Involvement of polyamines in sucrose-induced tolerance to atrazine-mediated chemical stress in Arabidopsis thaliana

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Abstract

Treatment of *Arabidopsis thaliana* seedlings with the PSII-inhibiting herbicide atrazine results in xenobiotic and oxidative stress, developmental arrest, induction of senescence and cell death processes. In contrast, exogenous sucrose supply confers a high level of atrazine stress tolerance, in relation with genome-wide modifications of transcript levels and regulation of genes involved in detoxification, defense and repair. However, the regulation mechanisms related to exogenous sucrose, involved in this sucrose-induced tolerance are largely unknown. Characterization of these mechanisms was carried out through a combination of transcriptomic, metabolic, functional and mutant analysis under different conditions of atrazine exposure. Exogenous sucrose was found to differentially regulate genes involved in polyamine synthesis. *ARGININE DECARBOXYLASE ADC1* and *ADC2* paralogues, which encode the rate-limiting enzyme (EC 4.1.1.19) of the first step of polyamine biosynthesis, were strongly upregulated by sucrose treatment in the presence of atrazine. Such regulation occurred concomitantly with significant changes of major polyamines (putrescine, spermidine, spermine). Physiological characterization of mutant affected in ADC activity and exogenous treatments with sucrose, putrescine, spermidine and spermine further showed that modification of polyamine synthesis and of polyamine levels could play adaptive roles in response to atrazine stress, and that putrescine and spermine had antagonistic effects, especially in the presence of sucrose. This interplay between sucrose, putrescine and spermine is discussed in relation with survival and anti-death mechanisms in the context of chemical stress exposure.

Abbreviations

ADC, Arginine Decarboxylase; AIH, Agmatine Iminohydrolase; ATAO, Amine Oxidase; Atz, atrazine; DAO, Diamine oxidase; DW, dry weight; Man, mannitol; NPL, N-Carbamoylputrescine Amidase; PAO, Polyamine Oxidase; Put, putrescine; ROS, reactive oxygen species; SAMDC, S-Adenosylmethionine Decarboxylase; Spd, spermidine; SPDS, spermidine synthase; Spm, spermine; SPMS, spermine synthase; Suc, sucrose

Keywords: atrazine herbicide, chemical stress, molecular regulations, polyamine dynamics, sucrose-induced tolerance

Introduction

Adaptation of plants to challenging environments involves complex processes associating plasticity of gene expression, regulation of protein levels and activities, and physiological and developmental modifications. Signaling molecules play a central role in these adaptive mechanisms by converting the perception of environmental or endogenous cues into stress defence and repair processes. In addition to its major roles as carbon metabolite and as carbon transport molecule, sucrose (Suc) is now recognised to have important regulatory and signaling effects on transcriptional and post-transcriptional control of gene expression, not only in the context of plant development (León and Sheen, 2003; Lastdrager et al., 2014), but also in the context of stress responses (Loreti et al., 2005; Couée et al., 2006).

Most of the sugar signaling pathways studied by forward and reverse genetic approaches are related to general sensing of carbohydrates rather than to specific sensing of sucrose (Jang et al., 1997; Ruan, 2014; Li and Sheen, 2016). Sucrose-specific signaling is indeed difficult to apprehend since sucrose is involved in active metabolic exchanges with hexoses, hexose phosphates and trehalose (Tiessen and Padilla-Chacon, 2013). Nevertheless, there is ample evidence that sucrose is perceived as a distinct sugar signal, which cannot be substituted by hexoses. Indeed, the existence of sucrose-specific signaling pathways can thus be indirectly established from several processes where equimolar amounts of glucose and fructose fail to induce the same responses as sucrose itself (Li and Sheen, 2016). Furthermore, when sucrose is externally supplied to aerial tissues, emergence of lateral roots is induced in Arabidopsis (Macgregor et al., 2008). Application of sucrose to somatic embryos of spruce results in higher frequency of embryos without ectopic cell division (Iraqi et al., 2005). An Arabidopsis bZIP transcription factor gene (*bZIP11*) in a promoter-GUS reporter gene construct presented an expression specifically affected by sucrose, whereas other sugars, such as glucose and fructose, alone or in combination, are ineffective (Rook et al., 1998). Whole-genome transcript profiling also reveals that complex metabolisms, such as flavonoid and anthocyanin biosynthetic pathways, are strongly and specifically up-regulated following sucrose treatment (Solfanelli et al.,

2006). At a whole plant level, manipulating the rates of synthesis, transport or degradation of sucrose affects plant growth, development and physiology (Wind et al., 2010). Finally, Loreti et al. (2005) have shown that exogenous sucrose, to a much greater extent than glucose, enhances anoxia tolerance of Arabidopsis seedlings, thus indicating that the specific regulatory effects of sucrose are relevant to environmental stress tolerance.

Soluble sugars have also been shown to confer to Arabidopsis plantlets tolerance to atrazine (Atz)-mediated xenobiotic and oxidative stress (Sulmon et al., 2004, 2006, 2007). Binding of this widely-used triazine herbicide to the thylakoid D1 protein results in inhibition of photosystem II (PSII), by blocking electron transfer to the plastoquinone pool, thus leading to production of triplet chlorophyll and $^1O_2$ and therefore to massive oxidative stress, bleaching and plant death (Rutherford and Krieger-Liszkay, 2001). In contrast, exogenous sugar treatment or natural variation of endogenous sugars result in a high level of tolerance to atrazine-mediated stress, with maintenance of chlorophylls, carotenoids, and D1 protein, and protection of photosystem II (Sulmon et al., 2004; Ramel et al., 2009a, 2009b). The protective effects of sugar treatment could not be ascribed to mere carbon feeding of preexisting pathways or to carbon compensation of photosynthesis, and, as in the case of induction of tolerance to anoxic stress (Loreti et al., 2005), exogenous sucrose was more effective than exogenous glucose (Sulmon et al., 2004; Ramel et al., 2009a, 2009b). Moreover, analysis of the impact of natural variation of endogenous sugars showed that sucrose, rather than glucose or fructose, showed the highest correlation with xenobiotic and oxidative stress tolerance (Ramel et al., 2009b). Induction of tolerance was associated with important modifications of gene expression related to photosynthesis and chloroplast biogenesis, to reactive oxygen species (ROS) defense and repair mechanisms, to cell death protection and to metabolic re-orientation, and seemed to result from complex interactions between sugar and xenobiotic signaling (Ramel et al., 2007, 2009a, 2012). The functional categories of these regulatory effects were in accordance with adaptive biochemical processes of atrazine tolerance, including thylakoid reorganization (Mattoo et al., 1984) and ROS quenching and scavenging (Ramel et al., 2009a).

In the present work, characterization of sucrose-induced mechanisms of xenobiotic stress tolerance was carried out through a combination of transcriptomic, metabolic, mutant and exogenous treatment approaches under different conditions of atrazine exposure. Exogenous sucrose was thus found to differentially regulate genes involved in polyamine synthesis. Both *ARGININE DECARBOXYLASE* (*ADC*) paralogues, *ADC1* and *ADC2*, which encode the rate-limiting enzyme (EC 4.1.1.19) at the beginning of the polyamine biosynthesis pathway (Fuell et al., 2010), were strongly transcriptionally upregulated, in terms of transcript accumulation and promoter activity, by sucrose treatment in the presence of atrazine. The potential impact of this gene expression pattern was further characterized through the study of the quantitative variations of major polyamines (putrescine, spermidine, spermine), of xenobiotic stress responses of *spe1-1* mutant affected in overall ADC enzyme activity

(Watson et al., 1998; Kasinathan and Wingler, 2004), and of the effects of exogenous treatments with varying levels of sucrose, putrescine, spermidine and spermine. The adaptive role that can be inferred from this interplay between sucrose, putrescine and spermine is discussed in relation with survival and anti-death mechanisms in the context of chemical stress exposure.

