Biological activities of methanolic extract from *Tuber aestivum*, *T. borchii*, and *T. brumale* f. *moschatum*

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Abstract: The potential allelochemical, antimicrobial and antioxidant activities of MeOH extracts of *Tuber borchii*, *T. aestivum*, and *T. brumale* f. *moschatum* were investigated. The allelopathic effect of the truffle extracts was evaluated using a Petri dish assay. Twenty seeds of each plant species (*Hieracium pilosella*, *Lotus corniculatus*, *Melica ciliata*, *Silene vulgaris*) were sown on filter paper in a Petri dish with 7.5 ml of the test solution of the MeOH extracts. The antibacterial activity of the truffle MeOH extract against *Streptomyces* species was determined by broth microdilution method. The MeOH extracts of truffle had a dose-dependent inibitory effect on seedling height and root length and showed significant antibacterial activity against *Streptomyces* species. The antioxidant activity of the methanolic extracts was tested with the DPPH test. The results showed that between 38.7 and 19.2 mg were necessary to reduce 50% of the DPPH coloration. There was no linear correlation between antioxidant activities and the polyphenol content.

Zusammenfassung: Potentielle allelochemische, antimikrobielle und antioxidative Wirkungen der Methanolextrakte von *Tuber borchii*, *T. aestivum* und *T. brumale* f. *moschatum* wurden untersucht. Der allelopathische Effekt der Trüffelextrakte wurde in Petrischalen geprüft. 20 Samen jeder Pflanzenart (*Hieracium pilosella*, *Lotus corniculatus*, *Melica ciliata*, *Silene vulgaris*) wurden auf Filterpapier in einer Petrischale mit 7,5 ml Testlösung der Methanolextrakte gesät. Die antibakterielle Wirkung des MeOH-Trüffelextraktes gegen *Streptomyces*-Arten wurde mit Verdünnungsreihen (broth microdilution method) bestimmt. Die Trüffelmethanolextrakte haben eine dosisabhängige Wirkung auf Höhe und Wurzellänge der Sämlinge und zeigten signifikante antibakterielle Wirkung gegen *Streptomyces*-Arten. Die antioxidative Wirkung der Methanolextrakte wurde mit dem DPPH-Test geprüft. Es waren 38,7-19,2 mg für eine Reduktion der DPPH-Färbung um 50% nötig. Es gab keine lineare Korrelation zwischen der antioxidativen Wirkung und dem Polyphenolgehalt.

Resumée : Les possible activitées allelochimiques, antimicrobiques et antioxidantes ont été étudiées par les extracts en MeOH de *Tuber borchii*, *T. aestivum* et *T. brumale* f. *moschatum*. Les effects allelopathiques des extracts de la truffe ont été évalué en utilisant des essays en boîte de Petri. 20 semence de chaque éspece de plants (*Hieracium pilosella*, *Lotus corniculatus*, *Melica ciliata*, *Silene vulgaris*) ont été

ensemencées sur le papier de filtrage dans un boîte de Petri avec 7,5 ml de la solution contenant de l'extract méthanolique. L'activité antimicrobique de l'extract méthanolique de la truffe vers *Streptomyces* spp. a été déterminé avec la méthode de microdilution en culture liquide. Les extracts méthanoliques de la truffe ont montré un effect d'inhibition, en dependant de la concentration, vers l'hauteur des semis et la longuer de leurs racines et ont montré une rémarquable activité antimicrobique vers *Streptomyces* spp. L'activité antioxydante des extracts méthanoliques a été essayée avec le text DPPH. Les résultats ont montré que entre 38,7 et 19,2 mg il était nécessaire de réduire du 50% de la coloration DPPH. Il n'y avait pas une correlation lineaire entre les activité antioxydantes et le contenu de polyphenol.

