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Olive trees response to lead stress: Exogenous proline provided better tolerance than glycine betaine

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ABSTRACT

Glycine betaine (GB) and proline (Pro) function as compatible solutes and are upregulated in plants under abiotic stresses. The objective of this study was to investigate whether exogenous GB and Pro could improve lead (Pb) tolerance in young olive trees (*Olea europaea* L.). Comparison between the effect of GB and Pro on Pb-stressed olive trees was realized. Two-year-old olive trees were subjected for five months to two lead-stress levels (150 and 450 mg Pb $(NO_3)_2$ kg^{-1} soil). GB and Pro were supplied through the irrigation water at 20 mM concentration. In both root and leaf tissues, an increase in electrolyte leakage and in oxidative stress markers, such as hydrogen peroxide and thiobarbituric acid reactive substances was observed despite the elevation of antioxidant enzymes activities as well as non-enzymatic antioxidants. Interestingly, GB and Pro supplementation mitigated the adverse Pb effects on *O. europaea* trees. Indeed, they reduced Pb content and increased the enzymatic and non-enzymatic antioxidants parameters. Thus, oxidative damage was reduced and better levels of plant biomass were obtained. The exogenous Pro appeared to be a better ameliorator than the GB in protecting young *O. europaea* trees against Pb toxicity.

1. Introduction

The heavy metals pollution is one of the major environmental problems because of their increasing level caused mainly by anthropogenic activities, such as industrial production, mining activities, agriculture and transportation. The continuous discharging of these pollutants resulted in dangerous problems on the mankind and ecosystem (Amari et al., 2017; Marques et al., 2017). The Pb does not play a known function in plant metabolism, but their exposure to Pb often delays their growth and development. In fact, Pb stress causes morphological disorders and plant growth perturbation (Akhtar et al., 2017). In addition, it causes inhibition on physiological and biochemical mechanisms including alteration of enzymatic function and water relations.

Although some reactive oxygen species (ROS) can act as signal molecules that alter the genes controlling expression of defense responses, these molecules can be harmful at high concentration (Bharwana et al., 2014). The ROS possess a strong oxidative power allowing them able to react with most of the biological molecules, which has harmful consequences for the cell integrity. Indeed, some ROS are able to diffuse through the cells walls and can thus cause multiple damages such as proteins oxidation. Membrane lipids are also subject to multiple modifications that give rise to peroxidation, which could lead to changes in cell membrane permeability (Kumar et al., 2013; S. Li et al., 2016). Several systems of antioxidant defenses, such as antioxidant enzymes, small antioxidant molecules and trace elements are able regulate the ROS production (S. Li et al., 2016).

Several abiotic stresses, such as drought, salinity, cold, heat and chemical pollution, lead to an increase of compatible solutes concentration in plants (Vinocur and Altman, 2005; Habib et al., 2012). The common characteristic of these molecules is that they can be accumulated at high levels without disrupting the intracellular biochemistry. These compatible solutes include mainly proline (Pro) and glycine betaine (GB). The accumulation of compatible solutes in plant cells correlates with an enhanced stress tolerance through: (i) decreasing potential water in plant cell, which increase the water absorption from the rhizosphere (ii) scavenging free radicals (iii) activation of enzymatic and non-enzymatic antioxidant systems as well as (iv) protection of photosynthetic apparatus against damage (Dawood et al., 2014; Zouari et al., 2016b; Yadu et al., 2017; Youssef et al., 2018).

In earlier studies, it has been demonstrated that exogenous application of osmolytes like Pro and GB mitigates the detrimental effects of several abiotic stresses. It has been reported that Pro and GB that were supplied exogenously can enhance the antioxidant defense system and osmotic balance in plants exposed to various stress conditions. Rasheed et al. (2014) also reported that exogenous Pro and GB

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application provide a protection against Cd-induced oxidative stress in two wheat cultivars (*Triticum aestivum*).

Olive tree (*Olea europaea*) is the most widely cultivated tree species in Tunisia. With a total cultivated area of about 1.7 million ha and more than 70 million olive trees (Gargouri et al., 2013). This plant is characterized by its resistant to water, salt and heat stresses. Nowadays, the Sfax region (Southern Tunisia) accommodates one of the most important industrial complexes, among which the lead smelter and phosphate fertilizer factories constitute the principal pollution source. Heavy metals (particularly Pb) are among the most pollutants discharged from these industries. The continuous discharging of Pb into the soil caused serious problems on the ecological balance. Therefore, the selection of appropriate plants that can be grown and expected to perform well under such conditions is very important for the physical stability of the soil and the development of greener environment. The objectives of this study were to examine the comportment of olive tree exposed to Pb individually or simultaneously with exogenous application of Pro or GB. The parameters regarding the plant biomass, oxidative stress indicators, antioxidant enzymes activities, phenolics content and DPPH• radical-scavenging activity were studied. The comparison between Pro and GB was assessed to know their effectiveness in alleviating the Pb stress.

