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INVOLVEMENT OF PHENOLIC COMPOUNDS IN WALNUT TREES RESPONSES TO SOIL FLOODING

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Walnut trees have been shown to be very sensitive to abiotic stresses, especially to soil hypoxia consequence of soil flooding or waterlogging. The aim of study was to search for correlation between polyphenols and tolerance to flooding stress. Two-three years old walnut trees, *Juglans regia*, *J. nigra* and interspecific hybrids, grown in pots were subjected to soil flooding in summer at full leaves expansion. In high sensitive *J. regia* trees, when flooded, the net CO_2 assimilation rapidly decreased and 3-4 days of flooding were enough to block the recovery when soils was drained. In walnut hybrid (*J. nigra* x *J. regia*) and less sensitive *J. regia* genotypes, a longer resistance was observed; after 13 days of treatment leaves culd still be green and trees can recovered till to the normal photosynthesis when drained. *J. nigra* when flooded reduced net photosynthetic rate but maintained leaves without damages and with the capacity to recover CO_2 assimilation. The data showed resistance of *J. nigra* and hybrid trees and of some *J. regia* genotypes to the stress. The HPLC analyses of polyphenols showed a modification of the patterns during the stress. The plants less tolerant to hypoxia have higher content of polyphenols distributed in a lot of compounds. Trees more tolerant, *J. nigra*, showed a very simple HPLC pattern. All samples contained juglone, more in less tolerant genotypes. Hydroxy juglone glucoside was detectable in all genotypes, but only in low quantity, it increased in trees with high resistance to hypoxia and decreased in *J. regia.* Less flooded tolerant *J. regia* genotypes have higher polyphenols and juglone content. The metabolism of hydroxyl juglone glucoside could be involved in a mechanism of juglone detoxification during the hypoxia to give stress tolerance.

Keywords: Juglans regia, *Juglans nigra*, polyphenols, juglone, photosynthesis, abiotic stress, soil hypoxia.

Introduction

Persian or common or Greek walnut (*Juglans regia* L.) is one of the most economically important members of the genus *Juglans*. It is a large, wind-pollinated, monoecious, dichogamous, long-lived, perennial tree, with seed dispersal by animals over short distances and by human over large distances. *Juglans regia* is cultivated for its high quality wood and edible nuts throughout the temperate regions of the world [4]. Although the center of origin of Persian walnut is obscure, it is considered native to the mountain ranges of Central Asia, and its diffusion followed the ancient trade routes passing from China into India, Persia, Greece and thus into Europe [9].

In western European countries the decline of walnut cultivation after the second world war is mainly due to the changing rural structure, both technically and socially (depopulation, land abandonment and high labour costs). In the last decades, new attention is given to human and environmental safety, eco-sustainable development and protection of the national resources in order to promote the redevelopment of the rural areas. In the light of these problems, there has been an increasing demand for new walnut genotypes for both wood and nut production [14]. For this reason, several studies considering genetic, physiologic and biochemical variability between walnut genotypes and varieties have been carried out to explain the high variation in morphology and phenology of adult trees that has been reported [10].

In walnut polyphenols are a characteristic biochemical compounds. Polyphenol contents gave indication of possible their utilisation for identification of walnut tree genotypes [5, 7]. The major polyphenol belong the hydroxycinnamic, hydroxybenzoic and flavonoid types the more characteristic in walnut trees is the naphthoquinone juglone and its biochemical related compounds. The content of polyphenols other then the genotypes changed also with stage of growing and in plant organs. If the phenols are mainly recognised for their antioxidant actions [1, 12], the juglone is well known for its allelopathic and inhibitory effect in many plants [2, 8, 13]. Walnut trees have been shown to be very sensitive to stress environmental condition especially to soil hypoxia consequence to soil waterlogging [2, 11]. Different walnut genotypes have shown different degree of resistance, from few days to weeks. The aim of this study has been to detect the presence of polyphenols and to correlate their presence with different tolerance to waterlogging stress shown by different walnut genotypes.

