Putrescine differently influences the effect of salt stress on polyamine metabolism and ethylene synthesis in rice cultivars differing in salt resistance

Muriel Quinet, Alexis Ndayiragije, Isabelle Lefèvre, Béatrice Lambillotte, Christine C. Dupont-Gillain and Stanley Lutts

1 Groupe de Recherche en Physiologie végétale (GRPV), Université catholique de Louvain, 5 (Bte 13) Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium
2 Unité de Chimie des Interfaces, Université catholique de Louvain, 2 (Bte 18) Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium
† Present address: International Rice Research Institute (IRRI), East and Southern Africa Regional Office, Av. Das FPLM, n 2698-Recinto do IIAM, Maputo-Mozambique
‡ Present address: Centre de Recherche Public Gabriel Lippmann, Departement EVA, 41 rue du Brill, L-4422 Belvaux, Luxembourg.
* To whom correspondence should be addressed. E-mail: stanley.lutts@uclouvain.be

Received 20 January 2010; Revised 15 March 2010; Accepted 29 March 2010

Abstract

Effects of salt stress on polyamine metabolism and ethylene production were examined in two rice (Oryza sativa L.) cultivars [I Kong Pao (IKP), salt sensitive; and Pokkali, salt resistant] grown for 5 d and 12 d in nutrient solution in the presence or absence of putrescine (1 mM) and 0, 50, and 100 mM NaCl. The salt-sensitive (IKP) and salt-resistant (Pokkali) cultivars differ not only in their mean levels of putrescine, but also in the physiological functions assumed by this molecule in stressed tissues. Salt stress increased the proportion of conjugated putrescine in salt-resistant Pokkali and decreased it in the salt-sensitive IKP, suggesting a possible protective function in response to NaCl. Activities of the enzymes ornithine decarboxylase (ODC; EC 4.1.1.17) and arginine decarboxylase (ADC; EC 4.1.1.19) involved in putrescine synthesis were higher in salt-resistant Pokkali than in salt-sensitive IKP. Both enzymes were involved in the response to salt stress. Salt stress also increased diamine oxidase (DAO; 1.4.3.6) and polyamine oxidase (PAO EC 1.5.3.11) activities in the roots of salt-resistant Pokkali and in the shoots of salt-sensitive IKP. Gene expression followed by reverse transcription-PCR suggested that putrescine could have a post-translational impact on genes coding for ADC (ADCa) and ODC (ODCa and ODCb) but could induce a transcriptional activation of genes coding for PAO (PAOb) mainly in the shoot of salt-stressed plants. The salt-resistant cultivar Pokkali produced higher amounts of ethylene than the salt-sensitive cultivar IKP, and exogenous putrescine increased ethylene synthesis in both cultivars, suggesting no direct antagonism between polyamine and ethylene pathways in rice.

Key words: Ethylene, Oryza sativa, polyamine, putrescine, rice, salinity, salt resistance.

Introduction

The diamine putrescine (Put) and the polyamines (PAs) spermidine (Spd) and spermine (Spm) are low molecular weight organic cations that are implicated in various physiological and developmental processes in all living organisms. In plants, these processes include regulation of cell division, rhizogenesis, embryogenesis, senescence, floral development, and fruit ripening (Galston et al., 1997; Kakkar et al., 2000; Arena et al., 2005). In addition, PAs have been shown to afford protection against a large number of environmental biotic and abiotic stresses...
Each of these enzymes is encoded by a low copy number of genes exhibiting both transcriptional and post-translational regulation (Hummel et al., 2004; Delis et al., 2006). For a given enzyme, the corresponding genes are differently regulated according to developmental and environmental cues (Hummel et al., 2004; Groppa and Benavides, 2008) but stress-induced modifications in enzyme activities were not necessarily correlated with modifications in gene expression (Bouchereau et al., 1999; Groppa and Benavides, 2008). PAs themselves were shown to modulate post-translational regulation of ADC (Borrell et al., 1996) and ODC (Palanirumugan et al., 2004). Page et al. (2007) demonstrated that alteration of the cellular Put content may differently affect the expression of the SAMDC and SPDS gene families but that there was no feedback of the expression of ODC and ADC genes. On the other hand, it has been hypothesized that PAs might also influence the expression of genes coding for Put synthesis or PA oxidation (Kasukabe et al., 2004), but data concerning rice exposed to salinity remain scarce in this respect.

Previous data demonstrated that long-term application of exogenous Put reduced Na⁺ and Cl⁻ accumulation in salt-treated rice calli (Ndairajige and Lutts, 2006a) and improved grain yield of a salt-sensitive cultivar exposed to NaCl (Ndairajige and Lutts, 2007). In the present work, the tested hypotheses were that (i) ion discrimination is influenced by exogenous Put on a short-term basis, even after a few days of treatment; and (ii) the impact of exogenous Put on salt-treated rice depends on the cultivar in relation to the influence of exogenous Put on endogenous PA metabolism. Therefore, the impact of salt stress on PA accumulation and metabolism was analysed in a salt-sensitive I Kong Pao (IKP) and a salt-resistant (Pokkali) rice cv. The free, conjugated, and bound forms of PA were quantified in salt-treated plants exposed or not to exogenous Put and were analysed in relation to plant growth, ethylene synthesis, osmotic potential (Ψₛ), monovalent cations (Na⁺ and K⁺ as primary targets of salt stress), and malondialdehyde (MDA; an indicator of oxidative stress) concentrations in roots and shoots. The activities of ADC, ODC, DAO, and PAO were also determined and the implications of these enzymes in PA metabolism under salt stress conditions are discussed in relation to the impact of salt and/or exogenous Put on the corresponding gene expression.

Materials and Methods

Plant culture and growth conditions

Seeds of two rice cvs (O. sativa L.; salt-sensitive cv IKP and salt-resistant cv Pokkali) were obtained from the IRRI (International Rice Research Institute, Philippines). The seeds were germinated on two layers of moistened Whatman No. 2 filter paper in a growth chamber at 25 °C under a 12 h daylight period (120 μmol m⁻² s⁻¹).

Ten-day-old seedlings of the two cultivars were transferred into a phytotron and fixed on polystyrene plates floating on nutritive solution (Yoshida et al., 1976). Illumination was provided by...
Sylvania fluorescent tubes (F96T12/CW/HO) for 12 h 1 at a photon flux density of 290 μmol m⁻² s⁻¹. Daytime humidity was between 60% and 80%, and the temperature was maintained at 29 °C during the day and 26 °C during the night. The solutions were readjusted every 2 d and renewed weekly. After 2 weeks of acclimatization in control conditions, seedlings were exposed for 5 d and 12 d to 0.50, or 100 mM NaCl in the absence or presence of 1 mM exogenous Put. For each treatment, seedlings were distributed among four pots (eight seedlings per pot) containing 2.0 l of solution in a complete randomized block design.

Sixteen plants per treatment were collected after 5 d and 12 d of treatment. Osmotic potential (Ψ) was estimated on tissular sap collected by centrifugation (15 000 g for 15 min at 4 °C) of frozen fresh leaf segments (four plants per treatment) according to Lefèvre et al. (2001) using a vapour pressure osmometer (Wescor 5500). Leaf stomatal conductance (gs) was determined after 5 h of light in the photoperiod (at 11.00 am) on the abaxial surface of the third and fourth leaves on five plants per treatment using an automatic diffusion porometer (AP4 Delta-T Devices Ltd, Cambridge, UK). Leaf water potential (Ψw) was measured on the same leaves with a Scholander-type pressure chamber on excised leaf blades. Roots and shoots of four other plants were separated and weighed before and after incubation for 48 h in an oven at 70 °C in order to determine fresh weight (FW) and dry weight (DW), respectively. The remaining material was immediately frozen in liquid nitrogen and maintained at −80 °C for further PA, MDA, and enzyme extraction and quantification.

