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Phenolic Compounds in Olive Explants and their Contribution to Browning During the Establishment Stage *in vitro*

Phenolische Verbindungen in Ölbaumexplantaten und ihr Beitrag zu der Verbräunung während der Etablierungsphase *in vitro*

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Abstract

Shoot tips and single node explants from glasshouse and field growing 'Koroneiki' olive trees, were assayed for their browning potential at the establishment stage *in vitro*, during the growing season. Measurements of the total phenol content, the total o-diphenol content and six major o-diphenols found in olive tissues were conducted, in order to determine the relationship of these parameters to the browning of explants. Shoot tips, from both explant sources, exhibited higher browning percentage than the nodes, while both total phenol content and the o-diphenol content were found to be higher in shoot tips than those in the nodes. Explants from the glasshouse showed a higher survival rate than those from the field, while the total phenol content and the o-diphenol content from the glasshouse deriving explants were lower than those from the field. Individual phenolic compounds exhibited various correlation coefficients with the browning percentage. Luteolin-7-glucoside, luteolin, and quercetin, all three of them, exhibited strong positive relationship, while oleuropein, chlorogenic acid and rutin did not show any significant one. Single nodes from the glasshouse proved to be superior to all other explants, concerning the survival rate, during the establishment stage.

Zusammenfassung

Sproßspitzen und einzelne nodale Explantante von im Gewächshaus und im Freiland gewachsenen 'Koroneiki' Ölbaumen wurden bezüglich ihres Verbräunungspotentials während der *in vitro* Etablierungsphase zu verschiedenen Jahreszeiten untersucht. Messungen des Gesamtphenolgehaltes, des o-Diphenolgehaltes und von sechs einzelnen o-Diphenolen, wurden durchgeführt, um die Beziehung zwischen diesen Parametern und der Explantatverbräunung zu bestimmen. Sproßspitzen von beiden Standorten zeigten höheres Verbräunungspotential und höheren Gehalt an Gesamtphenolen so wie o-Diphenolen als die Nodien. Explantate aus dem Gewächshaus zeigten eine höhere Überlebensrate als solche aus dem Freiland. Der Gehalt an Gesamtphenolen und o-Diphenolen war bei Explantaten aus dem Gewächshaus niedriger als bei denen aus dem Freiland. Einzelne phenolischen Verbindungen beeinflussten das Verbräunungspotential unterschiedlich. Luteolin-7-glucoside, Luteolin und Quercetin zeigten starke positive Beziehung, während Oleuropein, Chlorogensäure und Rutin keine signifikante Beziehung ergaben. Nodien aus dem Gewächshaus erwiesen sich während der *in vitro* Etablierungsphase hinsichtlich Überlebensrate besser als alle anderen Explantate.

Introduction

A major problem in *in vitro* culture of many plant species including olive (*Olea europaea* L), is the browning and death of explants during the establishment stage.

Various attempts have been made to alleviate this problem, including pretreatment of explants with antioxidants, incorporation of antioxidants into the culture medium, incubation of cultures in the dark and frequent subculture to fresh medium, etc. These manipulations have proved to be either successful or not depending on the species and type of explant propagated (BHAT and DHAR 2000, BLOCK and LANKES 1995, JORDAN et al. 1991). According to RUGINI (1986) and CANAS et al. (1992) it is not possible to control the browning of olive meristems, even with the most active anti-oxidants such as ascorbic acid or reduced glutathione etc., although SEYHAN and OZZAMBAK (1994) reported full overcome of this problem, when employing single node explants.

In this study we examine i) the fluctuation and distribution of some phenolic compounds according to explant type, explant origin and season of explant collection and ii) the possible contribution of these phenolic compounds in olive explant browning. The major aim was to better conceive some of the factors controlling explant browning along with some factors influencing phenol biosynthesis in explants, in order to be able to predict the possible phenol distribution in the shoot and consequently select explants which will present the minimum browning reaction.