Material and Methods

Plant material

Wild-type *Arabidopsis thaliana* accessions [Columbia (Col-0) and Wassilewskija (Ws) ecotypes] were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Mutant with reduced activity of arginine decarboxylase [*spe1-1* (Watson et al., 1998; Kasinathan and Wingler, 2004)] was a kind gift from Professor Russell Malmberg (University of Georgia, Athens, USA). Arabidopsis *pADC1::*GUS and *pADC2::*GUS transgenic lines were previously described by El Amrani et al. (2002) and Hummel et al. (2004a). Analysis of the *ADC1* and *ADC2* promoter activities were carried out on T_3 homozygotic lines. Wild-type, mutant and transgenic lines were homogenized by single-seed propagation and were bulk-amplified prior to utilization.

Plant growth conditions

Seeds were surface-sterilized for 5-10 min in 50 % bayrochlore/50 % ethanol containing 0.05 % Triton X-100, rinsed twice in absolute ethanol and dried overnight prior to plating on Petri dishes under axenic conditions. After sowing, Petri dishes were kept at 4 °C for 48 h to break dormancy and homogenize germination. Germination and growth under controlled conditions in vertical Petri dishes were carried out at 22 °C under a 16 h light period with a light intensity of 85 µmol m^{-2} s^{-1}. Standard growth media consisted of 0.8 % agar in 0.5X or 1X Murashige and Skoog (MS) basal salt mix (Sigma, St. Louis, MO, USA), pH 5.8. Sucrose (Suc) treatment medium consisted in standard growth medium containing from 15 to 80 mM sucrose. For transcriptomic analysis, sucrose treatment (80 mM) was compared with a control medium consisting in standard growth medium containing 80 mM mannitol (Man) as osmoticum. Atrazine (Atz) treatment medium consisted in standard, Suc-containing or Man-containing growth medium in the presence of varying concentrations of atrazine, as indicated in the Results section. Prior to addition, atrazine was sterilized by microfiltration through 0.2 µm cellulose acetate filters (Polylabo, Strasbourg, France) and added axenically to the melted MS-agar medium. Polyamine treatments consisted in standard growth medium, in the absence or presence of sucrose, and in the presence of varying concentrations of putrescine (Put), spermidine (Spd) or spermine (Spm), as indicated in the Results section. Prior to addition, polyamines were dissolved in water, sterilized by microfiltration through 0.2 µm cellulose acetate filters (Polylabo, Strasbourg, France) and added axenically to the melted MS-agar medium. Atrazine, Put, Spd and Spm were purchased from Sigma (St. Louis, MO, USA). The various combinations and modalities of exogenous xenobiotic and biochemical treatments are described in the Results section and in the Figure legends.

Growth and photosynthetic parameters

At the end of the experiments, length of primary root and fresh weight of seedlings were measured. Pigments were extracted by grinding shoots of four pooled seedlings in 80 % (v/v) acetone, and the absorbance of the resulting extracts was measured at three wavelengths: 663, 646, and 470 nm. Levels of chlorophylls in these extracts were determined from the equations given by Lichtenthaler and Wellburn (1983). Results are given as the mean (±S.E.M.) of these determinations.

Determination of polyamines

Seedlings were harvested, immediately frozen in liquid nitrogen and then lyophilized. Polyamine analyses were performed on pools of whole seedlings, corresponding to at least five individual plants to reach the range of 1 to 10 mg dry weight (DW). Free amines were extracted and analysed, as their dansyl derivatives, by HPLC [column with reverse phase spherisorb ODS-2 (particle size 5 mm, 4.6x250 mm, Waters, Milford, USA), elution with methanol:water solvent gradient from 60 % to 95 % over 23 min, flow rate: 0.8 ml min^{-1}] and spectrofluorimetry (365 nm and 510 nm as excitation and emission wavelengths respectively), as described in Hummel et al. (2002). Amine levels were calculated as mean (±SEM) from measurements on three pools.

Microarray data

Gene expression data were extracted from previous transcriptomic profiling experiments (Ramel et al., 2007) registered as E-MEXP-411 in Array-Express (http://www.ebi.ac.uk/arrayexpress/). These transcriptomic experiments compared the RNA profiles of 1.02 development stage seedlings (Boyes et al., 2001) grown on standard growth medium and then transferred to control and treatment media consisting of various combinations of mannitol, atrazine and sucrose (Ramel et al., 2007). The transcriptomes of Arabidopsis seedlings transferred to control condition (Man, 80 mM mannitol), to condition of atrazine stress (Man-Atz, 10 µM atrazine in the presence of 80 mM mannitol), to condition of exogenous sucrose feeding (Suc, 80 mM sucrose), and to condition of stress tolerance to atrazine exposure (Suc-Atz, 10 µM atrazine in the presence of 80 mM sucrose) were compared after 24 h of treatment. The four conditions were compared pairwise, so that the complete analysis consisted of six comparisons. Differentially expressed genes were those genes showing a P-value ≤ 0.05 after Bonferroni's correction. Conversely, genes with a Bonferroni's P-value higher than 5 % were considered as being not differentially expressed (nde). Current annotations of genes were updated from The Arabidopsis Information Resource (Lamesch et al., 2012).

Expression quantification of polyamine-related genes

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) analysis was performed for 5 candidate genes on the same samples as those described in our previous work (Ramel

et al., 2007). Each sample, corresponding to the Man, Man-Atz, Suc, Suc-Atz conditions described above, consisted in a pool of 3 independent biological replicates (Ramel et al., 2007). Primers were designed with Primer3 (http://fokker.wi.mit.edu/primer3/), with an optimal length of 21 nt, and an optimal temperature of 60 °C, and tested for their PCR efficiency, which ranged between 90 % and 99 % (Supplementary Table A1). Four independent cDNA synthesis reactions were realized. Reverse transcription was performed on 1 µg of total RNA with oligodT primer (18 mer) and the Superscript II Rnase H- reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Three PCR replicates for each of the cDNAs were included in every run. Three template controls were included in every qPCR experiment. Quantitative PCR reactions were performed with SYBRGreen PCR master mix (Eurogentec, Seraing, Belgium). All reactions were performed using the ABI PRISM 7900 HT sequence Detection System (Applied Biosystem, Pleasanton, CA) as follows: 95 °C, 10 min; 40x (95 °C, 15 s; 60 °C, 1 min) and a dissociation step to discriminate primer dimers from the PCR product. Data were then analysed by using the SDS software provided by the manufacturer. Gene expression was quantified relative to the level of expression of the housekeeping genes *ACTIN2* and *RPN7* (Supplementary Table A1).

Histochemical analysis

Histochemical GUS staining was performed as described previously by Jefferson et al. (1987). Transgenic *pADC1::GUS* and *pADC2::GUS* homozygous plants (El Amrani et al., 2002) were grown as described above and stained for GUS activity at 37 °C during eight hours, in 50 mM potassium phosphate buffer, pH 7, containing 0.1 % Triton X-100, 10 mM $K_3Fe(CN)_6$ and 10 mM $K_4Fe(CN)_6$ to avoid diffusion of the intermediary reaction product. Plantlets were washed with 50 mM sodium phosphate buffer (pH 7) and then left in 70 % ethanol overnight.

