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Essential elements as a distinguishing factor between mycorrhizal potentials of two cohabiting truffle species in riparian forest habitat in Serbia

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Abstract

True truffles (*Tuber sp.*) that establish ectomycorrhizal symbiosis (ECM) with trees in the Mediterranean and temporal regions have species specific abilities to assimilate soil born elements. Suitable habitats are usually inhabited by few truffle species, while distinguishing their symbiotic potentials appeared very difficult. Two species that commonly inhabit riparian forests in Serbia are the most prized one, *Tuber magnatum* Pico (Piedmont white truffle) and not so highly valued *Tuber brumale Vitt.* In order to assess potential differences between their assimilation and accumulation abilities, the differences between contents of elements that may be the subjects of the symbiotic trade between the host plant and fungi were evaluated in accumulation target (ascocarps) and their source (the soil). Essential macro (K, Na, Ca, Mg, Fe, P, S and Zn) and essential trace (Co, Cr, Cu, Mn and Se) elements in truffles and soil samples were determined by means of inductively coupled plasma with optical emission spectrometry (ICP–OES). Their concentrations (mg/kg) in ascocarps were in the range from 1.364±0.591 (Cr) to 10760.862±16.058 (K), while in soil ranged from 23.035±0.010 (Cr) to 20809.300±122.934 (Fe). Element accumulation potential (bioaccumulation factor) was calculated in the system truffle/soil. The statistical approaches were used for establishing the differences, while the possible differentiation between symbiotic potentials of two mycelia in the defined soil conditions was discussed.

Keywords: *Tuber magnatum* • *Tuber brumale* • essential elements • essential trace elements • ICP–OES

Introduction

Macro fungi, mostly belonging to the orders of *Asco*-and *Basidiomycota*, form spore dispersal organs (sporocarps) visible by naked eye and have saprobic or symbiotic way of life (with few exceptions being tree parasites). As a result of co adaptations on seasonal climates, some of them establish mutualistic relationship with trees, mostly in temporal and boreal regions, called ectomycorrhizal (ECM) symbiosis. Special small rootlets enveloped by the layer of fungal hyphe that grow into the epidermis of the roots are formed in order to establish a symbiotic organ called ectomycorrhiza. Here the exchange of nutrients and water occurs in the manner that the plant receives nutrients from the soil (water, N, P, K and other macro and micro elements) while fungi acquire carbohydrates, mostly simple sugars, from the plant.[1] In general, having high ability of absorption, fungi are able to take up from the environment many inorganic substances in a species specific way.[2] Accordingly, different ECM fungi also have different abilities of assimilation and accumulation of soil born elements and water, but this process is, in addition, controlled by host plant demands. Most ECM fungi form highly nutritious above ground (epigeic) sporocarps, aimed to be consumed by different animals for spore dispersal, but some species have specialized in surviving harsh conditions and adapted to forming sporocarps within the soil (hypogeic). In order to attract potential spore dispersing subjects, they usually produce plenty of strong aromatic substances so that animals would be able to detect them.[3]

True truffles are the Ascomycetes fungi that establish ECM with different tree species mostly in the Mediterranean and temporal regions. Ascocarps of some European species can reach high prices on the market, while in Serbia four of those have officially been traded with (*T. magnatum Pico., T. aestivum Vitt, T. macrosporum Vitt, T. brumale Vitt*).[4] Even though common demands of majority of truffle species have been reported (neutral to alkaline soils with high content of cations, specific ECM hosts, mild seasonal climate), every one of them has distinct ecological niche.[5] Usually, in suitable habitats, there are few cohabiting species and distinguishing their demands can be very challenging – the problem that appears quite often in truffle plantations where one of the species is always more desired. [6,7]

Two species that most commonly inhabit lowland forests in Serbia are, the most prized and the most endangered ones, *Tuber magnatum* (Piedmont white truffle) and not so highly valued, but one of the most widespread in natural habitats of Serbia, *Tuber brumale*.[5] Typical habitats for both species are old riparian forests in the flooding planes of big rivers, dominated by *Quercus robur, Populus sp, Fraxinus sp*., on the alluvial soils strongly influenced by the fluctuating water tables.[5,8] The plant hosts, soil conditions and the time of fructification (in autumn and early winter) for these species appear to be the same, but it is clear that they must be playing different roles in symbiotic relationships of the shared ecosystems. *T. brumale* is a questionable species that has been reported from very different habitats,[6,7,9] this is why its metabolic symbiotic possibilities are expected to be quite different in comparison to dominant in production *T. magnatum*. ECM is usually assumed as dominating symbiosis type in the ecosystems where nutrient limiting the plant growth is N, even though P and also some other nutrients may be symbiotically assimilated as well.[1] However, *T. magnatum* productive soils have been reported to be nitrogen rich implying that its shortage is hardly the main reason for ECM establishment.[5,8] Therefore, some other factors may be playing crucial roles in competition between cohabiting species for plant root originated C source. These factors of competition may be anywhere between inorganic nutrients for which host plant may be in demand. As it was almost impossible to measure nutrient transport through different ECM associations in the natural conditions, we reached for the only source of information that was available – truffle ascocarps.

Truffle ascocarps have been explored as a source of nutrients of health benefits,[10-13] toxic elements,[14,8] or the most often, volatiles that are responsible for their specific and appreciated aroma.[3,15,16] However, if an ascocarp essential element content is compared to its soil content, it may be possible to estimate differences in assimilation of specific inorganic nutrients that may be beneficial for the host plant, at least at the time of truffle fructification. Such data originating from different areas of investigation or different truffle species are very scarce.

Therefore, in order to assess potential differences between assimilation and accumulation abilities between two cohabiting truffle species (*Tuber magnatum* and *Tuber brumale*) in specific habitat in Western Serbia, we aimed to evaluate differences between contents of elements that may be the subject of mycorrhizal trade off between host plant and fungi in accumulation target (ascocarps), and their source (the soil). In that respect, elements that have been already detected as limiting in such ecosystems[8] and those that are usually abundantly present in truffle productive soils and responsible for their buffering capacity, like calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), sulphur (S) and zinc (Zn), as well as essential trace elements, cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn) and selenium (Se) have been chosen. Along with that, bioaccumulation factors of elements for both *Tuber* species were determined.