Materials and Methods

Plant material

The experiment was conducted during the growing season (March – November) of two successive years (1998 – 1999) with two groups of trees each year. The first

group consisted of trees growing in the field while the second group of trees growing in a glasshouse. All trees were heavily pruned in winter, to promote sprouting of shoots with juvenile physiological characteristics. These shoots were collected at one-month intervals every season (three samples per season). Immediately after collection the shoots were transferred to the laboratory.

Measurement of browning

Approximately 30 explants from each kind - single node and shoot tip explants - per group of trees were withdrawn and surface sterilized with 10 % (v/v) sodium hypochlorite (BDH) plus three drops of Tween 20 as detergent, for 10 min. After sterilization, the explants were washed, at least three times, with sterile distilled water. The explants were then incubated aseptically on a modified OM medium (RAMA and PONTIKIS 1990) and grown in a growth chamber at 24 ± 1 °C and a 16 h photoperiod provided by cool daylight lamps (38 µmol m^{-2} s^{-1}). Explants showing brown discoloration to more than 1/3 of their length were recorded as browning in this experiment, as these were the explants, which failed to survive. Observations over the browning of explants were recorded one week after the establishment of the aseptic culture, while explant growth was monitored for at least one month, to ensure that browning, measured by this way, was directly related to explant survival.

Extraction and purification of phenolic compounds

The remaining explants were lyophylized and the dry explants were grounded into a fine powder by a centrifugal mill. Phenolic compounds were extracted with 50 ml 50 % methanol in water (pH 3 with 1N HCl) in an ultra sound ice bath for 30 min. The extract was then centrifuged at 4000 g for 6 min and the residue was discarded. The purification of phenolic compounds from the supernatant was done according to AMIOT et al. (1995). The final residue was dissolved in 1 ml methanol, filtered through a 0.25 µm nylon syringe filter and analyzed with high performance liquid chromatography. Each sample was assayed twice.

Colorimetric determination of total phenols and o-diphenols

Colorimetric determination of total phenols was based on the procedure of Folin-Ciocalteau as described by WATERMAN and MOLE (1994) and results are expressed as mg (tannic acid equivalents)/g dry weight. Briefly, 50 µl of the supernatant were added at 960 µl water and agitated thoroughly. Thereafter, 250 µl of Folin reagent (BDH) and another 750 µl of saturated Na_2CO_3 were added and thoroughly mixed. The mixture was let to stand at room temperature for 2 h and the absorbance at 760 nm was measured against a reagent blank.

Spectrophotometric determination of o-diphenols was based on the procedure of GUTFINGER (1981) and results are expressed as mg (caffeic acid equivalents)/g dry weight. Briefly, 100 µl of the supernatant were added at 900 µl water and agitated thoroughly. Thereafter, 1 ml of phosphate buffer pH 6.5 and 2 ml 5 % Na_2MoO_4 H_20 were added and stirred. The mixture was let to stand at room temperature for 15 min and the absorbance at 350 nm was measured against a reagent blank.

Separation and quantification of phenolic compounds by HPLC

The extracts were analyzed by HPLC (Hewlett-Packard 1050, Germany) and the separation was performed on an Inertsil ODS 2 (GL Sciences, Japan) column. Six o-diphenols, commonly distributed in olive tissues, were assayed (quercetin, luteolin, rutin, luteolin-7-glucoside, chlorogenic acid and oleuropein). Identification of the phenolic compounds was achieved by comparison with retention times of standards and calculation of UV absorbance ratios after co-injection of samples with standards. Each phenolic compound was quantified in comparison with a multipoint calibration curve obtained from the corresponding standard. The results of the HPLC analysis are expressed in mg or µg(phenolic compound)/g dry weight.

Statistical analysis

The experiment was designed as a factorial in a completely randomized design, with three replications. The significance of effects of type of explant, source of explant and season was estimated by analysis of variance. Regression analysis was performed in order to assay the relationships of the measured variables to the browning percentage. For the figures, standard errors (SE) were calculated from the residual variances. Statistical analysis was performed using Statgraphic plus, version 2.1 for Windows 95/98. Due to the overall similarity of the results of the two seasons, data presented here are of the 1998 growing season.

