EFFECT OF NUTRIENT SOURCES AND PLANT HORMONES ON MYCELIAL MORPHOLOGY OF THE BLACK PERIGORD TRUFFLE *TUBER MELANOSPORUM*

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ABSTRACT

Physiological studies on strain Mel-28 of *Tuber melanosporum*, a highly prized edible ectomycorrhizal truffle species having a pungent and earthy fragrance, were undertaken to standardize its growth under *in vitro* conditions as it grows very slowly under cultural conditions. A total of ten media were evaluated, of which, minimal medium with slight modifications supported the best growth, followed by malt extract PVP medium. The media with pH 6.5 and 7.0 were optimal for the fungal growth. Preferred carbon and nitrogen sources were sucrose and organic nitrogen, followed by nitrates. Effect of the strigolactone analogue Gr-24 and auxin (Indole-3-acetic acid) was also studied on the growth, mycelial morphology and branching pattern of the fungus using confocal microscopy. Supplementation of the medium with Gr-24 resulted in highly branched mycelium, whereas elongation of hyphal tips was noticed in auxin-supplemented medium along with increased number of nuclei per cell. On the other hand, the radial growth of the fungus was lower in Gr-24, whereas it was higher in auxin-supplemented medium.

Keywords: Truffles; *Tuber melanosporum*; Growth conditions; Strigolactones; Auxin

INTRODUCTION

The true truffles are a group of several valuable and highly sought-after edible species of underground ascomycetes belonging to the genus Tuber [1]. All are ectomycorrhizal and are found in close association with tree roots. There are hundreds of truffles, and while none are known to be poisonous only a few of them are considered to be delicacies by humans. Among them, there are four commonly known edible taxa: the white autumn truffles (*Tuber magnatum*), the black winter truffles (*Tuber melanosporum*), the white spring truffles, and the black summer truffles (*Tuber aestivum/uncinatum*). The black winter truffles are also known as Perigord truffles and are the most expensive and sought together with *T.magnatum* among the truffle species. They can be described as "gourmet mushroom" and have a pungent, intense, earthy fragrance, which offers a unique flavor to food. *T. melanosporum* grows naturally as mycorrhized fungus on roots of oak (*Quercus* spp) and other trees in certain parts of France, Italy and Spain, and to a lesser extent in other countries [2, 3]. The annual harvest of black Perigord truffles from France, Italy and Spain combined is currently 50-80 tons [1].

The worldwide demand for this truffle has fuelled intense efforts for improving cultivation and production. Identification of processes that condition and trigger fruit body and symbiosis formation, ultimately leading to a more efficient production, will be facilitated by the knowledge of genomic traits and by a thorough analysis of the fungal physiology. While the first step has been reached with the sequencing of the truffle genome [4] data on the nutrient requirements in cultural conditions are still limited [5, 6, 7]. In the present study, the physiological requirements of *T. melanosporum* (strain Mel-28) were studied so as to standardize a medium for *in vitro* growth of the fungus for further studies. The effects of two growth promoters like auxins and strigolactone analogue Gr-24 on the growth and the branching pattern of the fungus were investigated by using confocal microscopy.

MATERIAL AND METHODS

Germplasm: Mel-28 strain of *Tuber melanosporum was* provided by INRA, Nancy [4] and the other strain Rey-t from Dipartimento di Biologia Vegetale dell'Università, Sezione di Torino, Torino.

Growth studies: For physiological studies, a basal medium is a prerequisite. For the identification of the black truffle basal medium, a total of ten media were tested (Table 1). Out of the ten media used a minimal medium with some modifications was selected for further studies (Tables 2, 3).

Table 1: Cultivation media used

Medium	Composition	Medium	Composition
HM medium	Standard composition	Malt extract PVP agar	Malt extract 10g; dextrose- 5g; PVP-1g;KH_2PO_4-1g; $MgSO_4.7H_2O$-0.5g
Hill & Kafer medium	Standard composition	MMN medium with mannose	Standard composition
Potato Dextrose Agar	Standard composition	MMN medium with Glucose	Glucose instead of mannose
Malt extract Agar	Malt extract 20g; dextrose-20g	MMN with malt extract	Malt extract instead of mannose
Potato dextrose malt agar	100g Potato extract; 10g malt extract	Modified Minimal medium	$Ca(NO_3)_2.4H_2O$ in place of $NaNO_3$; Gellan gum in place of agar

For identification of optimal pH the fungus was grown in a range of five pH values i.e. 6.0, 6.5, 7.0, 7.5 and 8.0. Radial growth of the fungal strains was recorded at a weekly interval up to four weeks to assess the growth rates and optimum pH for the fungus.