Results

Content of elements in truffles and soil

Among 13 elements analyzed in this study, Co and Se were below the limit of detection in both truffle ascocarp and soil samples. Concentrations of other elements, expressed in fresh matter, are presented in Table 1. The content of the studied elements, with the exception of chromium and manganese, in ascocarps of *T. brumale* and *T. magnatum* was significantly different at level $P \leq 0.01$ (Table S1, Supporting information). Major element was K, in both, *T. magnatum* and *T. brumale*, followed by P, S and Ca, with other elements being represented in markedly lower contents. The content of analyzed elements in soil was in the range of 52.827±0.532 mg/kg for Cr to 20809.300±122.934 mg/kg for Fe. The most abundant elements, besides iron, were calcium, magnesium, potassium and manganese. Other elements were measured in concentrations less than 500 mg/kg, Table 1. Bioaccumulation factor (BAF) of each element in the truffle species was calculated as the ratio between its content in the system truffle/soil. In both *T. magnatum* and *T. brumale,* the highest BAF values were for phosphorous P and potassium K, followed by Na and S. In addition, Cu and Zn in *T. magnatum* were accumulated in high degree (BAF>1) (Table 1).

Table 1. Content of elements in truffles and soil (mg/kg fm) and bioaccumulation factor (BAF)

Element	*Tuber* spp.		Soil	BAF	
	T. magnatum	*T. brumale*		*T. magnatum*	*T. brumale*
Ca	557.943±7.679[a,b]	1358.387±16.170	4342.578±21.252	0.128	0.313
Cr	1.364±0.591	1.957±0.000	52.827±0.532	0.026	0.037
Cu	34.445±0.591	15.659±0.000	23.035±0.000	1.495	0.680
Fe	349.226±3.593	232.270±5.565	20809.300±122.934	0.017	0.011
K	10760.862±16.058	8640.308±15.172	2263.583±14.105	4.754	3.817

Mg	300.116±3.873	395.381±6.851	4091.956±6.644	0.073	0.097
Mn	7.162±0.001	6.851±0.001	746.645±1.407	0.010	0.009
Na	118.341±2.130	67.528±2.936	48.834±0.921	2.423	1.383
P	2769.593±8.742	2155.021±2.589	380.233±7.836	7.284	5.668
S	1183.071±9.284	1318.588±3.437	388.525±3.236	3.045	3.394
Zn	168.133±0.591	72.747±1.495	79.855±5.320	2.105	0.911

[a] data are expressed as mean ± st. deviation. (n=3); [b] Fresh matter (fm)

The results obtained for Ca and Mg in the studied truffles indicated considerable variations between the species; concentration of Ca was 2-fold higher in *T. brumale* than in *T. magnatum.* The most dominant element, K, as well as Fe, were measured in significantly higher concentrations in *T. magnatum* when compared to *T. brumale* ($P \leq 0.01$) (Table 1). The obtained results indicated that both *Tuber* species were a good source of these elements. Also, both ascocarps were accumulating Na and P, both considerably higher in *T. magnatum.* On the other hand, the concentration of S was significantly higher in *T. brumale* ($P \leq 0.01$) (Tables 1 and S1). Among essential trace elements, Cu and Zn, were measured at twice as high concentrations in *T. magnatum* compared to *T. brumale* ($P \leq 0.01$) whereas concentration of Cr was higher in *T. brumale*, but statistically not significant ($P \geq 0.05$) (Tables 1 and S1).

Based on the physical and chemical parameters (Table 2), soil was characterized as clay loam, with high percentage (62.51%) of particiles smaller than 0.02 mm. The pH value indicated that soil was weakly alkaline (7.81) with medium content of humus (3.36%). The soil was well supplied with available K (22.7 mg expressed as content of K_2O/100g of soil) whilst the content of the available P was low (2.7 mg expressed as content of P_2O_5/100g of soil). The results of the exchangeable adsorbed cations content showed that Ca (22.8 cmol/kg) was the most prevalent one, followed by Mg (7.5 cmol/kg), K (0.5 cmol/kg) and Na (0.4 cmol/kg), while the Ca/ Mg ratio was 3.04 (Table S2).

Table 2 . Major physical and chemical properties of the soil

Properties	**Texture**	**Content of mechanical fractions (%)**						**Hygroscopic moisture (%)**
Physical	**Clay loam**	2-0.2 (mm)	0.2-0.05 (mm)	0.05-0.02 (mm)	0.02-0.002 (mm)	<0.002 (mm)	Σ of fractions <0.02 (mm)	
		6.56	17.65	13.28	29.76	32.76	62.51	5.21
Chemical		**pH/H_2O**	**pH/KCl**	**$CaCO_3$ (%)**	**Organic matter (%)**	**Humus (%)**	**mg K_2O/100g**	**mg P_2O_5/100g**
		7.81	6.82	6.87	1.95	3.36	22.7	2.7

The correlation coefficients for the elemental content were studied in the system truffle/soil, as well as in each truffle species and soil (Tables 3 and 4). In the system *T. magnatum*/soil following could be noticed: positive correlations between Ca-Na and S-Mn in *T. magnatum* and soil, respectively ($P \leq 0.05$). Highly significant negative correlations were observed for Cu-Cr, Fe-S, Mn-Cr and Zn-Zn ($P \leq 0.01$) (Table 3). Elements Ca-Na and Mg-Ca were positively correlated ($P \leq 0.05$), whereas significant negative correlations, at both levels, for Ca-S ($P \leq 0.05$), S-S ($P \leq 0.01$) were noticed in the system *T. brumale/*soil (Table 4).

