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ALLELOPATHIC INVESTIGATIONS IN THE *FRAXINO ANGUSTIFOLIAE-QUERCETUM ROBORIS* (JOV. ET TOMIĆ 1979) FOREST COMMUNITY WITH THE AUTUMNAL TRUFFLE (*TUBER MACROSPORUM* VITT.)

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Abstract - Both the total phenols and phenolic acids (PA) were analyzed in the litter and soil of the mixed *Quercus robur-Fraxinus angustifolia* forest with the autumnal truffle (*Tuber macrosporum* Vitt.). These compounds are the main allelopathic products of host plants of ectomycorrhizal fungi.

Fresh litter, mostly composed of falling leaves of pedunculate oak (*Quercus robur*) and ash tree (*Fraxinus angustifolia*), is rich in bound (12.39 *mg/g*) as well as free (12.43 *mg/g*) forms of phenolics. Partially decomposed litter had significantly lower amounts of phenolics.

The soil in investigated forest is non-calcareous. This is in disagreement with available literature sources which emphasize that autumnal truffle exclusively grows on calcareous soils.

Amounts of both PA and total phenolics decrease with increasing soil depth. The greatest proportion of the truffle's fruit bodies (nearly 30 *kg*/ha/year) is located in surface (phenolics-rich) soil horizon, so it may be assumed that *Tuber macrosporum* Vitt. is well adapted to high concentrations of phenolic compounds.

The fruit body of autumnal truffle is rich in free (243.6 *μg/g*) and bound 349 *μg/g*) phenolics. Considering PA, however, only small amounts of vanillic acid were detected in the truffle.

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INTRODUCTION

Truffles in a narrower sense (*Tuber* sp.) represent underground ectomycorrhizal fungi (subclass *Ascomycotina*) that are closely associated with numerous tree species. The most frequent host plants for truffles are various trees and shrubs that are included in the following genera: *Quercus, Populus, Fraxinus, Carpinus, Corylus, Fagus* and *Rosa* (Chatin 1892; Pagnol 1983; Hrka 1984, 1988; Milenković *et al.* 1992). Different aspects of truffles' biology (taxonomy, morphology, anatomy, biogeography, mycorrhization, biochemistry, genetics, ecology, *etc.*) have been investigated by numerous authors (Chatin 1892; Delmas and Poiton 1973; Chevalier 1974, 1979; Papa 1980; Chevalier *et al.* 1985; Pargney-Leduc *et al.* 1987; Talou *et al.* 1987; Biocca *et al.* 1988). Analyzing available literature sources, we found that the allelopathic effects of host plant metabolites (especially phenolics) on this group of ectomycorrhizal fungi were not considered so far.

Phenolics represent a widely distributed group of compounds in the plant tissues. Several hundred phenolic compounds have been discovered so far. They are located in all plant organs, both as free forms and associated with other compounds such as lignin, the commonest compound of tree species (Bate-Smith 1969; Saunders and MacClure 1976; Harborne 1980; Schtte 1985; Kögel and Bochter 1985; Kögel 1986) and polysaccharides of cell walls (Whitmore 1976). Phenolics are transferred from plants into the litter and soil by foliage and stem leaching, leaf falling, as well as by exudates of roots and glandulose trichins. Moreover, the phenols are accumulated in soil by microbial decomposition of plant remains, especially by degradation of lignin. Considering abundance and primary productivity, dominating trees in forest ecosystems have a great allelopathic significance (Al-Naib and Rice 1971; Lodhi and Rice 1971; Lodhi 1975, 1976; Alexander 1977;

Tang and Young 1982; Whitehead *et al.* 1983; Sterling *et al.* 1987).

The accumulation of phenolic toxins in soil inhibits seed germination as well as growth of seedlings. Phenolic compounds act as inhibitors of some fungi (Harrison 1971). However, other fungi may grow in phenol solutions, using the phenolic compounds as the only source of carbon and energy (Martin *et al.* 1972).

