

Comparative Study of Leaf Anatomy and Certain Biochemical Traits in Two Olive Cultivars

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Summary

Olive (*Olea europaea* L.) is one of the most cultivated trees in Dalmatia (Croatia). The aim of this work was to compare leaf anatomy, guaiacol peroxidases activity and soluble polyphenols content in two olive cultivars: Oblica and Leccino, to get preliminary data for further investigations of stress influence on their growth and productivity. Leaves from five two-year-old trees of each, *Olea europaea* L. cv. Oblica and cv. Leccino were used in this study. Composed sample of each cultivar was used for determination of guaiacol peroxidases activity (GPOD), content of soluble polyphenols (PHEN) and dry weight (DW). The presence of lignin, suberin and callose were analyzed by histochemical reactions on fresh hand-made leaf sections. The leaf anatomy was studied on 3 μm thin sections stained with Toluidine blue O. There were significant differences of tissue areas between two olive cultivars. Bigger areas of upper and lower epidermis in cultivar Leccino leaves as well as bigger areas of schlerenchyma, idioblasts and intercellulars in leaves of cultivar Oblica were measured. Oblica had higher activity of GPOD and portion of DW than Leccino. Higher PHEN content was measured in Leccino leaves. Histochemical reactions showed more lignin and suberin in cultivar Oblica leaves. Based on the presented results, cultivars Oblica and Leccino should response differently on cold, salt and drought stress that should be the scope in further investigations.

Key words

olive, leaf anatomy, guaiacol peroxidase, phenolics, callose

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Introduction

Olive is one of the most cultivated trees in Dalmatia (Croatia). *Olea europaea* L. cv. *Oblica* is autochthonous species and most frequent olive cultivar identified by prolific crown and dark green leaves. *Olea europaea* L. cv. *Leccino* originating from central Italy (Toscana) grown in Croatia since 1960 (Miljković, 1991), has prolific crown and oval-laniform green leaves brighter than cv. *Oblica* (Perica et al., 2004). During the summer olives are exposed to high solar irradiance, high air temperatures and limited water availability. Under these extreme conditions the excess of reactive oxygen species (ROS) arises. ROS comprise different compounds such as superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radical. These reactive species can cause oxidation of proteins, chlorophyll degradation, lipid peroxidation and inhibition of enzymatic activity (Mittler, 2002). The level of ROS in plants is controlled by enzymatic and non enzymatic systems. The enzymatic antioxidant system includes several enzymes as superoxide dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase (GPOD) (Sofo et al., 2004). The non enzymatic system includes ascorbat, tocopherol, carotenoids and different phenolic compounds having antioxidant activity.

Phenolics as plant secondary metabolites are primary synthesized through the pentose phosphate pathway, shikimate and phenylpropanoid pathways (Randhir and Shetty, 2005). Soluble phenolic compounds can be stored in the vacuoles or in the apoplastic spaces, while non soluble phenolic compounds, such as suberin and lignin, are embedded in cell walls. Their biosynthesis is, among others, regulated by guaiacol peroxidases, heme containing enzymes. Besides lignification (Hatzilazarou et al., 2006) and suberinization (Franke and Schreiber, 2007) it was shown that peroxidases play a role in wound healing (Bernards et al., 1999), auxin catabolism (Hiraga et al., 2001., Tyburski et al., 2006), UV-B and ozone protection (Rao et al., 1996), heavy metals loads (Geebelen et al., 2002), pathogen defences (Sedlářová and Lebeda, 2001) and aging (Lepeduš et al., 2005).

Changes in lignification and suberinization processes could be expected as the consequence of different GPOD activity provoked by stress situations (Stobrawa and Lorenc-Plucińska, 2007; Lee et al., 2007). Also, other histochemical modifications (e.g. different callose deposition) as well as changes in tissue structure could take place in distinct growth conditions (Cesar et al., 2004; Lepeduš and Cesar, 2004). The aim of this study was to compare leaf anatomy and some biochemical characteristics of two olive cultivars, 'Oblica' and 'Leccino', to get preliminary data for further investigations of cold, salt and drought stress influence on their growth and productivity.