Statistics

All results were given as the mean (±SEM). Statistical analysis was carried out with the Minitab version 17 software. Pairwise comparisons of means used Student's t-test and Mann-Whitney test.

Results

Genome-wide analysis reveals regulatory effects of exogenous sucrose on polyamine metabolism genes under conditions of atrazine xenobiotic stress

As previously characterised (Ramel et al., 2007, 2009a), 10 µM atrazine treatment in the absence of sucrose induced complete bleaching of seedlings after 6–7 days of stress application, thus leading to seedling death within 8 days. In contrast, addition of exogenous sucrose during atrazine treatment allowed seedlings to maintain growth and development beyond 8 days of transfer (Ramel et al., 2007, 2009a). Genome-wide analysis of gene expression through a CATMA approach was therefore carried out after 24 h atrazine treatment in the presence of exogenous Suc or Man as control. In this

microarray experiment, atrazine and Suc showed important effects on transcriptome dynamics (Ramel et al., 2007, 2009a), while global development and photosynthetic activity were slightly affected. The atrazine treatment that led to injury and plant death was associated with the repression of genes involved in protein translation and ROS defence. Conversely, the situation of sucrose protection against atrazine stress and injury was associated with activation of genes involved in transcription processes, cellular repair and protection, signal transduction, cellular communication, photosynthesis, and anti-ROS defence. Closer analysis of differentially-expressed genes reveals that, surprisingly, expression of genes involved in polyamine biosynthesis pathway showed differential patterns of response to Suc-Atz, Suc, and to Man-Atz treatments (Fig. 1A), specifically highlighting the contrasted patterns of expression of *ADC* genes. Indeed, the two *ADC1* and *ADC2* paralogues that encode the ADC enzymes at the start of the biosynthesis pathway were highly activated by exogenous sucrose in the absence or presence of atrazine, whereas atrazine in absence of Suc either induced or repressed significantly *ADC* genes. In particular, focusing on combined sucrose and atrazine treatment, *ADC2* gene exhibited the strongest induction of expression, which was also confirmed by RT-qPCR (Fig. 1B). In contrast, expression of *AGMATINE IMINOHYDROLASE* (*AIH*) and *N-CARBAMOYLPUTRESCINE AMIDASE* (*NLP1*), as well as of *SPERMIDINE SYNTHASE* (*SPDS*) and *SPERMINE SYNTHASE* (*SPMS*) genes, which encode the enzymes involved in subsequent steps of, respectively, Put, and Spd and Spm synthesis, showed no significant variation, whether under conditions leading to injury and plant death or under conditions of sucrose protection (Fig. 1). Moreover, one *S-ADENOSYLMETHIONINE DECARBOXYLASE* (*SAMDC)* encoding gene was highly activated by Man-Atz and Suc treatments. Considering Put, Spd, and Spm back-conversion and catabolism, three *POLYAMINE OXIDASE* (*PAO*) genes were significantly repressed under atrazine exposure in the absence of exogenous sucrose, and one *DIAMINE OXIDASE* (*DAO*) gene was significantly repressed under atrazine treatment in the presence of exogenous sucrose (Fig 1A).

Effects of exogenous sucrose on the regulation of *ADC1* and *ADC2* promoter activities in the presence of atrazine

The increase of *ADC* gene expression under conditions of sucrose-induced protection against atrazine injury was further analysed by a promoter::reporter gene approach. Promoter activities were studied in stable homozygous transformants harboring promoter::reporter gene fusions. Homozygous plants expressing GUS as reporter gene, under the control of *ADC1* or *ADC2* promoters, including the transcribed 5'UTR, were generated by El Amrani et al. (2002). Exogenous sucrose alone was found to induce significantly both *ADC1* and *ADC2* promoter activities (Fig. 2). In the presence of atrazine alone, promoter activities of both *ADC1* and *ADC2* were either not detected or at very low level. In Suc-Atz treatments, promoter activities were found to be positively correlated with the level of sucrose in a dose-dependent manner, as shown in Fig. 2. Particularly, *ADC2* promoter exhibited, in such conditions, a stronger response than *ADC1* promoter. Moreover, comparing *ADC* promoter responses

observed under similar Suc treatment (25 mM) but in the absence and presence of atrazine, highlighted, as found in gene expression results (Fig. 1), a repressive effect of atrazine on *ADC1* promoter activity, which was not observed for *ADC2*. These results strongly suggested that the *ADC2* paralogue gene may play a major role under conditions of Suc-induced protection against atrazine injury. This was reinforced by the pattern of transcriptional activity of *ADC2*, which was more pronounced and generalized in both roots and shoots, whereas GUS staining was localized only in the aerial parts in *pADC1*::GUS transgenic lines. This important and specific regulatory effect of exogenous sucrose on *ADC1* and *ADC2* gene expression suggested that the dynamics of polyamine metabolism may be significantly affected under conditions of sucrose-induced protection against atrazine injury.

Impact of mutation affecting ADC enzyme activity on sucrose-induced atrazine tolerance

To analyse the physiological role of ADC activity in atrazine sensitivity and sucrose-induced atrazine tolerance, we determined the effects of reduced ADC activity on atrazine and sucrose responses in Arabidopsis *spe1-1* mutant. This mutant, which has been previously described (Watson et al., 1998, Kasinathan and Wingler, 2004), exhibits decreased ADC activity. Leaves of *spe1-1* have been shown to contain about one quarter of wild-type ADC activity (Watson et al., 1998) and reduced levels of both ADC1 and ADC2 proteins (Kasinathan and Wingler, 2004). Whole plants of *spe1-1* mutant also show perturbations of polyamine dynamics under salt stress conditions in comparison with wild-type plants (Kasinathan and Wingler, 2004). *spe1-1* mutant phenotype was analysed after 15 days of growth on atrazine and sucrose treatments. Under conditions of atrazine sensitivity (atrazine alone), *spe1-1* seedlings exhibited the expected growth inhibition resulting from atrazine exposure (Fig. 3). Moreover, growth in the presence of sucrose similarly led to the expected enhancement of root growth for this mutant. However, *spe1-1* mutant exhibited contrasted phenotype in comparison to WT, under conditions of sucrose-induced protection against atrazine. Indeed, *spe1-1* failed to develop atrazine tolerance despite the presence of exogenous sucrose (Fig. 3). Thus, the atrazine sensitivity of the *spe1-1* mutant under conditions of sucrose treatment agreed with the involvement of ADC induction in sucrose-induced protection against atrazine.

Role of polyamine dynamics in atrazine sensitivity and sucrose-induced atrazine tolerance

In order to compare the differential dynamics of polyamines under conditions of atrazine sensitivity and sucrose-induced atrazine tolerance, Arabidopsis seedlings were exposed, during 15 days starting from germination, to a sublethal concentration of atrazine (0.5 µM) in the absence or presence of sucrose. Under conditions of sensitivity, Arabidopsis responses to atrazine were restricted to a strong increase of Spd levels (Fig. 4). In contrast, all the polyamines analysed were found to be highly responsive to exogenous sucrose, with Put, Spm, and Spd levels being significantly increased. However, in the situation of sucrose-induced atrazine tolerance, atrazine specifically modulated

sucrose-response patterns of the three polyamines. Whereas Spm levels were strongly decreased under atrazine tolerance in comparison to sucrose treatment, Put levels were found to be significantly increased. In contrast, endogenous concentrations of Spd remained unchanged. Sucrose-induced atrazine tolerance could thus be directly linked to differential regulatory effects of sucrose on the different steps of polyamine synthesis leading to increased level of Put and decreased level of Spm, and to induction of stress-tolerance and survival processes. In other words, it was tempting to hypothesise that sucrose-induced differential dynamics of polyamines may play a major role in alleviation, by sucrose addition, of the deleterious effects of atrazine.

