regarded as an important mechanism in maintaining higher $\mathrm{K}^{+}/\mathrm{Na}^{+}$ ratios and, therefore, better salt stress tolerance.

In PA-primed seeds, at 24 h Put followed the same trend as in WP ones, suggesting that the decline in Put concentration under saline



Fig. 1. Percentage germination of quinoa seeds primed with Spm (A) and Spd (B) on media containing 0, 450, or 600 mM NaCl. Data represent means \pm S.D. of three replicates, each one consisting of 100 seeds.

conditions is an essential process (Fig. 3D). Although P-SPD seedlings maintained higher levels of Spd than WP ones on NS medium, levels were much lower than at 4 h, indicating that degradation of the accumulated PA was still on-going (Fig. 3E). Although no upregulation of CqPAO1 and CqPAO4 genes was observed, one cannot exclude enhanced PAO enzyme catabolic activity. In P-SPD samples, there were no longer any significant differences in endogenous Spd levels between NS and saline media (Fig. 3E). In P-SPM seedlings, the Spm that accumulated on NS medium was again partially degraded in the presence of salt (Fig. 3F); however, these seedlings accumulated more Spd on NaClcontaining medium than on the NS one (Fig. 3E), possibly as a result of back-conversion type PAOs that convert Spm back to Spd and Spd to Put. Paul and Roychoudhary (2017) also reported that priming with 2.5 mM Spm increased both Spm and Spd levels in two indica rice varieties under salt stress. Finally, at 168 h, no differences in Put, Spd, and Spm levels were observed in WP vs PA-primed seeds in response to salinity (Fig. 3G–I).

Overall, priming with PAs, except for the transient accumulation

observed at the end of priming (30 min) and 4, and 24 h after transfer on NS medium, did not determine major changes in response to salinity in terms of Put, Spd, and Spm levels. Perhaps, it is not the amounts of free PAs accumulated that determine the salinity response, but the amount that was conjugated and/or oxidatively degraded. Further research is needed to check the metabolic fate of exogenously supplied PAs.

8.3. Proline

At 4 and 24 h, salt had no significant effects on proline content in both WP and PA-primed seedlings (data not shown), whereas at 168 h (Fig. 4), WP and P-SPD seedlings accumulated proline (*ca.* 8-fold) on medium containing 450 mM NaCl; priming with Spm enhanced this response (21-fold on 450 mM *vs* 0 mM NaCl). Similarly, with 600 mM NaCl, more proline accumulated in P-SPM seedlings than in the others. In a previous study in which four Chilean landraces of quinoa with varying levels of drought/salt tolerance were compared, we showed that in 7-day old seedlings, 300 mM NaCl induced an accumulation of proline WP P-SPM



Fig. 2. Dry weight (g) of quinoa seedlings after seed priming with Spm (P-SPM) or Spd (P-SPD) at 168 h after transfer to non-saline medium (0 mM) or media containing 450 mM or 600 mM NaCl. Different letters indicate statistically significant (P < 0.05) differences.

Table 1
Endogenous Spd and Spm contents (μ mol g ⁻¹ DW) in quinoa seeds after 30 min
priming with either water (WP) 1 mM Spd (P-SPD) or 0.5 mM Spm (P-SPM)

Priming treatment	Spd	Spm				
WP	1.05 ± 0.16	$\textbf{0.15}\pm\textbf{0.03}$				
P-SPD	13.00 ± 1.92	0.17 ± 0.04				
P-SPM	1.07 ± 0.23	$\textbf{8.36} \pm \textbf{0.95}$				

in all accessions; however, they could be distinguished into those that exhibited a moderate increase, and those that accumulated this osmolyte 3- to 5-fold more than control levels and the extent of accumulation correlated positively with the more salt-tolerant accession (Ruiz Carrasco et al., 2011). Proline accumulation under salt stress is often involved in osmotic adjustment, but also exerts other protective functions by acting as a ROS scavenger and singlet oxygen quencher (Szabados and

Savouré, 2010). Present results confirmed that salt increased proline levels at 168 h; the greater accumulation with 450 mM NaCl as compared with 600 mM may suggest that the seedlings' capacity for coping with the higher concentration of salt was compromised. The salt-induced effect on proline biosynthesis was confirmed by the enhanced expression of the key proline biosynthetic gene Cq*P5CS* at 168 h in salt-treated (WP) seedlings (see below Table 2). P-SPM (but not P-SPD) seedlings exhibited significantly higher proline accumulation relative to WP ones under both saline treatments, suggesting that these seedlings would be better protected. These results are in line with those showing that exogenous Spm improved osmotic regulation, growth, and photosynthesis of tomato seedlings under salt stress by enhancing accumulation of proline (Ahanger et al., 2019).