Material and methods

Plant material

Leaves from five two-years-old trees of each, *Olea europaea* L. cv. *Oblica* and *Olea europaea* L. cv. *Leccino* were used in this study. Trees were grown in natural conditions. Healthy one-year-old leaves from the middle crown were sampled in March 2007. Composed sample contained twenty five leaves five from each tree of single cultivar. Five repetitions were made from composed sample of each cultivar separately.

Biochemical analyses

Fresh leaves were cut into small pieces and macerated in liquid nitrogen with addition of polyvinylpyrrolidone (PVP). About 0.5 g of obtained fine powder was used for the extraction of proteins with 1 ml of 0.1 M Tris-HCl buffer, pH=8.0. The extracts were centrifuged at 18000 x g at 4 °C for 10 min. Activity of guaiacol peroxidases (GPOD; EC 1.11.1.7) was determined according to Siegel and Galston (1967) by measuring the absorbance increase at 470 nm. Reaction mixture contained 5 mM quaiacol with equimolar H₂O₂ in 0.2 M phosphate buffer, pH = 5.8.

Fine powder obtained by maceration in liquid nitrogen without PVP was used, partially for the extraction of polyphenols and partially for the determination of dry weight by drying at 105 °C for 24 h. The extraction of polyphenols was done with 2.5 ml 95 % EtOH at -20 °C for 72 h and homogenates were centrifuged at 10000 x g at 4 °C for 10 min. The polyphenols content was determined according to Liang et al. (2003) by measuring absorbance at 540 nm. All absorbance measurements were done by Analytik Jena Specord 40.

Leaf anatomy investigation and histochemical analysis

For anatomy investigations middle parts of leaves were cut in appropriate pieces, fixed in 1 % - glutaraldehyde in 0.05 M phosphate buffer, pH = 6.8 at +4 °C for 24 h, dehydrated in 2-methoxyethanol, ethanol, n-propanol and n-butanol (two changes in each) and embedded in methacrylate resin (Historesin, Leica). Three μm thin sections were cut on microtome (Leica RM 2155) and stained with Toluidine blue O in benzoate buffer, pH = 4.4 (Feder and O'Brien, 1968). Areas of different tissues were measured using Motic Images software. Number of idioblasts in spongy parenchyma was counted per leaf cross-section.

To demonstrate the presence of suberin 10 μm thin leaf sections were cut on cryostat (Leica CM 1100) and coloured with Sudan III reagent.

Fresh hand-made leaf sections were used for histochemical determination of lignin and callose. Lignin was proved by phloroglucinol-HCl (Purvis et al., 1964) while presence of callose was established with 0.05 % aniline blue in Hepes buffer, pH=9.25 under UV light (O'Brien and McCully, 1981; Cesar and Bornman, 1996).

The sections were studied using light microscope (Carl Zeiss Jena) and photographed by Olympus μ300.

Statistical analysis

The data were analyzed by t-test modified for a small sample (Petz, 1997). Each cultivar was treated as a single statistic sample that contained leaves from five olive trees (n=5). P-values < 0.05 were considered to be significant.

Results and discussion

Anatomical characteristics of olive leaves, in general, are well established (Bacelar et al., 2006; Bosabadalis and Kofiodis, 2002). Between upper and lower epidermis there are few layers of elongated cells building multilayer palisade parenchyma and spongy parenchyma containing intercellulars, veins and idioblasts. Schlerenchyma tissue is placed directly below the upper epidermis. Leaf cross-sections of olive cultivars *Oblica* and