To identify the best carbon source used by *T. melanosporum*, four types of carbon sources i.e. three monosaccharides (Mannose, Glucose and Galactose) and the disaccharide maltose were used while sucrose was used as a control as the minimal medium contained sucrose.

Table 2: Minimal Medium composition (100 ml)

Sucrose	1.0g	Stock-4	0.5 ml
Stock-1	10 ml	Stock-5	0.5 ml
Stock-2	0.1 ml	Stock-6	0.1 ml
Stock-3	10 ml	pH	6.5
Gellan gum	0.4 g		

Table 3: Compostion of the Stock Solutions used in Minimal Medium

Stock-1 (100 ml)		**Stock-2 (25ml)**		**Stock-3 (100 ml)**	
$MgSO_4.7H_2O$	0.731 g	$MnCl_2.4H_2O$	0.15 g	$Ca(NO_3)_2.4H_2O$	0.288 g
KNO_3	0.080 g	$ZnSO_4.7H2O$	0.118 g	**Stock-4 (150 ml)**	
KCl	0.065 g	H_3BO_3	0.0375 g	Na.Fe.EDTA	0.24 g
KH_2PO_4	0.0048 g	$CuSO_4.5H_2O$	0.033 g	**Stock-5 (150 ml)**	
		$(NH_4)_6Mo_7O_{27}.4H_2O$	0.0058 g	Glycine	0.09 g
Stock-6 (20 ml)				Thiamine HCl	0.003 g
Potassium Iodide	0.015 g			Pyridoxal HCl	0.003 g
				Nicotinic acid	0.015 g
				Myo-Inositol	1.5 g

To determine the preferred nitrogen source five nitrogen sources were used i.e. two ammonium sources [$(NH_4)_2SO_4$, $(NH_4)_2H.PO_4$], two amino acid sources (Alanine, Asparagine) and one amide source (Urea) along with one nitrate control. The amount of carbon and nitrogen were equalized according to their weight present in the control medium. A total of 3.4 mg of nitrogen was supplemented through these sources replacing stock-3.

Effect of Indole-3-acetic acid and strigolactone analogue Gr-24: For the study, four concentrations of Gr-24 viz. 10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} Molar were used along with a negative control. The radial growth of *T. melanosporum* was measured placing the growing fungal hyphae in the centre and Gr-24 in a well at a distance of 1.7 cm from the growing mycelium. To see the effect of auxins on the truffle growth, Indole-3-acetic acid was used in three concentrations (0.136μM, 1.36 μM and 2.72 μM) along with one negative control. The medium used was the Minimal Medium. Solution of various concentrations of Indole-3-acetic acid was supplemented in the medium by sterilizing it through syringe filter of 0.22μ porosity. The growth of the fungus was recorded along two predefined axis on weekly basis up to 6 weeks. Each treatment was replicated five times.

Microscopic studies: The effect of the Gr-24 and Indole-3-acetic acid on branching pattern of the fungus was studied using confocal microscopy. For microscopic studies, the fungus was grown on a dialysis membrane treated with EDTA. The membrane was boiled with 1mM solution of EDTA for 30 minutes and then washed with distilled water several times. The membrane was sterilized and placed over the solid medium before fungal inoculation. After two weeks of growth the membrane was lifted and cut in small pieces using surgical blade under a stereo microscope and was left overnight for recovery from injury. Propidium iodide was used as a stain. The cut membrane containing the fungus was stained and viewed under a Leica confocal microscope (Laboratory of Advanced Microscopy, Department of Plant Biology, Torino).

Statistical Analysis: Statistical analysis was done using one-way analysis of variance (ANOVA) and critical difference (CD) was calculated by multiplying standard error with the value of two-tailed t-distribution on n-1 degree of freedom at 5%.

RESULTS AND DISCUSSION

A total of 10 media were tested for the growth of *T. melanosporum*. Only two media, Malt extract PVP agar and a slightly modified Minimal medium supported the fungal growth. Malt extract PVP agar supported the maximum growth with a growth rate of 8 mm per week. The results obtained are shown in Table 4.