According to Molich (1937), the allelopathy may be considered as a biochemical interaction among plants, including microorganisms. In order to reveal allelopathic relations between the donors (host plants) and acceptors (ectomycorrhizal fungi) of phenolic compounds, we analyzed content of both total phenolics and PA in litter, soil and fruit body of *Tuber macrosporum* Vitt.

MATERIAL AND METHODS

1. Descrption of investigated forest

The investigated forest is located at 200 m a. s. l. near the Danube river, in a close proximity of Belgrade. The forest is developed on a temporary water logged soil.

Pedunculate oak (*Quercus robur*) almost exclusively forms the highest stratum of the forest. A few dispersed individuals of *Fraxinus angustifolia* and *Populus alba* also occur in this stratum. *Fraxinus angustifolia* dominates in the shrub and lower trees strata.

The greatest species diversity was recorded in the stratum of herbaceous plants. The most abundant herbaceous species in the analyzed forest are: *Stellaria media, Cerastium glomerata, Taraxacum officinale, Urtica dioica, Galium aparine, Cardamine pratensis, Lamium purpureum, Fraxinus angustifolia, Lythrum salicaria* and *Glechoma hederacea.*

2. Soil analyzes

The soil acidity was analyzed in H_2O and 1 M KCl solution (1:2.5), respectively. The concentration of $CaCO_3$ was determined using the volumetric method (Allison and Moodie 1965). The percentage of humus and carbon was detected using the Tiurin's method (Beljchikova 1975). Total nitrogen was determined by "Semimicro-Kieldahl's" method (Bremner 1965). The soil texture was analyzed extracting soil particles of different size using the "pipette method" (Day 1965). The adsorptive complex properties which include hydrolytic acidity (Y_1), cation exchange capacity (T), sum of base cations (S), sum of acid cations (T-S) and percentage base saturation (V=100 x S/T) were detected according to Kappen's method after Askinazi (1975a, b).

2. Sampling of litter and soil material

a) Litter

The same quantities (1 *kg*) of both, slightly transformed (uppermost) and partially decomposed (a lower) litter layers were collected. The sampled litter was dried at 60 °C, milled and sieved through the sieve with 0.5 *mm* diameter holes.

b) Soil

Amounts of phenols and phenolic acids were detected in all horizons of a soil profile. Moreover, these compounds were analyzed in additional topsoil samples. After removal of partially decomposed litter, topsoil samples were collected from the surface layer (up to 5 cm depth) including the "fermentation" (Of) and the organo-mineral "humic" (Ah) horizons. Topsoil samples were divided in two groups (samples with and without fruit bodies of *Tuber macrosporum*). After removal of visible plant remains, the soil was dried, milled and sifted through the sieve with 0.5 *mm* diameter holes.

4. Identification of phenolic compounds

a) Analysis of phenolics in the litter and fruit bodies of Tuber macrosporum

Free forms of phenols and phenolic acids were extracted from dried material (4 *g* for litter and 2 *g* for truffle) in boiling 80% ethyl alcohol and ethylacetate. The bound phenolics were extracted in ethylacetate after the pretreatment procedure involving the one hour boiling of dried material in 2 N HCl (Mijdla *et al.* 1975).

b) Analysis of phenolics in soil

Free forms of phenolics were extracted from 30 *g per* sample of dried soil in boiling ethylacetate (3x50mL), during 24 *h*. Residual soil was treated with 15 mL of 2 N NaOH, and after boiling for 24 *h* the bound phenols were determined (Hennequin and Juste 1967; Katase 1981a, 1981b). The quantity of total phenols (free and bound forms) in truffle, litter and soil was detected spectrophotometrically, using the Folin-Ciocalteau's reagent (Feldman and Hanks 1968). The calibration curve was constructed on the basis of different concentrations of ferulic acid.

