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ANALYSIS OF PHENOLIC COMPOUNDS FOR DETERMINATION OF CAMBIUM DIFFERENTIATION AND TRACHEAL ELEMENTS IN OLIVE GRAFT COMBINATIONS

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KEYWORDS

Gemlik

Olive

Parancymatic cell

Sclerenchyma tissue

Histology

ABSTRACT

The aim of this investigation was to evaluate the effect of phenolic compounds during differentiation of cambium and tracheal elements in olive cultivars 'Ayvalik', 'Domat', 'Gemlik', 'Memecik', 'Nizip Yaglik' and 'Sari Ulak. These cultivars were grafted onto one year-old cv. 'Gemlik' rootstock. This rootstock has been propagated by cuttings. According to the results of histological studies for two years; new cambium cells were initiated in first three months while the formation of vascular bundles and sclerenchyma cells were initiated in six months. Large numbers of undifferentiated parancymatic cells were also determined in both side grafts of the cultivars 'Ayvalik', 'Domat' and 'Nizip Yaglik' after 3, 6and 12 months of grafting. Further, High levels of 4 hydroxyphenylacetic acid, vanillic and ferulic acids content were determined in the scion of the cultivars Ayvalik, Nizip Yağlik and Domat. As a result, during recovery of graft zone, development of new cambium tissues, vascular connections and sclerenchyma tissues occurred imperfectly in 'Ayvalik', 'Domat' and 'Nizip Yaglik' cultivars and graft zones of these combinations were found weak. Moreover level of 4Hydroxyphenylacetic acid and Ferulic acidphenolic compounds had high concentration in scions of 'Ayvalik' and 'Domat', respectively. These results illustrated existence of problem in differentiation of cambium and vascular systems in graft interface of 'Ayvalik' and 'Domat' cultivars.

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1 Introduction

Olive is one of the most important crops all over the world, especially in Mediterranean region (Aguilera et al., 2005). It is widely cultivated for the production of olive oil and table olives. Grafting is one of the most common plant propagation methods in fruit trees for control the characteristics of scion growth (Tworkoski & Miller, 2007). This method is considerable in the adaptation of important cultivars in different areas. Successful formation of graft depends on various complexes of biochemical and structural procedures, which includes callus formation, establishment of new vascular tissue, and formation of an active vascular system across the graft interface (Hartmann et al., 1990; Errea et al., 2001; Pina & Errea, 2005).

Many researchers used different methods for determination of graft incompatibility, such as phenol analysis (DeCooman et al., 1996; Errea 1998; Musacchi et al., 2000;Usenik & Štampar, 2001; Usenik et al., 2006; Mng'omba et al., 2008), histological studies (Errea et al., 1994c; Ermel et al., 1995; Ermel et al., 1999; Mng'omba et al., 2007), isozyme analysis (Fernandez-Garcia et al., 2004; Gülen et al., 2005) and accumulation of carbohydrates (Moing & Gaudillere, 1992; Ciobotari et al., 2009).

According to Zucker (1983) phenolic compounds have an important role in determination of plants graft incompatibility especially with regard to ecological interactions. Further, Errea et al. (1994b) reported accumulation of two phenols in incompatible combinations in apricot. Effect of phenol compounds in incompatible combinations was reported by Treutter & Feucht (1986) in cherry trees (*Prunus avium*). Prunin and p-coumaryl-glucoside were found in the phloem of less compatible combinations of cherry (*Prunus avium*) on sour cherry (*Prunus cerasus*). Errea (1998) believed that quality and quantity of phenol patterns in rootstock-scion parts explained decreasing of metabolic functions at the graft union. Moreover, existences of various phenolic compounds have been signified procedures of division, development and differentiation into new tissues at the graft union.