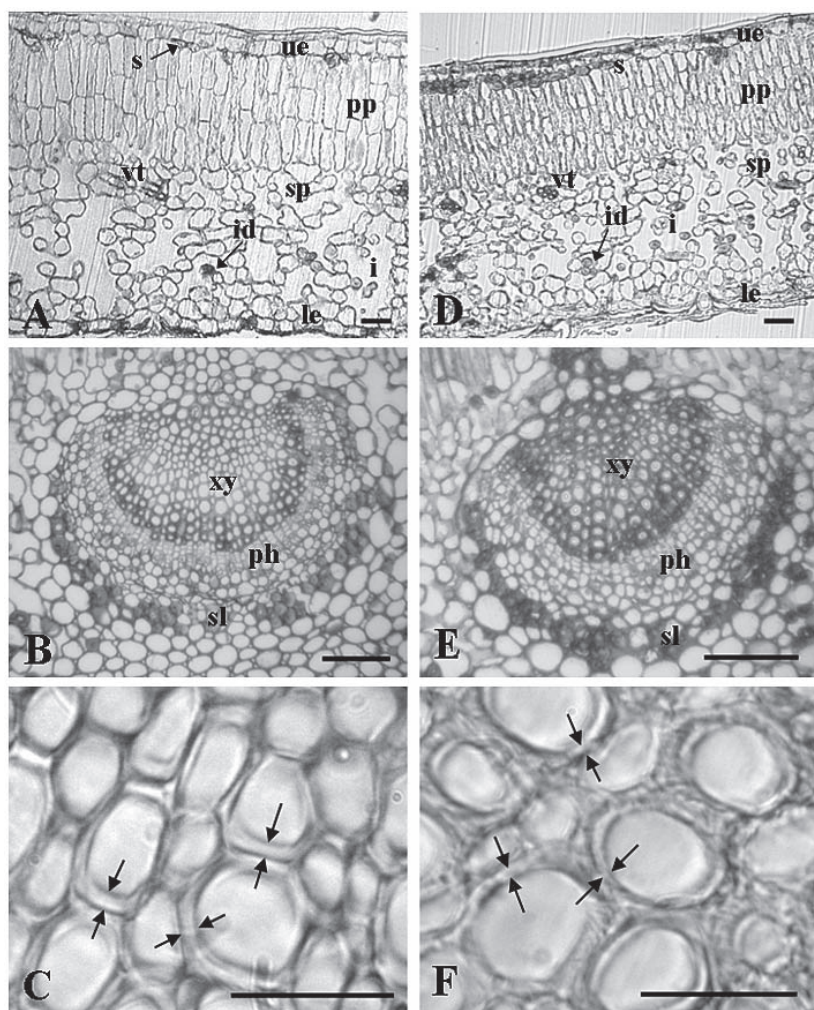


Figure 1.

Three μm thin leaf sections stained with Toluidine blue O. A, B, C – cultivar Leccino; D, E, F – cultivar Oblica. Bar = 50 μm (A, D - Leaf anatomy (ue - upper epidermis, s - schlerenchyma, pp - palisade parenchyma, sp - spongy parenchyma, i - intercellulars, id - idioblasts, vt - vascular tissue, le - lower epidermis). Specific characteristics of olive leaves are multilayer palisade parenchyma and idioblasts located in spongy parenchyma. Leaves of Oblica are thinner compared to Leccino leaves and have coherently arranged cells of palisade parenchyma; B, E – Xylem and sclerenchyma layer in veins stained with Toluidine blue O (xy - xylem, ph - phloem, sl - sclerenchyma layer); C, F – Cell walls of xylem. They were thicker in Oblica compared to those in Leccino leaves (\uparrow))

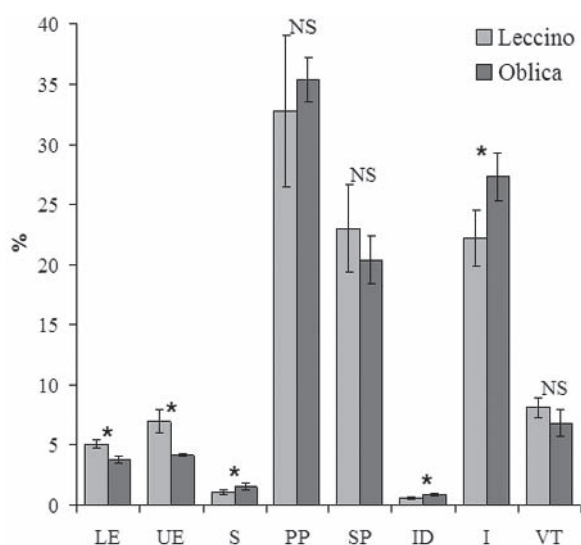


Figure 2. Areas of different leaf tissues (LE - lower epidermis, UE - upper epidermis, S - schlerenchyma, PP - palisade parenchyma, SP - spongy parenchyma, ID - idioblasts, I - intercellulars, VT - vascular tissue) expressed as a percentage of total leaf section. Bars represent standard deviations (n=5), * - significant and NS - not significant differences.