Quantitative and qualitative analyses of phenolic acids were performed using the ascending two-dimensional paper chromatography. This method separates five PA including two derivatives of cinnamic acid

(ferulic or 4-hydroxy-3-methoxy cinnamic acid and *p*-coumaric or *trans*-4-hydroxycinnamic acid) and 3 derivatives of benzoic acid (*p*-hydroxybenzoic acid, vanillic or 4-hydroxy-3- methoxy benzoic acid and syringic or 3,5-dimethoxy-4-hydroxybenzoic acid). These components were separated using two mixtures: 1. Isopropanol : Ethylacetate : NH_4OH : H_2O (30 : 50 : 1 : 19) and 2. 2% acetic acid.. Dried chromatograms were sprayed with *p*-nitroanaline and 20% Na_2CO_3. Obtained chromatographic spots were eluted with 45% ethyl alcohol. The quantity of PA was determined spectrophotometrically, measuring the optical density for each acid on a characteristic wave-length (M i j d l a *et al.* 1975).

RESULTS AND DISCUSSION

1. Amount of PA and sum of phenolic compounds in the litter

Fresh litter in the analyzed forest is rich in free (12.43 *mg/g*) and bound (12.39 *mg/g*) forms of phenolic compounds. Comparing to fresh litter, partially decomposed litter had significantly lower amounts of both free (1.49 *mg/g*) and bound (5.99 *mg/g*) phenolics.

Four phenolic acids (*p*-coumaric, ferulic, vanillic and syringic) were detected in the analyzed litter. Free syringic acid and bound vanillic acid dominate in fresh litter (Table 1). Considerably lower amounts of these compounds were detected in partially decomposed litter. Decrease of total phenols during the initial stage of the litter decomposition is caused by the leaching of water-soluble phenols. As the consequence of the free phenols leaching, the insoluble and less degradable compounds such as lignin, cellulose and hemicellulose prevail in the litter during the later stages of decaying process (Y a v i t t and F a h e y 1986). Analyzing litter decomposition, K ö g e l and B o c h t e r (1985) and K ö g e l (1986) detected 5 phenolic acids and 3 phenolic aldehydes as a result of the oxidative lignin degradation. Allelopathic effects of different oak (*Quercus*) species are mainly caused by phenolic phytotoxines (PA and flavonoids) which are accumulated in the leaves (A l-N a i b and R i c e 1971, R i c e and P a n c h o l y 1973; L o d h i 1976,). F e e n y (1968) detected significant amount (up to 5%) of tannins in the leaves of *Quercus robur*.

2. Physico-chemical properties of soil

The soil in the forest belongs to the non-calcareous swampy soil (mullic humogley) on loess. The soil is heavy, and throughout the profile it belongs to the clay texture type. A high percentage of both colloidal clay (46.12 to 52.80 %) and total clay and silt (77.7 to 86.27 %), as well as very low proportion of total sand (from 13.73 to 22.30 %) indicates unfavorable aerating and draining properties of the soil (Table 3).

Table 1. Total phenolics and phenolic acids in fruit bodies of autumnal truffle and in litter and soil of the analyzed forest

		Litter		Soil profile			Fruit body of truffle
		Fresh	Partially decomposed	Depht (cm) 0-15	15-30	30-45	
Tothal phenols (µg/g)	free	12,433.04	1,487.32	74.06	30.10	15.99	243.64
	bound	12,387.12	5,988.28	752.87	328.80	183.55	394.03
	Sum	24,820.60	7,475.60	826.93	358.90	199.54	637.67
PA (µg/g)							
p-Coumaric	free	-	trace	trace	-	-	-
	bound	-	-	20.91	6.98	2.00	-
Ferulic	free	-	29.54	trace	-	-	-
	bound	-	11.67	28.44	10.04	1.81	-
p-Hudroxybenzoic	free	-	-	0.57	0.09	0.07	-
	bound	-	-	6.64	1.33	2.11	-
Vanillic	free	19.67	11.08	1.31	0.17	0.10	1.78
	bound	203.75	131.54	26.84	7.54	3.34	4.76
Syringic	free	131.17	38.05	1.07	-	-	-
	bound	-	trace	21.45	6.19	2.78	-
Total PA	free	150.84	78.67	2.95	0.26	0.17	1.78
	bound	203.75	143.21	104.28	32.08	12.04	4.76
	sum	354.59	221.88	107.23	32.34	12.21	6.54

Table 2. Total phenolics and phenolic acids in soil samples with and without fruit bodies of autumnal truffle.