Fig. 3. Endogenous free polyamine levels in quinoa seedlings after seed priming with water (H_2O), 1 mM Spd or 0.5 mM Spm. Determinations were carried out at 4, 24, and 168 h after transfer to non-saline medium (0 mM) or media containing 450 mM or 600 mM NaCl. Different letters indicate statistically significant (P < 0.05) differences.



Fig. 4. Proline content 168 h after transfer to non-saline medium (0 mM) or media containing 450 mM or 600 mM NaCl in quinoa seedlings after seed priming with water (WP), Spd (P-SPD) or Spm (P-SPM). Different letters, within each salt treatment, indicate statistically significant (P < 0.05) differences.

8.4. Gene expression

(a) *Effect of salt in WP seedlings* – Before examining the effect of priming with PAs, we checked for salt-induced changes in gene expression in WP seedlings (Table 2). Transcript levels were, therefore, calculated as fold changes (FC) relative to 0 mM NaCl. The earliest salt-induced response (4 h) was the downregulation of *CqABA8H3-4* and *CqACS1. ABA8H3-4* encodes for an ABA hydroxylase, a member of a family of enzymes that degrade ABA to phaseic acid, while 1-aminocy-clopropane-1-carboxylate synthase (ACS) is the key enzyme for ethylene biosynthesis. The DR of the ABA hydroxylase gene may have

contributed to the accumulation of ABA, a common response under salt stress. This was accompanied by the transient upregulation of *CqNCED* at 24 h, which could have further increased production of ABA via enhanced biosynthesis.

Many studies have demonstrated that differential transcription of *ACS* gene family members is an important factor regulating ethylene production in response to different stimuli, and, in this case, the process seems to have been inhibited, at least at the transcriptional level. Ethylene is a key regulator of plant G&D, but is also regarded as a positive regulator of salt stress responses in *Arabidopsis* and maize, while it was reported to be a negative regulator in rice (Yang et al., 2015). In a

Table 2

Salt-induced changes in gene expression in WP seedlings after 4, 24, and 168 h exposure to 450 and 600 mM NaCl. Values are fold changes relative to 0 mM NaCl.

Gene	4	450 mM NaCl			600 mM NaCl		
	4 h	24 h	168 h	4 h	24 h	168 h	
CqNCED	=	+4.6	-2	=	+5.5	-3.7	
CqABA8H	-8.8	-2.4	-120	-6.8	-3.7	-57	
CqP5CS	=	=	+3.8	=	=	+3.5	
CqAOX1	=	=	-2	=	-3.4	=	
CqACS1	-11.5	=	-2	-26	=	-14.4	
CqERF1	=	=	+6	=	+2.7	+6	
CqGA3ox1	-2.4	+6.5	-8	-4	+11.2	-7	
CqGA20ox	=	=	-8	=	+5	-6.5	
CqDOG1	=	=	+60	=	+7.5	+55	
CqDAO1	+3	+2.5	+8	+2	+2.4	+8	
CqDAO2	+3	=	-3	+2.6	=	-3.6	
CqPAO1	=	-3	=	=	-4	=	
CqPAO4	=	-3	=	=	-3.7	=	
CqSPDSYN	-5	+3	-10	-30	+3.8	-5.8	
CqSPMSYN-like	+2	=	=	=	=	=	
CqACL5	-	=	-2.8	=	=	-4.7	

recent study by Vita et al. (2021), exposing quinoa plants to ethephon caused increased sensitivity to salt in quinoa, suggesting that enhanced ethylene production under salinity stress might negatively affect salt tolerance.