The optimal pH was investigated keeping malt extract with PVP as the basal medium. The growth of the two strains of *T. melanosporum* (Rey-t and Mel-28) was recorded for their respective pH requirements. The growth patterns of the fungal strains were recorded at a weekly interval up to four weeks to assess the growth rates and optimum pH for the fungus (Table 5). The results indicated that the two strains differed in their pH requirements. The strain Rey-t grows optimally at the pH 6 whereas the strain Mel-28 prefers pH 6.5.

Table 4: Radial growth of *Tuber melanosporum* on different growth medium

Medium	Radial growth (mm)				
	2nd week	3rd week	4th week	5th week	6th week
HM medium	--	--	--	--	--
Kafer medium	--	--	--	--	--
Potato Dextrose Agar	--	--	9.00	11.00	12.00
Malt extract Agar	--	11.00	14.00	18.00	20.00
Potato dextrose malt agar	--	--	9.00	10.00	12.00
MMN medium with mannose	--	--	--	--	--
MMN medium with Glucose	--	--	--	--	--
MMN with malt extract	13.00	19.00	24.00	29.00	33.00
Malt extract PVP agar	14.00	20.00	28.00	34.00	40.00
Modified Minimal medium	15.00	22.00	31.00	39.00	47.00
SE					**1.16**
CD (0.05)	**--**	**--**	**--**	**--**	**2.46**

Table 5: Radial growth of *Tuber melanosporum* on different pH

pH	Radial growth (mm)									
	2nd week		3rd week		4th week		5th week		6th week	
	Mel-28	Rey-t	Mel-28	Rey-t	Mel-28	Rey-t	Mel-28	Rey-t	Mel-28	Rey-t
6	9	10	14	17	18	23	21	27	23	32
6.5	14	8	19	13	26	17	32	20	39	23
7	11	7	15	12	21	15	28	18	34	23
7.5	----	----	----	----	12	10	15	13	19	17
8	----	----	----	----	----	----	----	----	----	----

Further study was carried out on Mel-28 strain since the strain was a sequenced one. The carbon sources utilization was studied using modified minimal medium without any carbon source. Four carbon sources viz. mannose, glucose, galactose and maltose were tested and sucrose was used as control. Fungal bit of 3 mm dia was cut using cork borer from actively growing fungal culture on potato dextrose agar medium and inoculated in the test media. Interestingly, none of the carbon source except the sucrose supported the growth of *T. melanosporum*. Till 3 weeks of time, the fungus even did not start to grow in the carbon sources except sucrose. In the medium containing sucrose as sole carbon source the fungus could show 23.4 mm radial growth in 3 weeks of time.

To determine the preferred nitrogen source by *T. melanosporum*, five nitrogen sources were tested along with calcium nitrate as control. The results showed the best growth of *T. melanosporum* on amino acid alanine followed by the amide source urea (Table 6). However, the fungus showed good growth on almost all the nitrogen source. Minimum growth of the fungus was recorded on Ammonium phosphate.

Table 6: Effect of various nitrogen sources on growth of *T. melanosporum*

Treatments	Growth (Radial in mm)		
	1st Week	2nd Week	3rd Week
Ammonium sulfate	10.4	18.6	23.7
Ammonium phosphate	9.6	14.2	20.2
Asparagine	10.2	19.6	24.5
Alanine	12.2	21.5	28.2
Urea	11.0	19.6	26.6
Control ($Ca(No_3)_2$)	11.6	20.4	26.9
SE	**0.06**	**0.07**	**0.11**
CD (5%)	**0.12**	**0.15**	**0.21**

The studies on the effect of the strigolactone analogue Gr-24 on the branching and growth pattern of the fungus *T. melanosporum* indicated a negative effect and growth inhibition of the mycelium even at 10^{-9} M concentration (Figure 1). By contrast, the Indole-3-acetic acid revealed a positive effect on the radial growth as well as the biomass of the fungus (Table 7 and 8).