Secondary metabolites produced by fungi are one of the largest classes of natural products. Such compounds may not play a fundamental biochemical role in the normal growth or development of fungal cells, but they can clearly play an ecological role (EFREMENKOVA & al. 2003). Secondary metabolism is important in many fungal processes, including stimulation or inhibition of organisms by allelochemicals, regulation of symbiotic and protective interactions with microbes and other interacting root. Furthermore, they represent these natural products that are a new potential source of antifungal, antibacterial and other curative activities (JANG & AN 2000, SPLIVALLO 2008, FOX & HOWLETT 2008). *Tuber* spp. are ascomycete fungi belonging to *Pezizales* living in symbiosis with host plant roots in order to accomplish their life cycle (TRAPPE 1979). Truffle species have common ecological features, such as a wide range of host plant species and the need for calcareous soil (except *T. borchii* and *T. aestivum* which tolerate slightly acidic soils). The natural geographic distribution of known truffle species (about 100) mainly covers the temperate zones of the northern hemisphere, with at least three differentiation areas: Europe (CHEVALIER 2008), Soustheast Asia (YUN 2008) and North America (LEFEVRE 2008). The culinary and commercial value of such edible fungi is mainly due to their particular aroma, resulting from a blend of hundreds of volatile compounds (GIOACCHINI & al. 2005). The great demand for black and white truffles, the increasing interest in other local species with respect to the rural economy (e.g., *Tuber borchii*, *T. aestivum*, and *T. brumale* f. *moschatum*) and the decline in productivity, have stimulated research to better understand the ecology of truffles by exploiting the effects of allelopathic interactions of truffle metabolites with plants and microrganisms. The allelopathic effect of volatile substances released by truffles on *Arabidopsis thaliana* were investigated by SPLIVALLO & al. (2007). PACIONI (1991) reported that several species of *Tuber* produce a burned area around their symbiotic plants as a result of a phytotoxic action of truffle volatile compounds. TIRILLINI & GRANETTI (1995) and TIRILLINI & STOPPINI (1996) reported the presence of phenolic derivatives in methanolic (MeOH) extracts of truffle.

The aim of this research was to study the allelopathic, antibacterial and antioxidant activities induced by methanolic extracts of *Tuber borchii* VITTAD., *T. aestivum* VITTAD. and *T. brumale* VITTAD. f. *moschatum* (FERRY) CERUTI, truffle species that are found throughout Europe (RIOUSSET & al. 2001).

Material and methods

Plant material. *Lotus corniculatus* L. and *Silene vulgaris* (MOENCH) GARKE seeds and *Melica ciliata* L. and *Hieracium pilosella* L. fruits were collected from truffles in Umbria (Italy). Voucher specimens were identified (PIGNATTI 1982) and deposited in the Herbarium Laboratory of the Department of Applied Biology (University of Perugia, Italy).

Carpophores. Carpophores from *Tuber borchii*, *T. aestivum*, and *T. brumale* f. *moschatum* were purchased from the company "Tartufi di Paolo" of Spello (Perugia, Italy). Voucher specimens were identified morphologically (GRANETTI & al. 2005) and deposited.

Table 1. Activity of methanolic extract of *Tuber* spp. on germination of plant seeds 5 d after sowing. Data in the row followed by different letters are significantly different ($p \leq 0.05$). The values are the means of three repetitions ± standard error.

Plant species	% of germination			
		Tuber aestivum		
	10 mg /ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	0 a	58.3±0.5 b
Lotus corniculatus	0 a	0 a	16.6±0.3 b	63.3±0.5 c
Melica ciliata	0 a	0 a	21.6±0.5 b	81.6±0.5 c
Silene vulgaris	0 a	8.3±0.7 b	33.3±0.7 b	70.0±1.1 d
		Tuber borchii		
	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	10.0±1.1 b	53.3±1.1 c
Lotus corniculatus	0 a	0 a	28.3±1.7 b	66.6±0.6 c
Melica ciliata	0 a	0 a	8.3±0.5 b	76.6±3.4 c
Silene vulgaris	0 a	0 a	16.6±0.5 b	78.3±1.7 c
		Tuber brumale f. *moschatum*		
	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	18.3±0.7 b	60.0±0.5c
Lotus corniculatus	0 a	0 a	5.0±0.2 b	75.0±2.8 c
Melica ciliata	0 a	0 a	48.3±1.1 b	78.3±1.7 c
Silene vulgaris	0 a	6.6±0.3 b	23.3±1.7 c	73.3±1.7 d