Table 3 . Pearson's correlations of elements between *T. magnatum* and soil

Tuber magnatum	**Soil**										
	Ca	**Cr**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**P**	**S**	**Zn**
Ca	0.965	0.885	.$^{/c}$	0.225	0.158	-0.314	0.291	0.999^{a}	0.927	-0.999^{a}	0.846
Cr	-0.676	-0.500	.$^{/}$	-0.710	-0.660	-0.240	-0.756	-0.866	-0.984	0.822	-1.000^{b}
Cu	-0.976	-1.000^{b}	.$^{/}$	0.255	0.321	0.721	0.189	-0.866	-0.645	0.904	-0.500
Fe	0.975	0.904	.$^{/}$	0.182	0.115	-0.355	0.249	0.997	0.910	-1.000^{b}	0.822
K	0.494	0.294	.$^{/}$	0.849	0.811	0.451	0.883	0.733	0.920	-0.674	0.975
Mg	-0.939	-0.991	.$^{/}$	0.381	0.443	0.806	0.317	-0.792	-0.538	0.840	-0.381
Mn	-0.976	-1.000^{b}	.$^{/}$	0.255	0.321	0.721	0.189	-0.866	-0.645	0.904	-0.500
Na	0.896	0.971	.$^{/}$	-0.480	-0.539	-0.866	-0.419	0.721	0.442	-0.775	0.277
P	0.315	0.101	.$^{/}$	0.936	0.910	0.617	0.958	0.585	0.826	-0.517	0.912
S	0.091	-0.127	.$^{/}$	0.991	0.980	0.779	0.998^{a}	0.386	0.676	-0.309	0.795
Zn	-0.676	-0.500	.$^{/}$	-0.710	-0.660	-0.240	-0.756	-0.866	-0.984	0.822	-1.000^{b}

[a, b] - Correlations are significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. [c]- Can not be computed as one of the variables is constant.

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Table 4. Pearson's correlations of elements between *T. brumale* and soil

Tuber brumale	**Soil**										
	Ca	**Cr**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**P**	**S**	**Zn**
Ca	0.964	0.883	./[c]	0.227	0.161	-0.312	0.293	0.999[a]	0.928	-0.999[a]	0.847
Cr	./[c]	./	./	./	./	./	./	./	./	./	./
Cu	./	./	./	./	./	./	./	./	./	./	./
Fe	0.846	0.711	./	0.499	0.438	-0.024	0.556	0.967	0.996	-0.943	0.965
K	0.548	0.354	./	0.814	0.773	0.394	0.852	0.774	0.943	-0.719	0.987
Mg	0.997[a]	0.990	./	-0.115	-0.182	-0.614	-0.047	0.929	0.747	-0.956	0.619
Mn	0.976	1.000[b]	./	-0.255	-0.321	-0.721	-0.189	0.866	0.645	-0.904	0.500
Na	0.954	0.866	./	0.262	0.196	-0.277	0.327	1.000[b]	0.941	-0.997	0.866
P	-0.931	-0.988	./	0.403	0.464	0.820	0.339	-0.778	-0.518	0.827	-0.359
S	0.975	0.904	./	0.182	0.115	-0.355	0.249	0.997	0.910	-1.000[b]	0.822
Zn	-0.397	-0.189	./	-0.901	-0.870	-0.545	-0.929	-0.655	-0.872	0.590	-0.945

[a, b] - Correlations are significant at $P≤0.05$ and $P≤0.01$, respectively. [c]- Can not be computed as one of the variables is constant.

Correlation matrix for elements in each *Tuber* specie, pointed out the differences between them; in *T. magnatum,* Ca and Fe were positively correlated ($P≤0.05$), whereas highly significant correlations were observed between Cr and Zn as well as Cu and Mn ($P≤0.01$) (Table S3). On the other hand, in *T. brumale* Ca was significantly positive correlated with both Na and S ($P≤0.05$) (Table S4). In the soil, positive correlations between Fe and both K and Mn were observed, significant at $P≤0.05$ (Table S5).

Principal component analysis (PCA)

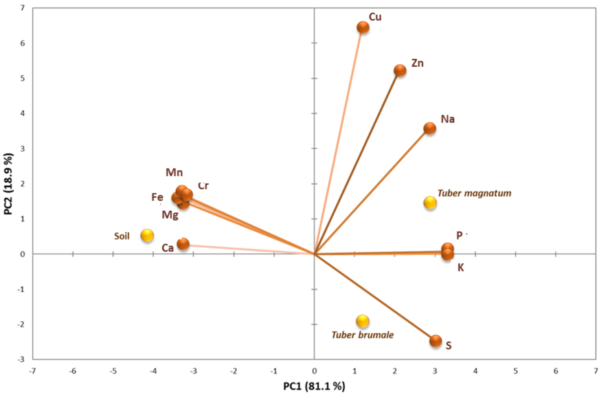

Figure 1. Biplot for elemental composition of *T. magnatum, T. brumale* and soil

By applying the PCA to the correlation matrix of data related to the elemental composition of *T. magnatum* and *T. brumale* ascocarps and soil, samples were clearly differentiated. The soil was separated from the truffle samples by the highest concentrations of Ca, Cr, Fe, Mg and Mn. The two species of ascocarps' positions within the biplot indicate lower concentration of these elements and higher concentrations of K, P and S. In addition, *T. magnatum* was distinguished from *T. brumale* and soil by the highest concentrations of Cu, Na and Zn, Figure 1. Regarding the correlation of certain elements, a positive correlation could be noticed between Ca, Cr, Fe, Mg and Mn, on one hand, and P, K and S on the other hand, while the elements from these two groups are negatively correlated with each other.

Discussion

In this contribution the ascocarps of two co-habiting truffle species from a specific habitat in Western Serbia (*T. magnatum* and *T. brumale*) were differentiated concerning total content of macro and micro nutrients that may be supplied to the host plant through ectomycorrhizal symbiosis, or used by fungi. These differences may imply differences in assimilation potentials of truffle mycelia. Additionally, distribution of the total content of such nutrients in soils appeared very different to those in ascocarps, implying strong active control of input or possible exclusion by the truffle mycelia.