Material and methods

Two-three years old walnut trees, *Juglans regia*, *J. nigra* and interspecific hybrids (*J. major* x *J. regia* and *J. nigra* x *J. regia*), grown from seeds sowing in 80 l pots were used in the study. Five to eight trees of each walnut genotype were selected for uniformity in height, diameter and number of leaves to use in each experiment set. In summer, at beginning of July when the full leaves expansion were reached, the trees were subjected to waterlogging increasing and maintaining the water level at two-three cm above the soil. The treatment mimics the period and conditions that walnut trees can experience in nature after summer heavy rains especially around river basins.

To evaluate stress level, leaf gas exchanges (photosynthetic rate, transpiration rate, stomatal conductivity) were measured with an open system LCA3 (ADC, England). Measurements were made daily, during the period of water treatment and in the week later of soil draining, to follow the plants recovery.

Walnut leaflets were collected at different length period of stress treatment were used for analysis of polyphenols. Polyphenols were extracted from freeze-dried and ground leaves with 100 acetone (1 g 30 ml) during 30 min in ultrasound bath and with frequently hand shaking. The extract was then filtered, evaporated to dryness under reduced pressure (10 °C), and redissolved in methanol. Polyphenols had been analysed by HPLC updating essentially the method described [6]. Trirotar VI pump with online degasser DG3510 and Diode Array Detector (DAD) MD910, all of Jasco Co., Japan, were the HPLC instrument components. Column Purospher

RP18 (5 μm) 4 × 250 mm (Merck, Germany) was eluted at 0.6 ml /min with mixture of water:methanol:acetonitrile in 30 min gradient from 85:7:8 to 10:45:45 and 20 min more at final composition (10:45:45). The chromatograms at 340 nm and 525 nm had been used for quantification compounds. The spectra collected by DAD in the range 230-500 nm wavelength continuously during column elution permitted identification of polyphenols, at least grouped in chemical groups.

Results and discussion

Comparing different walnut genotypes was possible to have indication of different resistance capacity to root anaerobiosis. The stress effect can be visible after few days of soil hypoxia caused by soil waterlogging, when leaves became wilted. To quantify the stress level the leaf gas exchange was measured. In some *J. regia* genotypes, when flooded, the CO_2 assimilation rapidly decreased and after 3-4 days of flooding the plants did not recover the photosynthetic capacity when soil drain conditions were restored (see example in Fig 1 left). When interspecific hybrids (*J. nigra* × *J. regia*) were subjected to waterlogging a higher resistance capacity was observed. Also in these plants a decrease of CO_2 assimilation was recorded but after 13 days of treatment the leaves recovered to normal photosynthesis level when drained and plants recover to normal growth. The *J. nigra* trees when waterlogged reduced the net photosynthetic rate but they maintain leaves without evident damages and the capacity to recovered CO_2 assimilation to level of the control plants (Fig. 1 right). From the comparison of several genotypes was possible distinguished *J. regia* genotypes that after 3-4 days of stress were totally damaged, other genotypes showed damage after 6-8 days and we had recorded one genotype, named Soraloviner, that was severe affected by stress only after 13-14 days of soil waterlogged. In the hybrid trees only 50% of trees showed visible stress effect only after two weeks of soil flooded. The *J. nigra* trees showed a low visible damages in prolonged stress period and them were able to recover the photosynthesis to the level of control trees also after 16-20 days of soil hypoxia (the longer period tested). Those indication on walnut genotypes permitted a scale definition from flooding high sensitive to flooding high resistant.