**Determination of Na⁺, K⁺, and MDA concentration**

The roots of harvested plants were quickly rinsed in sterile deionized water to remove ions from the free space and gently blotted dry with a paper towel. For quantification of Na⁺ and K⁺ in shoots and roots, tissues were oven-dried at 70 °C for 48 h and 50 mg DW were digested in 35% HNO₃. Na⁺ and K⁺ were resuspended in 1 ml of HCl (0.1 N) and the solutions were filtered. Ions were quantified by flame atomic absorption spectrophotometry (VARIAN spectra-300).

Lipid peroxidation was estimated as the amount of the thiobarbituric acid-reactive substances (TBARS; mainly MDA from lipid peroxidation) determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). For each sample, 250 mg FW for shoots and for roots were separately frozen in liquid nitrogen, ground in a pre-chilled mortar, and homogenized in 5 ml of 5% (w/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 10 000 g for 10 min and filtered through Whatman No. 1 filter paper. A 2 ml aliquot of TBA [0.67% (w/v)] was added to 2 ml of supernatant: the mixture was heated at 100 °C for 30 min and then quickly cooled on ice. Samples were centrifuged at 5000 g for 1 min and the absorbance was measured at 532 nm. The non-specific absorption of 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

**Free, conjugated, and bound polyamine extraction and determination**

Free, conjugated (PA conjugates with phenolic acids and other low molecular weight compounds), and bound PAs (PA conjugates with macromolecules) were extracted according to Piqueras et al. (2002). Tissues were frozen in liquid nitrogen and ground in a pre-chilled mortar: samples (~500 mg FW for shoots and 250 mg for roots) were then ground with a pestle with 10% perchloric acid (PCA) (at a ratio of 100 mg tissue ml⁻¹ 10% PCA), vortexing vigorously, and left to stand for 1 h in an ice bath at 4 °C. The homogenate was centrifuged at 23 100 g at 4 °C during 20 min. The supernatant (containing free and PCA-soluble conjugated PAs) was stored at 4 °C, and the pellets (containing PCA-insoluble bound PAs) were resuspended in an equal volume (5 ml) of 1 N NaOH.

For free and conjugated PA analysis, 200 μl of the supernatant was mixed with 200 μl of 12 N HCl and heated at 110 °C for 16 h in tightly capped glass tubes. After acid hydrolysis, HCl was evaporated from the tubes by further heating at 80 °C and the residue was resuspended in 200 μl of 10% PCA and used for dansylation. To extract PCA-insoluble bound PAs, the pellet was dissolved by vigorous vortexing in 5 ml of 1 N NaOH. The mixture was centrifuged at 23 100 g at 4 °C for 20 min, and the supernatant, including the solubilized bound PAs, was hydrolysed under the same conditions as above. Aliquots of 200 μl of supernatant (free PAs), hydrolysed supernatant (conjugated PAs), and hydrolysed pellet (bound PAs) were dansylated, along with PA standards, as previously described by Lefèvre et al. (2001). Samples were resuspended in 1 ml of methanol, centrifuged at 13 000 g during 15 min, and filtered through microfilters (Chromafil PES-45/15, 0.45 μm; Macherey-Nagel). Aliquots (20 μl) were injected into a Bio-Rad HPLC system equipped with a Nucleosil 100-5 C18 MN 250/04 column (particle size: 5 μm, 4.6×250 mm²). Elution was performed at 35 °C at a flow rate of 1 ml min⁻¹ using a methanol/water stepped gradient program changing from 60% to 100% methanol over 25 min. The column was washed with 100% methanol for 15 min. Detection of dansylated PAs was performed with a Shimadzu RF-10Axl fluorimeter, with an excitation wavelength of 320 nm and an emission wavelength of 510 nm. For a given treatment, each quantification was performed on three independent samples.

**Enzyme analysis**

Extraction and assays of ADC and ODC were performed on fresh extracts according to Lee et al. (1996). Shoots and roots (~0.5 g FW) were frozen in liquid nitrogen, ground to a fine powder, and homogenized with 1.5 ml of grinding buffer containing 25 mM potassium phosphate, 50 μM EDTA, 100 μM phenylmethylsulphonyl fluoride (PMSF), and 25 mM ascorbic acid (pH 8.0). The homogenate was then centrifuged at 5000 g for 20 min at 2 °C and the supernatant collected and dialysed at 4 °C against 2.0 l of grinding buffer for 24 h in darkness. Dialysed extract (50 μl) was used in the enzyme assay. Enzyme activity was determined by measuring CO₂ evolution from the decarboxylation reaction. The reaction buffers for ADC and ODC assays were 0.1 ml of 200 mM Tris-HCl (pH 8.0) and 0.1 ml of 200 mM potassium phosphate (pH 7.5), respectively. After reincubation of enzyme extract and reaction buffer at 0 °C for 5 min, 10 μl of the respective substrate solution, 3.66 mM arginine (containing 185 kBq ml⁻¹ [L-¹⁴C]arginine) or 21.55 mM ornithine (containing 185 kBq ml⁻¹ L-¹⁴Cornithine) were added to the reaction mixture. The 15 ml reaction tubes containing two filter paper discs impregnated with 2 M KOH were then sealed with rubber caps and incubated at 40 °C on a rotating shaker for 2 h. The released [⁰¹⁴CO₂ was trapped by the two filter paper discs. The reaction was stopped by injecting 0.2 ml of 10% (w/v) trichloroacetic acid (TCA) with a syringe, and trapping was continued for 1 h. The paper discs were then allowed to dry and put in 5 ml of scintillation liquid.

Radioactivity on the discs was measured with a Beckman LS-1810 liquid scintillation spectrometer (Beckman Instruments, Inc., Irvine, CA, USA). The enzyme activity was expressed as nmol [¹⁴CO₂ released (mg protein)⁻¹ h⁻¹]. Protein content was determined according to the protein–dye binding method of Bradford (1976) using bovine serum albumin as a standard. L-[¹⁴C]Arginine monochloride and L-[¹⁴C]ornithine monochloride were purchased from Amersham (Buckinghamshire, UK). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

DAO (EC 1.4.3.6) and PAO (EC 1.5.3.11) activities were estimated spectrophotometrically by a method based on the colorimetric assay of Δ-pyrroline using Put (for DAO) or Spd (for PAO) as substrates (Holmstedt et al., 1961). For extraction, tissues were frozen in liquid nitrogen and ground in a pre-chilled...
Reverse transcription-PCR (RT-PCR)

Genes coding for ADC, ODC, DAO, and PAO in rice were searched on the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and Rice Genome Annotation (http://rice.plantbiology.msu.edu/) databases. With the exception of ADC, the results obtained were sequences of hypothetical proteins and for this work they were renamed according to their function, as described in Table 1. Primers were designed using Primer 3 software (version 0.4.0) under default parameters (Rozen and Skaletsky, 2000). The primers used for UBQ5 were chosen based on Mukesh et al. (2006)

Total RNA was prepared from 150 mg of plant material using the TRI Reagent Solution (Ambion, Austin, TX, USA). Reverse transcription was performed with 1 μg of total RNA using the ‘Revert Aid H minus first strand cDNA synthesis kit’ (Fermentas, St Leon-Rot, Germany), and following the manufacturer’s instructions. The amplification procedure consisted of 28 cycles for UBQ5, 30 cycles for ADC, ADCa, ODCa, PAOa, PAOb, and DAO, and 32 cycles for ODCb. After an initial denaturation step at 94 °C for 2 min, each cycle consisted of 30 s at 94 °C, 30 s at an annealing temperature depending on the primer combination, and 1 min extension at 72°C, followed by a final extension of 5 min at 72 °C. The primer pairs and the annealing temperature used for each amplification are presented in Table 1. Amplifications were conducted using GoTaq DNA polymerase (Promega Benelux b.v). The PCR products were resolved on agarose gels.