Mng'omba et al. (2008) showed that high phenol concentration was obtained from less compatible combinations versus compatible combinations. High peaks obtained above the grafts union were r-coumaric acid. So, phenols especially r-coumaric acids and flavonoids caused poor callus formation at the union. This is the explicit sign of graft incompatibility. High percentage of lipids, phenols, small cell size and disorganized arrangement cells were observed in incompatible combinations (Errea et al., 2001). Accumulation of two phenols in incompatible combinations of apricot was monitored by high performance liquid chromatography (HPLC) and light microscopy (Errea et al., 1994b). Increase of phenylpropanoid metabolism in the incompatible unions result stress situations between scion-rootstock partners (Pina & Errea, 2009). It's believed that a lot of phenolic compounds accumulate in wounded plants, which is main problem ingraft compatibility and specific phenols could cause graft incompatibility. The objective of this work was to determine the relationship between phenol compounds production and presence or absence of graft success in five major Turkey olive cultivars onto Gemlik cultivar at the graft zones and how the differentiation of cambium and tracheal elements was occurred in these cultivars.

2 Materials and Methods

2.1 Plant material

This investigation was carried out in 2011-2012 at Horticulture department of Agricultural, University of Ankara, Turkey. Olive cultivars 'Ayvalik', 'Domat', 'Gemlik', 'Memecik', 'Nizip Yaglik' and 'Sari Ulak' were obtained from Olive Research Institute, Bornova, Izmir, Turkey and were used as plant materials in this study. These plants were used as scions and were grafted (T-budding) onto one year-old 'Gemlik' cv. as rootstock. 'Gemlik' cv. was propagated by cuttings at Edremit Olive Nursery Station, Balikesir, Turkey. These grafts were maintained in the field for recording of grafting success of each sample.

2.2 Histological preparation

The samples collected from grafted plant were analyzed 3, 6 and 12 months after grafting. Three plants were used for each graft combination, at each stage of sampling for preparation sections. For each plant, one cm above and one cm below of the grafting region were used for analysis. Collected section were protected by using formalin aceto alcohol (FAA) solution (96% ethyl alcohol 900 ml; 5% glacial acetic acid 50 ml; 10% formaldehyde 50 ml: v/v/v). The fixed samples were sliced by using a microtome (Thermo Shandon Finesse 325), to obtain 30 µm-thick sections for microscopic operations(Leica EZ4D and Leica DM500 microscope). The cross sections were stained with safranin-fast green (60 seconds for each stain) and covered with a thin glass cover slip after addition of 10% glycerin (Espen et al., 2005).

2.3 Phenol extraction

One year old grafted plants of 'Ayvalik', 'Domat', 'Gemlik', 'Memecik', 'Nizip Yaglik' and 'Sari Ulak' on to 'Gemlik' cultivar were used for phenol extraction. Three plants were used for each graft combination. Bark of scion and rootstock of each plant were cut one cm above and one cm below of the graft. The bark samples that comprise the vascular cambium and the phloem were ground by using a mortar and pestle.

For phenol extraction, 0.50g of fine powder was placed in Ependorf tubes and added 20 ml of methanol (60%) solution on tubes (Meirinhos et al., 2005). Samples were kept in dark conditions for 24 hours on shakers at room temperature. It was followed by storing this mixture at 4^{o}C and centrifuged 8000 rpm for 10 min by a bench centrifuge (Sigma 3K30). This procedure repeated two times with 20 and 10 ml methanol (60%) solution respectively. Final obtained volume of extraction was 50 ml. After mixing of all the supernatants, 10 ml was taken for total soluble phenol qualification.

2.4 HPLC-high performance liquid chromatography

Separation of different phenolic compounds was carried out on a Shimadzu HPLC (LC- 10A) system with diode array detector, Thermo (250 mm × 4.5 mm) column. The mobile phase consisted of solvent A: 100% methanol and solvent B: 2% acetic acid. Gradient was linear from 0% to 90% of solvent B and its duration was 60 min, by flow rate of 1 ml*min-1.Cafeic, Ferulic, ρ -coumaric, Vanilic acids, Quersetin, Quersetin 3 β- D Glucoside, Rutin trihydrate and 4Hydroxyphenylacetic acidwere individual phenol compounds as standards(Meirinhos et al., 2005).

2.5 Statistical analysis

Experimental design for phenol analysis was randomized complete block design with three replications. Statistical analysis was done with the IBM SPSS Statistics Version 21 Software.