Results

Effect of the type, source and season of explant collection on the browning percentage of olive explants

The type and the source of the explant affected significantly the survival of olive explants during the establishment stage of the *in vitro* culture (Table 1). Shoot tips exhibited higher browning percentages than the nodes during the growing season (Fig. 1a). Similarly, explants from trees growing in the field presented higher necrosis percentage than explants from the glasshouse.

Effect of the type, source and season of explant collection on total phenol and o-diphenol content of olive explants

All three variables tested in this experiment had a significant effect on total phenol content of olive explants, without any significant interaction, while only the type of explant and the season of collection had significant effects on o-diphenol content of the various explants (Table 1). Shoot tips exhibited higher total phenol and o-diphenol content than the nodes, while at the same time explants from trees growing in the

Table 1. Probabilities of effects of type of explant, source of explant and season of explant collection, as a factorial experiment, on browning percentage during the establishment stage *in vitro* and on concentration of phenolic compounds of 'Koroneiki' olive explants.
Faktoranalyse der Wirkungen der Explantatorte, der Explantatquelle und der Zeit der Explantatentnahme, auf den Verbräunungsprozentsatz während der Etablierungsphase in vitro *und auf den Gehalt der phenolischen Verbindungen in 'Koroneiki' Ölbaumexplantaten.*

Variables	**Source of variation**						
	TE	SE	SEA	TE*SE	TE*SEA	SE*SEA	TE*SE*SEA
Browning percentage	**<0.001**	**<0.001**	>0.05	>0.05	>0.05	**<0.05**	>0.05
Total phenols	**<0.001**	**<0.001**	**<0.05**	>0.05	>0.05	>0.05	>0.05
o-diphenols	**<0.001**	>0.05	**<0.001**	>0.05	>0.05	>0.05	>0.05
Chlorogenic acid	**<0.001**	>0.05	**<0.001**	>0.05	>0.05	>0.05	>0.05
Rutin	>0.05	>0.05	**<0.01**	**<0.05**	>0.05	>0.05	>0.05
Luteolin-7-glucoside	**<0.01**	**<0.01**	**<0.05**	>0.05	>0.05	**<0.01**	>0.05
Quercetin	**<0.001**	>0.05	**<0.05**	**<0.05**	>0.05	>0.05	>0.05
Luteolin	**<0.001**	**<0.05**	**<0.05**	>0.05	>0.05	**<0.001**	**<0.001**
Oleuropein	**<0.001**	**<0.01**	>0.05	>0.05	>0.05	>0.05	**<0.001**

TE = Type of Explant; SE = Source of Explant; SEA = Season of Explant-Collection. * = Interaction.