Ethylene biosynthesis

Ethylene biosynthesis was estimated on the youngest fully unfolded leaf of six distinct plants according to Kevers and Gaspard (1985). The leaves were cut into segments and a sample containing two segments (~30 mg) was incubated in 10 ml vials containing filter paper moistened with 1 ml of deionized water and sealed with rubber serum caps. The vials were incubated in darkness at 27 °C on a rotating shaker (100 rpm) for 12 h; a gas sample (1 ml) was then withdrawn from the headspace of the flask and ethylene was assayed using a gas chromatograph (Intersmath IGC120DFL) equipped with an alumina column (Porapak Q) and a flame ionization detector.

Statistical analysis

Two independent experiments were performed and exhibited similar trends. For a given sample, each quantification was performed in triplicate. Three-way analysis of variance (ANOVA III) was conducted separately for each duration of stress considering cultivars, salt doses, and Put treatment as main factors, as well as their interactions. Differences between means were scored for significance according to the Scheffe F-test (SAS system for Windows V8) by using 95% confidence intervals.

Results

Plant growth and water status

The salt-resistant cv Pokkali exhibited a higher DW for shoots and roots than the salt-sensitive cv IKP in both the absence and presence of salt (P <0.05; Fig. 1). Exogenous Put application had no impact on shoot DW in the absence or presence of NaCl in IKP compared with the plants maintained in the absence of Put. Exogenous Put slightly but significantly increased shoot growth of salt-resistant cv Pokkali after 12 d of treatment at 50 mM NaCl (Fig. 1B).

Table 1. Primers and annealing temperature (AT) used in reverse transcriptase-PCR

| Gene  | Accession no. | Gene description                  | Primer sequence                                                                 | AT  | Size (bp) |
|-------|---------------|-----------------------------------|--------------------------------------------------------------------------------|-----|-----------|
| UBQ5  | AK061988      | Ubiquitin 5                       | 5'-ACGCGTACGGTGGGACCA-3'                                                        | 60 °C | 69        |
|       |               |                                   | 5'-TGTCGAGAGTGCTG-3'                                                            |     |           |
| ADC1  | AY604047      | Arginine decarboxylase (EC 4.1.1.19) | 5'-ACCCGCGCTGGTGGGACCA-3'                                                      | 60 °C | 445       |
| ADCa  | NM_0010558553 | Arginine decarboxylase (EC 4.1.1.19) | 5'-TGTCGAGAGTGCTG-3'                                                            | 60 °C | 711       |
| ODCa  | NM_001070362  | Ornithine decarboxylase (EC 4.1.1.17) | 5'-GGGTCAGGAGAAGAGTGCTG-3'                                                     | 58 °C | 206       |
| ODCb  | NM_0010533985 | Ornithine decarboxylase (EC 4.1.1.17) | 5'-GGGTCAGGAGAAGAGTGCTG-3'                                                     | 58 °C | 460       |
| PAOa  | NM_001065782  | Polyamine oxidase precursor (EC 1.5.3.11) | 5'-CTACGAGTCGGCTGATCCAC-3'                                                     | 55 °C | 429       |
| PAOb  | NM_001069545  | Polyamine oxidase precursor (EC 1.5.3.11) | 5'-TGCACAATTTTATTTATTTTATTTTATTT-3'                                           | 58 °C | 283       |
| DAO   | NM_001066591  | Copper amine oxidase precursor (EC 1.4.3.6) | 5'-ACGTGACCTACACCCACTC-3'                                                      | 55 °C | 478       |
An exogenous application of Put induced a significant decrease in root DW for IKP in the absence of salt (Fig. 1D). As shown in Table 2 for plants exposed to NaCl during 12 d, both $\Psi_s$ and $\Psi_w$ were lower in Pokkali than in IKP ($P < 0.01$). Exogenous application of Put had no impact on $\Psi_s$ and $\Psi_w$ values in the salt-sensitive IKP but significantly reduced this parameter in Pokkali in both control and stressed plants. Salt stress decreased stomatal conductance ($g_s$) in the two studied cultivars. Exogenous application of Put significantly increased $g_s$ values in the absence of stress in salt-sensitive IKP and in the presence of the highest NaCl dose in salt-resistant Pokkali, confirming a highly significant interaction among salinity and cultivars ($P < 0.01$).

**Monovalent cations and MDA accumulation**

Salt stress induced an increase in Na$^+$ in roots and shoots (Fig. 2). In both organs, the sodium concentration was higher in IKP than in Pokkali except in roots after 5 d of treatment at 50 mM NaCl (Fig. 2C). Exogenous Put reduced Na$^+$ accumulation in shoots ($P < 0.05$) and roots ($P < 0.01$) of salt-treated IKP but had no significant impact on Pokkali. Salt stress also reduced K$^+$ concentration in the

### Table 2

| Cultivar | NaCl (mM) | $\Psi_s$ (MPa) | $\Psi_w$ (MPa) | $g_s$ (mol m$^{-2}$ s$^{-1}$) |
|----------|-----------|----------------|----------------|-----------------------------|
|          | − Put     | + Put          | − Put          | + Put                      | − Put          | + Put          |
| IKP      | 0         | −0.77±0.06 a   | −0.75±0.04 a   | −0.31±0.02 a               | 0.62±0.04 a   |
|          | 50        | −1.45±0.09 c   | −1.44±0.05 c   | −0.58±0.02 c               | 0.44±0.03 c   |
|          | 100       | −2.04±0.12 d   | −2.29±0.13 d,e | −1.09±0.05 d               | 0.38±0.06 c,d |
| Pokkali  | 0         | −0.86±0.10 a   | −1.02±0.01 b   | −0.45±0.05 b               | 0.66±0.05 a   |
|          | 50        | −1.89±0.09 d   | −2.71±0.02 f   | −1.12±0.01 d               | 0.52±0.04 b   |
|          | 100       | −2.32±0.11 e   | −3.05±0.07 g   | −1.58±0.07 e               | 0.41±0.02 c   |

Values sharing a common letter are not significantly different at $P < 0.05$. Each value is the mean of seven replicates ± SE. For a given parameter, means followed by the same letter are not significantly different at $P = 0.05$. 

**Fig. 1.** Dry weights of shoots (A, B) and roots (C, D) of rice (Oryza sativa) plants belonging to the salt-sensitive cv IKP or to the salt-resistant cv Pokkali and exposed during 5 d (A and C) or 12 d (B and D) to 0 (white bars), 50 (grey bars), or 100 mM NaCl (black bars), in the absence or presence of 1 mM Put (n=4; vertical bars are the SE). Note that vertical scales are not the same for shoots and roots.
roots but not in the shoots (data not shown). Exogenous Put contributed to K+ reduction at the root level in both cultivars (Table 3). Salt stress induced a significant increase in shoot MDA concentration after 5 d in IKP and after 12 d of treatment in both cultivars (Fig. 3A, B). In the presence of NaCl, the salt-resistant cv Pokkali usually exhibited a lower shoot MDA concentration than the salt-sensitive cv IKP ($P < 0.01$). Exogenous Put had no impact on shoot MDA in IKP ($P = 0.32$) while it reduced shoot MDA in salt-treated Pokkali after 12 d of treatment ($P < 0.01$). Salt stress had no clear impact on root MDA concentration (Fig. 3C, D): exogenous Put increased root MDA concentration in control plants of IKP and reduced root MDA concentration in salt-stressed plants of Pokkali after 12 d of treatment.