Leccino are shown in Fig. 1. Cultivar Oblica leaves were thinner and had more coherently arranged cells of palisade parenchyma than leaves of cultivar Leccino (Figs 1A and 1D). Also, significant differences between tissue areas of two olive cultivars were

found in upper and lower epidermis, schlerenchyma, intercellulars and idioblasts (Fig. 2). Cultivar Leccino had for 35 % higher areas of lower epidermis and 67 % higher areas of upper epidermis than Oblica. In contrary, cultivar Oblica had higher areas of

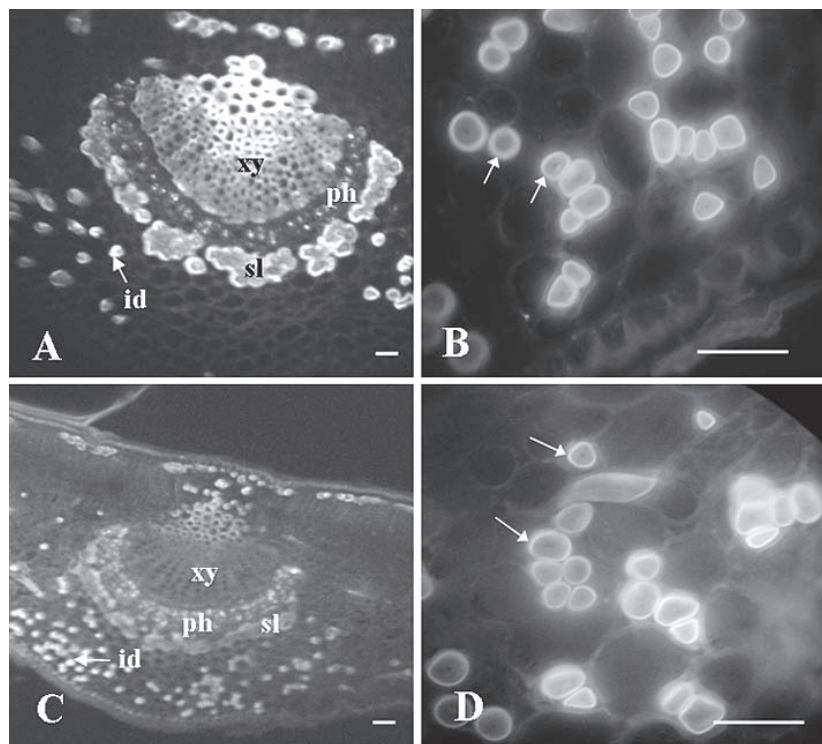


Figure 3.

The presence of callose on hand-made leaf sections stained with aniline blue in Hapes buffer. (A, B – cultivar Leccino; C, D – cultivar Oblica. Bar = 50 μ m (A, C – Callose deposits in phloem of vascular tissue and idioblasts (xy - xylem, ph - phloem, sl - sclerenchyma layer, id - idioblasts); B, D – Cell walls of idioblasts of spongy parenchyma loaded with callose (\uparrow))