Table 7: Biomass enhancement of *T. melanosporum* by IAA

Treatment	Growth of mel-28 on membrane (in mg) in 4 weeks
0.136mM	84.33
1.36mM	83.66
2.72mM	64.67
Control	42.66
SE	**7.10**
CD (5%)	**14.87**

Table 8: Effect of strigolactone and IAA on the growth of *T. melanosporum*

Treatment	Growth of *T. melanosporum* (in mm)			
	1st week	2nd week	3rd week	4th week
Gr-24 (10^{-9}M)	3.8	10.2	11.6	12.4
IAA (0.136mM)	3.6	10.8	15.4	18.2
Control	4.2	10.5	12.4	15.6
SE	**0.10**	**0.24**	**0.32**	**0.56**
CD (0.05)	**0.22**	**0.41**	**0.68**	**1.21**

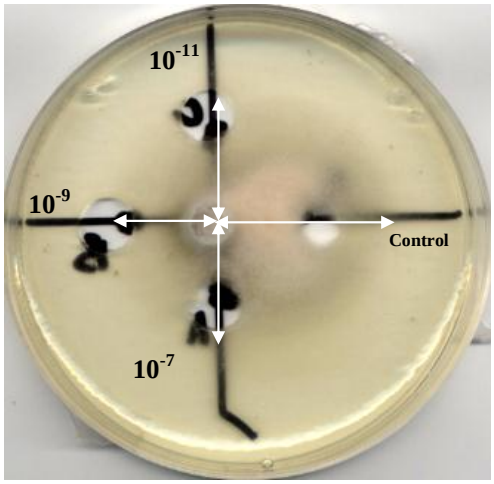

Figure 1: Growth of *T. melanosporum* as affected by Gr-24

To analyze the results obtained from Indole-3-acetic acid and strigolactone experiments, the fungal hyphae and their nuclear distribution were observed under confocal microscope. In the medium supplemented with the Indole-3-acetic acid, the length of the hyphal tips increased (Figure 2), while in Gr-24-treated mycelia, a more intense branching was observed, which may be the reason for the reduced radial growth (Figure 3).

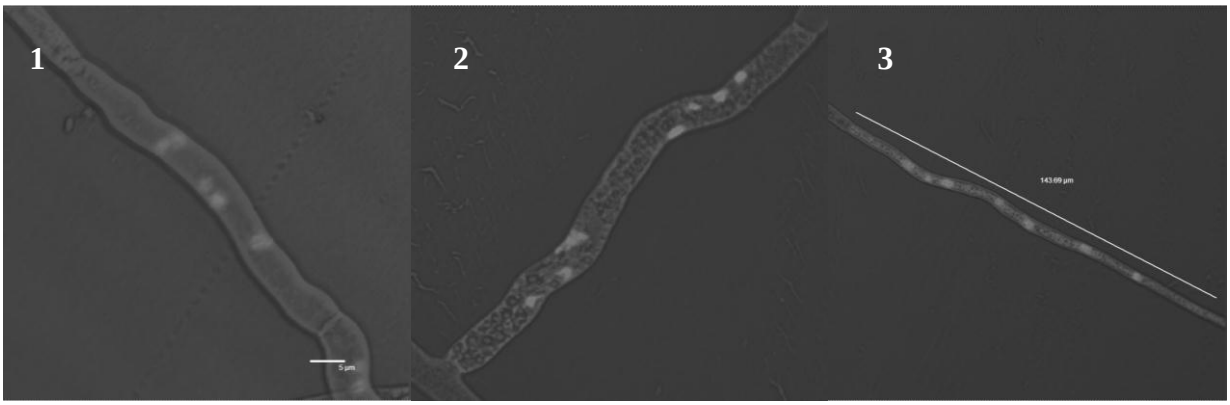

Figure 2: Elongation of hyphal tip in auxin treated mycelium (1 = Control; 2 & 3 = Auxin treated mycelium)