Microorganisms. The following strains of bacteria were used: *Streptomyces griseus* subsp. *griseus* (DSMZ 40236, Deutsche Sammlung von Mikroorganismen und Zellkulturen), *S. anulatus* (DSMZ 40361, Deutsche Sammlung von Mikroorganismen und Zellkulturen), *S. albus* subsp. *albus* (DSMZ 41209, Deutsche Sammlung von Mikroorganismen und Zellkulturen), *S. parvus* (DBVPG 8605, Industrial Yeast Collection DBVPG), *S. prasineus* (DBVPG 8609, Industrial Yeast Collection DBVPG) and *S. virginiae* (DBVPG 8615, Industrial Yeast Collection DBVPG). Quality control testing was performed according to NCCLS (2003) guidelines using *Staphylococcus aureus* ATCC (American Type Culture Collection, Manassas, Va., USA) 29213.

Bioassays. A bioassay based on seed germination and subsequent radicle and seedling growth was used to study the allelopathic effects of the methanolic extracts of *Tuber* spp. Seeds and fruits were surface-sterilized with 1% NaClO for 30 min and sown in Petri dishes (∅ = 9 mm), containing five layers of Whatman filter paper, impregnated with 7.5 ml of distilled water (control) or 7.5 ml of the test solution of the methanolic extract of *Tuber* spp. at known concentrations (10.5 and 2.5 mg/ml). Methanolic extracts that have a low solubility in water, were dissolved in a water-acetone mixture (7 : 0.5). Controls performed with this mixture alone showed no appreciable differences in comparison with the controls in water alone. The Petri dishes were incubated at 25 °C in a greenhouse with 10 h of artificial light (250 µmol photons m^{-2} s^{-1}) daily. The seed germination process was observed directly in the Petri dishes, every 8 h, using a stereomicroscope. A seed was considered germinated when the protusion of the radicle became evident (BEWLEY & BLACK 1985). The root length and seedling height of

the tested plant species were measured after 7 and 14 d. Each determination was repeated three times.

In statistical analysis the relative standard error was determined. Analyses of variance (Anova), followed by LSD post hoc determinations, were performed. All computations were done using the statistical software SuperAnova for Mac Plus (1989-90, Abacus Concepts, Inc).

Determination of total phenolic compounds. The total soluble phenolic content in the mushroom methanolic extracts was determined with Folin-Ciocalteu reagent according to the method of SINGLETON & al. (1971) using gallic acid as a standard. One ml of extract solution (containing 2000µg/ml) in a volumetric flask was diluted with glass-distilled water (46 ml). Folin-Ciocalteu reagent (1 ml) was added and the contents of the flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2%) were added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the mushroom methanolic extracts were quantified by calibration curve obtained by measuring the absorbance of known concentrations of gallic acid standard (25 to 150 µg/ml of 50% methanol). The concentrations are expressed as mg of gallic acid equivalents per 100 g of dry weight. The total phenolic assay of mushrooms was measured three times for each *Tuber* species.

DPPH assay. The scavenging free radical potentials were tested in a methanolic solution of 1.1-diphenyl-2-picrylhydrazyl (DPPH) (HATANO & al. 1988). The degree of discoloration of the solution indicates the scavenging efficiency of the added sample. For the methanolic extract, 100 ml were added to 0.9 ml of pure methanol and 4 ml of DPPH solution (final concentration of DPPH = 2.0×10^{-4} M). 30 min later, the absorbance was measured at 517 nm. A blank solution was prepared with 100 ml of methanol. The scavenging activity of the DPPH radical was expressed as EC50, which is the concentration of the test extract required to have 50% less in absorbance than that of the blank solution.