Site description

Investigated sites and soil appeared very similar to previously reported descriptions for *T. magnatum* and *T. brumale* habitats - typically, available K was quite high, and available P very low (Table 2).[17, 8] The habitat is located in the final sedimentation zone of the river Sava, which explains high clay content, and where the chemical and physical characteristic of soil (Table 2) reflect the origin of the sediments from the weathering zone, which is in calcareous Slovenian Alps.[18,19] Indeed, it is expected that such soils will have highly effective adsorptive complexes and therefore high element content.[18] Concentrations of analysed elements in soil (Table 1) were in accordance with values measured for Cu, Cr and Zn in the same area where the truffle habitats are common.[19,5] Therefore, it may be assumed that the investigated truffle habitat is representative for Serbia concerning soil inorganic nutrient status.

Truffle ascocarps element content in comparison to soil

In habitats supporting *T. magnatum* in Serbia, this species is usually dominant in fructification and commonly accompanied by other truffle species, while sporocarps of epigeic ECM species are detected very rarely, especially in autumn/early winter when fructification of white truffle is the most abundant.[20] On the level of symbiotic root mycobiome, the situation is very different,[21] but when it comes to truffles, sporocarp productivity is of a major interest. Therefore, the ECM species that are co-currently producing sporocarps may be supposed as the major competitors for inorganic nutrients and in lowlands of Serbia, in autumn fructification season, *T. brumale* most commonly competes with *T. magnatum.*[5]

The mycelium of ECM fungi is the absorbing part for essential macro and microelements of the symbiotic relationship.[22] While mechanisms and pathways of symbiotic assimilation of N, P and water were systematically explored,[8,23,24] the data on assimilation of other macro- and microelements are scarce.[25] Mycelium of *T. magnatum* is very hard to obtain as there are no published results of experimental results, while *T. brumale* has rarely been investigated.[26] However, high economical value and small and highly jeopardized area of distribution raise the interest in *T. magnatum*, hence the importance of distinguishing its mycorrhizal potential in comparison to competitive species.

What differentiates most truffles from other ECM fungal species is the ability to form ECM in high soil content of total and available N and K, and very low availability of P, besides usually neutral pH in the soil.[8,17] Therefore, it is logical to hypothesize that not N, but P and maybe some other soil born elements may be the fungal subjects of exchange for ECM establishing. Inability to set up an experiment that could provide direct information on this topic induced the need to try elucidating easily measurable variables – ascocarp and soil element content. But such data are not common in available literature. In recent study of Orczán and co-workers, chemical contents of a cumulative sample of different *Tuber spp.* ascocarps were compared to the cumulative samples of other hypogeic genera, as well as with the data base for epigeic sporocarps.[27] Even though variations in measured values between samples were very high, concentrations of the majority of elements were detected on average lower or similar to those in epigeic sporocarps. Compared to data obtained in this study (Table 1), apart for K, the most abundant element in fungal sporocarps,[28] even the proportion of measured elements was very different, while individual elements could not be compared. Still, it is hard to draw any conclusions from the data on the cumulative genus samples, as every species has different physiological abilities and ecological demands, plus the information on soils from which the sporocarps originated was not provided. Kruzselyi and Vetter reported values much higher than data presented in this work (Table 1) (not providing soil data), but in similar proportion, for ascocarps of *T. aestivum.*[29] In the study of Odiss and co-workers,[30] ascocarp contents of potentially toxic trace elements in 6 truffle species were indeed compared to soil contents, but the values reported were to a great extent lower than any other data for fungi or soils that we managed to find in the literature, including our observations (Table 1).[28,31] The authors evaluated the possible hazard for human consumption and soil characterisation of the sampling points was not provided, this is why it was not possible to compare their findings with samples from the well characterized soils of Serbia (Table 1).[8] Obviously, literature data on chemical content of truffle ascocarps were not comparable, implying that different environmental conditions may influence the ascocarp chemical content. In general, the chemical composition of the plant/mycorrhizal fungi reflects the elemental composition of the soil which they inhabit , taking into account the close link between the soil and plants/mycorrhizal fungi, it is clear that various factors that influence the soil properties have an indirect effect on their composition as well.[32]

On the other hand, differences between element contents in two cohabiting truffle species from this study were highly statistically supported except for Mn and Cr (P≤0.01) (Tables 1 and S1). Obviously, truffle ascocarps expressed high ability for accumulation of some elements. As expected, both species accumulated K and P, [33] but also Na and S, while *T. magnatum* additionally accumulated Cu and Zn and BAF values for these elements were higher than 1 (Table 2). This implies that these elements were actively taken up from the environment where they were present in much smaller concentrations. The mechanisms of element immobilization by ECM fungi from the soil was reviewed in the literature.[34] One of the mechanisms was utilized by biosorptive processes due to the high percentage of cell-wall material that shows excellent metal-binding properties.[35] Apart from Ca that is known to be associated with cell walls,[36] this is hard to imagine for other biogenic elements as the actively mediated import/exclusion membrane bound systems must be the part of functional transportome of ECM fungi.[37] Indeed, metal transporters are the most populated class of metal homeostasis-related gene products in *T. melanosporum*, but the total number of predicted metal transporters in this fungus is significantly lower than in the non-mycorrhizal ascomycetes.[38] Therefore, it can be postulated that essential elements here studied that were accumulated in truffle ascocarps probably have functional roles and are not only accumulated in cell walls. Additionally, PCA analysis clearly differentiated two truffle species and elements that influenced this differentiation (Figure 1). Interestingly, K and P, elements that have been reported as objects of symbiotic assimilation, appeared equally important for both truffle species (Figure 1), but strongly negatively correlated with soil sample, implying that they cannot be factors of physiological differentiation between two truffle species, but rather factors of competition between them.[39,40] However, Cu, Zn and Na were strongly related with *T. magnatum*, while S was strongly related with *T. brumale*, and this is exactly where we may expect differences in symbiotic potential (Figure 1). Sulphur is not only the biological element with numerous biological roles, but also the common component of volatile substances that form the specific truffle aroma.[41] Sulphur related metabolism appeared to be strikingly active in ascocarps of *T. melanosporum*, especially the part related to production of volatiles.[42] The ascocarp odours of two species investigated here are markedly different, but from the data on total S content it is impossible to know where in ascocarps this S is incorporated.