Soil samples	Total phenols (μg/g)		Phenolic Acids (μg/g)									
			p - Coumaric		Ferulic		*p* - Hydroxybenzoic		Vanillic		Syringic	
	free	bound	free	bound	free	bound	free	bound	free	bound	free	bound
Without truffle	93.62	1,528.0	1.96	18.86	trace	31.10	1.17	10.12	5.39	20.81	6.33	63.11
With truffle	78.81	1,413.1	1.74	23.39	2.70	33.40	0.79	0.89	4.14	40.51	3.28	76.72

Table 3. Physical properties of the soil in the analyzed forest

Deph	Hygroscopic moisture	Soil particles size (mm)				Total clay + silt	Total sand
		Very coarse sand 2.0-0.2	Fine sand 0.2-0.02	Silt 0.02-0.002	Clay <0.002		
(cm)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
0-15	6.24	2.03	11.70	35.97	50.30	86.27	13.73
15-30	6.33	0.54	13.92	32.74	52.80	85.54	14.46
30-45	4.73	0.29	22.01	31.58	46.12	77.70	22.30

Table 4. Chemical properties of the soil in the analyzed forest

Deph	pH		$CaCO_3$	Y_1	Adsorptive complex properties				Humus	C	N	C:N
	H_2O	KCl			(T-S)	S	T	V				
(cm)			(%)	ccm	(*mg* eqv/100g)			(%)	(%)	(%)	(%)	
0-15	6.40	5.22	-	10.70	3.92	40.36	44.28	91.15	6.60	3.83	0.35	10.94
15-30	6.95	5.62	-	4.26	1.46	37.88	39.34	96.29	2.95	1.71	0.19	9.00
30-45	7.35	6.09	-	1.54	0.46	41.80	42.26	98.91	1.91	1.11	0.16	6.94

The soil is neutral to slightly acid and non-calcareous (Table 4). The presence of autumnal truffle in this soil is in disagreement with literature sources (M a l e n c o n 1938; R e b i e r 1982; H r k a 1988) which emphasize requirements of truffles for calcareous soil. This discrepancy may be explained by insufficient knowledge of ecological requirements of autumnal truffle. Moreover, it may be assumed that ecological amplitude of this fungus is much broader than it was described in the literature.

Adsorptive complex of the soil is characterized by a high percentage of base saturation (91.15 to 98.91 %). Due to high content of humus and clay, total adsorption capacity in the uppermost horizon is very high. The soil is rich in both humus and total nitrogen. The transformation of organic matter is characterized by alternating aerobic and anaerobic phases. Nevertheless, the process of peat formation is absent since several artificial channels enable efficient drainage of the sufficit water. Due to a prolonged period of the aerobic transformation of organic matter, the humus has mullic properties, and C:N ratio indicates that the mineralization process is slowed, but not blocked.

3. Total phenolics and phenolic acids in the soil and fruit bodies of truffle

The greatest amounts of PA and total phenolics are localized in the surface layer of the soil profile. The quantity of both PA and total phenols decreases with increasing depth. Free forms of *p*-coumaric, ferulic and syringic acids were not detected in the lowermost horizons (Table 1). As in the case of topsoil, the lowermost horizon is characterized by a high ratio of bound to free phenols. Compared to our results, K u i t e r s and D e n n e n m a n (1987) detected significantly lower amounts of free phenols (155 μg/g) and free PA (0.19 to 1.07μg/g) in a humic layer of the soil beneath *Quercus robur*. However, L o d h i (1975, 1978) found considerably greater content of bound phenolic acids in the soil developed below *Fraxinus pensilvanica, Quercus macrocarpa* and *Celtis laevigata*, where concentrations of *p*-coumaric and ferulic acids were almost 50 times greater than in the soil of the forest investigated in the present study.

