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Research article

Resveratrol and its combination with α-tocopherol mediate salt adaptation in citrus seedlings

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ABSTRACT

Resveratrol, a phytoalexin found in red wine, has the potential to impact a variety of human diseases but its function in plants exposed to stressful conditions is still unknown. In the present study the effect of exogenous application of resveratrol (Res), α-tocopherol (α-Toc) and their combination (Res + α-Toc) in salt adaptation of citrus seedlings was investigated. It was found that Res, α-Toc or Res + α-Toc treatments reduced NaCl-derived membrane permeability (EL), lipid peroxidation (MDA) and pigments degradation, whereas companied Res and α-Toc application also reduced H_2O_2 accumulation in leaves and restored the reduction of photosynthesis induced by NaCl. Application of Res under salinity retained Cl^- in roots while Res + α-Toc reduced the translocation of Na^+ and Cl^- to leaves. Carbohydrates and proline, phenols, total ascorbic acid and glutathione were remarkably affected by NaCl as well as by chemical treatments in leaves and roots of citrus. NaCl treatment increased the activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR), polyphenol oxidase (PPO) in leaves while SOD and POD activities were decreased in roots by this treatment. Also, Res, α-Toc or Res + α-Toc treatments displayed tissue specific activation or deactivation of the antioxidant enzymes. Overall, this work revealed a new functional role of Res in plants and provided evidence that the interplay of between Res and α-Toc is involved in salinity adaptation.

1. Introduction

Soil salinity is one of the most severe abiotic stresses with detrimental effects in plant productivity, especially in salt-sensitive species as citrus (Navarro et al., 2014). Salinity arises through osmotic stress, ion toxicity and consequently oxidative stress with enhanced formation of reactive oxygen species (ROS) (Gill et al., 2013b; Lee et al., 2013). In response to salinity stress, plants express various adaptive mechanisms (Lee et al., 2013; Mittal et al., 2012). Particularly, the control of Cl^- transport is crucial for citrus plants and represents an indicator of salt tolerance (Moya et al., 2003; Teakle and Tyerman, 2010). Accumulation of compatible organic solutes like carbohydrates and proline is a common mechanism essential for effective adaptation to salt stress (Mittal et al., 2012). To cope with salt-induced oxidative stress, plants have also developed a non-enzymatic defense system including phenols, carotenoids (Car), ascorbic acid (AsA), glutathione (GSH) as well as a network of antioxidant enzymes, including superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2) and polyphenol oxidase (PPO; EC 1.30.3.1) (Gill and Tuteja, 2010).

Resveratrol (3, 5, 4′-trans-trihydroxystilbene) is a member of the stilbene family of phenolic compounds produced in grapevines, red wine, berries, Japanese knotweed, peanuts and their derivatives (Turan et al., 2012; Shi et al., 2014). Resveratrol besides acting as a phytoalexin has a variety of diverse biochemical and physiological functions mostly due to its intrinsic antioxidant capacity (Fernández-Mar et al., 2012). It was shown that Res is able to scavenge ROS and as a consequence to decrease oxidative stress in various cell systems (Fernández-Mar et al., 2012; Kovacic and Somanathan, 2010; Leonard et al., 2003; Soares et al., 2003). To the best of our knowledge, however, external application of resveratrol in plant science is limited and is restricted to its function as natural pesticide in grapes (Montero et al., 2003) and as a

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; Car, carotenoid; Chl, chlorophyll; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; EL, electrolyte leakage; GSH, glutathione; GR, glutathione reductase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; PMSF, phenylmethanesulfonylfluoride; POD, peroxidase; PPO, polyphenol oxidase; PVPP, polyvinylpolypyrrolidone; Res, resveratrol; ROS, reactive oxygen species; SE, standard error; SOD, superoxide dismutase; α-Toc, α-tocopherol.

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postharvest treatment in grapes and apples (Gónzalez-Urena et al., 2003). α-Tocopherol, the major vitamin E compound in plant leaves, is a lipophilic compound located in the chloroplast envelope and thylakoid membranes (Havaux et al., 2000). The endogenous status of α-tocopherol is significantly modulated by environmental stresses (Munné-Bosch, 2005) and associated with the protection of plants against photooxidative stress (Havaux et al., 2005) and salinity (Queirós et al., 2011). Experimental evidence indicated that α-tocopherol as being an efficient chain breaking antioxidant, can be used as external application molecule to enhance the adaptation of plants to stress conditions (Uchendu et al., 2010), including salinity (Sakr and El-Metwally, 2009).

Based on the antioxidant functions of both biomolecules mentioned above, along with the fact that resveratrol and vitamin E synergistically reduce ROS-induced lipid peroxidation in micelles (Fang et al., 2002), the objective of the present study was to investigate the impact of exogenous application of resveratrol or α-tocopherol and their combination in salinity adaptation of citrus plants.