Antagonistic effects of exogenously-applied putrescine and spermine on the responses to atrazine xenobiotic stress in the absence or presence of sucrose

Given the involvement of polyamine dynamics in atrazine sensitivity and sucrose-induced atrazine stress tolerance, we tested whether exogenous treatments with different polyamines (putrescine, spermidine, spermine) could have differential impacts on atrazine responses in the absence or presence of sucrose.

The effects of exogenous Put, Spm, and Spd on Arabidopsis atrazine sensitivity in the absence of sucrose were analysed under conditions of sublethal (0.5 µM) and lethal (1 µM) atrazine treatments (Fig. 5A). Considering primary root growth, Put and Spd exogenous treatments conferred, in the absence of sucrose, and after 15 days of growth, atrazine tolerance at least for the sublethal concentration. In contrast, Spm exposure increased atrazine sensitivity, with seedlings exhibiting at 0.5 µM atrazine the same length of primary root as that of 1 µM atrazine treatment (Fig. 5A). Such differential impacts resulting from exogenous polyamine treatments were associated with modifications of endogenous polyamine levels (Supplementary Figure A1). Indeed, Put, Spd, and Spm exogenous treatments were found to strongly induce their corresponding polyamine endogenous level in seedlings. Moreover, Spd and Spm treatments were also found to similarly increase endogenous levels of Put in comparison to control condition. Nevertheless, these increases remained lower than that observed under exogenous Put treatment. In the same way, Spm slightly increased endogenous Spd content.

The effects of exogenous polyamines on sucrose-induced atrazine protection were also tested by applying an additional step of 20 h of 1 mM polyamine pre-incubation treatment, in order to maximize polyamine-related regulations. After 5 days of treatment, plant growth and development were determined. In the presence of sucrose, roots (Fig. 5B), cotyledons and shoot meristem escaped atrazine injury and the first leaves appeared and underwent normal development (Supplementary Figure A2). Exogenous treatments with the three different polyamines resulted in strikingly different effects on sucrose-induced atrazine protection, in the same way as those observed under atrazine sensitivity conditions (Fig. 5). Thus, whereas exogenous Spd did not show any significant effect, exogenous Spm was found to prevent sucrose-induced atrazine tolerance and even enhanced atrazine-

related stress with cotyledon bleaching after 5 days of treatment (Supplementary Figure A2). In contrast, exogenous Put significantly increased sucrose-induced root development in the presence of atrazine, with as much as a 25 % increase in comparison with sucrose plus atrazine treatment (Fig. 5B).

The specific effect of Put was further investigated under conditions of strong Put chemical priming. Chemical priming was performed by pre-incubating 4 day-old seedlings for 20 h in standard growth medium containing 6 mM Put, whereas unprimed seedlings were pre-incubated in standard growth medium. Seedlings were then transferred on treatment media corresponding to 1 µM atrazine in the presence or absence of sucrose and Put, and were analysed after 18 days of growth (Fig. 6). Under conditions of atrazine exposure, high level of Put, in combination with sucrose, positively modulated several markers of sucrose-induced atrazine tolerance (fresh biomass, shoot chlorophyll contents, primary root growth; Fig. 6). Moreover, exogenous Put also activated lateral root formation in comparison with plantlets grown in the presence of sucrose without additional Put (Fig. 6C). Lateral roots are derived from the deep pericycle layer within parent root tissues *(*Malamy and Benfey, 1997*),* and the first stages of Lateral Root Primordia (LRP) are therefore an inconspicuous process. Histological analysis of stage VI LRPs (Malamy and Benfey, 1997) showed that exogenous Put in combination with the presence of sucrose had therefore an important effect on LRP initiation and development under atrazine exposure, with an 8-fold increase in the number of LRPs per plantlet (Fig. 6C). Put was therefore shown to have specifically strong effects on atrazine responses, reducing atrazine sensitivity in the absence of sucrose (Fig. 5A), and enhancing sucrose-induced atrazine tolerance in the presence of sucrose (Figs. 5-6), in sharp contrast with the negative effects of Spm (Fig. 5).

Discussion

Exogenous sucrose modifies polyamine dynamics under conditions of xenobiotic exposure through transcriptional regulation of *ADC1* and *ADC2* expression

Exposure to abiotic stresses such as drought, cold, heat, and pollutants, including herbicides and heavy metals, generally gives rise to excess accumulation of ROS (Price et al., 1989; Bowler et al., 1992; Stohs and Bagchi, 1995; Schützendübel and Polle, 2002; Ramel et al., 2009a). Previous investigations showed that exposure to atrazine results in a typical situation of abiotic stress with increases of ROS production leading to plant death (Rutherford and Krieger-Liszkay, 2001; Ramel et al., 2009a). However, when exogenous sucrose is added or when endogenous sucrose increases, ROS accumulation is reduced (Ramel et al., 2009a, 2009b). The molecular mechanisms involved in this process seem to be complex as condition of sucrose-induced atrazine tolerance specifically induced and repressed 1107 and 921 genes, respectively, compared with atrazine condition (Ramel et al., 2007). Comparison of different sugar feeding conditions, mutant analysis and the dynamics of ROS production show that sucrose enhancement of atrazine tolerance must be ascribed to regulatory and

signaling effects, rather than to mere nutritional and metabolic effects (Sulmon et al., 2004, 2007; Ramel et al. 2009a). This reflects the idea that sucrose plays pivotal roles as a signaling molecule. Although no sucrose sensor has been identified yet, accumulating transcriptomic data have shown that sucrose specifically and dramatically affects plant development and stress tolerance by controlling a myriad of genes (Loreti et al., 2005; Solfanelli et al., 2006; Ramel et al., 2007, 2009a).

The present study shows that genes involved in polyamine biosynthesis present a very contrasted pattern of differential expression under conditions of sucrose-induced atrazine tolerance. In particular, genome-wide transcriptomic analysis, gene expression quantification and promoter activity studies (Figs. 1-2) reveal that *ADC1* and *ADC2* genes are transcriptionally controlled by exogenous sucrose under conditions of atrazine exposure. The transcripts of *ADC1* and *ADC2* paralogues were greatly increased under conditions of sucrose-induced atrazine tolerance, and promoter::reporter gene approaches in homozygous transgenic plants expressing *pADC1*::GUS and *pADC2*::GUS further showed that this regulation was due to a transcriptional control. The corresponding ADC-catalysed reaction, which produces agmatine from arginine, is placed at the beginning of the polyamine synthesis pathway that leads to Put, Spd and Spm synthesis. In higher plants, Put can be produced by ADC- or Ornithine Decarboxylase(ODC)-catalysed reactions. However, no ODC-encoding gene was found in the sequenced genome of the model plant *Arabidopsis thaliana* (Hanfrey et al., 2001; Liu et al., 2015). This is why, in Arabidopsis, the rate-limiting ADC-catalysed reaction represents the key connection between amino acid metabolism, putrescine production and polyamine metabolism.

