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Enzymes of the glutathione–ascorbate cycle in leaves and roots of rhizobia-inoculated faba bean plants (*Vicia faba* L.) under salinity stress

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ABSTRACT

Grain legumes such as the faba bean (*Vicia faba* L.) used for human as well as animal nutrition, are of great importance especially in countries under Mediterranean climate like Morocco, which is mainly characterized by semi-arid and arid climates and by saline soils. The objective of the present study was to evaluate the effects of salt stress on growth, nodulation and the enzymes of the glutathione–ascorbate cycle of faba bean plants (Moroccan cultivar "Aguadulce"). The experiments were carried out under greenhouse conditions, where faba bean plants were grown under control (nutrient solution) or salt stress conditions (nutrient solution containing 75 mM or 150 mM of NaCl). The activity of enzymes of the glutathione–ascorbate cycle was studied in leaves and roots of faba bean plants inoculated by rhizobia RhOF4 or RhOF6, isolated from nodules of faba bean cultures in the region of Marrakech. The growth of faba bean plants decreased with increasing salt concentrations. Total weight and length of shoots significantly decreased under NaCl stress ($p < 0.05$), especially at 150 mM. Also the nodulation of the roots was strongly decreased under salinity stress, with reductions of 91.35% and of 88.67% in the total nodule number of plants exposed to 150 mM of NaCl and inoculated by RhOF4 and by RhOF6 respectively. NaCl effects on the glutathione–ascorbate cycle were more pronounced in roots inoculated by RhOF6. Stress responding glutathione *S*-transferase (GST) activity was generally increased in roots inoculated by RhOF6 and submitted to salt stress. On the contrary, specific activities of glutathione peroxidase (GPOX), superoxide dismutase (SOD), ascorbate peroxidase (APOX) and monodehydroascorbate reductase (MDHAR) were reduced in roots of faba bean plants inoculated by RhOF6 exposed to saline treatment as compared to the controls. Our results show the importance of several enzymes of the ascorbate–glutathione cycle and the reduced glutathione (GSH) during some faba bean –rhizobia symbiotic combinations, in root defence and adaptation against salt stress conditions.

1. Introduction

Legume plants play a key role in sustainable agriculture and present economic and environmental benefits due to their important capacity to fix atmospheric nitrogen in the root nodules in symbiotic relationship with rhizobia. Symbiotic rhizobia can increase yields, accelerate flowering/fruit ripening and contribute to the improvement of the soil nitrogen balance for the benefit of legumes and associated species [1,2]. Hence, the symbiosis with soil rhizobia could, by growing legumes, exempt farmers at least partly from the costly chemical fertilizers which have been shown to pollute agro-ecosystems.

Abbreviations: GSH, glutathione; GST, glutathione *S*-transferase; GR, glutathione reductase; GPOX, glutathione peroxidase; YEM, Yeast Extract–Mannitol medium; CFU, colonies forming units; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; NBC, 4-nitrobenzyl-chloride; NBOC, *p*-nitrobenzoyl chloride; SOD, superoxide dismutase; APOX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase.

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The legume–rhizobia symbioses constitute an important area of agricultural research because they produce seeds and fodder with high protein content and exert beneficial effects on crop productivity in diverse cropping ecosystems over the world [3]. However, legume plants as well as their symbionts are exposed to various biotic and abiotic environmental stresses especially in poor soils [4–9].

Salinity is currently one of the most troublesome environmental factors in agriculture [9]. The FAO [10] has reported that over 70 million ha of farmland are affected by salinity. Saline conditions inhibit growth and metabolism of legumes [11], affect the infection process and nodule function [12–14], they limit the symbiotic development and nitrogen fixation [14–16] and on top of that affect microbial populations of soil [17,18]. Salinity in soil or water is hence one of the main environmental stress parameters, especially in arid and semi-arid regions, and can severely reduce legume production [19,20].

The aim of the present work is to investigate the characteristics of enzymatic stress response, especially of metabolizing enzymes of the glutathione–ascorbate cycle, and the damage caused by salinity in some rhizobia–faba bean symbiotic combinations. *Vicia faba* L. (faba bean) is the most important legume grown in Morocco, occupying about 40% of the total area reserved for legumes [21]. The faba bean is rich in proteins, mineral elements and vitamins, and hence used for human as well as animal nutrition. The research on salt stress is important since salinity increasingly affects agricultural areas throughout the world [22,3]. The enzymes of the glutathione–ascorbate cycle represent important physiological and biochemical markers for plants under environmental stress.

Glutathione (GSH), the tripeptide γ-glutamylcysteinylglycine, occupies a pivotal role in the ascorbate–glutathione cycle for the destruction of photosynthetically generated H_2O_2. Furthermore, GSH contributes to scavenge lipid peroxides and to detoxify xenobiotics [23,24].

