Scientia Horticulturae 231 (2018) 194–200

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Olive tree glutathione *S*-transferase and its response against the herbicides oxyfluorfen and glyphosate

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ARTICLE INFO

Keywords:
Glutathione *S*-transferase
Olea europaea
Oxyfluorfen
Glyphosate
Olive tree
Olive

ABSTRACT

Glutathione *S*-transferases (GST, EC 2.5.1.18) are a group of detoxifying enzymes that catalyze the conjugation of glutathione to certain toxins or xenobiotics. These enzymes are very well characterized in mammals and plants in general but not in the olive tree. In this work the GST specific activity, kinetic behavior, substrate specificity, and protein expression levels have been determined in the leaves, fruits, stems, and roots of olive tree cv. Picual. The results reveal the presence of GST in all the organs, the leaves and roots are containing the highest activity levels. Substrate specificity and molecular mass of subunits differ between organs. The use of the herbicides oxyfluorfen and glyphosate caused barely significant changes in the kinetic behavior and GST protein-expression levels, in general. Only in the stems of the olive tree treated with glyphosate were greater GST specific activity and protein expression detected. As conclusions, GST has been located and characterized in leaves, fruits, stems and roots of olive tree and the action of this enzyme, implied in the detoxification processes in this species, is lightly affected by the soil application of oxyfluorfen and glyphosate.

1. Introduction

Glutathione *S*-transferase (GST, EC 2.5.1.18) is a highly conserved superfamily of enzymes that catalyze the conjugation of electrophilic substrates with the glutathione (GSH). GST acts on many types of substrates with different specificity, participating in a great number of detoxification processes (Andrews, 1999). This biotransformation process generates more water-soluble and more easily removed products. GST is a multifunctional enzyme widely studied in prokaryotes and eukaryotes (Fahey and Sunquist, 1991). In plants, the presence of GST and its relation with herbicide detoxification was recognized in maize when Frear and Swanson (1970) demonstrated that the GST activity was responsible for the conjugation of chlore-S-triazine and GSH and thus protects the culture against the herbicide atrazine. Currently, the plant GST superfamily is divided into eight classes: phi, tau, zeta, theta, lambda, dehydroascorbate reductase and tetrachlore hydroquinone dehalogenase, and one microsomal GST (Sasan et al., 2011). The active enzyme forming GST is a dimer composed of two 26–30 kDa subunits that form a 50 kDa hydrophobic protein with an isoelectric point of 4–5 (Dixon et al., 2002). The highest amount of GST is found in the cytosol although activity has also been found in nuclei, plastids, and microsomes (Andrews, 1999). GST is unusually frequent in plants and is involved in many functions, such as the regulation of the plant response against the oxidative stress (Alscher, 1989; Dudler et al., 1991; Marrs, 1996), the union and transport of hydrophobic compounds as auxins (Dean et al., 1995; Watahiki et al., 1995), and tolerance to herbicides (Frear and Swanson, 1970; Jepson et al., 1994; Rossini et al., 1996).

Herbicide tolerance in plants is related to their capacity to metabolize and transform these chemicals into less toxic substances. Processes such as hydrolysis, oxidation, dealkylation, and conjugation are involved (Cole, 1994). Detoxification of herbicides in plants by conjugation with GSH catalyzed by GST was first observed when the abundance and activity of GST in atrazine-resistant plants such as maize and sorghum was far higher than in sensitive plants such as pea, wheat, and barley (Frear and Swanson, 1970). This was confirmed also in maize (Rossini et al., 1996). Breaux et al. (1987) demonstrated that weeds resistant to herbicides metabolized GST more easily than did sensitive weeds. Moreover, the presence of specific GST isoenzymes has been related to the selectivity against herbicides by different plants (Cole, 1994).

The olive tree, an evergreen tree constituting the mainstay of the economy of Jaén and other provinces in southern Spain, is cultivated

Abbreviations: CDNB, 1-chlore-2,4-dinitrobenzene; DCNB, 1,2-dichlore-4-nitrobencene; EPNP, 1,2-epoxy-3-(*p*-nitrofenoxy)propane; EA, ethacrynic acid; GSH, glutathione; GST, glutathione *S*-transferase; *h*, Hill coefficient; K_m, Michaelis-Menten constant; RI, ripeness index; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecylsulphate; TBS-NFM-T, TBS containing 1.5% non-fat milk 0.05% Tween20; V_{max}, maximum velocity; V_{ss}, subsaturating velocity

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https://doi.org/10.1016/j.scienta.2017.12.044
Received 5 October 2017; Received in revised form 18 December 2017; Accepted 20 December 2017

between 30° and 45° of latitude in both hemispheres (Barranco et al., 2010). Currently, in this crop, herbicides such as oxyfluorfen and glyphosate are commonly used to control weeds. Oxyfluorfen (2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene) is used mainly in controlling pre-emergence weeds while glyphosate (N-(phosphonomethyl)glycine) is applied to control post-emergence weeds. The presence of GST has not been described in olive tree (*Olea europaea*), and our hypothesis is that GST must be present in the organs of this plant and must be involved in the detoxification of herbicides by this plant. For this, the aims of this work were to determine the kinetic and molecular characteristics of GST in olive organs and to investigate the effects of soil application of oxyfluorfen and glyphosate on GST kinetic behavior and protein expression level. This study sheds light on the little-known effects of these two herbicides on the olive tree, which have stirred major concern and controversy regarding their effects on the environment and the human health (Myers et al., 2016; Portier et al., 2016).