On the other hand, in Arabidopsis and in other Brassicaceae species, the two *ADC* paralogues, generally called *ADC1* and *ADC2*, exhibit complex patterns of differential expression and potential subfunctionalization (Hummel et al., 2004a, 2004b). In *Arabidopsis thaliana*, induction of *ADC2* is associated with osmotic stress, wounding, light, and salinity (Hummel et al., 2004a; Podlešáková et al., 2019), whereas *ADC1* is particularly responsive to chilling (Podlešáková et al., 2019). The present work shows that, under conditions of xenobiotic exposure and potential stress, *ADC2* was the most sucrose-responsive of the genes related to polyamine biosynthesis and was therefore likely to play an important role in the sucrose- and atrazine-dependent variations of polyamine levels (Fig. 4). Moreover, *ADC2* was found to be significantly more responsive to exogenous sucrose than *ADC1* (Fig. 2), as has been shown to occur under conditions of optimal growth (Hummel et al., 2004a), thus reflecting important differences of regulation (El Amrani et al., 2002) and suggesting substantial functional differences between *ADC1* and *ADC2*.

At the protein level, analysis of the responses of *spe1-1* mutant clearly showed that mutation decreasing both ADC1 and ADC2 protein levels and affecting global ADC activity (Watson et al., 1998; Kasinathan and Wingler, 2004) led to an impairment of sucrose induction of atrazine tolerance (Fig. 3). This thus confirmed the adaptive importance of *ADC* gene expression and metabolic impact in such context of atrazine tolerance.

Importance of sucrose/putrescine/spermine crosstalk as a mechanism of xenobiotic and oxidative stress tolerance

In parallel with the regulation of *ADC1* and *ADC2* expression, carbon status (absence or presence of exogenous sucrose) and exposure to xenobiotic stress resulted in significant and antagonistic changes of polyamine levels in a differential manner, such as the increase of Put and the decrease of Spm (Fig. 4). Such differential dynamics demonstrated that additional regulations were active downstream of the ADC-catalysed step. In the case of atrazine exposure and of sucrose addition in the absence of atrazine, regulations of *PAO* and/or *SAMDC* gene expression (Fig. 1A), involved respectively in polyamine catabolism and in DcSAM production, could, at least in part, explain these differential dynamics. However, the weak responsiveness of non-ADC genes, and in particular of *SPDS* and *SPMS* genes, to the situation of sucrose-induced atrazine tolerance (Fig. 1) strongly suggested that, besides the transcriptional regulation of *ADC1* and *ADC2*, the differential dynamics of Put, Spd and Spm was likely to depend also on post-transcriptional and post-translational regulations (Chang et al., 2000; Guerrero-González et al., 2014). Thus, even though sucrose signaling is a complex and poorly understood process, the present work emphasises that sucrose addition can lead to sucrose-dependent regulations modulating polyamine dynamics in parallel with enhanced abiotic stress tolerance.

There is accumulating evidence that polyamines are involved in abiotic stress responses, senescence processes and programmed cell death (Handa and Mattoo, 2010; Moschou and Roubelakis-Angelakis, 2014; Liu et al., 2015). Polyamines can act as general protective molecules as they enhance tolerance to various abiotic stresses such as salt (Liu et al., 2015), heavy metals (Groppa et al., 2007), chilling (Hummel et al., 2004c; Liu et al., 2015), and flooding (Yiu et al., 2009), which are all known to generate ROS and oxidative stress. Yiu et al. (2009) reported that exogenous application of Put resulted in reduced superoxide radical ($O_2^{\cdot-}$) and H_2O_2 contents, and thereby less oxidative stress in plant cells. Their findings suggested that Put can confer abiotic stress tolerance through inducing the activities of various anti-oxidative systems. Polyamines have also been shown to be free radical scavengers and protectants against ozone damage (Bors et al., 1989). Moreover, polyamines are interconnected with other metabolic pathways involved in the formation of various signaling molecules and metabolites that are involved in plant stress responses and development (Liu et al., 2015; Podlešáková et al., 2019). Thus, polyamines and ethylene biosynthesis are metabolically connected through S-adenosylmethionine (SAM) which acts as a common precursor (Takahashi and Kakehi, 2010; Podlešáková et al., 2019). Different connections also link polyamines with ROS dynamics (Moschou and Roubelakis-Angelakis, 2014). Additionally, Put, Spd and Spm are known to have differential impacts on plant metabolism, whether under conditions of optimal development or under conditions of abiotic stress (Handa and Mattoo, 2010). Finally, polyamines have been listed as promising naturally-occurring metabolites that can promote chemical priming and hardening against multiple abiotic stresses (Savvides et al., 2016). However, the exact mechanisms underlying the roles played by polyamines in stress responses remain to be fully elucidated.

The various modalities of exogenous treatments with polyamines (Figs. 5-6) clearly showed that variations of polyamine levels directly acted on the sensitivity or tolerance responses to atrazine exposure. However, whereas the tolerance response to salt stress has been associated with parallel increases of Put, Spd and Spm (Kasinathan and Wingler, 2004), the relationship between polyamine dynamics and the responses to atrazine was more complex. Indeed, atrazine tolerance was associated with increase of Put and decrease of Spm, and Spm was associated with enhancement of sensitivity (Figs. 4-5). In contrast, Spm has been shown to exert, in wheat, anti-oxidant and protective effects under conditions of heavy-metal-induced oxidative damage (Groppa et al., 2007), and to have, in Arabidopsis, anti-senescence properties under conditions of dark-induced senescence (Sequera-Mutiozabal et al., 2016). In other words, the polyamine response involved in sucrose-induced atrazine tolerance reflected the general involvement of putrescine in stress tolerance and the versatile relationship of Spm with stress tolerance (Takahashi and Kakehi, 2010), versatility probably related to specific molecular and physiological contexts. Put was thus found to alleviate atrazine-mediated stress and injury, and more particularly under priming experiments (Figs. 5-6). Indeed, Put preexposure strongly increased the tolerance to atrazine conferred by sucrose treatment (Figs. 5-6), thus confirming the interest for chemical priming proposed by Savvides et al. (2016). In line with the impact of soluble sugars on responses to abiotic stresses (León and Sheen, 2003; Sulmon et al., 2004; Loreti et al., 2005; Couée et al., 2006; Solfanelli et al., 2006; Li and Sheen, 2016), it was therefore shown that sucrose/polyamines cross-talk was important for the responses to xenobiotic stress, and regulated root growth and development processes (Figs.5-6), in line with the effects of sucrose (Macgregor et al., 2008) and polyamines (Couée et al., 2004) on root development.