Pearson's correlation coefficients highlighted highly supported ($P \leq 0.01$) negative correlation between soil and ascocarp contents of Zn for *T. magnatum* and S for *T. brumale*, and positive of Na for *T. brumale* ($P \leq 0.05$) (Tables 3 and 4). This was also the case of two elements that expressed no significant differences between ascocarp contents, Cr and Mn (Table S1), but appeared strongly connected to soil content, although in the opposite way; negative correlation for *T. magnatum*, and positive for *T. brumale* (Tables 3 and 4). Interestingly, both species expressed the same correlations between soil Na and S and ascocarp Ca contents; positive with Na and negative with S ($P \leq 0.05$, Tables 3 and 4). In *T. magnatum*/soil relationships, Cr was also negatively correlated to Cu and Zn ($P \leq 0.01$), implying that specific uptake/exclusion mechanisms could occur (Table 3). All this additionally suggested the differentiation in assimilation ability between two species.

During the vegetation time, transporting processes within ECM mycelia are more or less clear – sugars are taken in from the plant and transported towards mycelia periphery to support mycelial growth and intake of inorganic nutrients and water, while those nutrients are transported towards plant roots.[37] In the time of fructification, ECM mycelia experience redistribution of transporting process, as sporocarp appears as an additional sink for both sugars and inorganic nutrients.[42] Intensive biomass production that is obvious for *T. magnatum* (it can produce ascocarps in large amounts and more than 1kg weight),[8] certainly demands abundant sources of sugars and nitrogen for biosynthetic processes, K for generating osmotic pressure that will enable mechanical ascocarp enlargement in narrow soil environment and macro/microelements to support enzymes function.[36] But, for obtaining enough sugars from the plant, truffle mycelia must concurrently fulfil its requirements for inorganic nutrients as well. In the soil, there is plenty of N and K for both,[8] but P is hardly available (Table 2) and therefore in high demand. Strong accumulation of P in both species of ascocarps imply existence of very high affinity P transporters in mycelia as reported previously for ECM fungi,[37] but 30% surplus in *T. magnatum* ascocarps may imply competitive advantage (Table 1). Additionally, it appears that this species has also strong affinity transporters for some trace elements (Table 1, Figure 1). It was observed that the mycelia of *T. melanosporum* and *Tuber brumale* rapidly accumulated copper ions.[26] Indeed, ZIP proteins (key uptake transporters in eukaryotic cells) were marked as the most populated family of *Tuber* metal transportome. A rather promiscuous transporter capable of mobilizing various essential metals (e.g., Cu and Zn) was detected in the genome of *T. melanosporum.*[38] Also, a homolog of yeast transcription factor Zap1, involved in the regulation of numerous metal transporters, which plays a direct role in controlling Zn-responsive gene expression, was recently identified in *T. melanosporum.*[43] Similar mechanisms may be present in *T. magnatum* as well, but much more sophisticated methods must be applied for their detection, which is becoming feasible after very recent publication of genome of this species.[44] This resource will enable strong development of knowledge on *T. magnatum* metabolic specificities that could not be so far elucidated.

Conclusions

In this contribution the clear differences between two cohabiting truffle species were detected for the majority of investigated ascocarp elements (Ca Co, Cu Fe, K, Mg, Na, P, S and Zn, apart from Mn and Cr). As expected, the most abundant element in both ascocarps was K, followed by P, S and Ca while others were detected in substantially lower concentrations. In comparison to soil, concentrations of some elements were higher, implying active uptake and accumulation by the truffle mycelia. *T. magnatum* appeared more competitive, being able to more efficiently assimilate/accumulate Cu, K, Na, P, and Zn, while *T. brumale* was more successful in accumulating/assimilating S. Additionally, Ca, Cr, Fe, Mg and Mn were actively excluded by the mycelia of both truffle species (*T. brumale* contained more Ca and Mg, while *T. magnatum* contained more Fe), being detected in lower concentrations in ascocarps then in soil samples. The obtained data gave an insight into the differences between possible symbiotic assimilation of two cohabiting and co-fructifying species in a specific ecosystem in Serbia. On the other hand, the present study provided the directions for further investigations that are certainly necessary for the real comparison and elucidation of both truffle mycelia potentials, while the enlargement of the sampling area on ecologically different habitats and involving of the other cohabiting species in the analyses would be the first steps.

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Experimental Section

Study area and sample collection

Ascocarps of *Tuber magnatum* and *Tuber brumale* were collected in late autumn 2018, in the area of the southern Srem, Republic of Serbia, by using a trained dog. The sampling area was located nearly 1.5 km from the river Sava in the vicinity of the town Šabac, covered by the natural riparian forests dominated by *Quercus robur, Carpunus betulus* and occasionally *Populus alba*. The majority of soils from the area belong to Fluvisols and Gleysols (hydromorphic soils), the common soil types in Serbian province Vojvodina, which are affected by both ground and surface water.[45] Truffle samples were determined by Dr Žaklina Marjanović, and voucher specimens were deposited in the Institute for Multidisciplinary Research, Belgrade University (IMSI-SFH 75, IMSI-SFH 76). Soil samples, at depth 0–30 cm, were collected from several sites (in total ~5 kg), and then taken to a chemistry laboratory where the representative sample was made using a random square method.[46] Fresh truffles and soil were analysed for the content of essential elements (K, Na, Ca, Mg, Fe, P, S and Zn), and trace essential elements (Co, Cr, Cu, Mn and Se), by means of inductively coupled plasma-optical emission spectroscopy (ICP–OES) analysis.

ICP-OES analysis

The truffle and soil samples digestion

Wet digestion with acids is the most commonly applied technique for the decomposition of organic substances, in particular food. Truffle ascocarp samples were prepared for the ICP analysis by the modified literature procedure.[47] Fresh ascocarps were first cleaned from the ground, washed with deionized water and dried in the air. For the preparation of representative samples, 8-10 fruiting bodies (50-70 grams of truffles) were used. The samples were ground in a porcelain mortar using a pestle. Around 0.5 g of the representative samples of each truffle species was measured on the analytical scale and placed into 25 ml glasses. Nitric acid (7 ml) and hydrogen-peroxide (1 ml) were added, and mixtures were heated on the water bath for 6 hours, at constant temperature (80 ^{o}C). After the digestion of samples was completed, mixtures were transferred into volumetric flasks and diluted with deionized water. The same procedure was applied for a blank. Prior to ICP-OES analysis sample solutions were filtered through a quantitative paper (pore size 2-4 μm) into stand-alone cuvettes.