Susceptibility testing. Susceptibility testing was performed by the broth microdilution method in cation-adjusted Mueller-Hinton broth, as recommended by CLINICAL AND LABORATORY STANDARDS INSTITUTE/NCCLS (2003) guidelines. Prior to testing, the isolates and controls were inoculated on YMD (0.4% yeast extract, 5.0% malt extract, 0.44% dextrose, pH 7.4) agar plates and incubated at 35 °C to ensure optimal growth and purity (PRIDHAM & LYONS 1980). A small loopful of growth was then scraped off the medium and inoculated to 5 ml of Mueller-Hinton broth. The broth tubes were incubated at 35 °C in air and periodically vortexed at high speed for 2 to 3 min before preparing the standard inoculum in order to assist in achieving a homogenous suspension. When the optical density of broth reached 0.5 McFarland, it was used to prepare the inoculum and susceptibility results were assessed after 48 h of incubation.

Methanolic extract of *Tuber* spp. had a minimum inhibitory concentration (MIC) range of 0.625 to 10 mg/ml and sulfamethoxazole had a MIC range of 0.06 to 32 µg/ml. MIC end-points (mg/ml) were determined after 48 h of incubation in ambient air at 35 °C and were defined as the lowest concentration that prevents any visually discernible growth.

Growth (methanolic extract-free) and sterility controls were included for each isolate tested. These experiments were performed in triplicate. Quality control isolates were tested in the same manner.

Results and discussion

Allelopathic activity

Metabolites of many fungi may have adverse or stimulatory effects on plants (HEISEY & al. 1985, RICE 1995) such as suppression of seed germination, malformation and retardation of seedling growth (LYNCH & CLARK 1984, EATON & AYRES 2002). To evaluate possible allelopathic effects of the *Tuber* spp. methanolic extracts, extract concentrations from 2.5 to 10 mg/ml were used to test the in vitro effects on germination, root length and plant height. Table 1 shows the effects of *Tuber* spp. extract on the germination of tested plant seeds, in a dose-dependent manner, 5 d after the treatment. Germination was inhibited by 100% at the highest concentration tested (10 mg/ml) and by 30-40% at the lowest concentration (2.5 mg/ml). The extracts inhibited both root length

and seedling height of the tested plant species, in a dose-dependent manner. The test species showed different responses to methanolic extracts. Plant growth was less in *Hieracium pilosella*, *Silene vulgaris*, and *Lotus cornicolatus*, while the growth of *Melica ciliata* plant was stimulated with *T. borchii* extract (10 mg/ml) (Tables 2, 3). The extracts also induced leaching in the cotyledon leaves and tissue darkening in the roots.

Table 2. Activity of methanolic extract of *Tuber* spp. on root length, 14 d after sowing. Data in the row followed by different letters are significantly different ($p \leq 0.05$). The values are the means of three repetitions ± standard error.

	Root length			
			Tuber aestivum	
Plant species	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	0 a	0.4±0.1 b
Lotus corniculatus	0 a	0 a	0.3±0.1 b	0.6±0.2 c
Melica ciliata	0 a	0 a	0.7±0.3 b	1.2±0.1 c
Silene vulgaris	0 a	0 a	0 a	0.5±0.1 b
			Tuber borchii	
	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	0.2±0.1 b	0.4±0.1 c
Lotus corniculatus	0 a	0 a	0.5±0.1 b	0.6±0.2 b
Melica ciliata	0 a	0 a	1.2±0.1 b	1.2±0.1 b
Silene vulgaris	0 a	0 a	0 a	0.5±0.1 b
			Tuber brumale f. *moschatum*	
	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	0.2±0.1 b	0.4±0.1 c
Lotus corniculatus	0 a	0 a	0.6±0.3 b	0.6±0.2 b
Melica ciliata	0 a	0 a	0.6±0.2 b	1.2±0.1 c
Silene vulgaris	0 a	0.2±0.1 b	0.3±0.1 b	0.5±0.1 c