Importance of sucrose/putrescine/spermine crosstalk in survival and death of the chemically-stressed plant

Molecular and physiological analysis of the effects of atrazine and of exogenous sucrose on Arabidopsis (Ramel et al., 2007, 2009a) showed that initiation of atrazine-mediated chemical stress and toxicity involved inefficient regulation of singlet oxygen ($^1O_2$) quenching and ROS scavenging, thus intensifying $^1O_2$ and ROS accumulation. This situation was also shown to evolve into disruption of cellular homeostasis, induction of senescence-like processes and activation of cell death programs, in line with the programmed cell death effects of singlet oxygen (Wagner et al., 2004) and of some xenobiotics (Ramel et al., 2012). The growth arrest and bleaching events that were observed under conditions of atrazine toxicity (Figs. 5-6) reflected these programmed cell death effects. Conversely, exogenous sucrose, which was associated to more efficient $^1O_2$ quenching and lower $^1O_2$ accumulation in plantlets (Ramel et al., 2009a), and also exogenous putrescine, which was found to reduce $O2^{\cdot-}$ and H_2O_2 contents, and thus oxidative stress, in plant cells (Yiu et al., 2009), could lift growth arrest effects and activate development. In contrast, Spm enhanced developmental arrest and bleaching in the

absence, but also, in the presence of sucrose (Figs. 5-6). Spermidine was found to have an intermediate behaviour in the sense that it did not enhance further sucrose-induced atrazine tolerance (Fig. 5B), but had a slight positive effect on tolerance to low levels of atrazine (Fig. 5A). Since exogenous sucrose and polyamine treatments did not lead to similar trends, and since polyamines could alter positively (Put) or negatively (Spm) the induction of atrazine tolerance by sucrose (Figs. 5-6), it was therefore clear that the sucrose/polyamines crosstalk had important effects on programmed cell death and survival, as schematically hypothesized in Fig. 7. The pro-survival and anti-death effects of exogenous sucrose could be ascribed to this mechanism of sucrose/polyamines crosstalk (Fig. 7), through the regulation of polyamine metabolism by sucrose (Figs. 1,4), the context-dependent functions of polyamines in survival or programmed cell death (Moschou and Roubelakis-Angelakis, 2014), the increased antioxidative status of sucrose-treated plants (Couée et al., 2006), and the potential role of sucrose in preventing programmed cell death (Tognetti et al., 2013). Crosstalk with abscisic acid has also been shown to be important for the effects of polyamines on abiotic stress responses (Shi and Chan, 2014). The regulatory effects of polyamines and sucrose may thus be connected through abscisic acid (Rook et al., 2006). However, the specificities of sucrose/polyamine crosstalk in the present work would require further characterization with mutant studies.

Author contributions

A.E.A, I.C., G.G. and C.S. conceived the study and designed experiments. A.E.A, R.B., F.R., G.G. and C.S. performed experiments. A.E.A, I.C., R.B., G.G. and C.S. carried out analysis and interpretation of experimental data including bioinformatics and statistical analyses. The article, which all authors edited and approved, was written by A.E.A, I.C., R.B., G.G. and C.S.

Acknowledgements

This work was supported by the interdisciplinary program "Ingénierie écologique" (CNRS, France), by Rennes Métropole (France) local council, and by recurrent funding from the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (France) and the Centre National de la Recherche Scientifique (France). We gratefully acknowledge the kind gift of *spe1-1* mutant seed from Professor Russell Malmberg (University of Georgia, Athens, USA). We also gratefully acknowledge Dr Françoise Hennion (University of Rennes, ECOBIO UMR 6553, Rennes, France) for help in polyamine analysis.

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Figure legends

Figure 1: Effects of atrazine and sucrose on transcript levels of genes involved in the metabolic pathway of major polyamines (putrescine, spermidine and spermine). Atz (10 µM) and Suc (80 mM) treatments were carried out as described in material and methods. Man (80 mM) was used as osmotic control. Microarray data are shown as $\log_2$ ratio of gene expression under indicated conditions (A). Differentially expressed genes were those genes showing a P-value ≤ 0.05 after Bonferroni's correction. Conversely, genes with a Bonferroni's P-value higher than 5 % were considered as being not differentially expressed (nde). The nomenclature of *DAO* genes is as suggested by Shelp et al. (2012). For RT-qPCR (B), RNAs were reverse-transcribed and cDNAs were used. Three replicates were used for each experimental condition. Man: Mannitol, Atz: Atrazine, Suc: Sucrose.

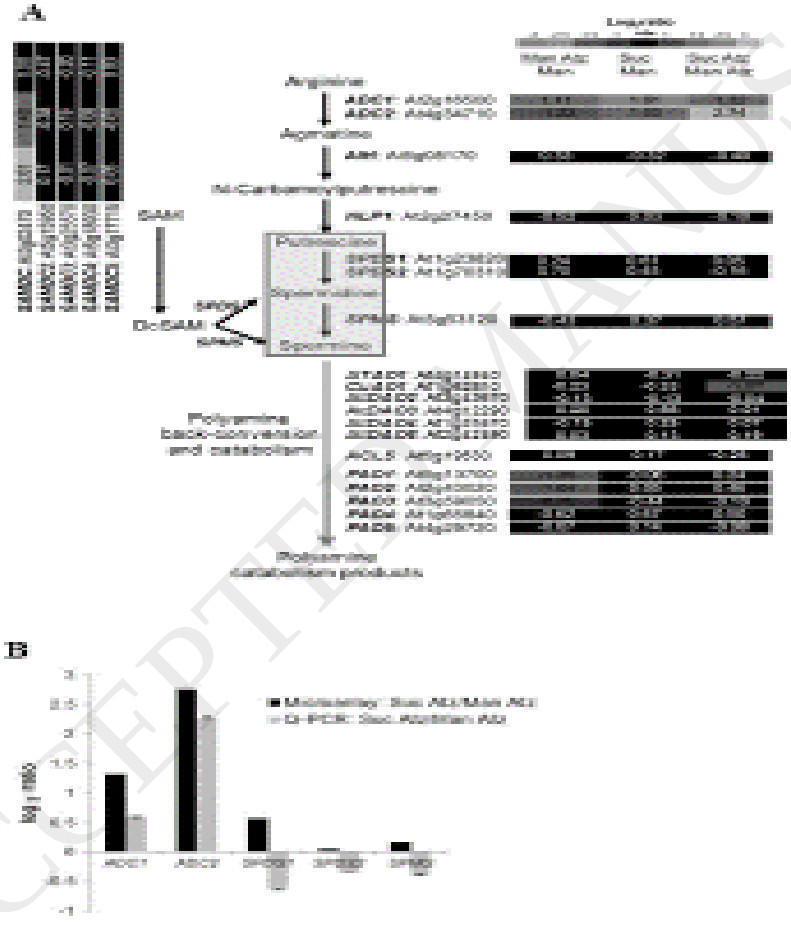

Figure 2: Histochemical staining of *ADC* promoter::GUS expression in homozygous transgenic Arabidopsis (Ws) plants under atrazine or sucrose+atrazine conditions. Promoter activities of ADC1 and ADC2 were studied in stable homozygotic transformants harboring promoter::GUS reporter gene fusions. Homozygotic lines were grown for 3 days on MS medium, then transferred for 4 days on the indicated medium (black bar represents 1 mm).