**PA concentration (Fig. 4)**

A salt stress-induced decrease in the Put concentration was noticed for shoots of IKP after 5 d and 12 d but not in Pokkali exposed during 12 d to 50 mM and 100 mM NaCl. The proportion of bound Put decreased and the proportion of conjugated Put increased in response to salinity in Pokkali while an opposite trend was recorded for IKP. As far as Put was concerned, the conjugated fraction remained the most important from a quantitative point of view in all treatments. Salt stress reduced the root Put concentration after 5 d of exposure in the salt-sensitive IKP (Fig. 4C) and such a decrease affected both free and bound forms, as well as the conjugated fraction at high NaCl dose. Salt-resistant Pokkali exhibited a higher titre of Put in the roots than the salt-sensitive IKP, except after 12 d of exposure to 50 mM NaCl (Fig. 4D). When the plants were exposed to exogenous Put, the endogenous concentration of Put increased in the roots of both cultivars ($P < 0.01$) and to a lower extent in the shoots ($P < 0.05$). Such an increase mainly concerns the free soluble fraction after 5 d: in control plants, the conjugated fraction remained unaffected and the bound fraction was even slightly decreased in IKP.

**Table 3.** Potassium concentration (in mmol g$^{-1}$ DW$^{-1}$) in roots of two rice cultivars (IKP, salt sensitive; and Pokkali, salt resistant) exposed during 5 d and 12 d to 0, 50, or 100 mM NaCl in the presence (+ Put) or absence (– Put) of 1 mM putrescine in the nutrient solution

| Cultivar | NaCl (mM) | 5 d | 12 d |
|----------|-----------|-----|-----|
|          | – Put     | + Put | – Put | + Put |
| IKP      | 0   0.98±0.09 a | 0.68±0.04 b | 1.10±0.01 a | 0.76±0.08 a |
|          | 50  0.55±0.03 c,d | 0.41±0.02 d | 0.84±0.02 c | 0.60±0.04 c |
|          | 100 0.50±0.03 d | 0.31±0.03 e | 0.43±0.05 e | 0.34±0.01 d |
| Pokkali  | 0   0.83±0.00 b | 0.81±0.01 a | 0.94±0.05 b | 0.80±0.02 a |
|          | 50  0.45±0.03 d | 0.50±0.02 c | 0.75±0.06 d | 0.72±0.05 b |
|          | 100 0.60±0.01 c | 0.53±0.01 c | 0.50±0.04 e | 0.42±0.05 d |
There was no significant difference between the two studied genotypes for root Spd concentration (data not shown), and salt stress had no significant impact on this parameter \( (P=0.57) \). In the shoots of non-stressed plants, free, conjugated, and bound Spd concentrations were higher for the salt-sensitive cv IKP than for Pokkali (Table 4). Salinity induced a decrease in free and conjugated Spd in IKP but an obvious increase of conjugated Spd in Pokkali \( (P<0.001) \). The exogenous application of Put increased the conjugated and bound Spd concentration in the shoots of the salt-sensitive cv IKP after 5 d of treatment but had no similar impact on the salt-resistant cv Pokkali.

Total Spm in the roots obviously decreased with the age of the plant but there was no significant difference between cultivars (Table 5). Salt stress slightly increased the root Spm \( (P<0.05) \) and there was no obvious difference between cultivars in this respect \( (P=0.47) \). In the absence of salt, the shoot Spm concentration was higher in IKP than in Pokkali. Salt stress induced an increase in free, conjugated, and bound Spm in IKP and Pokkali after 12 d of exposure, and total Spm concentration therefore always remained higher in IKP than in Pokkali. Exogenous Put had no impact on endogenous Spm concentration, whatever the cultivar, the considered organ, or the NaCl dose (detailed data not shown).

**Enzyme activities**

ADC activities increased in response to salinity in the shoots of both cultivars and remained higher in the salt-resistant cv Pokkali compared with IKP (Fig. 5A, B). As far as salt-sensitive cv IKP is concerned, it is noteworthy that exogenous Put stimulated ADC activities in control plants only and had no impact or even decreased ADC activities in salt-treated plants. ADC activities were always higher in the roots than in the shoots. Salt stress slightly increased ADC activities in roots after 5 d of treatment in the absence of exogenous Put (Fig. 5C). Salt stress decreased root ADC activities after 12 d of treatment in Put-treated plants only (Fig. 5D). Shoot ODC activities were stimulated by salt stress in both cultivars after 5 d of treatment (Fig. 6A), although ODC activity was higher at 50 mM than at 100 mM in IKP. It has also to be mentioned that exogenous Put either had no impact on ODC activity or clearly increased it. In the roots of salt-treated plants, ODC activity was always higher in Pokkali than in IKP, especially after 12 d of exposure to 100 mM NaCl (Fig. 6D). Salt stress increased the root ODC activity in IKP only after 5 d of exposure to 100 mM NaCl.

Root DAO remained unaffected by exogenous salt or Put in IKP but clearly increased in response to NaCl in Pokkali (Fig. 7). In contrast, the shoot DAO activity was slightly increased by salt stress in IKP in both the absence and presence of Put, while the shoot DAO increase in Pokkali was reported in the presence of exogenous Put in 100 mM NaCl-treated plants only. Root PAO activity was higher in Pokkali than in IKP, especially in salt-treated plants. In IKP, exogenous Put decreased root PAO in the presence of 50 mM NaCl but increased it in...
the presence of 100 mM NaCl. At the shoot level, PAO activities were similar in control plants of the two considered cultivars. Salt stress increased shoot PAO activity in IKP ($P < 0.01$) but not in Pokkali ($P = 0.63$). Exogenous Put increased PAO activity in 100 mM NaCl-treated plants.

Table 4. Spd concentrations [in nmol g$^{-1}$ FW$^{-1}$; free, conjugated, and bound fractions] in the shoots of two rice cultivars (IKP, salt sensitive; and Pokkali, salt resistant) exposed during 5 d or 12 d to 0, 50, or 100 mM NaCl in the absence or presence of 1 mM Put ($n = 4$; vertical bars are the SE). Note that vertical scales are not the same for shoots and roots.