Prior to analysis, the soil samples were air-dried, sieved through stainless sieve and ground to a fine powder with a pestle in an agate mortar. Wet digestion was performed by modified procedure described in our previous work.[48] Briefly, in ~1 g of the representative sample conc. nitric acid (15 ml) and hydrochloric acid (5 ml) were added. The mixture was heated on the water bath for 6 hours, at constant temperature (80 ^{o}C). After cooling, the sample was transferred into volumetric flask, diluted with deionized water, and filtered through a quantitative paper (pore size 2-4 μm) prior to ICP-OES analysis.

Samples were analysed for the content of thirteen elements by means of inductively coupled plasma–optical emission spectrometry (SPECTROMETAR ICP-OES SPECTRO BLUE TI - SPECTRO Analytical Instruments GmbH, Germany) using US EPA Method 200.7.[49] Data of all measurements (acquired in triplicate) are expressed as the mean value with standard deviation. The method performance data are given in Table S6.

Bioaccumulation factor (BAF)

Bioaccumulation factor (BAF) of each element in truffles, as the ratio between its content in the fruiting body and the soil was calculated as reported in our previous work,[47] using the formula: BAF=Ct/Cs;
where Ct represents a concentration of the essential or trace element in the truffle, while Cs stands for the concentration of the same element in the soil.

Statistical analysis and Principal Component Analysis (PCA)

Data were analysed using statistical package IBM SPSS Statistics 20.0 (IBM Inc., New York, USA). Indicators of descriptive statistics (mean and standard deviation) were calculated for all the observed elements. Element concentrations between the investigated truffle species were compared using the t-test (significant at the level of $P \leq 0.05$ and $P \leq 0.01$). Pearson's correlation coefficients (significant at the level of $P \leq 0.05$ and $P \leq 0.01$) between the concentrations of elements in truffles and soil were also analysed.
For visualizing the correlations between observed parameters (elements) and examining similarities of individual samples principal component analysis (PCA) was used. This visualization has been achieved by the biplot construction.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

Jelena Popović–Djordjević designed the study, supervised chemical experiments, analysed the data, and wrote the manuscript; Žaklina Marjanović was involved in the biological aspect of the study and wrote the manuscript draft; Jelena Bogosavljević performed soil analyses; Tamara Adžić performed the preparation of samples for ICP analysis; Nemanja Gršić collected the truffle and soil samples; Blaženka Popović performed statistical analysis; Ilija Brčeski performed ICP analysis. Authors declare no conflict of interest.

References

[1] S. E. Smith, D. J. Read, 'Mycorrhizal Symbiosis', Academic Press, San Diego, USA, 1997.

[2] J. Falandysz, J. Borovička, 'Macro and trace mineral constituents and radionuclides in mushrooms: health benefits and risks', *Appl. Microbiol. Biot*. **2013**, *97*, 477–501.

[3] R. Splivallo, S. Ottonello, A. Mello, P. Karlovsky, 'Truffle volatiles: from chemical ecology to aroma biosynthesis', *New Phytol*. **2011**, *189*, 688–699.

[4] R. Mandić, M. Adžemović, Ž. Marjanović, 'Conservation and trade with wild edible mushrooms of Serbia – history, state of art and perspectives', *Nat. Conserv*. **2018**, *25*, 31-53.

[5] Ž. Marjanović, T. Grebenc, M. Marković, A. Glišić, M. Milenković, 'Ecological specificities and molecular diversity of truffles (genus *Tuber*) originating from mid-west of the Balkan Peninsula', *Sydowia* **2010**, 62, 67–87.

[6] I. Valverde-Asenjo, G. García-Montero, A. Quintana, J. Velázquez, 'Calcareous amendments to soils to eradicate *Tuber brumale* from *T. melanosporum* cultivations: a multivariate statistical approach', *Mycorrhiza* **2009**, *19*, 159–165.

[7] F. Ori, P. Leonardi, E. Stagnini, V. Balestrini, M. Iotti, A. Zambonelli, 'Is *Tuber brumale* a threat to *T. melanosporum* and *T. aestivum* plantations?', IFOREST. *11*, **2018**, 775–780.

[8] Ž. Marjanović, A. Glišić, D.Mutavdžić, E.Saljnikov, G.Bragato, 'Ecosystems supporting *Tuber magnatum* Pico production in Serbia experience specific soil environment seasonality that may facilitate truffle lifecycle completion', *Appl. Soil Ecol*. **2015**, 95, 179–190.

[9] Z. Merényi, T.Varga, Z.Bratek, in 'True Truffle (Tuber spp.) in the World', Eds. A. Zambonelli, M. Iotti**, C.** Murat, Springer, Cham, 2016, p. 49.

[10] B. Granetti, A. Angelis, G. Materozzi, 'Umbria terra di tartufi, Gruppo Micologico Ternano, Terni, Composizione chimica e valore nutritivo del tartufo', **2005**, 76–78.

[11] S. Wang, M. Marcone, 'The biochemistry and biological properties of the world's most expensive underground edible mushroom: Truffles', *Food Res. Int.* **2011**, 44, 2567–2581.

[12] I.N. Beara, M.M. Lesjak, D.D. Četojević–Simin, Ž.S. Marjanović, J. D. Ristić, Z.O. Mrkonjić, N.M. Mimica–Dukić, 'Phenolic profile, antioxidant, anti–inflammatory and cytotoxic activities of black *(Tuber aestivum* Vittad.) and white (*Tuber magnatum* Pico) truffles', *Food Chem.* **2014**, *165*, 460–466.

[13] S. Patel, A. Rauf, H. Khan, S. Khalid, M. Mubarak, 'Potential health benefits of natural products derived from truffles- a review' *Trends in Food Science & Technolog.* **2017**, *70*, 1–8.