3 Results and Discussion

3.1 Histologic analysis

Callus formation in all graft combinations mainly were take placed in the both side of grafts, and no differences have been recorded between any combinations. Formed Callus tissue, that fills space between scion and rootstock, is the essential stage for development of future cambium and vascular systems at graft interface (Errea et al., 1994a; Wang & Kollmann, 1996). When ‘Gemlik’, ‘Memecik’ and ‘Sari Ulak’ cultivars grafted on ‘Gemlik’ rootstock, in both years, 3 months after grafting in wide range the callus tissue and subsequent cambial zone formation have been occurred and vascular tissue were differentiated. Moreover sclerenchyma tissues were identified in ‘Sari Ulak’ on ‘Gemlik’ graft. Fontanazza & Rugini (1983) showed that new vascular tissue formed during 3 months after grafting, also after 4 months of grafting sclerenchyma ring at graft union start appearing. But in ‘Ayvalik’, ‘Domat’ and ‘Nizip Yaglik’ cultivars grafted onto ‘Gemlik’ rootstock, cambial zone formation were occurred in very restricted spaces (Figure 1).

After 6 and 12 months of grafting, widely undifferentiated parenchymateous cells were observed at both sides of ‘Ayvalik’, ‘Domat’ and ‘Nizip Yaglik’ cultivars grafts. Errea et al. (1994a) reported the presence of parenchymatic tissue in weak combination of incompatible *Prunus* species. Mng’omba et al. (2007) described existence of undifferentiated tissues (parenchymal cells) in the incompatible graft combinations of *Uapaca kirkiana* Müell Arg. Results showed that these parenchymateous cells were not determined or was very thin layers at both sides of grafts of ‘Memecik’ and ‘Sari Ulak’ cultivars grafted on ‘Gemlik’ rootstock. Differentiations of cambium cells were developed in ‘Gemlik’, ‘Memecik’ and ‘Sari Ulak’ cultivars on the ‘Gemlik’ rootstock. Although cambium cells were discontinues and scattered form, in graft zone, but these differentiated tissues were determined in ‘Ayvalik’, ‘Domat’ and ‘Nizip Yaglik’ cultivars combinations. Hartmann et al. (1990),mentioned that new cambial cells, derived from the newly formed callus, are differentiated and formed as a continuous cambial connection between rootstock and scion.

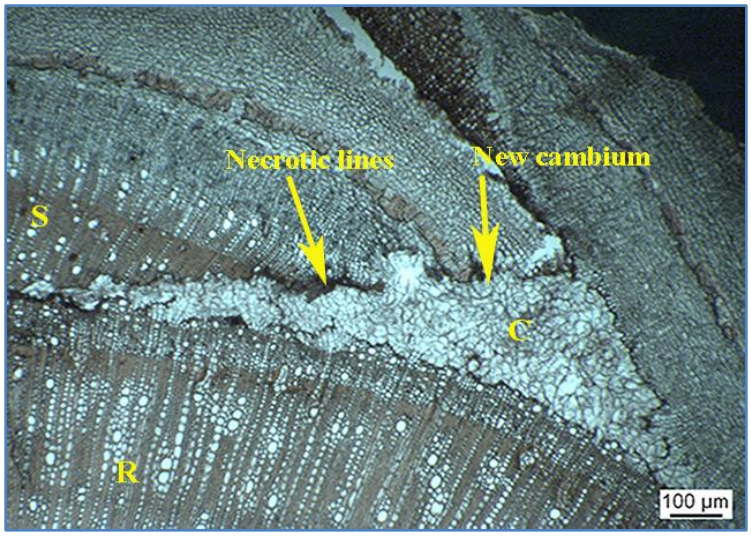

Figure 1 Three months old of budded graft of Ayvalik cultivar onto Gemlik rootstock ("The photograph was taken by bright field light microscopy. The C. shows callus; S. scion; R. rootstock")

Table 1 Mean comparison of some phenolic compounds.