Considering amounts of PA and total phenolics, both groups of topsoil samples (samples with and

without fruit body of autumnal truffle) are very similar. In both cases the ratio of bound to free phenols varied from 16 to 18 (Table 2). Content of free phenolic acids (which have a stronger physiological effects than bound PA) is very low (up to 6.33 μg/g). Compared to soil samples without autumnal truffle, the samples with truffles have considerably greater (up to 2 times) amount of bound vanillic acid. This indicates that fruit bodies may exudate the vanillic acid in the adjacent soil.

The fruit body of autumnal truffle is rich in free (243.64 μg/g) and bound (394.03 μg/g) phenols. Considering PA, however, only small amounts of vanillic acid were detected (Table 1).

Both *Macromycetes* and *Micromycetes* are able to synthesize PA, a simpler phenols and phenolic polymers (Bondietti *et al.* 1971; Martin *et al.* 1972; Tomaszewski and Wojciechowska 1973). Some fungi may grow in media containing high concentrations (up to 5 g/L) of orcinol, protocatechuic, *p*-hydroxybenzoic or vanillic acids, using these compounds as only source of carbon and energy (Martin *et al.* 1972). On the contrary, Harrison (1971) found that some fungi (14 species isolated from decomposed oak leaf litter) are inhibited by tannins of *Quercus petrea* and *Q. robur* leaves. Tannic acid and condensed tannins inhibit β-glucosidase, amylase and cellulase, the enzymes important for cellulose degradation (Goldstein and Swain 1965; Benoit and Starkey 1968; Alexander 1977).

The greatest amounts of both phenols and PA are localized in the surface horizon (0-5 cm) of the analyzed soil. The greatest proportion of the fruit bodies of autumnal truffle (nearly 30 kg/ha/year) was also found in this soil layer. Since the mycelia and fruit bodies of *Tuber macrosporum* Vitt. are surrounded by phenols-rich soil, it may be concluded that the autumnal truffle is well adapted to high concentrations of phenolic compounds.

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АЛЕЛОПАТСКА ИСТРАЖИВАЊА У ЗАЈЕДНИЦИ ЛУЖЊАКА И ПОЉСКОГ ЈАСЕНА *(FRAXINO ANGUSTIFOLIAE-QUERCETUM ROBORIS JOV. ET TOMIĆ 1979)* СА ЈЕСЕЊИМ ТАРТУФОМ *(TUBER MACROSPORUM* VITT.)

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У шуми лужњака и пољског јасена (*Querceto-Fraxinetum serbicum* Rudski) са јесењим тартуфом (*Tuber macrosporum* Vitt.) испитивана су фенолна једињења, која су идентификована као најчешћи алелохемијски продукти виших биљака-домаћина ектомикоризних гљива. Опало јесење лишће лужњака и пољског јасена је главни извор укупних фенола (по 12 *mg/g* слободних и везаних). Делимично разложена стеља садржи знатно мању количину фенола.

Земљиште у испитиваној заједници је безкарбонатно, што је у супротности са доступним литературним подацима, према којима тартуфи искључиво расту у карбонатним земљиштима.

Количина фенолних киселина и укупних фенола опада са повећањем дубине земљишног профила. Њихова количина је највећа у површинском слоју земљишта (0-5 cm) у коме је смештен и највећи број плодоносних тела тартуфа (око *30 kg/ha)*. Може се претпоставити да фенолна једињења лужњака и пољског јасена-домаћина јесењег тартуфа утичу позитивно на ову подземну ектомикоризну гљиву.

Плодоносна тела јесењег тартуфа такође садрже слободне (243.64 *μg/g*) и везане феноле (349.03 *μg/g*), а од фенолних киселина детектована је само ванилинска киселина у малој количини.