2. Materials and methods

2.1. Plant material and growth conditions

This research was carried out in Olive Tree Institute (Sfax, Tunisia) (34°43′N, 10°41′E). Two-year-old olive trees (*Olea europaea* L. cv Chemlali) with similar stem diameter and height were selected and planted in 5 l plastic pots (one plant per pot), which were filled with 5 kg soil. The soil was a sandy (85% sand, 5% clay and 10% silt) collected at 0–30 cm depth from a farmland field not exposed to pollution. Air-dried soil samples were passed through 2 mm sieve and then thoroughly mixed and characterized. The main characteristics of the study soil were: organic matter = 1.8%, electrical conductivity = 1.5 mS cm^{-1} and total Pb = 4.6 mg kg^{-1} soil. The soil pH is around 7 and no fertilizer was used in our experiment. The plants were grown under naturally conditions of temperature and relative air humidity. Air temperature, relative humidity and photosynthetically active radiations ranged between 16.4 ± 3.5 and 35 ± 2.5 °C, 53 ± 5.5 to 72 ± 6.5% and 1600 to 1100 (μmol m^{-2} s^{-1}) respectively.

All plants were subjected to the following treatments (from 15 April to 15 September 2013): (i) Three Pb levels: (control (Pb0), Pb150 and Pb450) distributed as 0, 150 and 450 mg $Pb(NO_3)_2$ kg^{-1} soil, (ii) Two exogenous applications of Pro: (Pb150 + Pro, Pb450 + Pro) and (iii) Two exogenous applications of GB (Pb150 + GB, Pb450 + GB). The Pro and GB were supplied into the irrigation water at 20 mM concentration. We selected these rates of Pb pollution, starting to Pb content in Sfax soil at approximately of lead smelter and phosphate fertilizer factory that resulted in growth inhibition of young olive trees. Pro and GB concentration were selected based on the literature.

Treatments were arranged in a completely randomized design. The number of young olive trees for each treatment was nine ($n = 9$). All measurements were done in the end of the experiment. Pots were regularly rotated and weeds were removed when they are present. The irrigation was performed daily to replace evapotranspiration water.

2.2. Lead content determination

The Pb content was measured in the leaf and root tissues following the procedure described by Bankaji et al. (2015). Dry leaf samples (1 g) were placed in an oven at 250 °C for 3 h and then digested with 10 ml of 1 M HNO_3. After that, the resultant solutions were adjusted to 25 ml using distilled water. After filtration, the Pb content was determined using atomic absorption spectrophotometry (Perkin Elmer A Analyst 300, USA).

The translocation factor was calculated by using Eq. (1).

$$TF = [\text{Pb leaf}]/[\text{Pb root}] \quad \text{Eq.(1)}$$

2.3. Oxidative stress indicators: hydrogen peroxide (H_2O_2), lipid peroxidation (TBARS) and electrolyte leakage (EL) determinations

Fresh leaf and root samples were used for H_2O_2, TBARS and EL determination. The H_2O_2 level was determined according to Sergiev et al. (1997) method. A 0.5 g of fresh sample was homogenized in an ice bath with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). Then, the homogenate was centrifuged at 12,000 × *g* for 15 min. After that, 1 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide. The absorbance of the supernatant was measured at 390 nm with uv/vis spectrophotometer (Perkin Elmer, Norwalk, USA). The H_2O_2 content was calculated using a standard curve.

The lipid peroxidation level in leaf and root tissues was estimated the thiobarbituric acid reactive substances (TBARS) content expressed as equivalents of malondialdehyde (MDAeq), which is a product of lipoperoxidation. The TBARS content was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968) with minor modifications reported by Zouari et al. (2016a). A 0.5 g of fresh sample was homogenized in 5 ml of 0.1% TCA. The homogenate was centrifuged at 12,000 × *g* for 5 min. Then, 4 ml of 20% TCA containing 0.5% TBA was added to 1 ml aliquot of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 12,000 × *g* for 10 min, the absorbance of the supernatant was measured with Helios βspectrophotometer (Perkin Elmer, Norwalk, USA) at 532 nm and the value of the nonspecific absorption at 600 nm was subtracted. The TBARS content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The degree of membrane integrity loss was estimated by measuring the amount of electrolyte leakage (EL) as previously described by Lutts et al. (1996). The fresh leaf and root samples (0.25 g) were cut in small parts, put into test tubes containing 10 ml of deionized water and incubated at room temperature on a rotary shaker for 24 h. Subsequently, the initial electrical conductivity of the medium (EC1) was assessed. The samples were placed in an oven (90 °C) for 2 h. Thereafter, they were cooled at 25 °C and a second measurement of the electrical conductivity (EC2) was determined. The EL was calculated by using Eq. (2).

$$EL\ (\%) = (EC1/EC2) \times 100 \quad \text{Eq.(2)}$$

2.4. Plant biomass measurements

At the end of the experiment and after the plant harvest, leaf and root fresh weights were determined. The plant material (leaves and roots) was dried in an oven at 70 °C for 72 h to determine the biomass.

2.5. Enzymatic antioxidant assays

Antioxidant enzyme activities (SOD, CAT, GPX and APX) of roots and leaves were determined spectrophotometrically. 500 mg plant tissue (roots and shoots) was crushed in 0.8 ml of 100 mM phosphate buffer (pH = 7.0) using pre-chilled mortar and pestle and centrifuged at 15,000*g* for 15 min at 4 °C. The resulting supernatant was collected and stored at 4 °C until use for enzyme activity estimations.

The SOD (E.C. 1.15.1.1) activity was determined according to the method of Giannopolitis and Ries (1977) following the inhibition of photochemical reduction due to nitro-blue tetrazolium (NBT). The reaction mixture was comprised of 40 mM K-phosphate buffer (pH 7.8),