Phenols Scions	4 Hydroxyphenylacetic acid (mg*l)	Vanilic acid (mg*l)	Cafeic acid (mg*l)	ρ -coumaric acid (mg*l)	Ferulic acid (mg*l)	Rutin trihydrate (mg*l)	Quersetin (mg*l)
Ayvalik	46.036 [a]	3.379 [c]	0.9022[a]	0.653[c]	10.924[a]	12.787[b]	31.653[ab]
Domat	0.000	14.395[b]	0.1880[c]	22.158[a]	10.691[a]	4.236[c]	42.665[a]
Gemlik	1.256 [b]	14.953[b]	0.8507[a]	15.482[b]	2.863[b]	34.799[a]	24.464[b]
Memecik	0.000	0.876[d]	0.6758[b]	20.492[a]	1.656[b]	12.068[b]	31.233[ab]
Nizip Yaglik	0.000	28.173[a]	0.7841[a]	25.059[a]	2.387[b]	4.088[c]	39.572[ab]
Sari Ulak	0.000	31.970[a]	0.7204[a]	23.657[a]	4.757[b]	4.873[c]	32.868[ab]

Means with the same letter are not significantly different at 0.05.

In the final step, formation of tracheal elements from new cambium cells is very important. These new differentiation tissues permit translocation of soluble material between the stock and the scion. Results showed that tracheal elements and sclerenchyma tissues were formed in graft union of 'Gemlik', 'Memecik' and 'Sari Ulak' cultivars onto 'Gemlik' rootstock but discontinuity of cambium cells leads to interruption of tracheal elements in 'Ayvalik', 'Domat' and 'Nizip Yaglik' cultivars combinations (Figure 2). Most researchers believed that a graft union can be considered successful and complete when several vascular connections cross exist on the graft interface (Moore, 1984; Simons, 1987).

Also the establishment of vascular connections and their function is principal step in determining the compatibility of graft combinations (Gebhardt & Goldbach, 1988). Likewise Ermel et al. (1997) showed that cell necrosis and lack of vascular connection continuity in graft union was the manifest symptoms of graft incompatibility. Errea et al. (1994a) reported that insufficient differentiation of callus cells in some areas of graft union affected the newly formed xylem and phloem activity. Results of this study support the view that said the formation of vascular connection is not a guarantee of a successful development union in grafted olive plants in this study.

3.2 Phenol Analysis

The presence of phenols accumulation at the union serves as an indicator of problems in grafting combinations. Stains of graft union with glutaraldehyde (%3) and osmium tetroxide-KI, confirmed the existence of phenolic compounds in all tissues of grafts unions (Figure 3). For this reason, in the first step content of phenolic compounds were determined above and below of graft union. Results of this study revealed the presence of phenolic compounds such as 4 hydroxyphenylacetic, vanillic acid, ferulic acid and routine trihydrate content from the scions of grafts and this concentration was significantly different among the treatments (Table 1). Scions of Ayvalik had the highest level of 4hydroxyphenylacetic acid (46.036 mg/l). Moreover concentration of ferulic acid was reported higher in Ayvalik and Domat scionsthan Gemlik, Memecik and Sari Ulak onto Gemlik rootstock. In callus cultures of *Prunus avium*, results showed that a high level of prunin limits the proliferation and differentiation of the cells (Feucht et al., 1988).