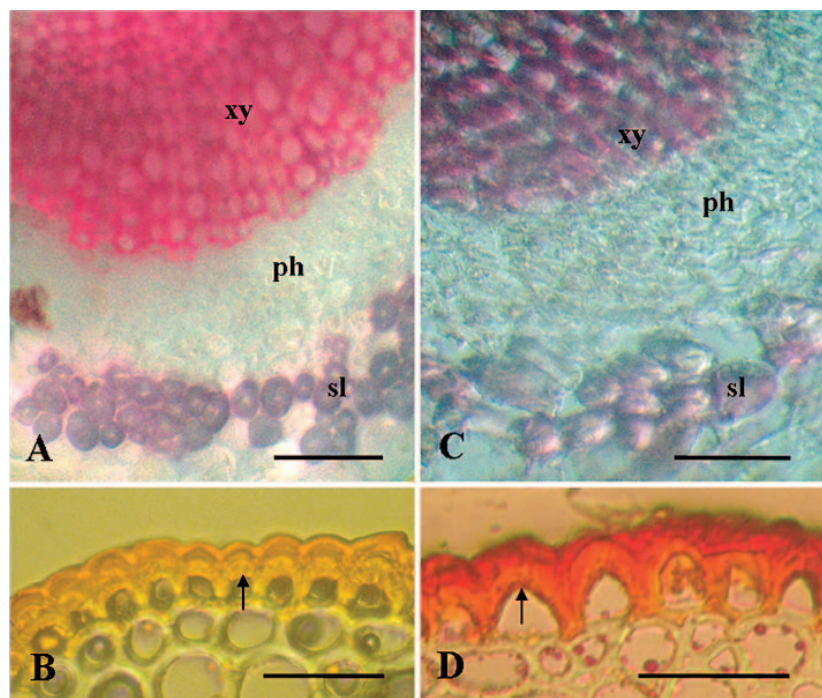


Figure 4.

The presence of lignin and suberin in leaf sections. A, B - cultivar Leccino; C, D - cultivar Oblica. Bar = 50 μ m. (A, C – Staining with phloroglucinol-HCl (pink to violet coloured) on hand-made sections confirmed the presence of lignin in the cell walls of xylem and sclerenchyma layer of leaf veins. There was more lignin in cell walls of xylem and sclerenchyma layer in Oblica leaves; B, D – Staining with Sudan III reagent indicates the presence of suberin (\uparrow) in cuticular layer of epidermis. Cuticular layer of Oblica epidermis was richer in suberin than cultivar Leccino)

sclerenchyma (for 48 %), idioblasts (for 54 %) and intercellulars (for 23 %) than Leccino. There were no significant differences between areas of palisade parenchyma, spongy parenchyma and vascular tissue in investigated leaves. Obtained results showed that Oblica and Leccino have different adaptations on water retaining. The similar results were shown by Bacelar et al. (2004; 2006) on Spanish olive cultivars. Cultivar Manzilla protects itself

against water loss by well developed upper and lower epidermis, while cultivar Cobrançosa is having small intercellulars and high density of foliar tissue. To reduce the transpiration, plants reduce intercellulars and increase number of mesophyll cells as well as idioblasts that are involved in light distribution within the mesophyll making photosynthesis, during the drought stress, possible (Bosabalidis and Kofidis, 2002).

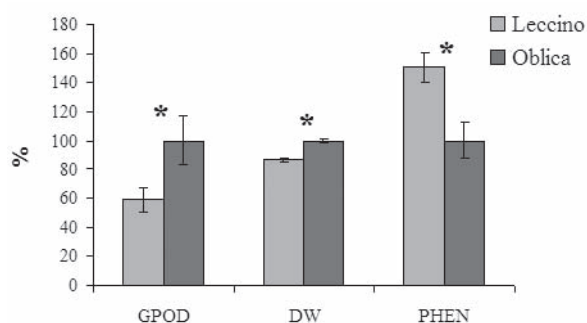


Figure 5. Guaiacol peroxidases activity (GPOD), portion of dry weight (DW) and total soluble polyphenols content (PHEN) in cv. Leccino. Obtained data for cv. Oblica is expressed as 100 %. Bars represent standard deviations (n=5), *- significant differences.

Presence of callose was indicated in phloem cell walls (Figs 3A and 3C). The callose deposits were quite abundant in cell walls of idioblasts (Figs 3B and 3D). No visible differences in callose deposits between idioblasts walls in spongy parenchyma of both cultivars were noticed, but there were significant differences ($p < 0.05$) in number of idioblasts per cross-section. Oblica had three-fold higher number of idioblasts per cross-section (103 ± 10.6) than Leccino (39 ± 3.7). Callose has the important role in plant tissues defence from stress factors such as temperature changes (Maeda et al., 2006), mechanical wounding (Levina et al., 2000) and pathogens attack (Sedlářová and Lebeda, 2001; Gindro et al., 2003). Callose deposition in leaf cells is also connected with Mn toxicity (Wissemeyer and Horst, 1987) as well as with presence of cement dust (Cesar et al., 2004).