| Cultivar | NaCl (mM) | Put (mM) | 5 d Free | 5 d Conjugated | 5 d Bound | 12 d Free | 12 d Conjugated | 12 d Bound |
|----------|-----------|----------|----------|---------------|-----------|-----------|----------------|-----------|
| IKP      | 0         | 0        | 93.12 a  | 119.62 b      | 58.41 b   | 83.55 a   | 117.13 a       | 25.66 b,c |
|          | 50        | 0        | 88.80 a,b| 43.65 d       | 27.44 d   | 63.23 b   | 87.69 c        | 19.44 c,d |
|          | 100       | 0        | 72.25 c  | 77.26 c       | 9.65 e    | 48.68 c   | 46.32 f        | 28.07 b   |
|          | 0         | 1        | 82.56 b  | 142.81 a      | 75.00 a   | 88.53 a   | 114.40 a       | 32.09 b   |
|          | 50        | 1        | 86.49 a,b| 144.68 a      | 84.19 a   | 85.18 a   | 52.55 ef       | 44.51 a   |
|          | 100       | 1        | 55.64 d  | 153.04 a      | 47.68 c   | 64.75 b   | 107.81 a,b     | 13.77 d,e |
| Pokkali  | 0         | 0        | 38.75 e  | 18.10 f       | 3.55 e    | 49.85 c   | 57.05 e        | 24.16 b,c |
|          | 50        | 0        | 38.02 e  | 77.56 c       | 45.68 c   | 62.54 b   | 72.24 d        | 24.71 b,c |
|          | 100       | 0        | 44.02 e  | 74.34 c       | 41.54 c   | 53.33 c   | 73.27 d        | 5.76 e    |
|          | 0         | 1        | 55.05 d  | 31.77 e       | 5.84 e    | 86.57 a   | 81.83 c        | 26.70 b,c |
|          | 50        | 1        | 39.08 e  | 77.12 c       | 32.31 d   | 68.19 b   | 99.04 b        | 9.16 e    |
|          | 100       | 1        | 39.42 e  | 78.79 c       | 43.67 c   | 68.09 b   | 94.41 b        | 8.39 e    |

Fig. 4. Impact of salt stress on shoot (A, B) and root (C, D) endogenous Put concentrations [free (black), conjugated (light grey) and bound (dark grey) fractions] in rice (Oryza sativa) plants belonging to the salt-sensitive cv IKP or to the salt-resistant cv Pokkali and exposed during 5 d (A and C) or 12 d (B and D) to 0, 50, or 100 mM NaCl, in the absence or presence of 1 mM Put ($n = 4$; vertical bars are the SE). Note that vertical scales are not the same for shoots and roots.
Steady-state mRNA levels

As shown in Fig. 8, DAO transcripts were observed in both IKP and Pokkali. In Pokkali, the highest expression level was observed for plants exposed to 100 mM NaCl in the presence of Put. In IKP, DAO was mainly expressed in control plants and in plants exposed to 100 mM NaCl in the presence of Put after 5 d of treatment (Fig. 8A, C) while there was no expression difference between treatments after 12 d of stress (Fig. 8B, D).

PAOa was only weakly expressed in IKP shoots exposed to 100 mM NaCl after 12 d of stress (Fig. 8B) and in Pokkali roots exposed to 100 mM NaCl in the presence of Put after 5 d of treatment (Fig. 8C). PAOb was mainly expressed in the shoot of IKP (Fig. 8A, B) and in the roots of Pokkali (Fig. 8C, D). The PAOb expression level increased with the salt treatment in both cultivars. The Put treatment increased the expression level in IKP shoots exposed to 100 mM NaCl (Fig. 8A, B) and in Pokkali shoots exposed to 100 mM salt treatment during 12 d (Fig. 8B).

The transcripts of ODCa were mainly observed in the absence of salt. After 12 d of treatment, the expression level was higher in Pokkali compared with IKP in both shoot and root (Fig. 8B, D). In contrast, ODCh was expressed in the shoot of both cultivars after 12 d of NaCl exposure (Fig. 8B). Transcripts of ADCa were present in roots (Fig. 8C, D). After 5 d of treatment, the highest expression levels were observed for the salt treatments in the absence of Put in both cultivars (Fig. 8C). After 12 d of stress, the Put treatment increased ADCa expression in salt-treated Pokkali roots while ADCa was mainly expressed in the absence of salt in IKP. ADCI was constitutively expressed.

Ethylene biosynthesis

As shown in Fig. 9A, ethylene biosynthesis after 5 d of treatment was higher in the salt-resistant cv Pokkali than in the salt-sensitive cv IKP. Salt stress induced a slight increase in ethylene synthesis in the salt-sensitive cv IKP. Exogenous Put application had no effect on ethylene production for either cultivar except for Pokkali plants in the presence of

| Cultivar | NaCl (mM) | Roots 5 d | Shoots 5 d | Roots 12 d | Shoots 12 d |
|----------|-----------|-----------|------------|------------|------------|
| IKP      | 0         | 49.37 c   | 6.04 c     | 71.45 b    | 56.28 c    |
|          | 50        | 51.53 b,c | 16.87 a,b | 46.58 c    | 94.57 b    |
|          | 100       | 65.80 a   | 12.33 b,c | 131.75 a   | 112.7 a    |
| Pokkali  | 0         | 37.59 d   | 7.11 c     | 47.23 c    | 64.59 c    |
|          | 50        | 58.15 b   | 17.84 a,b | 58.15 b    | 47.23 c    |
|          | 100       | 68.95 a   | 26.09 a    | 70.48 b    | 87.80 b    |

Fig. 5. Arginine decarboxylase (ADC) activities in shoots (A, B) and roots (C, D) of rice (Oryza sativa) plants belonging to the salt-sensitive cv IKP or to the salt-resistant cv Pokkali and exposed during 5 d (A and C) or 12 d (B and D) to 0 (white bars), 50 (grey bars), or 100 mM NaCl (black bars), in the absence or presence of 1 mM Put (n=4; vertical bars are the SE). Note that the vertical scales are not the same for shoots and roots. Values sharing a common letter are not significantly different at P<0.05.

Table 5. Total Spm concentration (free+conjugated+bound fractions; in mmol g^(-1) FW^-1) in roots and shoots of two rice cultivars (IKP, salt sensitive; and Pokkali, salt-resistant) exposed during 5 d and 12 d to 0, 50, or 100 mM NaCl

| Cultivar | NaCl (mM) | Roots 5 d | Shoots 5 d | Roots 12 d | Shoots 12 d |
|----------|-----------|-----------|------------|------------|------------|
| IKP      | 0         | 49.37 c   | 6.04 c     | 71.45 b    | 56.28 c    |
|          | 50        | 51.53 b,c | 16.87 a,b | 46.58 c    | 94.57 b    |
|          | 100       | 65.80 a   | 12.33 b,c | 131.75 a   | 112.7 a    |
| Pokkali  | 0         | 37.59 d   | 7.11 c     | 47.23 c    | 64.59 c    |
|          | 50        | 58.15 b   | 17.84 a,b | 58.15 b    | 47.23 c    |
|          | 100       | 68.95 a   | 26.09 a    | 70.48 b    | 87.80 b    |
Fig. 6. Ornithine decarboxylase (ODC) activities in shoots (A, B) and roots (C, D) of rice (Oryza sativa) plants belonging to the salt-sensitive cv IKP or to the salt-resistant cv Pokkali and exposed during 5 d (A and C) or 12 d (B and D) to 0 (white bars), 50 (grey bars), or 100 mM NaCl (black bars), in the absence or presence of 1 mM Put (n=4; vertical bars are the SE). Values sharing a common letter are not significantly different at P <0.05.

Fig. 7. Activities of diamine oxidase (DAO; A and B) and polyamine oxidase (PAO; C and D) in shoots (A and C) and roots (B and D) of two rice cultivars (IKP, salt sensitive; and Pokkali, salt resistant) exposed during 12 d to 0 (white bars), 50 (grey bars), or 100 mM NaCl (black bars), in the absence or presence of 1 mM Put (n=4; vertical bars are the SE). Values sharing a common letter are not significantly different at P <0.05.
100 mM NaCl where a significant increase in ethylene synthesis was observed in response to Put application. In the absence of NaCl after 12 d of treatment, ethylene biosynthesis was still slightly higher in the salt-resistant cv Pokkali than in the salt-sensitive cv IKP (Fig. 9B). In the absence of exogenous Put, salinity induced an increase in ethylene synthesis, but no difference could be recorded among cultivars after 12 d of exposure to 100 mM NaCl.