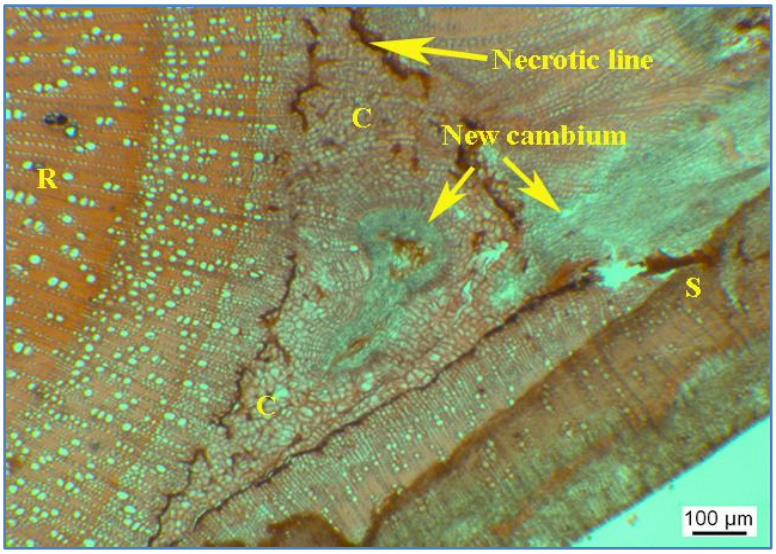

Figure 2 Six months old of budded graft of Domat cultivar onto Gemlik rootstock. ("The photograph was taken by bright field light microscopy. The C. shows callus; S. scion; R. rootstock")

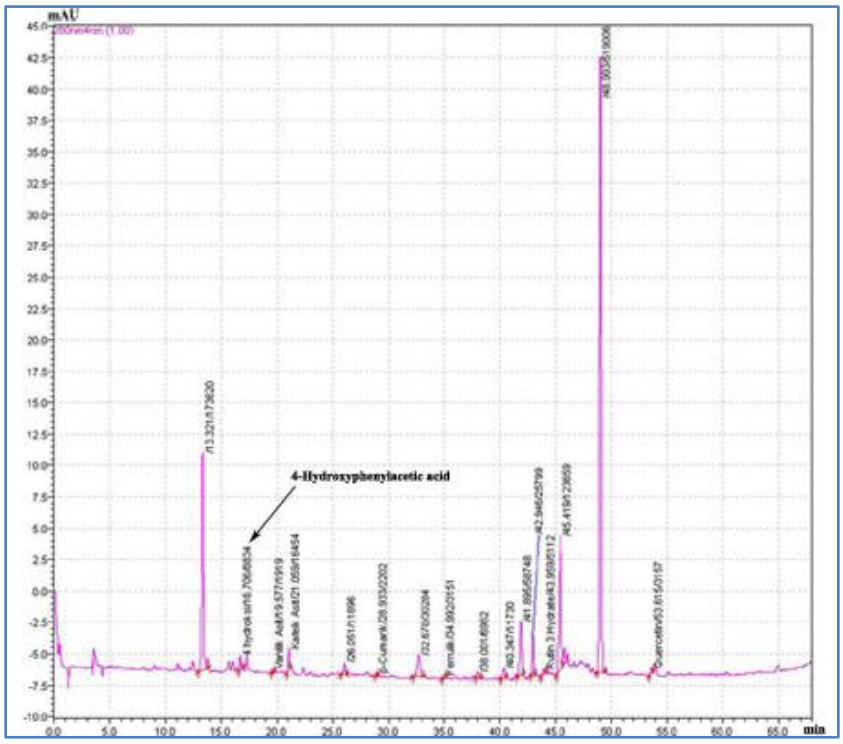

Figure 3 HPLC phenolic profile of Ayvalik scion.

De Cooman et al. (1996) reported that ρ-coumaric acid accumulation in graft zone of incompatible combinations of *Eucalyptus gunnii*. Usenik et al. (2006) reported high level of ρ-coumaric acid accumulation in the upper part of the graft union in incompatible combination of apricot. Also Mng'omba et al. (2008) observed high level of phenolic compounds accumulation and necrotic lines in *Uapaca kirkiana* Müell Arg. incompatible combination streak. May be, Existence of high concentration of these phenolic compounds in scions of graft combinations informs as reason of cambium tissues differentiation inhibitors.

Conclusion

Results of study revealed that cambium cell formation and differentiation were slow in Ayvalik, Domat and Nizip Yaglik cultivars grafted on Gemlik rootstock as compared to Memecikand Sari Ulak cultivars. Furthermore, discontinuity of cambium cells leads to interruption of tracheal elements. Accordingly, differentiation of callus tissues on cambium and vascular elements had problem in graft interface of these cultivars. Moreover numbers of phenolic compounds such as 4-Hydroxyphenylacetic acid and Ferulic acid had high concentration in scions of 'Ayvalik' and 'Domat', respectively. These results demonstrated existence of problem in dedifferentiated of cambium and vascular systems in graft interface of 'Ayvalik' and 'Domat' cultivars.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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