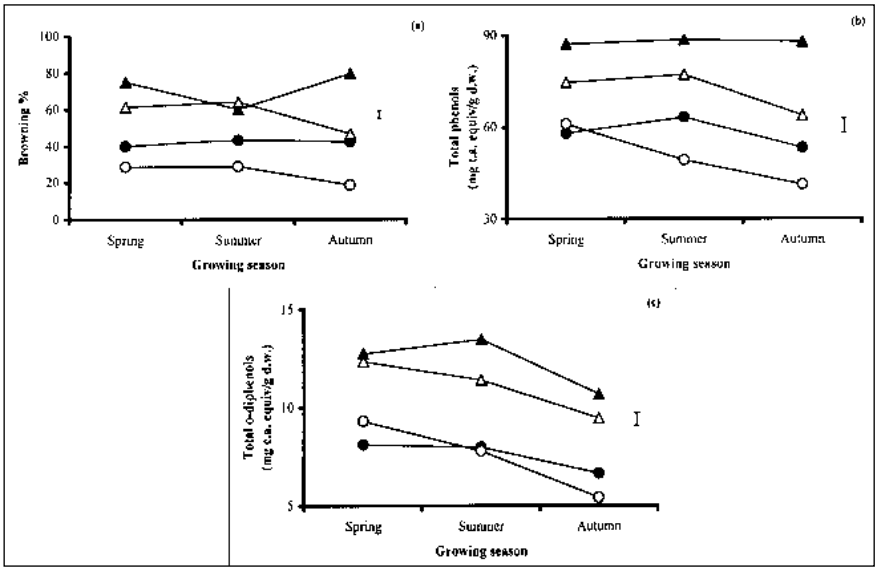


Fig. 1. Seasonal fluctuation of browning percentage, total phenols and total o-diphenols of 'Koroneiki' olive explants. —●—: single node explants from the field; —▲—: shoot tips from the field; —○—: single node explants from the glasshouse; —△—: shoot tips from the glasshouse. The vertical bar is the SE (n=3).
Jahreszeitliche Fluktuation des Verbräunungspotentials, der Gesamtphenolgehalte und des Gehalts an o-Diphenolen von 'Koroneiki' Ölbaumexplantaten. —●—: Nodale Explantate aus dem Freiland; —▲—: Sproßspitzen aus dem Freiland; —○—: Nodale Explantate aus dem Gewächshaus; —△—: Sproßspitzen aus dem Gewächshaus. Die vertikale Linie entspricht SE (n=3).

field had higher amounts of total phenols than explants from the glasshouse (Fig. 1b). It seems that the levels of both total phenols and total o-diphenols decrease significantly during autumn, while they were quite steady during spring and summer, especially in field derived explants (Fig. 1c).

Effect of the type, source and season of explant collection on the concentration of the various phenolic compounds of olive explants

The type of the explant used as well as the season of explant collection seemed to be the major factors influencing the relative concentration of the various phenolic compounds. There were also significant interactions between the variables (Table 1).

The concentration of both chlorogenic acid and oleuropein was significantly higher in the nodes than the shoot tips (Fig. 2a, f), while the opposite stood for luteolin-7-glucoside, quercetin and luteolin (Fig. 2c, d, e). rutin concentration was higher in the shoot tips from the field than the nodes from the same source, while the opposite occurred in the explants from the glasshouse (Fig. 2b).

Explants from the field exhibited higher concentration of luteolin-7-glucoside and luteolin than explants deriving from trees growing in the glasshouse, while the opposite happened with quercetin.

As the seasons progressed there was a significant decrease in the concentration of chlorogenic acid, rutin and luteolin-7-glucoside in explants from the glasshouse during summer, which was followed by a significant increase in autumn (Fig 2a, b, c). The concentration of the same phenolic compounds in explants from the field, seemed to be rather unaffected during spring and summer - except the concentration of luteolin-7-glucoside in shoot tips which increased significantly -, while all three displayed a significant increase during autumn. The concentration of quercetin was decreasing during the growing season.

The concentration of oleuropein in the shoot tips from the field continued to increase during the growing season, while in the nodes, after a small increase during summer, it exhibited a significant decrease in autumn. In the explants from the glasshouse, the concentration