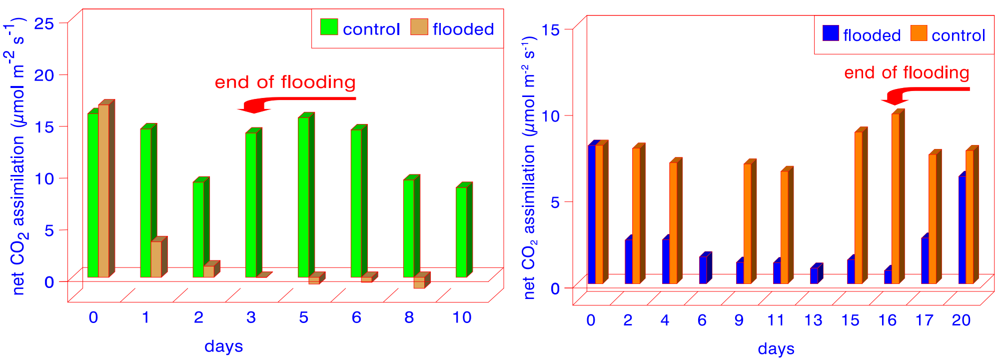

Fig. 1. Comparison of photosynthesis rate in *J. regia* Lozeronne (left panel) and *J. nigra* (right panel), in control trees and soil flooded trees and after returning to the drained soil at the “end of flooding”

The HPLC analyses of polyphenols in leaves showed, as expected, complex and different chromatographic pattern between the genotypes (Fig. 2, Fig. 3A, and 3B), but also the pattern modification of such compounds during the flooding stress Fig. 3C and 3D). The plants less tolerant to hypoxia had higher content of polyphenol distributed in a lot of compounds as separated by HPLC. In *J. regia* Lozeronne, twenty-seven compounds were identified (Fig. 3A, Table 1), 10 of which were characteristic of genotypes, and in total accounted to 189 μg/mg DW (Table 2). Trees more tolerant, *J. nigra*, showed a very simple HPLC pattern with only 13 components and 39 μg/mg DW (Tables 1, 2).

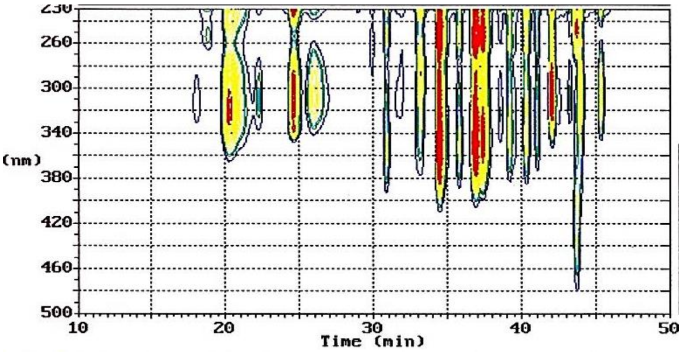

Fig. 2. Typical walnut leaf polyphenols HPLC separation and evidenced by Diode Array Detector with full wavelength (230-500 nm) registration for spectra identification of components The figure shows chromatogram of *J. regia* Solaroviner control leaf extract

