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Research article

Polyamines contribute to salinity tolerance in the symbiosis *Medicago truncatula-Sinorhizobium meliloti* by preventing oxidative damage

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ABSTRACT

Polyamines (PAs) such as spermidine (Spd) and spermine (Spm) are small ubiquitous polycationic compounds that contribute to plant adaptation to salt stress. The positive effect of PAs has been associated to a cross-talk with other anti-stress hormones such as brassinosteroids (BRs). In this work we have studied the effects of exogenous Spd and Spm pre-treatments in the response to salt stress of the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti* by analyzing parameters related to nitrogen fixation, oxidative damage and cross-talk with BRs in the response to salinity.

Exogenous PAs treatments incremented the foliar and nodular Spd and Spm content which correlated with an increment of the nodule biomass and nitrogenase activity. Exogenous Spm treatment partially prevented proline accumulation which suggests that this polyamine could replace the role of this amino acid in the salt stress response. Additionally, Spd and Spm pre-treatments reduced the levels of H_2O_2 and lipid peroxidation under salt stress. PAs induced the expression of genes involved in BRs biosynthesis which support a cross-talk between PAs and BRs in the salt stress response of *M. truncatula-S. meliloti* symbiosis.

In conclusion, exogenous PAs improved the response to salinity of the *M. truncatula-S. meliloti* symbiosis by reducing the oxidative damage induced under salt stress conditions. In addition, in this work we provide evidences of the cross-talk between PAs and BRs in the adaptive responses to salinity.

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1. Introduction

The diamine putrescine (Put) and the polyamines (PAs) spermidine (Spd) and spermine (Spm) are small ubiquitous polycations involved in numerous processes in all living organisms (Bouchereau et al., 1999). In plants, PAs are involved in cell division, embryogenesis, senescence, floral development and fruit ripening (Hussain et al., 2011). In addition, PAs play an important role in the response of plants to adverse environmental conditions due to their polycationic nature (Alcazar et al., 2010; Liu et al., 2007). Among these adverse conditions, soil salinity is one of the most important abiotic factors limiting crop productivity all over the world, especially in arid and semiarid regions, and is predicted to get worse under climate change conditions (Rozema and Flowers, 2008). Salt stress adversely affects plant development and productivity by generating ion toxicity, osmotic stress, water deficits and oxidative damage through the production of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) among others (Munns and Tester, 2008).

Legumes are classified as salt-sensitive crop species and their productivity is particularly affected by soil salinity because nodular nitrogenase activity, responsible for the nitrogen supply to the plant in symbiosis with soil bacteria known as rhizobia, markedly decreases upon exposure to saline conditions (López et al., 2008; Aranjuelo et al., 2014). Root nodules of legumes have been found to contain a high variety and concentration of PAs, some of them of bacteroidal origin (López-Gómez et al., 2014a), however, little is known about the role of PAs within the root nodules. A cross-talk between PAs and other plant growth regulators such as brassinosteroids (BRs), has been described in the response to abiotic stresses (López-Gómez et al., 2016a; Choudhary et al., 2012; Zheng

Abbreviations: PAs, polyamines; BRs, brassinosteroids; Put, putrescine; Spd, spermidine; Spm, spermine; Cad, cadaverine; Homspd, homospermidine; MDA, malondialdehyde; Pro, proline; NFR, nitrogen fixation rate; NFW, nodule fresh weight.

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et al., 2016). BRs biosynthesis is regulated at the transcriptional level of the biosynthetic genes by other plant hormones such as auxin (Chung et al., 2011) and in addition, BRs have been shown to be involved in the systemic regulation of the root nodule formation in soybean (Terakado et al., 2006) by a modification of the PAs levels in leaves (Terakado-Tonooka and Fujihara, 2008).

Exogenous addition of PAs has been extensively used as a strategy to enhance tolerance to salinity. There is a large body of evidence suggesting that exogenous application of PAs could preserve plant cell membrane integrity, minimize growth inhibition caused by stress, reduce superoxide radical and H_2O_2 contents and increase activities of antioxidant enzymes (reviewed by (Hussain et al., 2011)).

In this work we have studied the effects of exogenous Spd and Spm in the adaptation to salt stress of the symbiosis *M. truncatula-S.meliloti* by analyzing parameters related to nitrogen fixation, oxidative damage and cross-talk with BRs in the response to salinity.

2. Material and methods

2.1. Biological material and growth conditions

Medicago truncatula (var. Jemalong) seeds were scarified by immersion in concentrated H_2SO_4 for 5 min, washed with sterile water, surface sterilized by immersion in NaClO 50% (v/v) plus Tween-20 for 10 min and germinated onto 1.0% water-agar plates at 25 °C in the darkness. After 3 days, *Medicago* seedlings were transferred to sterile vermiculite:perlite (3:1) and watered with a modified nitrogen free (Puppo and Rigaud, 1975) nutrient solution. Two days later, *M. truncatula* seedlings were inoculated with *S. meliloti* 1021 strain (c. 10^9 cell ml^{-1}) grown in a TY medium.-Plants, in individual pots of about 200 ml, were grown in a controlled environmental chamber with a 16/8 h light-dark cycle, 23/18 °C day night temperature, relative humidity 55/65% and photosynthetic photon flux density (400–700 nm) of 450 μmol m^{-2} s^{-1} supplied by combined fluorescent and incandescent lamps. Six weeks after sowing plants were subjected to Spd and Spm treatments by the addition of 0.1 mM of each polyamine to the nutrient solution. Control plants were watered with the same nutrient solution without polyamines. Salt treatment was applied two weeks later by the addition of 75 mM NaCl to the watering nutrient solution. Control plants were watered with a NaCl-free nutrient solution. Plants were harvested 10 weeks after sowing with polyamines and NaCl treatments lasting 4 and 2 weeks, respectively. Nodules and leaves were frozen at −80 °C for further analyses. Samples of leaves, stems and roots were dried at 70 °C for 24 h and their dry weight determined.