At 168 h, *Ethylene Response Factor1* (*CqERF1*) was UR six-fold by salt at 450 mM. ERF1 is a key component in ethylene signaling, playing crucial roles in both development (Chen et al., 2021), and biotic and abiotic stress responses. It is an ethylene-responsive transcription factor and it may be that, in our case, although ACS1 transcript levels were not increased by salt, ethylene production was enhanced by post-transcriptional regulation of the enzyme (Chae and Kieber, 2005). As pointed out by Shi and Gu (2020) and Vita et al. (2021), although ERFs and other transcription factors regulate the expression of salt-responsive genes, their results and ours indicate the complexity of transcriptional networks involved in quinoa's responses to salt stress and the need for further investigation.

Consistent with the late (168 h) salt-induced increase in proline content (Fig. 4), *CqP5CS*, the gene coding for the rate-limiting proline biosynthetic enzyme, Δ -1-pyrroline–5-carboxylate synthetase, was also upregulated at 168 h.

A marked salt-induced upregulation of DELAY OF GERMINATION1 (CqDOG1) was observed, especially at 168 h, on both 450 and 600 mM NaCl. In Arabidopsis, expression of this gene determines the depth of seed dormancy (Huo et al., 2016). DOG1 promotes seed dormancy by up-regulating the ABA signaling genes ABI3 and ABI5, and repressing the gibberellin (GA) biosynthesis genes (Wu et al., 2020 and references therein). Thus, increased transcript levels of this gene could be associated with the inhibition of germination observed in quinoa seeds under the saline conditions to which they were exposed. Consistent with the salt-induced inhibition of germination, the GA biosynthetic genes CqGA3ox1 and CqGA20ox were downregulated at 168 h (with a transient upregulation at 24 h). In the absence of NaCl, maximum percent germination was reached at 24 h whereas with NaCl (both concentrations) there was a marked increase between 24 and 48 h (Fig. 1), indicating an acceleration between those two time points, which could account for the upregulation of this gene. GA20 oxidase is a key enzyme in the biosynthesis of gibberellins that catalyses the conversion of GA12 and GA53 to GA9 and GA20, respectively, while gibberellin-3-oxidase (GA3ox) catalyses the final step of the GA biosynthetic pathway, leading to formation of the active GAs. In a comparative transcriptomic analysis between a salt-sensitive and a salt-tolerant quinoa genotype exposed to 300 mM NaCl, Shi and Gu (2020) showed that the gene coding gibberellin-3-betadioxygenase (GA3ox4), involved in GA biosynthesis, was strongly inhibited by salt treatment. Moreover, GAs may not be essential in quinoa seed germination and may be involved in other stress signaling pathways. In fact, despite extensive knowledge about seed dormancy and germination regulatory networks in *Arabidopsis* and cereals, little is known about the corresponding networks in quinoa and in-depth knowledge of the relationships between phytohormones and quinoa seed dormancy is still lacking (Wu et al., 2020).

ALTERNATIVE OXIDASE (AOX) activity controls the level of mitochondrial signaling molecules, such as superoxide and NO, thereby ensuring signaling homeostasis in this organelle (Vanlerberghe, 2013). Evidence suggests that AOXs are particularly important during stress. The stress-induced nature of the alternative pathway in plants is supported by the fact that, in *Arabidopsis, AOX1a* and *AOX1d* are among the most stress-responsive genes encoding mitochondrial proteins (Clifton et al., 2006). In our study, neither salt treatment enhanced transcription of *CqAOX1*. Perhaps, in this salt-tolerant genotype of quinoa (Pandela) *AOX1* is not upregulated as in salt-sensitive plants (e.g., *Arabidopsis*).

Unexpectedly, *CqSPDSYN* was either downregulated or only slightly upregulated (at 24 h) and this is in contrast with the strong accumulation of Spd observed in salt-treated seedlings; the increased accumulation of Spd may derive from enhanced activity of the SPDSYN enzyme. *CqSPMSYN-like* and *CqACL5-1* were generally unaffected or downregulated, while upregulation of *CqDAO1/DAO2* is consistent with the decline in Put content and may have contributed to the production of H₂O₂ necessary for stress signaling and other stress-related functions.