This preliminary in vitro study has demonstrated the allelopathic potency of the methanolic extract of *Tuber* spp. and has put forth new questions, do the methanolic extract and active volatile compounds (SPLIVALLO & al. 2007) exert an allelopathic synergistic action and what are the effects of methanolic extract and allelochemicals on the soil and the related minerals. Active methanolic-extract compounds of *Tuber* spp. are particularly interesting, because they can act as allelochemicals to inhibit some plant species or be a signal to specific microbes. Allelochemicals are an important potential source for alternative agrochemicals and pharmaceuticals that could be used to solve the many of the problems that have arisen due to inadequate cultivation practices and/or abuse of synthetic herbicides (MACIAS & al. 2003).

Although allelopathic effects of methanolic extract of *Tuber* spp. have been demonstrated in the laboratory, understanding the interactions between allelochemicals and soil systems is needed to assess *Tuber* spp.-plant dynamics. Once a chemical enters the environment, a number of interacting processes such as retention, transformation, and transfer take place. Retention involves a reduced of the movement of the chemical from one location to another through soil, water, and/or air. Transformation changes the form or structures of the chemical, leading to a partial change or total decomposition of the molecule. Transport is the movement of chemicals in the environ-

ment. These processes are influenced by the nature of the chemical, the species present, the soil properties and environmental conditions. These processes could explain why only in *T. brumale* and *T. aestivum*, the continuous release of phytotoxins and accumulation of these compounds in the soil suppresses the growth of understory or causes the death of seedlings around the host tree which results in the formation of the "burnt" area (ANGELINI & al. 2010, PACIONI 1991). Further clarification of the interactions between *Tuber* spp. allelochemicals and soil microorganisms offers many potential implications and applications in *Tuber* ecosystems.

Table 3. Activity of methanolic extract of *Tuber* spp. on hypocotyl length, 14 d after sowing. Data in the row followed by different letters are significantly different ($p \leq 0.05$). The values are the means of three repetitions ± standard error.

	Hypocotyl height			
		Tuber aestivum		
Plant species	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	0 a	1.2±0.3 b
Lotus corniculatus	0 a	0 a	0.7±0.1 a	5.1±0.3 b
Melica ciliata	0 a	0 a	0.4±0.1 b	3.7±0.3 c
Silene vulgaris	0 a	0.9±0.2 b	1.1±0.1 b	3.4±0.3 c
		Tuber borchii		
	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	0.6±0.1 b	1.2±0.3 c
Lotus corniculatus	0 a	0 a	2.4±0.5 b	5.1±0.3 c
Melica ciliata	0 a	0 a	4.5±0.4 b	3.7±0.3 b
Silene vulgaris	0 a	2.8±0.3 b	3.2±0.3 b	3.4±0.3 b
		Tuber brumale f. *moschatum*		
	10 mg/ml	5 mg/ml	2.5 mg/ml	control
Hieracium pilosella	0 a	0 a	0.4±0.1 b	1.2±0.3 c
Lotus corniculatus	0 a	0 a	2.4±0.5 b	5.1±0.3 c
Melica ciliata	0 a	0 a	2.4±0.7 b	3.7±0.3 c
Silene vulgaris	0 a	0.9±0.1 a	2.2±0.1 b	3.4±0.3 c

Antibacterial activities

The antibacterial effect of methanol extracts of *Tuber* spp. was tested against six strains of *Streptomyces* spp. The MIC values are reported in Table 4. The most susceptible bacterium was *S. albus* subsp. *albus* DSMZ 41209 (MIC 0.72 mg/ml); while *S. griseus* subsp. *griseus* DSMZ 40236 was the least sensitive to the extracts (MIC 32 mg/ml). Nevertheless, *T. aestivum* extract proved to be more active.