Discussion
The impact of exogenous Put on plant response and PA conjugation depends on cultivars

Despite numerous experimental data available in the literature, the role of PAs in plant resistance to NaCl...
remains imprecise. The application of exogenous PAs is one of the possible strategies to study the implication of those molecules in stress response, but the present work suggests that their impact may vary depending on the considered genotype. Lefèvre et al. (2001) showed that the roots of the salt-resistant cv Pokkali contain high amounts of Put compared with the salt-sensitive cv IKP and it may thus be hypothesized that an exogenous application of Put could help the salt-sensitive genotype to cope with high external doses of salt. Ndaryragije and Lutts (2006b), however, demonstrated that although Put is efficiently absorbed and translocated to the shoots and had a positive impact on monovalent cation discrimination in this cultivar, the increase in Put did not allow the plant to overcome the deleterious effect of salt stress and even reinforced the negative impact of NaCl in terms of both shoot and root growth. The present work confirms those results for the salt-sensitive cv IKP but also underlines a different response for the salt-resistant cv Pokkali: although exogenous Put had no impact on control plants, it increased shoot growth of this cultivar exposed to 50 mM NaCl during 5 d and 12 d, and reduced Ψs but had no significant impact on Na+ accumulation in shoots and roots. Moreover, exogenous Put also reduced shoot MDA concentration for Pokkali exposed to NaCl, thus suggesting that the anti-peroxidative properties of PAs are not limited to Spm as reported elsewhere (Tadolini, 1988). The data therefore suggest that the two studied genotypes may differ not only in their mean levels of Put, but also in the physiological functions of this molecule in stressed tissues.

PA metabolism in higher plants exhibits several specificities. A first specificity of PA metabolism in plants is linked to the fact that PAs can be conjugated via an amide bond to hydroxycinnamic (mainly caffeic, ferulic, and p-coumaric) acids and that this conjugated PA pool can be larger than that of the free PA (Martin-Tanguy, 1997). As far as Put is concerned, the conjugated fraction is quantitatively the most important for the two studied cultivars, salt stress increased the proportion of conjugated Put in the salt-resistant cv Pokkali but decreased it in the salt-sensitive IKP, suggesting that this class of molecule may assume protective functions in response to NaCl. The inability of exogenous Put to afford protection in salt-treated IKP may be linked to the fact that such treatment only increased the free soluble Put fraction and not the conjugated forms. Similarly, conjugated Spd increased in response to salt stress in salt-resistant Pokkali but decreased in salt-sensitive IKP. Yang et al. (2007) reported that conjugated Spd and Spm are probably not involved in the drought resistance of rice, and the present data thus suggest that a different behaviour may occur in response to salt stress, which implies an important ionic component linked to Na+ and Cl− accumulation (Lefèvre et al., 2001). It could be argued that exogenous application of Put induced an increase in conjugated Spd but did not improve salt resistance in IKP. A possible explanation might be that the resulting conjugated Spd was not triggered to go the correct cell compartment, and further work is thus required to identify the precise site of conjugated PA accumulation in tissues.

Beside conjugation, PAs may also be covalently bound to macromolecules such as specific proteins or DNA (Watson and Malmberg, 1996; Roy et al., 2005). Surprisingly, bound Put decreased in response to salt stress in the salt-resistant cv Pokkali. Similarly, bound Spd in the shoots of plants exposed to 100 mM NaCl in the absence of exogenous Put (Fig. 5) was higher in IKP than in Pokkali, and a positive impact of exogenous Put in the latter genotype was not linked to any increase in bound Spd. Thus, there are no experimental data confirming that bound PAs assume crucial protection of salt-stressed tissues. On the other hand, there is no evidence that conjugated PAs are unable to interact with macromolecules: conjugated PAs are typically bifunctional compounds carrying properties of both amines and hydroxycinnamic acids, and possible interactions of those basic molecules with negatively charged sites in macromolecules and subcellular structures can still occur at the level of the protonated primary amino group.

Both ODC and ADC activities are involved in the response of rice to salinity and are modified by exogenous Put independently of gene expression

A second specificity of PA metabolism in plants is related to the fact that Put may be produced either through ornithine decarboxylation, as is the rule in animals, or also through arginine decarboxylation. ODC is usually associated with the regulation of plant growth and cell division since it is active in fast-growing tissues (Michael et al., 1996), while ADC is usually considered as the main enzyme involved in the response to abiotic stresses (Tiburcio et al., 1997). The present results, however, suggest that the situation is not so simple, at least in O. sativa. From a quantitative point of view, ODC activities were always higher than ADC activity, especially in salt-treated tissues, whatever the considered organ or stress exposure. Both ADC and ODC activities were higher in the salt-resistant genotype Pokkali than in the salt-sensitive genotype IKP, especially in the roots of salt-treated plants. Chattopadhyay et al. (1997) showed that rice ADC activity was higher in a salt-resistant cultivar than in a salt-sensitive one, but these authors did not consider ODC activities. On the basis of the present results, it is suggested that both enzymes may be involved in the response of rice to salinity and that the situation is similar to what was reported by Friedman et al. (1989) for Vigna radiata. This, however, does not imply that the considered enzymes assume similar functions since they are thought to be located in different cell compartments although conflicting reports are available in the literature concerning the subcellular localization of these enzymes (Bortolotti et al., 2004; Gemperlova et al., 2006).

ADC has been reported to be regulated mainly by post-translational mechanisms in relation to the cleavage of an inactive precursor of 60 kDa into two peptides, one of them located at the C-terminal end of the pre-protein exhibiting
the ADC activity. In *Avena sativa*, a feedback inhibition of ADC has been reported to be due to the inhibition of this post-translational maturation process by Spm, and to a lesser extent by Spd, but not by Put (Borrell *et al*., 1996; Tiburcio *et al*., 1997). The present results show that exogenous Put surprisingly increased ADC activity in unstressed plants of cultivar IKP, although Put is the product of the reaction catalysed by ADC. In some cases, stimulation of gene expression could partly explain a stress-induced increase in enzyme activities, as recorded for salt stress impacts of ODCa in the shoots after 12 d and in the roots after 5 d, or for ADCa in the shoots after 5 d. Similarly, the difference between the two considered cultivars could also be related to a difference in gene expression, as indicated by the higher levels of ODCa transcripts associated with higher ODC activities in roots of Pokkali after 12 d of treatment. In contrast, the impact of Put on ADC and ODC enzyme activities could not be related to any specific modification in the corresponding gene expression. These results suggest that Put could promote post-translational modifications of enzymatic proteins.

**Put increases PA oxidation through transcriptional activation**

The situation appeared somewhat different for enzymes involved in PA catabolism. Amine oxidases catalyse the oxidative deamination of PAs, and the production of hydrogen peroxide derived from PA oxidation has been correlated with cell wall maturation and lignification while cell wall modifications have been reported to be involved in the stress response (Cona *et al*., 2006). The present results showed a stimulation of these enzyme activities in the rice cultivars in response to salt stress but such an increase occurred in different organs depending on the cultivar (in roots for the salt-resistant cultivar and in the shoots for the salt-sensitive cultivar). It has been reported that an increase of PAO activities in photosynthetic tissues could lead to an oxidative burst if there is no concomitant activation of the antioxidant machinery (Moschou *et al*., 2008). This, however, should not have occurred in the present study since exogenous Put did not increase shoot MDA content (Fig. 3). In contrast to genes coding for ADC and ODC, there was a good correlation between DAO and PAO activities on the one hand (Fig. 7) and DAO or PAOb gene expression on the other hand (Fig. 5). These data also suggest that exogenous Put could increase PAO activity but that such an increase occurred mainly in the shoots of salt-treated plants and implies a transcriptional activation.