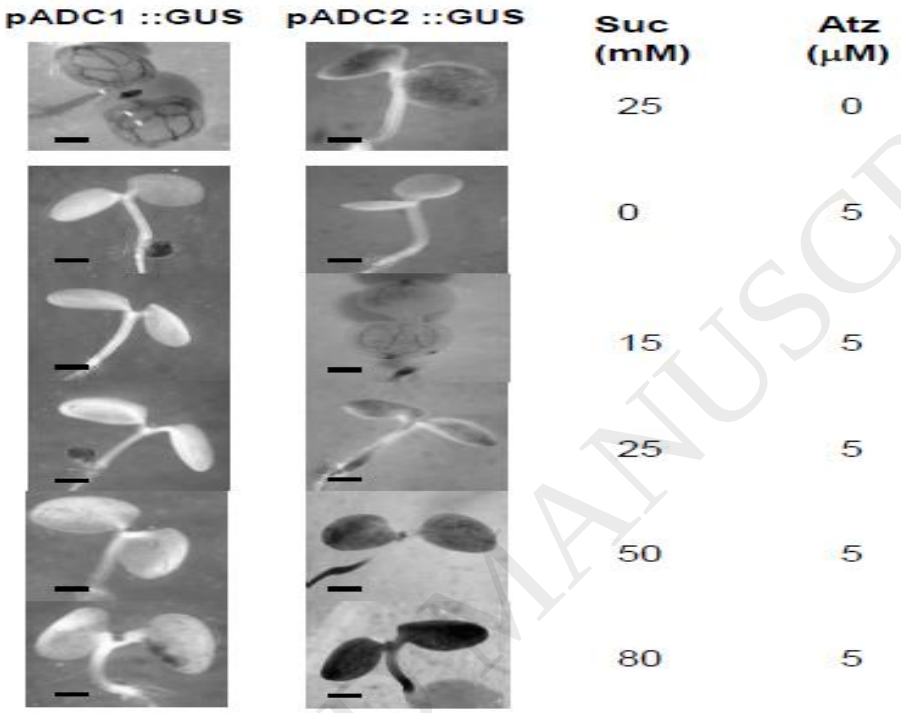

Figure 3: Responses of *spe1-1* mutant and of its isogenic WT (Col-0) to atrazine in the absence and in the presence of sucrose. Seedling morphology (A) and primary root length (mean ± S.E.M.) (B) are shown. Seed germination and seedling growth were carried out under the conditions of the different treatments, which consisted in the different combinations of absence (-) or presence (+) of 1 µM Atz and 80 mM Suc in MS medium. Seedlings were measured and photographed 15 days after germination. Statistical analysis between means was carried out using the Mann–Whitney test. Statistical significance of differences ($P \leq 0.05$) between treatments and plant lines is indicated by different letters above bars.

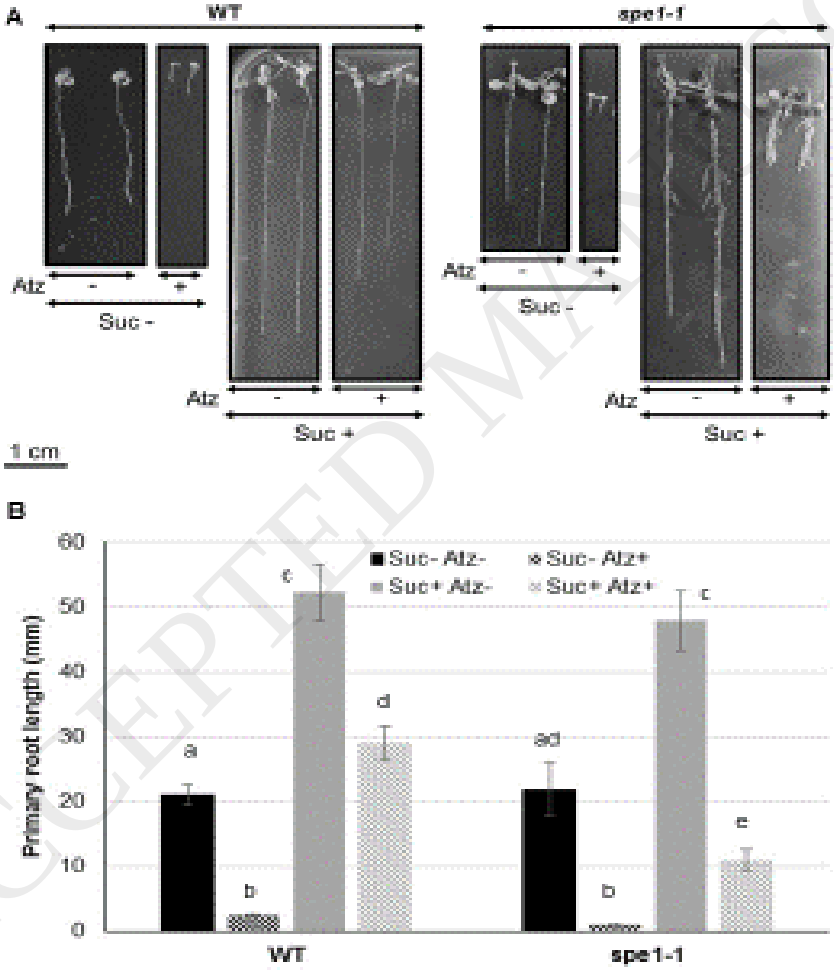

Figure 4: Effects of atrazine on polyamine levels in Arabidopsis (Ws) seedlings grown in the absence or presence of sucrose. The levels of putrescine, spermidine, and spermine are shown. Seed germination and seedling growth were carried out under conditions of the different treatments, which consisted in 0.5 µM Atz in the absence (Suc-) or presence (Suc+) of 80 mM Suc in MS medium. Values are the mean (±S.E.M.) of measurements of three independent replicates of pooled 15-day-old plantlets. Statistical analysis between means was carried out using the Mann–Whitney test. Statistical significance of differences ($P \leq 0.05$) between treatments is indicated by different letters above bars.