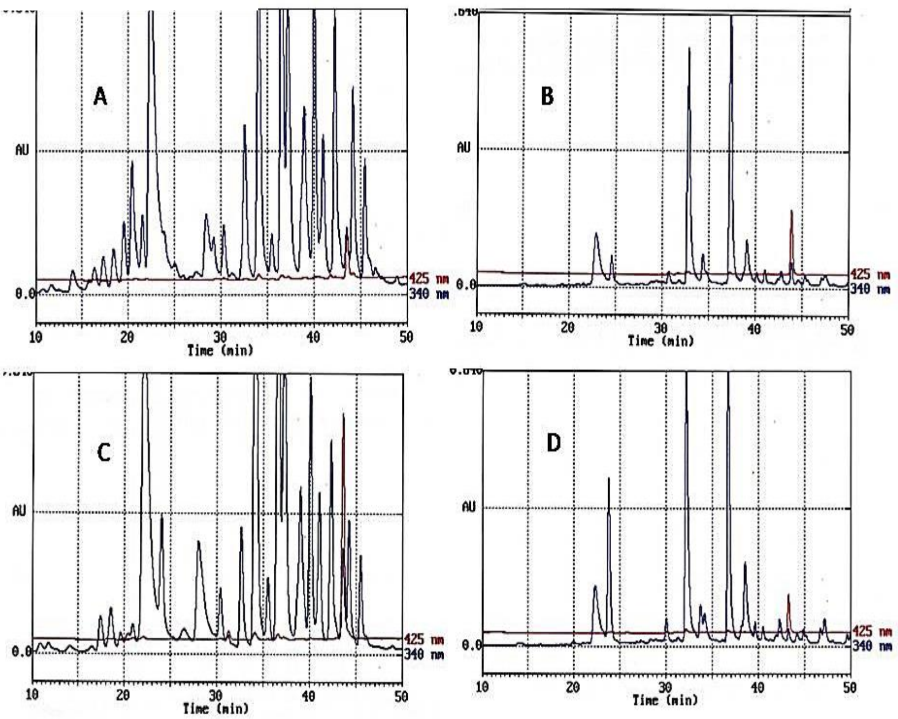

Fig. 3. Comparison of polyphenols HPLC separation: A, C = *J. regia* Lozeronne and B, D = *J. Nigra*. A, B = Control leaves, C, D = flooded leaves. Line at 340 nm total polyphenols, and 425 nm specific for juglone

Table 1

Characteristic and comparison between the three walnut genotypes of polyphenol compounds separated by HPLC expressed as the numbers of total, characteristic or induced by stress components

Indicators	*J. regia* Lozeronne	*J. regia* Soraloviner	*J. nigra*
Total polyphenols	27	17	13
Characteristics of genotype	10	4	3
Induced by stress	6	nd*	nd*

*Not detected.

Table 2

Total polyphenols content in leaves of different walnut genotypes and the quantities changed after the soil fooding

Genotype	Category of stability	Total Polyphenols, µg/mg DW		
		Control	Flooded	Days**
J. regia Lozeronne	High sensitive	189.20±9.70b*	209.40±34.30a	3
J. regia Feltre	High sensitive	76.30±8.43e	90.60±8.94d	4
J. regia Soraloviner	Low resistant	63.74±9.53f	59.49±4.46f	8
J. major x *J. regia*	Medium resistant	108.00±36.51c	100.40±32.19d	4
J. nigra x *J. regia*	Resistant	65.57±2.60f	62.38±5.68f	5
J. nigra	High resistant	39.27±4.17g	38.07±3.23g	8

*Values with different letters are significant different at $P \leq 0{,}05$ (Duncan test).
**Days after beginning of the flooding and when the leaves were collected and results presented in table.

The interspecific hybrids showed polyphenols content at intermediate level between high sensitive *J. regia* Lozeronne geneotype and *J. nigra* (Tables 2). Information about genetic background of *J. regia* used for interspecific hybrids were missed and can be the reason of the polyphenols content higher that in *J. regia* Soraloviner. Furthermore, during stress condition over then changes in the quantity of constitutive compounds the high sensitive genotype Lozeronne showed 6 new compounds induced by hypoxia (Table 1).

Considering the well known allelopathic and inhibitory effect in plants of the juglone, special attention was focused on the juglone and hydroxy juglone glucoside content. The leaf samples of all walnut species and genotypes investigated contained juglone, more in high stress sensitive Lozerone genotype comparing to all other low and high resistant genotypes (Table 3). Also the hydroxy juglone glucoside was detectable in all genotypes, but it was only in low quantity in leaves of tolerant plants and it increased in samples from *J. nigra* trees with high resistance to hypoxia as well as in *J. regia* Lozeronne high sensitive genotype (Table 3).

Table 3

Levels of juglone and hydroxyl juglone glocoside in leaves of different walnut genotypes and the modification after flooding stress

Genotype	Category of stability	Juglone (μg/mg DW)		Hydroxy juglone glucoside (μg/mg DW)	
		Control	Flooded	Control	Flooded
J. regia Lozeronne	High sensitive	11.83±0.15a*	7.38±2.92b	13.67±6.20a	18.62±2.65a
J. regia Soraloviner	Low resistant	1.49±1.28g	1.43±0.39g	6.83±1.43c	9.12±1.11b
J. major x *J. regia*	Medium resistant	4.72±0.07c	4.32±0.63e	4.81±0.38c	2.13±2.06d
J. nigra x *J. regia*	Resistant	4.65±0.76c	3.20±0.35f	2.03±0.13d	2.59±2.09d
J. nigra	High resistant	1.78±0.38g	0.93±0.45h	0.85±0.33e	3.63±1.59c

*Values with different letters are significant different at P $\leq$ 0,05 (Duncan test).