**Put interaction with ethylene synthesis depends on salt stress**

A third specificity of PA metabolism in plants may be due to their interaction with the hormonal status of the plant inasmuch as ethylene, on the one hand, and Spd or Spm on the other hand, share a common precursor (dSAM). The situation in this respect may be more or less particular in the case of rice which is a typical semi-aquatic species where ethylene may assume specific functions compared with classical terrestrial plants. In deep-water rice varieties, ethylene has been reported to act as a powerful stimulator of cell elongation rather than as a senescing hormone (Métraux and Kende, 1983). Some authors have already noticed that salt-resistant rice cultivars produce higher amounts of ethylene than salt-sensitive cultivars (Khan *et al*., 1987; Lutts *et al*., 1996). In classical terrestrial plants, ethylene and PA pathways are considered to be competitive (Muñoz de Rueda *et al*., 1994; Rea *et al*., 1995). In the present study, leaf segments were excised, and wounding could interfere to some extent with ethylene biosynthesis measurements. The present data nevertheless suggest that the salt-resistant cv Pokkali produced higher amounts of ethylene than the salt-sensitive cv IKP, and there was no clear relationship between endogenous concentrations of PAs and ethylene biosynthesis in plants exposed to salt stress in the absence of exogenous Put. Application of Put, however, increased ethylene synthesis, mainly in salt-treated plants of cv Pokkali after 5 d and in control plants of both cultivars after 12 d. These data suggest that the two considered pathways could be not strictly competitive: a hypothesis would be that Put increases the amount of total dSAM available for either ethylene or Spd and Spm synthesis. It has been reported, indeed, that SAMDC (EC 4.1.1.50) is the main limiting factor for PA biosynthesis (Walden *et al*., 1997) and that it is activated through a post-translational maturation process which is efficiently stimulated by Put (Schröder and Schröder, 1995).

According to Bouchereau *et al*. (1999), the relationships between PAs and ethylene may differ in stressed versus non-stressed tissues. It was noticed that ethylene synthesis induced by exogenous Put decreased after 12 d of exposure to a moderate stress intensity (50 mM NaCl), at a time when exogenous Put stimulated growth of salt-treated plants of cv Pokkali in relation to a decrease in Ψs. Other agents should thus be involved in the complex network of interactions between PA and ethylene pathways. Li *et al*. (2004) reported in wheat that oversynthesis of endogenous Put reduced the synthesis of reactive oxygen species which, in turn, reduced the synthesis of ethylene under moderate but not under severe stress conditions. In Pokkali, as far as the Put-treated plants are considered after 12 d of treatment, a negative impact of 50 mM NaCl in terms of ethylene synthesis (Fig. 8) was associated with a positive impact in terms of MDA, although the situation is rather different for plants exposed to 100 mM NaCl.

**Conclusions**

The present work demonstrates that exogenous Put reduces Na⁺ accumulation in root of a salt-sensitive rice cultivar already after a few days of salt exposure. Moreover, the impact of exogenous Put on salt-treated rice depends on the cultivar in relation to the influence of exogenous Put on endogenous PA metabolism. It is suggested that salt resistance was associated with an ability to increase Put...
synthesis as a consequence of higher ADC and ODC activities, and to maintain a high proportion of conjugated PAs within stressed tissues. Put had no feedback effect on ADC and ODC activities and could induce a transcriptional activation of genes coding for amine oxidase in the shoot of salt-treated plants. The salt-resistant cv Pokkali produced higher amounts of ethylene than the salt-sensitive cv IKP, and exogenous Put increased ethylene synthesis in both cultivars, suggesting no direct antagonism between PA and ethylene pathways in rice. Further work considering additional steps of the PA biosynthetic pathway (SPDS, Spm synthase, and transglutaminase) but also the subcellular distribution of accumulated PAs is required to increase our knowledge about PA involvement in plant response to salt stress.

Acknowledgements

The authors are grateful to the International Rice Research Institute (IRRI; Manilla) for providing the seeds, to Secrétariat à la Coopération (Université catholique de Louvain) for the research followship of AN, to Dr C Kevers (Université de Liège) for her valuable help in ethylene quantification and comments on the manuscript, and to FNRS (convention no. 1.5.090.08) for financial support. This work is dedicated to the memory of Professor Gilles Guerrier (Université d’Orléans).

References

Arena ME, Pastur GM, Benavides MP, Curvetto N. 2005. Polyamines and inhibitors used in successive culture media for in vitro rooting in Berberis buxifolia. New Zealand Journal of Botany 43, 373–380.

Bakhashavili M, Novitsky E, Levy I, Rahav G. 2005. The fidelity of DNA synthesis by human immunodeficiency virus type 1 reverse transcriptase increases in the presence of polyamines. FEBS Letters 579, 1435–1440.

Balestrasse KB, Gallego SM, Benavides MP, Tomaro ML. 2005. Polyamines and proline are affected by acridium stress in nodules and roots of soybean plants. Plant and Soil 270, 343–353.

Basu R, Ghosh B. 1991. Polymers in various rice (Oryza sativa) genotypes with respect to sodium chloride salinity. Physiologia Plantarum 82, 575–581.

Borrell A, Besford RT, Altabella T, Masgrau C, Tiburcio AF. 1996. Regulation of arginine decarboxylase by spermine in osmotically-stressed oat leaves. Physiologia Plantarum 98, 105–110.

Bortolotti C, Cordeiro A, Alcazar R, Borrell A, Culeiane-Macia FA, Tiburcio AF, Altabella T. 2004. Localization of arginine decarboxylase in tobacco plants. Physiologia Plantarum 120, 84–92.

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J. 1999. Polyamines and environmental challenges: recent development. Plant Science 140, 103–125.

Bradford MM. 1976. A rapid and sensitive method for determining microgram quantities of protein using the principle of protein–dye binding. Analytical Biochemistry 72, 248–254.

Chattopadhyay MK, Gupta S, Sengupta DN, Ghosh B. 1997. Expression of arginine decarboxylase in seedlings of indica rice (Oryza sativa L.) cultivars as affected by salinity stress. Plant Molecular Biology 34, 477–483.

Chen SL, Chen CT, Kao CH. 1991. Polyamines promote the biosynthesis of ethylene in detached rice leaves. Plant and Cell Physiology 100, 238–245.

Cona A, Rea G, Angelini R, Federico R, Tavoladoraki P. 2006. Functions of amine oxidases in plant development and defense. Trends in Plant Science 11, 80–88.

Delis C, Dimou M, Fiemetakis E, Aivalakis G, Katinakis P. 2006. A root- and hypocotyl-specific gene coding for copper-containing amine oxidase is related to cell expansion in soybean seedlings. Journal of Experimental Botany 57, 101–111.

Friedman R, Altman A, Levin N. 1989. The effect of salt stress on polyamine biosynthesis and content in mung bean plants and in halophytes. Physiologia Plantarum 75, 295–302.

Galston AW, Kaur-Sawhney R, Altabella T, Tiburcio AF. 1997. Plant polyamines in reproductive activity and response to abiotic stress. Botanica Acta 110, 197–207.

Gemperlova L, Novakova M, Vankova R, Eder J, Cvikrova M. 2006. Diurnal changes in polyamine content, arginine and ornithine decarboxylase, and diamine oxidase in tobacco leaves. Journal of Experimental Botany 57, 1413–1421.