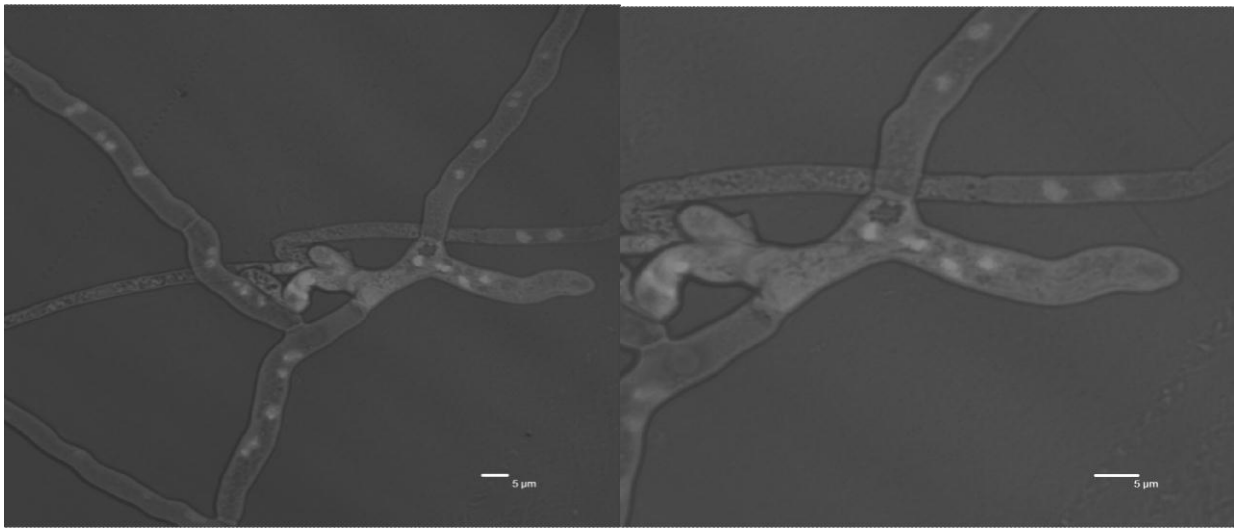

Figure 3: Change in branching pattern of Gr-24 treated mycelia of *T. melanosporum*

The genus Tuber groups ectomycorrhizal ascomycetes, which form mycorrhizas with a variety of hosts [8]. While the genome sequencing of the precious *T. melanosporum* has revealed the genomic traits, which are crucial for the establishment of the symbiosis [4, 9], a clear response to the slowness with which the fungus grows *in vitro* has not been found. This feature makes the study of the fungal physiology quite difficult. The results here reported largely confirm previous knowledge. Some studies regarding the use of different nutrient sources and culture media to optimize the growth of ectomycorrhizal fungi have been reported in the literature and, in particular, mycelium development has been assessed in relation to the carbon, nitrogen and phosphorus supply [10, 11, 12, 13]. Fontana agar medium [5] at either 20°C or 25°C (different growth chambers) was used for culturing *Tuber* mycelium. Use of modified Melin-Norkrans nutrient solution (MMN) (pH 6.6) by Molina [14] has been advocated for *in vitro* growth of truffles. MMN medium was also reported superior to Hagem + Modess medium [15] when growth was compared. As regards mycelial carbohydrate utilization, it was observed that ectomycorrhizal fungi utilize above all glucose and fructose [16], although sucrose and mannose each allowed substantial growth for *T. melanosporum* [7]. Mamoun and Olivier [7] suggested that the higher growth of *T. melanosporum* in mannose might be due to the fact that the fungus had adapted to environment, since mannose is an important constituent of plant photosynthetic sugars. In a previous study [17] using only *T. borchii* mycelium, utilization of glucose and fructose as carbohydrate sources was poor and stunted while good growth was recorded in sucrose [18]. Ceccaroli et al. [6] reported the utilization of mannose or mannitol as carbohydrate sources in culture by *T. borchii* strains. In contrast, during the present study the sugars other than sucrose did not support the growth of the fungus and we cannot propose any explanation for the same. Earlier all the studies on Mel-28 strain were conducted using potato dextrose medium, which also supported the growth of the fungus in this study. In fact, optimal production of mycelium is a fundamental step in the study of fungal metabolism, of the interaction between the truffle and its environment, of genetic variability and to obtain mycorrhization under controlled conditions. The hyphal morphology showed alterations in branching pattern when they were grown in mannose and mannitol [6, 19].

The above reports are in support of the present study that the morphology and branching pattern of the fungus *T. melanosporum* has shown changes with the change in the media and its composition. Interestingly, results obtained with the strigolactone (SLs) treatment suggest that *T. melanosporum* is sensitive to this novel class of plant hormones, similarly to arbuscular mycorrhizal fungi [19]. These fungi penetrate and colonize plant roots, where they develop highly branched structures called arbuscules, which are the sites of nutrient exchange. The natural SLs that have been identified so far have been examined for their activity on hyphal branching in the AM fungus *Gigaspora margarita*, and all the examined natural SLs were found to be active as branching factors. Although structural requirements for activity are very similar to those for germination stimulation of root parasites, some noticeable differences have been observed. For example, 3,6'-dihydro-GR24 was totally inactive as a germination stimulant but still showed distinct activity on hyphal branching (K. Akiyama, unpublished data).