Overall, except for the high FC in transcript levels of *CqDOG1*, none of the stress-related genes examined here were markedly upregulated by 450/600 mM NaCl in Pandela. In a recent transcriptomic study, two quinoa genotypes with contrasting tolerance to salinity were analyzed (Vita et al., 2021). Results showed that a greater number of genes were downregulated by salt in the salt-tolerant genotype, while higher expression levels were observed in the sensitive one. Overall, salt induced more transcriptomic changes in the sensitive genotype than in the tolerant one. It may be that even the high salinity levels used in our study did not cause severe metabolic changes, at least at the transcriptional level, in this *salares* landrace naturally preadapted to cope with salt stress.

(b) Effect of priming with PAs on the salt-induced response - Transcript abundance of the selected genes in response to 0, 450, and 600 mM NaCl was evaluated in P-SPD and P-SPM seeds as compared with WP ones (Figs. 5 and 6). Under NS conditions (0 mM NaCl), transcript levels of CqNCED were downregulated ca. 4-fold at 4 h both in P-SPD and P-SPM seedlings (Fig. 5A). With 450 mM NaCl, expression of this gene was differentially regulated by the two PAs: in P-SPD seedlings it was still DR, while in P-SPM seedlings, CqNCED was upregulated ca. 4-fold. At later sampling times, the gene was generally unaffected or slightly downregulated. Under NS conditions, transcript levels of CqABA8H were strongly (60-80-fold) decreased at 4 h by priming with either Spm or Spd, respectively (Fig. 5B), suggesting that the PAs could increase ABA levels by reducing its degradation. Whereas in WP seedlings, salt induced a decline in transcript levels of CqABA8H (Table 2) that probably contributed to the accumulation of endogenous ABA, the gene was upregulated (ca. 7-fold) by priming with both PAs in the presence of 450 mM NaCl (at 168 h) and downregulated (ca. 6-fold) only by Spm at 4 h with 600 mM NaCl. Overall, these data suggest that PAs have an effect on ABA levels by modulating expression of both a biosynthetic and degradative enzyme and that the effect is differentially modified by the presence of salt and by the two different PAs. On the whole, Spm would appear to induce a greater accumulation of ABA by upregulating CqNCED and downregulating CqABA8H.

Although salt enhanced transcript levels of the proline biosynthetic gene CqP5CS in WP seedlings at 168 h (Table 2), expression was not significantly affected by priming with PAs both under saline and non-saline conditions, except for a 3- to 5-fold downregulation by Spm at 450 mM NaCl and by Spd under NS conditions at 168 h (Fig. 5C). The protective effect of the PAs may substitute for the increase in proline levels that is usually associated with salinity. Priming with PAs did not



Fig. 5. Transcript levels of CqNCED, CqABA8H3-4, CqP5CS, CqAOX1, CqACS1, CqERF1, CqDOG1, and CqGA3ox1 expressed as fold changes relative to water-primed controls in P-SPD and P-SPM seedlings for each concentration of NaCl (0, 450 and 600 mM).

have any significant effects on *CqAOX1* expression levels, consistent with the scarce effect that salt had also on WP seedlings (Fig. 5D).

As regards *CqACS1* (Fig. 5E), the most relevant effects were observed at 4 h when, under NS conditions, the gene was strongly downregulated by priming with Spm and Spd (*ca.* 16- and 24-fold, respectively), whereas in the presence of 450 mM NaCl the gene was less downregulated (*ca.* 8.5-fold) in P-SPD and strongly upregulated (33-fold) in P-SPM seedlings. Overall, these results indicate that the two PAs modulate ethylene biosynthesis at the *ACS* transcription level and that the effect is strongly dependent on NS *vs* saline conditions. An increase in ACS transcripts in transgenic tomato fruits or *Arabidopsis* leaves overexpressing the PA biosynthetic genes SAMDC and SPMSYN has been reported (Anwar et al., 2015 and references therein), but the generally negative relationship between PAs and ethylene is also well documented. Expression of *CqERF1* was generally downregulated or not affected (Fig. 5F). The only significant effects exerted by priming with PAs were a 3-fold downregulation at 4 h in NS conditions and a 3- and 11-fold downregulation with Spm and Spd, respectively at 168 h in the presence of 450 mM NaCl. At the higher NaCl concentration, this decline in transcript levels was maintained at 168 h with exogenous Spd, while Spm had no effect. Thus, priming with Spd/Spm seems to reduce expression of this ethylene-responsive transcription factor, suggesting that PAs may negatively regulate ethylene-induced stress responses.