2.2. Nitrogen fixation

Nitrogenase activity (E.C. 1.7.9.92) was measured as the representative H_2-evolution in an open-flow system (Witty and Minchin, 1998) using an electrochemical H_2 sensor (Qubit System Inc., Canada). H_2 production was recorded in intact nodulated roots of plants. Apparent nitrogenase activity (ANA, rate of H_2 production in air) was determined under N_2:O_2 (80%:20%) with a total flow of 0.4 l min^{-1}. After reaching steady-state conditions total nitrogenase activity (TNA) was determined under Ar:O_2 (79%:21%). Nitrogen fixation rate (NFR) was calculated as (TNA-ANA)/3. Standards of high purity H_2 were used to calibrate the detector.

2.3. Determination of free polyamines in leaves and nodules

Nodule and leaves extracts were prepared from 0.2 g of fresh tissue with 0.6 ml of 5% (v/v) cold perchloric acid (PCA) and incubated 24 h at 4 °C. The homogenate was centrifuged (3,000×g, 5 min, 4 °C) and 0.2 ml aliquots of the supernatant were dansylated as described below. The analysis of free PAs was performed with HPLC (Agilent Technologies 1260) equipped with a reverse phase column (4.6 × 250 mm C18) after derivatization with dansyl chloride (Sigma). Derivatization was performed by mixing 0.2 ml aliquots of the extracts prepared as described above with 0.4 ml of dansyl chloride (prepared fresh in acetone, 10 mg/ml) and 0.2 ml of saturated sodium carbonate. After brief vortexing, the mixture was incubated in darkness at room temperature overnight. Excess dansyl reagent was removed by reaction with 0.1 ml (100 mg/ml) of added proline, and incubation for 30 min. Dansylpolyamines were extracted in 0.5 ml toluene. The organic phase was collected and evaporated to dryness under a stream of nitrogen, and redissolved in 0.1 ml acetonitrile.

Column flow was 1.5 ml min^{-1} and the elution gradient was prepared with eluent A (water) and eluent B (acetonitrile). The column was equilibrated with 70% B and 30% A before injecting 0.01 ml samples. This was followed by a linear gradient ending with 100% B after 9 min. The final step was held for 4 min before regenerating the column. Detection was done with a fluorometer using excitation and emission wavelengths of 415 and 510 nm, respectively, according to (Flores and Galston, 1982). A relative calibration procedure was used to determine the PAs in the samples, using 1,7- diaminoheptane (HTD) as internal standard and PAs standards amounts ranging from 0.3 to 1.5 nmol purchased from Sigma. Results were expressed as nmol g^{-1} fresh weight.

2.4. Proline determination

Proline was determined by the ninhydrine method (Troll and Lindsley, 1955). Briefly, 250 mg of plant material was homogenized in 3 ml of 95% (v/v) ethanol at room temperature and centrifuged at 2000×g for 5 min. 300 μl of distilled water and 2 ml of ninhydrine reagent were added to an aliquot of 200 μl of extract. The mixture was boiled for 60 min, and the reaction was stopped in an ice bath. The chromophore obtained was extracted with 6 ml of toluene by vigorous shaking for 20 s. Absorbance of the resulting organic layer was measured at 520 nm with Varian UV-VIS spectrophotometer. Calibration was made using L-Pro as a standard.

2.5. Lipid peroxidation

Lipid peroxidation was measured by the level of malondialdehyde (MDA), a product of lipid peroxidation, using a reaction with thiobarbituric acid (TBA) as described by (Hodges et al., 1999). Fresh samples (100 mg) were ground in a mixture of 1 ml trichloroacetic acid (TCA) (20% w/v) and 0.2 ml of 4% (w/v) butylatedhidroxytoluene in ethanol, at 4 °C. After centrifugation (10,000×g for 15 min), 0.25 ml aliquots of the supernatant were mixed with 0.75 ml of 0.5% (w/v) thiobarbituric acid in 20% TCA and the mixture was incubated at 94 °C for 30 min. The reaction was stopped by cooling in an ice bath for 15 min. Reaction tubes were centrifuged at 10,000×g for 15 min and supernatants were used to determine the absorbance at 532 nm. The value for non-specific absorption at 600 nm was subtracted.

2.6. Histochemical and quantitative detection of H_2O_2 in leaves

The histochemical analysis of H_2O_2 was performed in leaves of two weeks old plants grown axenically in glass test tubes. Plants were pretreated with Spm 0.1 mM 24 h before treatment with NaCl to a final concentration of 150 mM for 24 h. The H_2O_2 was localized histochemically by staining leaves with 1% 3,3-diaminobenzidine

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