2. Materials and methods

2.1. Plant material and salinity treatments

The experiment was conducted in the greenhouse at the Experimental Farm of the Aristotle University of Thessaloniki in 2012 using two- years- old *Citrus aurantium* L. seedlings. The experimental plants were transferred in plastic pots containing a mixture of soil:sand (1/1, v/v). The soil was clay loam with a pH value of 7.40, organic matter 0.70% and electrical conductivity (1:5) of 0.96 mS cm^{-1}. Initially, the experiment plants were irrigated with half-strength Hoagland's nutrient solution (NS) (Hoagland and Arnon, 1938) for one month. Then, plants were exposed to five different growth conditions: (1) control, only NS (C); (2) NaCl treatment, NS plus 100 mM NaCl (NaCl); (3) resveratrol treatment, NS plus 100 mM NaCl + 100 μM resveratrol (NaCl + Res); (4) α-tocopherol treatment, NS plus 100 mM NaCl + 0.50 mM α-tocopherol (NaCl+α-Toc) and (5) resveratrol + α-tocopherol treatment, NS plus 100 mM NaCl + 100 μM resveratrol + 0.50 mM α-tocopherol (NaCl + Res+α-Toc). Each group was consisted of 12 pots containing 24 plants (replicates) (2 plants per pot). Plants were irrigated three times per week, one of them with the supplementary additions of Res, α-Toc or Res + α-Toc. Resveratrol was diluted in small amount of ethanol, therefore during irrigation the same amount was added in all treatments. The experiment lasted for 30 days until visible typical leaf salt toxicity symptoms appeared.

2.2. Electrolyte leakage, malondialdehyde and hydrogen peroxide concentration analysis

Electrolyte leakage (EL) was measured according to Lutts et al. (1996). For malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentration analysis the leaves were extracted and measured according to Heath and Packer (1968) and Alexieva et al. (2001), respectively.

2.3. Chlorophyll and carotenoid concentration analysis

For chlorophyll (Chl) and carotenoid (Car) analysis the leaves were extracted with 96% ethanol. The total Chl (a+b) was calculated according to Wintermans and de Mots (1965) by measuring absorbance at 649 nm and 665 nm while the carotenoid concentration was read at 450 nm and quantified by the method of Strain and Svec (1996).

2.4. Photosynthetic rate measurement

Photosynthetic rate was determined on fully expanded leaves (3rd leaf from apex) with a portable LCprot System (ADC BioScientific Ltd, UK). Measurements were performed between 10 a.m. and 12 midday at a steady light intensity (>900 μmol m^{-2} s^{-1}), while leaf temperature varied between 28 and 32 °C.

2.5. Sodium and chloride ion analysis

The collected samples (leaves, shoots, roots) were washed initially with tap and afterwards with distilled water, oven-dried at 75 °C for 2 days, weighted [dry weight (DW)] and milled to a fine powder so as to pass a 30-mesh screen. For sodium concentrations 0.5 g of each sample was dry-ashed for 6 h at 550 °C, dissolved in 3 mL 6 mol L^{-1} HCl, diluted to 50 mL with deionized water and then determined by atomic-absorption spectrometry (Perkin–Elmer 2380, Norway) according to manufacturer's instructions. The chloride concentration was measured by titration with $AgNO_3$ according to Kolthoff and Kuroda (1951).

2.6. Carbohydrates and proline concentration analysis

For carbohydrates and proline analysis 0.1 g of tissue (leaves or roots) were extracted in 80% ethanol and their concentration (expressed as mg g^{-1} FW and μmol g^{-1} FW, respectively) was estimated using a standard curve with a range of 0–0.2 mM according to Fales (1951) and Bates et al. (1973), respectively.

2.7. Determination of phenols, ascorbic acid and glutathione

Phenols were extracted from 0.15 g of tissue (leaves or roots) in 80% methanol and assayed using the Folin-Ciocalteu reagent, following the method of Scalbert et al. (1989) and expressed as mg gallic acid equivalents (GAE) g^{-1} FW, which was used for standard curve with a range of 0–125 μM. For ascorbic acid determination 0.15 g of leaves and 0.2 g of roots were extracted in 5% metaphosphoric acid and determined according to Hodges et al. (1996) using a standard curve with a range of 0–37.5 μM and expressed as mg g^{-1} FW. The samples (0.1 g of leaves and 0.2 g of roots were extracted in 2% sulfosalicilic acid for glutathione determination according to Griffith (1980), using a standard curve with a range of 0–4 μM and expressed as μmol g^{-1} FW.

2.8. Extraction and assays of antioxidant enzymes

All operations were performed at 4 °C. For the extraction of polyphenol oxidase (PPO; EC 1.30.3.1) and peroxidase (POD; EC 1.11.1.7), tissue samples (0.1 g of leaves and 0.2 g of roots) were homogenized in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.05% (v/v) Triton X-100, 1 M NaCl and 1 mM phenylmethanesulfonylfluoride (PMSF). For the APX assay, 5 mM ascorbic acid was added to this mixture. For superoxide dismutase (SOD; EC 1.15.1.1) and glutathione reductase (GR; EC 1.6.4.2) tissue samples (0.1 g of leaves and 0.2 g of roots) were homogenized with a pre-cooled mortar and pestle in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA, 2% (w/v) PVPP, 0.05% (v/v) Triton X-100 and 1 mM PMSF. Each homogenate was centrifuged (4 °C, 20 min, 20 000 × *g*) and the resulting supernatant was used for the determination of enzymatic activity and protein content assays. Protein concentration was determined using the Coomassie brilliant blue dye with bovine serum albumin as the standard (Bradford, 1976).

Total SOD activity was assayed following the method of Beauchamp and Fridovich (1971) and the absorbance was recorded