2. Materials and methods

2.1. Chemicals

The chemical reagents used for making the assay media and buffers were of analytical grade and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The compounds and materials used for SDS-PAGE and Western-blot were from BioRad Laboratories (Hercules, CA, USA), GE Healthcare (Little Chalfont, UK) and Santa Cruz Biotechnology (Dallas, TX, USA). The herbicides used in the experiment (24% w:v oxyfluorfen; register number: 23804; 36% w:v isopropylamide glyphosate salt; register number:21793) were purchased from Agrofit Laboratories S. Coop. (Picassent, Spain).

2.2. Plant material and experimental design

Olive trees (*Olea europaea*) of cv. Picual 40 years old, growing in an orchard in Torredonjimeno (Jaén, Spain) at 655 m a.s.l. (37°45′61′′N, 3°57′12′′W) were used for this study. These trees were cultivated under traditional rainfed conditions. For the first part of this work, three trees periodically distributed in the orchard were used. In each tree, samples of leaves, fruits, stems, and roots were collected in triplicate 17 Oct 2014. From each orientation in each tree, five 25-cm segments of branch with fruits near the apical end were collected. All leaves and fruits were pooled and the stems cut into 5-cm segments. From each position in each tree, roots 2 mm in diameter located near the ground surface were removed. Leaf, fruit, stem, and root samples were frozen in liquid nitrogen until analyzed. The ripeness index (RI) of fruit was calculated using a color evaluation of the skin and flesh proposed by Uceda and Frías (1975). RI of this sample was 1.58.

For the second part of this work, the study of the effects of herbicides on GST, the ground under the olives tree was treated with oxyfluorfen, glyphosate or water (control) (Fig. 1). The day of application of herbicides was 16 Nov 2014, when weeds under the olive trees measured about 5 cm high. Herbicides were applied at the standard rate used in the area. That is, 200 mL of 24% (w:v) oxyfluorfen solution (Agroxifen, register number: 23804) or 150 mL of 36% (w:v) isopropyl amide glyphosate solution (Winnercoop, register number: 21793) was diluted in 16 L of water and applied into the ground using a Matabi compression sprayer. Each tree received 4 Liters of herbicide solution that were applied under the treetop. This meant one application of 0.34 g of oxyfluorfen per m^2 of soil or 0.39 g of glyphosate per m^2 of soil. The tree used were choose for avoid that herbicide reach the surface below control trees. After 9 days (26 November 2014) the effects of herbicide application on the weed viability was evident and samples from leaves, fruits, stems, and roots of the trees treated with oxyfluorfen, glyphosate or water were collected as indicated above. During the experimental time not rainfall occurred avoiding the leaching of herbicides.

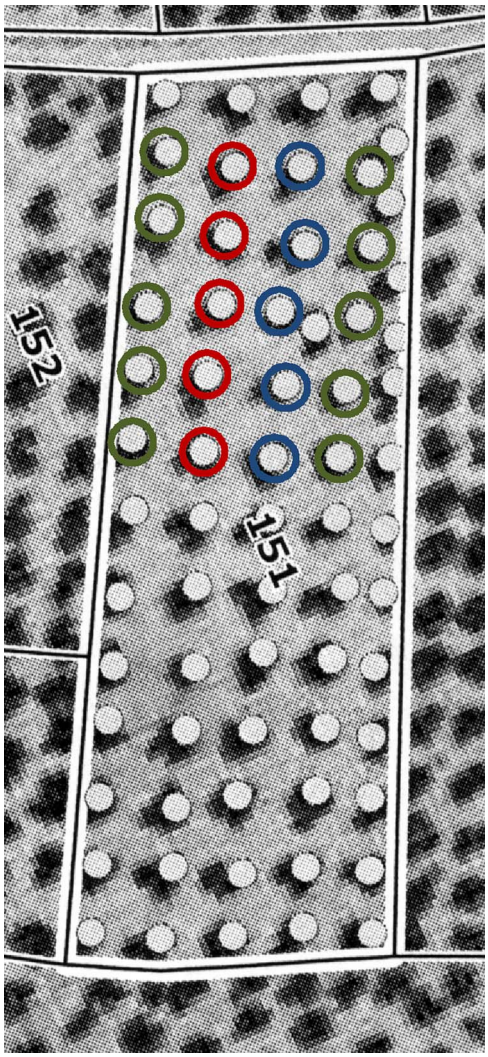

Fig. 1. Aerial photography of the olive cultivar studied. This cultivar, 'Picual', was growing in an orchard located in Torredonjimeno, Jaen (Spain). The control olive is marked with green circles, olives treated with oxyfluorfen were marked with red circles, and olives treated with glyphosate were marked with blue circles. The ground image was taken from the Servicio de Información Geográfica of the Ministerio de Agricultura, Pesca y Alimentación of Spain.

2.3. Protein extraction

Protein was extracted from leaves, fruits, stems, and roots following Ortega-García et al. (2008) with some modifications. Samples of the different organs were pulverized with liquid nitrogen in a mortar. For each organ, one homogenate was prepared mixing 4 g of powder −in the case of leaf, root and stem- or 10 g −in the case of fruit- with cold acetone and polyethylenglycol in a proportion of 1:4:1, w:v:v. In each case, the solid fraction was separated and re-extracted three times with the same volume of cool acetone. The resulting acetone powder was dried overnight in a drier.

Immediately before the assay, the acetone powder was resuspended in a proportion of 1:30 w:v for fruit; 1:20 w:v for leaf, and 1:15 w:v for the stem and roots in 0.1 M HCl-Tris buffer pH 7.5 containing 2 mM EDTA, 1 mM DTT, 2 mM PMSF, and 0.33 mg/mL plant type-II trypsin inhibitor. Samples were covered to avoid light and were maintained with mild stirring at 4 °C for 30 min. Afterwards, the suspensions were filtered through glass wool and filtrates were centrifuged at 20000xg for 20 min at 4 °C. The supernatants were used for the enzyme assays,