Groppa MD, Benavides MP. 2008. Polyamines and abiotic stress: recent advances. Amino Acids 34, 35–45.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. Archives of Biochemistry and Biophysics 125, 189–198.

Holmstedt B, Larsson L, Tham R. 1961. Further studies of spectrophotometric method for determination of diamine oxidase activity. Biochemica and Biophysica Acta 48, 182–186.

Hummel I, Gouesbet G, El-Amrani A, Aïnouche A, Couee I. 2004. Characterization of the two arginine decarboxylase (polyamine biosynthesis) paralogues of the endemic subantarctic cruciferous species Pringlea antiscorbutica and analysis of their differential expression during development and response to environmental stress. Gene 342, 199–209.

Kakkar RK, Nagar PK, Ahuja PS, Rai VK. 2000. Polyamines and plant morphogenesis. Biologia Plantarum 43, 1–11.

Kasukabe Y, He L, Nada K, Misawa S, Ihara I, Tachibana S. 2004. Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress regulated genes in transgenic Arabidopsis thaliana. Plant and Cell Physiology 45, 712–722.

Katyar S, Dubey RS. 1990. Changes in polyamines titer in rice seedlings following NaCl salinity stress. Journal of Agronomy and Crop Science 165, 19–27.

Kevers C, Gaspard T. 1985. Vitrification of carnation in vitro: changes in ethylene production, ACC level and capacity to convert to ethylene. Plant Cell, Tissue and Organ Culture 4, 215–223.
Khan AA, Akbar M, Seshu DV. 1987. Ethylene as an indicator of salt tolerance in rice. *Crop Science* 27, 1242–1248.

Lee TM, Shieh YJ, Chou CH. 1996. Role of putrescine in enhancing shoot elongation in Scirpus mucronatus under submergence. *Physiologia Plantarum* 96, 419–424.

Lefèvre I, Gratia E, Lutts S. 2001. Discrimination between the ionic and osmotic components of salt stress in relation to free polyamine level in rice (*Oryza sativa*). *Plant Science* 161, 943–952.

Li CZ, Jiao J, Wang GX. 2004. The important roles of reactive oxygen species in the relationship between ethylene and polyamines in leaves of spring wheat seedlings under root osmotic stress. *Plant Science* 166, 303–315.

Lin SS, Kao CH. 1995. Levels of endogenous polyamines and NaCl-inhibited growth of rice seedlings. *Plant Growth Regulation* 17, 15–20.

Lutts S, Kinet JM, Bouharmont J. 1996. Ethylene production by leaves of rice (*Oryza sativa* L.) in relation to salinity tolerance and exogenous putrescine application. *Plant Science* 116, 15–25.

Maiale S, Sanchez DH, Guirado A, Vidal A, Ruiz OA. 2004. Spermine accumulation under salt stress. *Journal of Plant Physiology* 161, 35–42.

Martin-Tanguy J. 1997. Conjugated polyamines and reproductive development: biochemical, molecular and physiological approaches. *Physiologia Plantarum* 100, 675–688.

Métraux JP, Kende H. 1983. The role of ethylene in the growth response of submerged deep water rice. *Plant Physiology* 72, 441–446.

Michael AJ, Furze JM, Rhodes MJc, Burtin D. 1996. Molecular cloning and functional identification of a plant ornithine decarboxylase cDNA. *Biochemical Journal* 314, 241–248.

Moschou PN, Delisq ID, Paschalidis KA, Roubelakis Angelakis KA. 2008. Transgenic tobacco overexpressing polyamine oxidase are not able to cope with oxidative burst generated by abiotic factors. *Physiologia Plantarum* 133, 140–156.

Mukesh J, Nijhawan A, Tyagi AK, Khurana JP. 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical and Biophysical Research Communications* 345, 646–651.

Muñoz de Rueda P, Matilla AJ, Sanchez Calle IM, Bueno M, Gallardo M. 1994. Thermoinhibition alters the polyamine levels in cotyledons and embryonic axes during germination of stratified chickpea seeds. *Plant Science* 101, 143–150.

Ndaiyiragije A, Lutts S. 2006a. Exogenous putrescine reduces sodium and chloride accumulation in NaCl-treated calli of the salt-sensitive rice cultivar I Kong Pao. *Plant Growth Regulation* 48, 51–63.

Ndaiyiragije A, Lutts S. 2006b. Do exogenous polyamines have an impact on the response of a salt-sensitive rice cultivar to NaCl? *Journal of Plant Physiology* 163, 506–516.

Ndaiyiragije A, Lutts S. 2007. Long term exogenous putrescine application improves grain yield of a salt-sensitive rice cultivar exposed to NaCl. *Plant and Soil* 291, 225–238.

Page AF, Mohapatra S, Minocha R, Minocha SC. 2007. The effects of genetic manipulation of putrescine biosynthesis on transcription and activities of the other polyamine biosynthetic enzymes. *Physiologia Plantarum* 129, 707–724.

Palanmurugan R, Scheel H, Hofmann K, Dohmen RJ. 2004. Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme. *EMBO Journal* 23, 4857–4867.

Pandey S, Ranade SA, Nagar PK, Kumar N. 2000. Role of polyamines and ethylene as modulators of plant senescence. *Journal of Biosciences* 25, 291–299.

Piqueras A, Cortina M, Serna MD, Casas JL. 2002. Polyamines and hyperhydricity in micropropagated carnation plants. *Plant Science* 162, 671–678.

Prakash L, Prathasenan G. 1988. Putrescine reduces NaCl-induced inhibition of germination and early seedling growth of rice (*Oryza sativa* L.). *Australian Journal of Plant Physiology* 15, 761–767.

Rea E, Di Monte G, De Agazio M. 1995. Inhibition of root growth by spermidine is not due to enhanced production of ethylene. *Plant Science* 108, 121–124.

Roy P, Niyogi K, SenGupta DN, Ghosh B. 2005. Spermidine treatment to rice seedlings recovers salinity stress-induced damage of plasma membrane and PM-bound H+-ATPase in salt-tolerant and salt-sensitive rice cultivars. *Plant Science* 168, 583–591.

Rozen S, Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics methods and protocols: methods in molecular biology*. Totowa, NJ: Humana Press, 365–386.

Schröeder G, Schröeder J. 1995. cDNAs for S-adenosyl-L-methionine decarboxylase from *Catharanthus roseus*, heterologous expression, identification of the proenzyme-processing site, evidence for the presence of both subunits in the active enzyme, and a conserved region in the 5′ mRNA leader. *European Journal of Biochemistry* 228, 74–78.

Tadolini B. 1988. Polyamine inhibition of lipoperoxydation: the influence of polyamines in iron oxidation in the presence of compounds mimicking phospholipids polar heads. *Biochemical Journal* 249, 33–36.

Tiburcio AF, Altabella T, Borrell A, Masgrau C. 1997. Polyamine metabolism and its regulation. *Physiologia Plantarum* 100, 664–674.

Walden R, Cordeiro A, Tiburcio AF. 1997. Polyamines: small molecules triggering pathways in plant growth and development. *Plant Physiology* 113, 1009–1013.

Watson MB, Maimberg RL. 1996. Regulation of *Arabidopsis thaliana* (L) Heynh arginine decarboxylase by potassium deficiency stress. *Plant Physiology* 111, 1077–1083.

Yang J, Zhang J, Liu K, Wang Z, Liu L. 2007. Involvement of polyamines in the drought resistance of rice. *Journal of Experimental Botany* 58, 1545–1555.

Yoshida S, Forno OA, Cock JH, Gomez KA. 1976. *Laboratory manual for physiological studies of rice*. Manila: International Rice Research Institute.