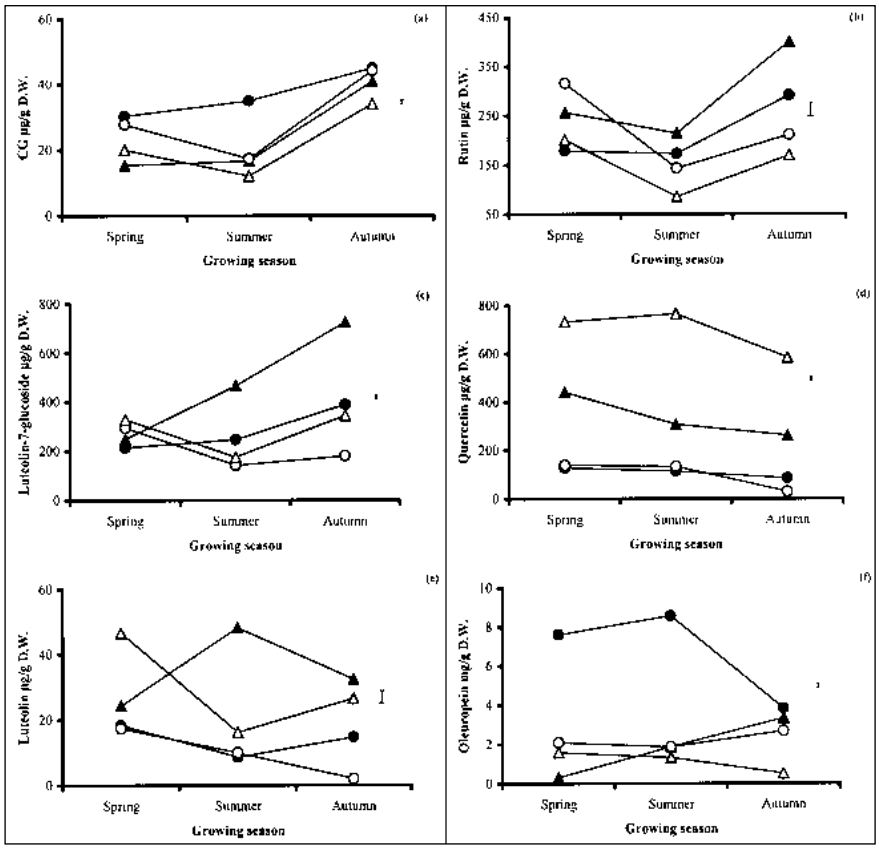


Fig. 2. Seasonal fluctuation of chlorogenic acid, rutin, lutrolin-7-glucoside, quercetin, luteolin and oleuropein concentration of 'Koroneiki' olive explants. —●—: single node explants from the field; —▲—: shoot tips from the field; —○—: single node explants from the glasshouse; —△—: shoot tips from the glasshouse. The vertical bar is the SE (n=3).
Jahreszeitliche Fluktuation von Chlorogensäure, Rutin, Luteolin-7-glucoside, Quercetin, Luteolin und Oleuropein in 'Koroneiki' Ölbaumexplantate. —●—: Nodale Explantate aus dem Freiland; —▲—: Sproßspitzen aus dem Freiland; —○—: Nodale Explantate aus dem Gewächshaus; —△—: Sproßspitzen aus dem Gewächshaus. Die vertikale Linie entspricht SE (n=3).

of oleuropein did not vary much during spring and summer, while in autumn it increased in the nodes and decreased in the shoot tips.

Correlation of the measured variables with the browning percentage of olive explants

The highest correlation coefficient was found for the total phenol content, as it explains 93% of the variation in olive explant browning percentage (Table 2). The total o-diphenol content and the concentration of luteolin in the explants showed a significantly strong positive correlation coefficient, while both luteolin-7-glucoside and quercetin showed a significant but a relatively weak relationship with the browning percentage. Neither the concentration of rutin, nor that of chlorogenic acid and oleuropein showed a significant relationship with the survival of olive explants *in vitro*.

Table 2. Correlation coefficients (r^2) between the various phenolic compounds and the browning of "Koroneiki" olive explants during the establishment stage *in vitro*
Korrelation zwischen verschiedenen phenolischen Verbindungen und der Verbräunung der 'Koroneiki' Ölbaumexplantate während der Etablierungsphase in vitro

Variables	Browning percentage
Total phenols	0.93[1]***
Total o-diphenols	0.78***
Chlorogenic acid	ns
Rutin	ns
Luteolin-7-glucoside	0.49**
Quercetin	0.49**
Luteolin	0.70***
Oleuropein	ns

[1] Correlation coefficients, **, $p<0.01$ – ***, $p<0.001$ – ns, not significant.