Conclusion

The data show the higher resistance to soil hypoxia for *J. nigra* and hybrid trees. Less waterlogged tolerant *J. regia* genotypes have higher total polyphenol and juglone content in leaves, the later decreases during stress condition, indicating that polyphenols content can be a biological marker for selection of stress resistant capacity. The metabolism of hydroxy juglone glucoside could be involved in a mechanism of juglone detoxification during the hypoxia to give stress tolerance. Indeed, in *J. nigra* the glucosylation of juglone could be a mechanism of detoxification of juglone during the hypoxia stress. Also in *J. regia* during hypoxia the hydroxy juglone glucoside increased during hypoxia condition, and mechanism of juglone detoxification through the glucosylation was enough for Soraloviner walnut genotype but the high content of juglone in high sensitive Lozerone genotype can not permit detoxification to a low and physiological compatible level.

In walnut polyphenol compounds have been reported to be associated to rejuvenation and clonal variability [7], now evidence of a possible role in abiotic stress (soil flooding, root hypoxia) degree of adaptation or resistance are added. Polyphenol components should take more attention in the future investigations on responses to stresses and to identify more resistant genotypes and the relationship to phonological stages or genetic background.

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Участие фенольных соединений в реакции грецкого ореха на затопление почвы

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Деревья грецкого ореха оказались более чувствительными к абиотическим воздействиям, особенно к гипоксии почвы вследствие ее затопления или заболачивания. Целью исследования являлся поиск взаимосвязи между полифенолами и стойкостью к затоплению. 2–3-летние деревья грецкого ореха (*Juglans regia, J. nigra*) и межвидовые гибриды, выращенные в горшках, были подвергнуты затоплению почвы летом

при полном облиствлении. При высокой чувствительности *J. regia*, при затоплении, количество поглощаемого CO_2 быстро сокращалось, и было достаточно 3-4-х дней затопления, чтобы блокировать восстановление дренирования почвы. У гибрида грецкого ореха (*J. nigra* × *J. regia*) и менее чувствительных генотипов *J. regia* наблюдались последствия; через 13 дней листья могли оставаться еще зелеными и не опадали до тех пор, пока деревья не восстановились до нормального фотосинтеза, соответствующего осушенной почве. При затоплении у *J. nigra* снижалась скорость фотосинтеза, но сохранялась без повреждений листва и возможность восстановить поглощение CO_2. Данные показали сопротивляемость *J. nigra* и гибридных деревьев, а также некоторых генотипов *J. regia* к воздействию затопления. Высокоэффективная жидкостная хроматография полифенолов показала изменение образцов в это время. Растения, менее устойчивые к гипоксии, имеют более высокое содержание полифенолов, распределенных во многих соединениях. У более устойчивых деревьев *J. nigra* получены очень простые образцы при высокоэффективной жидкостной хроматографии. Все образцы содержали юглон, его было больше в менее устойчивых генотипах. Гидроксиюглонгликозид обнаруживался во всех генотипах, но только в небольшом количестве, больше его содержится в деревьях с высокой устойчивостью к гипоксии, меньше – в *J. regia*. Менее устойчивые к затоплению *J. regia* генотипы имеют большее содержание полифенолов и юглона. Метаболизм гидроксиюглонгликозида может быть вовлечен в механизм детоксикации юглона при гипоксии для появления устойчивости к воздействию.

Ключевые слова: *Juglans regia, Juglans nigra*, полифенолы, юглон, фотосинтез, абиотическое воздействие, гипоксия почвы.

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