The highly branched morphology in the *Tuber* mycelium was also observed during the present study and indicates that the synthetic stregolactone analogue GR-24 can also induce the mycorrhization by the fungus more efficiently and can be used for artificial inoculations of *T. melanosporum* for efficient colonization of the plants.

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REFERENCES

[1] Murat C. et al. (2008). Edible mycorrhizal fungi: Identification, life cycle and morphogenesis. In *Mycorrhiza 3rd ed.* Ajit Varma Ed. pp. 707-732

[2] Giovannetti G. et al. (1994). Truffles and their cultivation. *Horticultural Reviews* 16: 1-107.

[3] Hall I.R. et al. (1994). *The Black Truffle: its History, Uses and Cultivation*. Crop & Food Research, Lincoln, New Zealand.

[4] Martin F. et al. (2010). Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. *Nature* 464: 1033-1038.

[5] Bonfante-Fasolo P. and Fontana A. (1973). Sulla nutrizione del micelia di *Tuber melanosporum* Vitt in coltura. *Atti Accad. Sci. Torino* 107: 713–741.

[6] Ceccaroli P. et al. 2001. Effects of different carbohydrate sources on the growth of *Tuber borchii* Vittad. mycelium strains in pure culture. *Mol. Cell. Biochem*. 218: 65–70.

[7] Mamoun M. and Olivier J.M. (1991). Influence du substrat carboné et de la forme d'azote minéral sur la croissance de *Tuber melanosporum* (Vitt.) en culture pure. Application à la production de biomasse mycélienne. *Physiol Vég* 11: 521–527.

[8] Trinci A.P.J. et al. (1994). The mycelium as an integrated entity. In *The Mycota I. Growth,Differentiation and Sexuality*. Wesser J.G.H. and Meinhardt F. Eds. pp 175–193.

[9] Bonfante P and Genre A (2010). Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. *Nat Commun* 1. 1–11.

[10] Antibus R.K. et al. (1992). Phosphatase activities and phosphorus uptake from inositol phosphate by ectomycorrhizal fungi. *Can J Bot* 70: 794–801.

[11] France R.C. and Reid C.P.P. (1984). Pure culture growth of ectomycorrhizal fungi on inorganic nitrogen sources. *Microbiol Ecol* 10: 187–195.

[12] Holligan P.M. and Jennings D.H. (1972). Carbohydrate metabolism in the fungus *Dendryphiella salina*. I.Changes in the levels of soluble carbohydrates during growth. *New Phytol* 71: 569–582.

[13] Mischiati P. and Fontana A (1993). In vitro culture of *Tuber magnatum* mycelium isolated from mycorrhizas. *Mycol Res* 97: 40–44.

[14] Molina R. (1979). Ectomycorrhizal inoculation of containerized Douglas-fir and lodgepole pine seedlings with six isolates of *Pisolithus tinctorius*. *Forest Sci* 25: 585-590.

[15] Pirazzi R. (1988). Micorrizazione articiale con miceli isolati *in vitro* di *Tuber melanosporum* Vitt. e *T. magnatum* Pico. In *Atti del Secondo Congresso Internazionale sul Tartufo, Spoleto, Italy 1988*. (Bencivenga M. and Granetti B. Eds. pp. 173-184.

[16] Hughes E. and Mitchell D.T. (1995). Utilization of sucrose by *Hymenoscyphusericae* (an ericoid endomycorrhizal fungus) and ectomycorrhizal fungi. *Mycol Res* 99: 1233–1238.

[17] Saltarelli R. et al. (1999). Strain differences in the mycelium of the ectomycorrhizal fungus *Tuber borchii*. *Mycol Res* 103: 1524–1528.

[18] Saltarelli R. et al. (1998). Biochemical and morphological modifications during the growth of *Tuber borchii* mycelium. *Mycol Res* 102: 403–409.

[19] Xie Xiaonan et al. (2010). The Strigolactone Story. *Ann. Rev. Phytopathol.* 48: 93–117.