Central vascular bundles in Oblica and Leccino leaves are shown in Figs 1B and 1E. Thicker cell walls of xylem (Figs 1C and 1F) were noticed in cultivar Oblica leaves. Presence of lignin was confirmed in cell walls of xylem and sclerenchyma layer of both cultivars (Figs 4A and 4C). Lignin is important for mechanical support of vascular and mechanical tissues. It gives rigidity to cell walls and makes tracheary elements impermeable thereby allowing the transport of water and solutes through the vascular system (Ros Barceló, 1997). Lignification is one of the final stages of xylem cell differentiation, where lignin is deposited within the carbohydrate matrix of the cell wall by infilling of interlamellar voids and by forming chemical bounds with non cellulosic carbohydrates (Donaldson, 2001). The last reaction in lignin biosynthesis pathway is oxidative polymerization of monolignols. The key enzymes in those reactions are peroxidases activated in the presence of H_2O_2 (Randhir and Shetty, 2005; Ros Barceló, 1997). Higher percent of dry weight (% DW) was measured in cultivar Oblica leaves (Fig. 5). According to Bacelar et al. (2006), increase in leaf dry weight percent can be related with more lignin in xylem and sclerenchyma layer, as well as with more idioblasts in spongy parenchyma, as was the case in cultivar Oblica leaves.

Cuticular layer of leaf epidermis contained suberin (Figs 4B and 4D). Oblica had more abundant suberin in cuticular layer of epidermis than Leccino. Usually, suberin occurs in cell walls

of tissues covering aerial surface or in primary roots where it is deposited in two distinct layers. Depositions of suberin significantly reduce uncontrolled transport of water, dissolved ions (e.g. nutrients and toxin) and gases (Franke and Schreiber, 2007). Also, suberin builds an outer barrier protecting the organ from pathogens (Franke et al., 2005). As reported by Franke and Schreiber (2007), final step in biosynthesis of suberin is connecting aliphatic polyester with polyaromatic domain (hydroxycinnamic acids and derivatives) synthesized in phenylpropanoid pathway by peroxidases activity in the presence of H_2O_2 .

The measurements of guaiacol peroxidases activity (Fig. 5) revealed differences in investigated leaves. Cultivar Leccino had 41.1 % lower activity of guaiacol peroxidases. Higher GPOD activity in cultivar Oblica could be related with its thicker lignified cell walls in vascular tissue (Figs 4A and 4C) and more suberin in cuticular layer of epidermis (Figs 4B and 4D). Hiraga et al. (2001) extensively described the relation between cell wall-bounded peroxidases, but, they also suggested that soluble peroxidases might be connected with cell wall biosynthetic processes. As reported by Ros Barceló (1997) peroxidases are located in lignifying cell walls mainly in the cell corners and middle lamella. Also, in the case of xylem elements, higher presence of soluble GPOD is detected at sites of secondary thickening as reported by Lepeduš et al. (2004).

The amounts of soluble phenolic compounds are given in Fig. 5. Higher soluble phenolic content (for 51 %) was measured in cultivar Leccino leaves. Soluble phenolic compounds are mostly localized in vacuoles (Takahama and Oniki, 2000). Some phenylpropanoids serve as substances to be polymerized and incorporated into the cell wall (lignin, suberin) by the peroxidases and some other enzymes (Sedlářová and Lebeda, 2001). Also, phenolics protect mesophyll cells from UV radiation (Luque-Rodríguez et al., 2007). Furthermore, they have antibacterial (Silici et al., 2007), antimutagenic and antioxidant activity (Santos-Cervantes et al., 2007). Because of these defence characteristics, some soluble phenolic compounds are stored in central vacuoles of epidermal cells as well as subepidermal cells of leaves.

Conclusion

Investigation of two olive cultivars, Oblica and Leccino, revealed differences in anatomical and biochemical characteristics of their leaves. Oblica was characterized with bigger areas of intercellulars, sclerenchyma tissue and higher number of idioblasts in spongy parenchyma. Higher GPOD activity in cultivar Oblica leaves could be connected with more comprehensive lignification of xylem and sclerenchyma layer, as well as wider suberinization of cuticular layer. More abundant lignin and suberin resulted with increased percentage of leaf dry weight. In the contrary, cultivar Leccino had increased areas of upper and lower epidermis. The presence of extended soluble phenolic content, supporting the defence leaf system, could be attributed to enlarged outer surface characterised by cuticular layer with reduced quota of suberin. Based on the presented results, leaves of 'Oblica' and 'Leccino' cultivars should develop different responses on cold, salt and drought stress what is to be expected as a result of further investigations.

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