[14] M. Giaccio, F. Di Giacomo, R. Marchegiani, 'Chromium, manganese, nickel, copper, zinc, cadmium and lead content in some species off truffles', Micol. Vegetazione Mediterr**.** VII (2), **1992**, 287–294.

[15] R. Splivallo, N. Valdez, N. Kirchhoff, M.C. Ona, J.P. Schmidt, I. Feussner, P. Karlovsky, 'Intraspecific genotypic variability determines concentrations of key truffle volatiles', *New Phytol.* **2012**, *194*, 823–835.

[16] R. Splivallo, L. Culleré, in 'True Truffle (Tuber spp.) in the World', Eds. A. Zambonelli, M. Iotti, C. Murat, Springer, Cham, 2016, p. 393.

[17] Ž. Marjanović, E. Saljnikov, M. Milenković, T. Grebenc, 'Ecological features of *Tuber magnatum* pico in the conditions of West Balkans – soil characterization'. Proceedings of 3rd International Congress on Truffles, 25-28 November 2008, Spoleto, Italy, **2010**, 190–195.

[18] A. Schnitzler, 'River dynamics as a forest process, interaction between fluvial systems and alluvial forests in large European river plains', *Bot. Rev.* **1997**, *63,* 40–64.

[19] P. Pavlović, M. Marković, O. Kostić, S. Sakan, D. Đorđević, V. Perović, D. Pavlović, M. Pavlović, D. Čakmak, S. Jarić, M. Paunović, 'Evaluation of potentially toxic element contamination in the riparian zone of the River Sava', *Catena* **2019**, *174*, 399–412.

[20] Ž. Marjanović (University of Belgrade, Institute for Multidisciplinary Research), 'personal communication'.

[21] M. Leonardi, M. Iotti, M. Oddis, G. Lalli, G. Pacioni, P. Leonardi, A. Zambonelli, 'Assessment of ectomycorrhizal fungal communities in the natural habitats of *Tuber magnatum* (Ascomycota, Pezizales)', *Mycorrhiza* **2013**, *23*, 349–358.

[22] G.D. Bending, D.J. Read, 'The structure and function of the vegetative mycelium of ectomycorrhizal plants. VI. Activities of nutrient mobilizing enzymes in birch litter colonized by Paxillus involutus (Fr.) Fr', *New Phyt*ol. **1995**, *130*, 411–417.

[23] H. Bücking, 'Phosphate absorption and efflux of three ectomycorrhizal fungi as affected by external phosphate, cation and carbohydrate concentrations', *Mycol. Res.* **2004**, 108, 599–609.

[24] M. Chalot, C. Plassard, in 'Ecological aspects of nitrogen metabolism in plants', Eds. J.C. Polacco, C.D. Todd, John Wiley & Sons, Inc. 2011, p. 69.

[25] S.E. Smith, D.J. Read, 'Mycorrhizal symbiosis', Elsevier, Academic Press, New York, 2008.

[26] N. Poitou, J. M. Olivier, 'Effect of copper on mycelium of three edible ectomycorrhizal fungi', *Agric. Ecosyst. Environ*. **1990**, *28,* 403–408.

[27] Á.K. Orczán, J. Vetter, Z. Merényi, É. Bonifert, Z. Bratek, 'Mineral composition of hypogeous fungi in Hungary', *J. Appl. Bot. Food Qual.* **2012**, 85, 100–104.

[28] P. Kalač, 'Chemical composition and nutritional value of European species of wild growing mushrooms: A review', *Food Chem.* **2009**, *113,* 9–16.

[29] J. Vetter, D. Kruzselyi, 'Complex chemical evaluation of the summer truffle (*Tuber aestivum* Vittadini) fruit bodies' *J. Appl. Bot. Food Qual.* **2014**, *87*, 291–295.

[30] M. Oddis, R. Cialfi, A. Iannarelli, M. Leonardi, G. Pacioni, 'Bioaccumulo di metalli pesanti nei tartufi con caratterizzazione qualitativa del prodotto Abruzzese', *Micol. e Veget. Medit.* **2009**, *25*, 33–46.

[31] R.M. Cenci, F. Sena, L. Cocchi, 'Elementi chimici nei funghi superiori', *European Commission Joint Research Centre Scientific and Technical Reports, European Union*. **2010**.

[32] A. Kabata-Pendias, 'Trace elements in soils and plants', CRC Press, Taylor and Francis Group, LLC, Boca Raton, 2011, p. 78.

[33] R. Saltarelli, P. Ceccaroli, P. Cesari, E. Barbieri, V.Stocchi, 'Effect of storage on biochemical and microbiological parameters of edible truffle species ', *Food Chem*. *109*(1), **2008,** 8–16.

[34] S. Zeppa, A. Amicucci, R. Saltarelli, G. Giomaro, V. Stocchi, 'In Edible Ectomycorrhizal Mushrooms', Eds. A. Zambonelli, G.M. Bonito, Springer-Verlag, Berlin, Heidelberg, 2012, p. 41.

[35] S. Zafar, F. Aqil, I. Ahmad,'Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil', *Bioresour. Technol*. **2007**, *98,* 2557–2561.

[36] F. J. Maathuis, 'Physiological functions of mineral macronutrients', *Curr.Opin. Plant Biol*. **2009**, *12,* 250–258.

[37] L. Casieri, N.A. Lahmidi, J. Doidy, C. Veneault-Fourrey, A. Migeon, L. Bonneau, P. E. Courty, K. Garcia, , M. Charbonnier, A. Delteil, A. Brun, 'Biotrophic transportome in mutualistic plant–fungal interactions', *Mycorrhiza*, **2013**, *23,* 597–625.

[38] A. Bolchi, R. Ruotolo, G. Marchini, E. Vurro, L. S. di Toppi, A. Kohler, S. Ottonello, 'Genome-wide inventory of metal homeostasis-related gene products including a functional phytochelatin synthase in the hypogeous mycorrhizal fungus *Tuber melanosporum*', *Fungal Genet. Biol*.**,** *48,* 573–584.

[39] C. Plassard, B. Dell, 'Phosphorus nutrition of mycorrhizal trees', *Tree Physiol*. **2010**, *30,* 1129–1139.