The most relevant effects on CqDOG1 transcript abundance were



Fig. 6. Transcript levels of CqDAO1, CqDAO2, CqPAO1, CqSPDSYN, CqSPDSYN-like, and CqACL5-1 expressed as fold changes relative to water-primed controls in P-SPD and P-SPM seedlings for each concentration of NaCl (0, 450 and 600 mM).

observed at 168 h when the gene was upregulated under NS conditions and, conversely, generally downregulated with 450/600 mM NaCl by priming with Spd or Spm (Fig. 5G). Thus, PAs inverted the salt-induced upregulation of the gene observed in WP seedlings (Table 2) and is consistent with the improved germination in PA-primed seeds. At 4 h, CqGA3ox1 was strongly downregulated by Spd and Spm under NS conditions (16- and 9.5-fold, respectively); however, with 450 mM NaCl the decline in transcript levels of this gene was attenuated by Spd (2.2fold relative to WP) and inverted to an almost 9-fold upregulation by Spm (Fig. 5H). This trend possibly contributed to the improved germination of P-SPD and P-SPM seeds.

At 24 and 168 h, the effects of priming were variable, depending on NaCl concentration and on the PA applied. As discussed above, these effects may or may not always be related to the germination process and are, therefore, difficult to explain. *GA20ox1* expression was not significantly affected by priming with PAs (data not shown).

All the genes encoding the di- and poly-amine oxidative enzymes, DAO1 (Fig. 6A), DAO2 (Fig. 6B), PAO1 (Fig. 6C) and PAO4 (data not shown), were generally unaffected, except at 168 h when they were strongly (DAOs) or mildly (PAOs) downregulated in P-SPD and P-SPM seedlings grown on 450 mM NaCl. Whereas in WP seedlings, salt caused an increase in transcript levels of DAO1/2 that could represent a stress

response (Table 2), it is noteworthy that in PA-primed seedlings this trend was inverted, possibly indicating the stress-protective action of the PAs. As expected, under NS conditions CqSPDSYN was strongly downregulated (20- to 40-fold) at 4 h by priming with PAs (Fig. 6D) concomitant with the accumulation of exogenously supplied ones. Under saline conditions (only 450 mM), this downregulation was attenuated by priming with Spd and inverted to an upregulation (ca. 23fold) by Spm. Perhaps, the catabolic activity leading to the marked decline in the accumulation of exogenously supplied PAs observed at 24 h in primed seeds (Fig. 3E, F) was accompanied by a biosynthetic activity necessary to maintain appropriate (Spd+Spm)/Put ratios required under these saline conditions. Seed priming with Spd and Spm also resulted in increased SPDSYN transcript accumulation in two rice varieties exposed to salt stress (Paul and Roychoudhary 2017). As regards CqSPMSYN-like and CqACL5-1, no significant effects or slight decreases in transcript levels were observed in PA-primed seeds as compared with WP ones (Fig. 6E, F). It has been reported that SPDS1, SPDS2 and SPMS in Arabidopsis is not responsive to exogenous PAs (Takano et al., 2012).

9. Concluding remarks

Polyamines can influence plant biochemical, physiological, and

molecular processes in many different ways, including reprogramming, either directly or indirectly, gene expression, thus making it difficult to assign a single mechanism of action to these PGRs. While basic research is still needed to gain even further insight into PA interactions with signaling pathways, protein functions, and so on, there is no doubt that their application to cultivated plants can provide benefits at multiple levels. The results of our case study confirm once again the triggering of differential responses between Spd and Spm, the latter being, in some cases, more effective than the former in inducing salt stress-related mechanisms in this *salares* landrace of quinoa. The usefulness of PAs as "biostimulants" has been emphasized and seed priming may be one of the most cost-effective and eco-friendly methods for applying them.

CRediT authorship contribution statement

Stefania Biondi: Writing – original draft, Writing – review & editing, Data curation. Fabiana Antognoni: Data curation, Formal analysis, Writing – review & editing. Lorenzo Marincich: Formal analysis. Mariacaterina Lianza: Formal analysis, Visualization. Ricardo Tejos: Writing – review & editing. Karina B. Ruiz: Conceptualization, Visualization, Data curation, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Supplementary materials

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