The MIC values of sulfamethoxazole for the strain *Staphylococcus aureus* (ATCC 29213) was within the established ranges (CLINICAL AND LABORATORY STANDARDS INSTITUTE/NCCLS 2003).

The antibacterial properties of *Tuber* spp. methanol extracts were not as effective as the commercial drug, but microrganisms become resistant to antibiotics overtime (NWOSU 2001). Researchers have reported the antimicrobial activity of several mushrooms (BARROS & al. 2007; DAMJAN & al. 2007 a, b; TAMBEKAR & al. 2006). The mechanism of antimicrobial activity is complex and could be attributed to a synergism between phenolic and other compounds. Polyphenols are a group of highly hydroxylated phenolic compounds present in the extractive fraction of several fungi (HATVANI 2001, HUR & al. 2004, ISHIKAWA & al. 2001, SHEENA & al. 2003). The microbicidal activities of these compounds against a huge number of pathogenic bacteria has been well documented (COWAN 1999, SCALBERT 1991).

Table 4. Minimum inhibitory concentration (MIC) of methanolic extract of *Tuber* spp. and commercial drug against *Streptomyces* spp. Values are the geometric mean of three repetitions.

Streptomyces spp.	MIC (mg/ml)			MIC (µg/ml)
	T. aestivum	*T. borchii*	*T. brumale* f. *moschatum*	Sulfamethoxazole
S. griseus subsp. *griseus* DSMZ 40236	3.96	5.00	6.29	32.00
S. anulatus DSMZ 40361	1.25	3.14	5.00	20.15
S. albus subsp. *albus* DSMZ 41209	0.72	2.50	3.14	10.07
S. parvus DBVPG 8605	1.98	3.96	6.29	25.39
S. prasineus DBVPG 8609	3.96	2.50	5.00	12.69
S. virginiae DBVPG 8615	3.14	1.91	3.14	16.00

Table 5. Percentage of polyphenols and antioxidant activity of methanolic extract of *Tuber* spp. The values are the means of three repetitions ± standard error.

Methanolic extract	% of polyphenols	DPPH EC50 (mg)
Tuber aestivum	15.71±2.34	38.73±2.69
Tuber borchii	17.61±2.45	19.16±0.66
Tuber brumale f. *moschatum*	23.19± 3.56	21.23±0.58

Polyphenol content

The phenolic content in the methanolic truffle extracts were 15.71, 17.6 and 23.19% respectively (Table 5). The concentrations are expressed as mg of gallic acid equivalents per 100 mg of dry weight. Phenolic compounds can be classified as simple phenols and phenolic acids (e.g., gallic, benzoic, syringic, chlorogenic) and polyphenols (e.g., flavonoids, tannins, stilbenes). Many studies have recently shown the presence of such compounds in medicinal mushrooms (KARAMAN & al. 2009).

Antioxidant activity of extracts

The methanolic extracts of *Tuber* spp. were screened for their possible antioxidant activity. DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of extracts. As antioxidants donate protons to these radicals, the absorbance decreases. The decrease in absorbance is used as a measure of the extent of radical scavenging. The free radical-scavenging capacities of the extracts, measured by DPPH assay, are shown in Fig. 5. The results showed that between 38.7 and 19.2 mg were necessary to reduce the DPPH coloration by 50%. Methanolic extracts of *T. borchii* exhibited a greater and more significant inhibitory activity against DPPH radical. These results suggest that the analysed fungi are potential sources of strong natural antioxidants that could be used in the food and nutrition industries. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (WAGNER & al. 1992). The phenolic compounds may contribute directly to antioxidative action (DUH & al. 1999), but, the scavenging activities on the DPPH radical obtained with *Tuber* spp. was very high if the low polyphenol content is considered. It is hypothesized that other classes of compounds could have a synergic action.

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