Plants have developed a complex antioxidant system in order to prevent damage to cellular components by reactive oxygen species (ROS). The primary components of this system include carotenoids, ascorbate, glutathione and tocopherols, in addition to enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPOX), peroxidases and the enzymes involved in ascorbate–glutathione cycle, such as ascorbate peroxidase (APOX) and glutathione reductase (GR). Many components of this antioxidant defence system can be found in various subcellular compartments [25–28].

Due to this central role of GSH, this study was performed to determine which role this tripeptide occupies in salinity tolerance and adaptation of the faba bean–rhizobia symbiosis. The effects of salinity stress on the enzymes of the glutathione–ascorbate cycle of *V. faba* L. have up to now not been covered in the literature.

2. Material and methods

2.1. Isolation and purification of rhizobial strains

Rhizobial strains RhOF4 and RhOF6 were isolated from root nodules of faba bean cultures grown in the Marrakech-Tensift-Al Haouz region (Morocco). These strains were isolated and purified by repeated streaking on Yeast Extract–Mannitol agar (YEM Agar) with Congo red, using standard microbiological methods [29]. After incubation for 48 h at 28 °C, the colonies of rhizobia characterized by a sticky aspect and off-white colour (without absorption of Congo red) were isolated and purified on YEM medium. Pure isolates were maintained at −20 °C in 30% glycerol and Yeast Extract–Mannitol broth (YEM broth). These local strains were verified for the nodulation of seedlings of faba bean. Working cultures were maintained on YEM agar slants at 4 °C. The inoculation of plants was performed with precultures grown on YEM broth at the rhizobial growth exponential phase.

2.2. Plant material and growth conditions

Faba bean seeds of the Moroccan cultivar “Aguaducle” were surface sterilized with ethanol for 3 min, rinsed several times with sterile distilled water and germinated for four days at 25 °C in the dark. The germinated seedlings, selected for uniformity, were placed during 30 min into a rhizobia liquid medium and separately inoculated with either RhOF4 strain or RhOF6 strain. The inoculum was prepared by growing the rhizobial strain in YEM medium at 28 °C for 2–3 days to obtain an OD of 1 at 600 nm (approximately 10^9 colonies forming units (CFU)/mL).

The seedlings were then grown on perlite in 5 L pots. Rigaud and Puppo nutrient solution in tap water was added to the trays (semi-hydroponic cultures). The trials were performed at the Helmholtz Zentrum greenhouse at 15 h day/9 h night, at a temperature of 23 °C/18 °C (day/night) and relative humidity of 65%.

The plants were initially supplied with 4 mM of urea to avoid N-deficiency during the nodule development. Afterwards they were grown in N-free Rigaud and Puppo nutrient solution. The treatments with or without NaCl were started at the 18th day (50 mM and 100 mM) reaching their final salt concentrations on the 21st day after sowing (75 mM and 150 mM). The harvest of plants was done at 36 days after sowing, so the plants were under NaCl treatment for 18 days.

The plants were harvested in the first stage of flowering and removed from the pots. The roots were then thoroughly rinsed with tap water. The plants were weighted (total fresh weight) and the aerial parts (shoots) of the plants were separated from the roots at the cotyledonary node. Their length was then measured and the number of nodules was counted.

2.3. Enzyme extraction and enzyme measurements

The enzyme extraction procedure followed protocols according to Schröder et al. [30].

2.4. Glutathione S-transferase (GST) assay

Spectrophotometer assays for determination of GST activity using the model substrates: 1-chloro-2,4-dinitrobenzene (CDNB, ε_{340nm} = 9.6 mM^{-1} cm^{-1}), 1,2-dichloro-4-nitrobenzene (DNCB, ε_{345nm} = 8.5 mM^{-1} cm^{-1}), 4-nitrobenzyl-chloride (NBC, ε_{310nm} = 1.8 mM^{-1} cm^{-1}), *p*-nitrobenzoyl chloride (NBOC, ε_{310nm} = 1.9 mM^{-1} cm^{-1}), followed the method of Schröder et al. [31]. Previous studies have noted that the glutathione *S*-transferase isoforms are not identical in different plant tissues, which results in differences in the substrate spectrum [24,32–34]. Assays with the substrate fluorodifen (ε_{400nm} = 3.1 mM^{-1} cm^{-1}) followed Schröder et al. [24]. The specific activity was expressed in μkat/mg protein.

2.5. Glutathione peroxidase (GPOX) assay

The GPOX activity was assayed spectrophotometrically according to Dixon et al. [35].

2.6. Superoxide dismutase (SOD) assay

The SOD activity was determined spectrophotometrically as the change from adrenalin to epinephrine was observed [36].