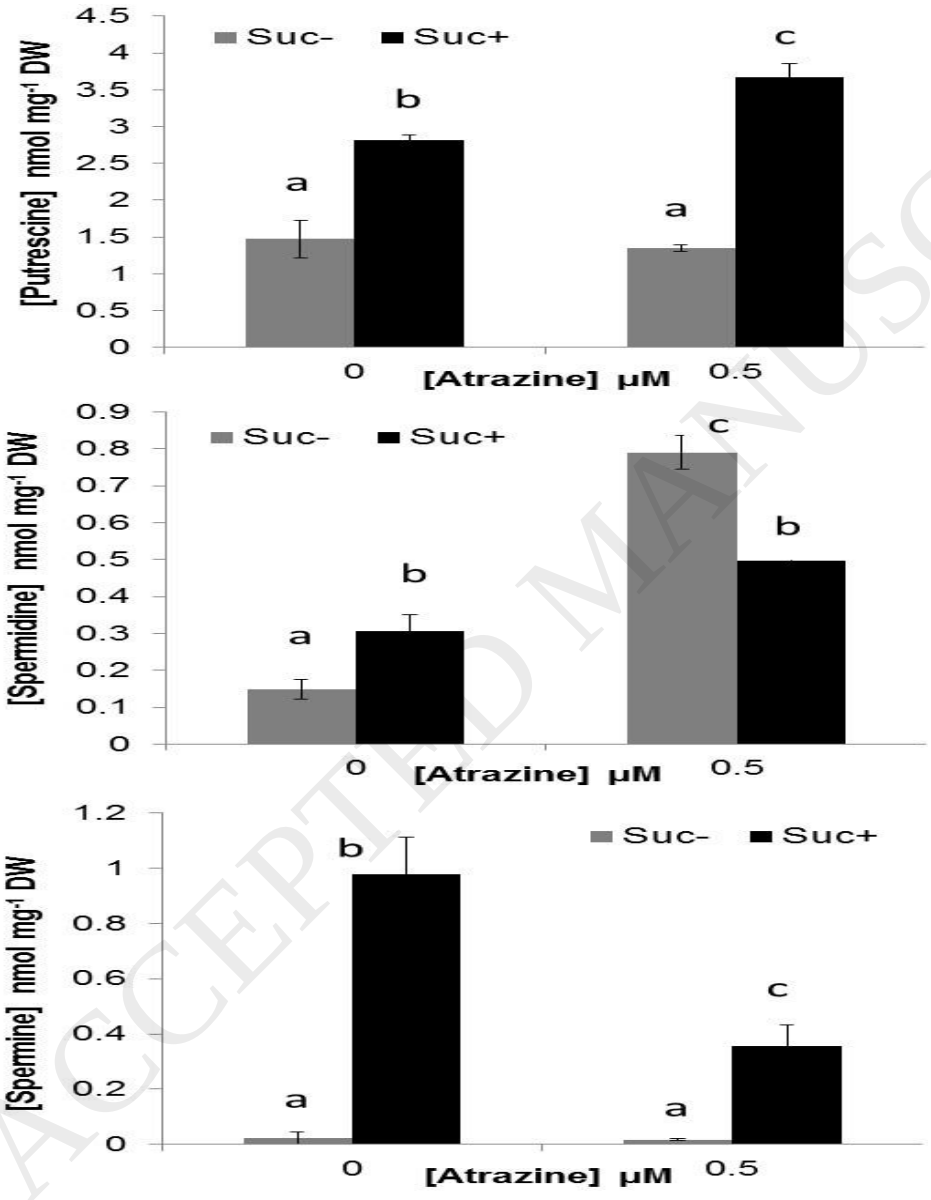

Figure 5: Effects of exogenous polyamine treatments on atrazine responses and sucrose-induced atrazine protection of Arabidopsis (Ws) seedlings. Primary root length is shown as means (± S.E.M.). (A) Seed germination and seedling growth were carried out under the conditions of the different treatments, which consisted in the different combinations of absence or presence of 0.5 or 1 µM Atz and of absence (Control) or presence of 0.5 mM of either Put, Spd, or Spm in MS medium. Seedlings were analysed after 15 days of treatment. Mean comparisons were conducted by Mann–Whitney test between atrazine treatments (0; 0.5; 1 µM) independently for each exogenous polyamine treatment. (B) Four-day old plantlets were grown on 0.5X MS medium and transferred either on 0.5X MS medium (Control), or on the same medium containing 1 mM Put, Spd or Spm for 20 h. Plantlets were then transferred on Suc (25 mM) and Atz (1 µM) containing media supplemented with 1 mM of either Put, Spd, or Spm. Seedlings were analysed after 5 days of treatment. Statistical analysis between means was carried out using t-test. Statistical significance of differences ($P \leq 0.05$) between compared means is indicated by different letters above bars (A, B).

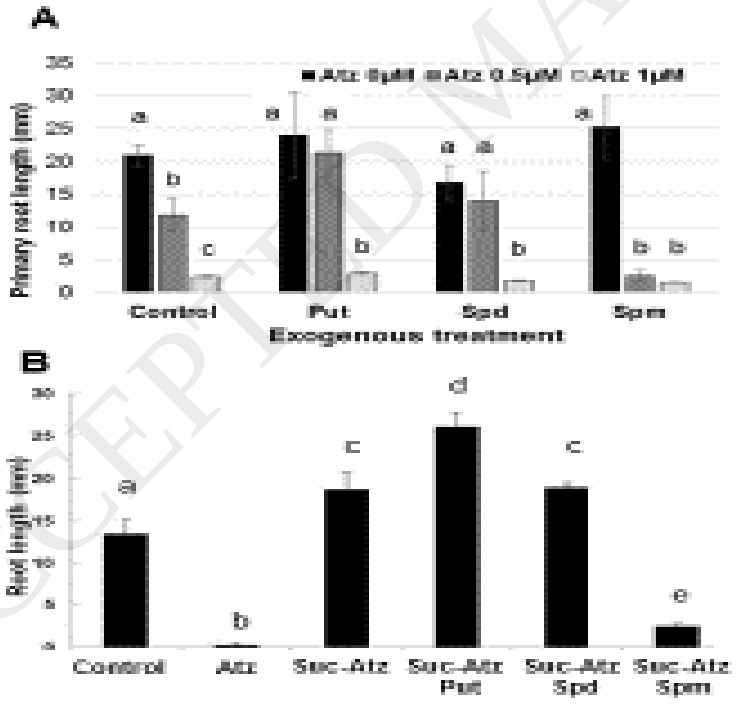

Figure 6: Putrescine-mediated enhancement of the protective effect of sucrose under atrazine exposure. Four-day old seedlings (Ws) were separated in two groups. In the first one, seedlings were transferred onto control medium (0.5X MS medium) for 20 hours, and then further grown in the presence of atrazine (1µM) alone or under atrazine-protected conditions (Suc 80 mM + Atz 1µM). In the second one, seedlings were transferred to the MS medium supplemented with Put (6 mM) for 20 hours, and then further grown in a medium containing either Atz (1 µM) and Put, or Atz (1µM), Put and Suc (80mM). Seedlings were analysed after 18 days of growth. Morphological changes (A), fresh weight and chlorophyll contents (B), and root length and lateral root primordium count (C) are presented. Values correspond to the means of at least 20 measurements and bars represent the standard errors of the means. Statistical analysis between means was carried out using the Mann–Whitney test. Statistical significance of differences ($P \leq 0.05$) between treatments is indicated by different letters above bars.

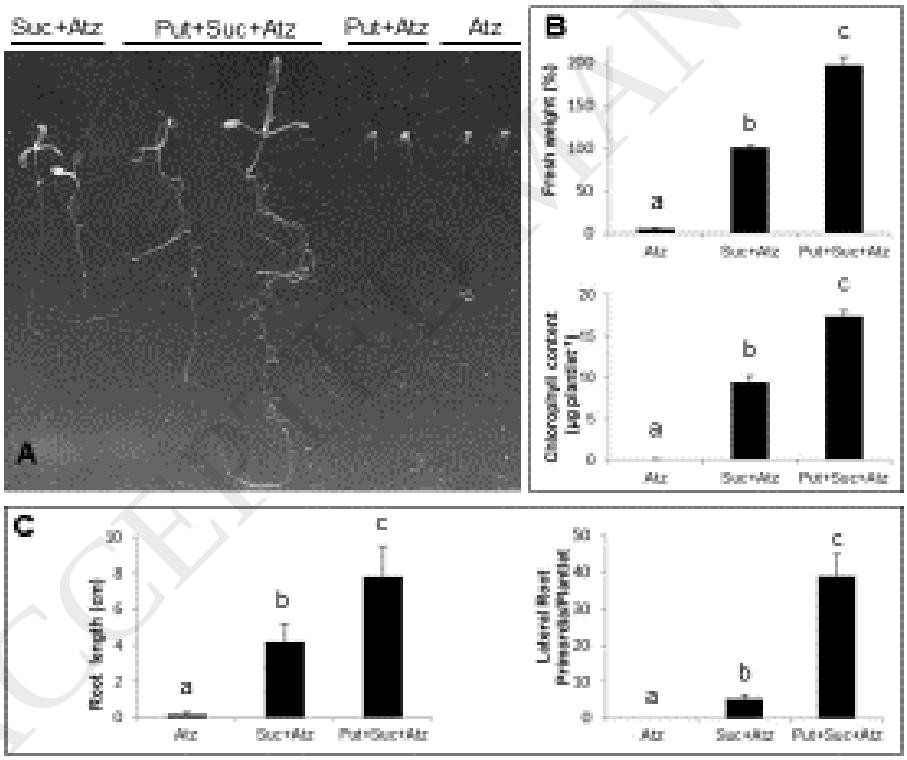

Figure 7: Hypothetical scheme of the regulatory effects of sucrose and polyamines on death and survival processes in the chemically-stressed plant.