[40] B. Benito, M.González-Guerrero, 'Unravelling potassium nutrition in ectomycorrhizal associations', *New Phytol.* **2014**, *201,* 707–709.

[41] F. Pelusio, T. Nilsson, L. Montanarella, R. Tilio, B. Larsen, S. Facchetti, J.Madsen, 'Headspace solid-phase microextraction analysis of volatile organic sulfur compounds in black and white truffle aroma', *J. Agr. Food Chem*. **1995**, *43,* 2138–2143.

[42] F. Martin, A. Kohler, C. Murat, R. Balestrini, P. M. Coutinho, O. Jaillon, B. Porcel, 'Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis', *Nature*, **2010**, *464,*1033 –1038.

[43] B. Montanini, E. Levati, A. Bolchi, A. Kohler, E. Morin, E.Tisserant, S.Ottonello, 'Genome-wide search and functional identification of transcription factors in the mycorrhizal fungus Tuber melanosporum', *New Phytol.* **2011**, *189,* 736–750.

[44] C. Murat, T. Payen, B. Noel, A. Kuo, E. Morin, J. Chen, A. Kohler, K. Krizsán, R. Balestrini, C. Da Silva, B. Montanini, 'Pezizomycetes genomes reveal the molecular basis of ectomycorrhizal truffle lifestyle', *Nat. Eecol. Evol.* **2018,** *2*, 1956–1965.

[45] V.I. Ćirić, N. Drešković, D.T. Mihailović, G. Mimić, I. Arsenić, V. Đurđević, 'Which is the response of soils in the Vojvodina Region (Serbia) to climate change using regional climate simulations under the SRES-A1B?', *Catena,* **2017**, *158*,171–183.

[46] R. Korunović, S. Stojanović, 'Praktikum iz pedologije, Poljoprivredni fakultet, Beograd', **1989**. (*in Serbian*)

[47] A. Duran, M. Tuzen, M. Soylak, 'Trace element levels in some dried fruit samples from Turkey', *Int. J. Food Sci. Nutr.* **2008**, *59*, 581–589.

[48] B. Stanimirović, J. Popović-Djordjević, B. Pejin, R. Maletić, D. Vujović, P. Raičević, Ž. Tešić, 'Impact of clonal selections on Cabernet Franc grape and wine elemental profiles', *Sci. Hort.* **2018**, *237*, 74–80.

[49] J. Popović–Dordevic, N. Bokan, A. Dramicanin, I. Brceski, A. Kostic, 'Content and weekly intake of essential and toxic elements in Serbian vegetables', *JEPE*. **2017**, *18*, 889–898.

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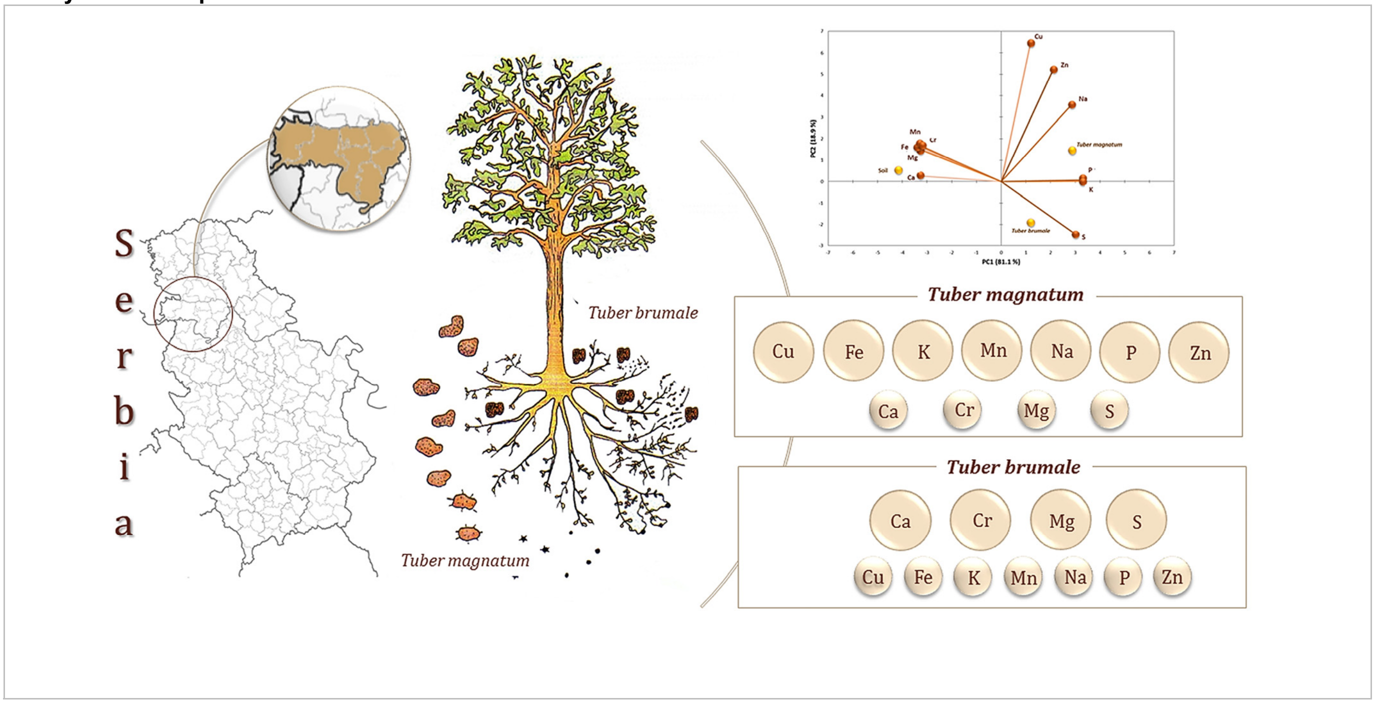
Entry for the Graphical Illustration



Twitter Text

Study presents the differences in symbiotic assimilation and accumulation of essential and essential trace elements in *Tuber magnatum* Pico and *Tuber brumale* Vitt. cohabitating in a specific ecosystem in Western Serbia.