Discussion

Culture initiation is the first critical step in the successful micropropagation of all plants. Obtaining a large number of viable explants is essential to commercial and laboratory production when relatively low multiplication rates are envisaged (MARKS and SIMPSON 1990). Olive is a typical example, as it presents a strong apical dominance, which up to now could not be reduced; only one shoot can develop from one bud (RUGINI et al. 1995).

Shoot tips from the various sources of olive explants presented higher BP than that of the nodes. Several attempts to culture olive meristems deriving from field or glasshouse growing plants failed due to the rapid necrosis of the explants (RUGINI 1986; CANAS et al. 1992). In addition to this, younger tissues of various species cultured *in vitro*, present much higher BP than older ones (THOMAS and RAVINDRA 1997; YU and MEREDITH 1986).

Moreover, as in many other species (YU and MEREDITH 1986, BLOCK and LANKES 1996), explants from the glasshouse showed higher survival percentages than those from the field.

Although the season of collection is in general a critical factor in explant survival (THOMAS and RAVINDRA 1997, BLOCK and LANKES 1996) in the present study it did not present any significant influence on explant survival.

The results from the various assays of phenol content determination indicated a significant relationship between the phenol content and the BP of a specific explant. Similar results have been reported in *in vitro* culture of other species too (BLOCK and LANKES 1995, AMIN DALAL et al. 1992, JORDAN et al. 1991, MARKS and SIMPSON 1990, YU and MEREDITH 1986, ZIV and HALEVY 1983). It is generally accepted that the enzyme that catalyzes the production of phenolic compounds, phenyl alanine ammonia lyase (PAL) presents much higher activity in the younger tissues than in the older ones (THOMAS and RAVINDRA 1997, FUNFGELDER et al. 1994).

Furthermore, explants from the glasshouse showed lower phenol content than these from field growing trees. The higher temperature in the glasshouse causes a kind of forcing to the shoots, which may influence the relative phenol concentration (AMIN DALAL et al. 1992) by influencing PAL activity (CLAUDOT et al. 1997).

Similarly, as the growing season proceeded, it seemed that both phenol and o-diphenol content decreased. This could be due to the lower light intensity and shorter photoperiod during autumn, factors influencing PAL activity, causing lower production rate of phenolic compounds in the tissue.

The different phenolic compounds, which were detected in this study, presented different fluctuation mode and different relationship with the BP of olive explants, revealing both the complexity of the browning process and the one of phenol biosynthesis. There is a number of reports where there was no apparent correlation between the type or concentration of phenolic compounds and the degree of browning of the tissue under study (COSETENG and LEE 1987, SPANOS and WROLSTAD 1992).

The higher correlation coefficient of luteolin against luteolin-7-glucoside and quercetin against rutin, could be due to the structure of their molecule. The aglycones forms of a flavonoid (luteolin and quercetin) are considered to have higher antioxidant activity – more oxidazable – than their corresponding glycosides (luteolin-7-glucoside, rutin) (ROBARDS et al. 1999, BENAVENTE-GARCIA et al. 2000).

The concentration of the different phenolic compounds changes during the growing season, presenting the great impact of environmental conditions on the total phenol content of a tissue and especially on individual phenolic compounds as it has been reported by LUX-ENDRICH et al. (2000). Moreover, as the concentration of individual phenolic compounds does not change in the same way during the growing season (HEIMLER et al. 1994), the simultaneous increases and decreases of the concentration of individual compounds, may keep the total phenol content relatively stable (COSETENG and LEE 1987).

The browning reaction of olive explants appears to be a complex process involving several factors, which influence not only the total phenol concentration of the explant but also the relative distribution of the different phenolic compounds. It seems that explants deriving from glasshouse growing plants present lower BP than the same ones from field growing trees, due to the lower phenol content. As the various treatments to reduce the browning phenomenon of explants of many woody plant species often fail to succeed, it is clear that treatments of the stock material in ways to keep the phenol content in low levels, such as growing them in a glasshouse, or the selection of explant type e.g. node explants, offer the best solution.

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