Mycorrhiza (1996) 6:15–19

ORIGINAL PAPER

Michèle Mamoun · Jean-Marc Olivier

Receptivity of cloned hazels to artificial ectomycorrhizal infection by *Tuber melanosporum* and symbiotic competitors

Accepted: 4 June 1995

Abstract The cultivation of the black truffle, *Tuber melanosporum* (Vitt.), is based upon plantation of seedlings artificially infected with *Tuber*. At the present time, neither the host-plant nor the fungal material used for planting truffle orchards are genetically well defined. Cloning the host-plant was performed as a first step in improvement. A clone of hazel (*Corylus avellana* L.) was artificially infected with *T. melanosporum*. The heterogeneity of the root volume was not completely suppressed by cloning but very extensive growth unfavorable to *Tuber* colonization rarely occurred. The root system was highly receptive to ectomycorrhizal fungi. The percentage of roots infected by *T. melanosporum* reached a higher level on cloned hazels than uncloned seedlings. The relation between *Tuber* and other symbionts appeared to depend on the morphology of the root system. *T. melanosporum* spread more easily on medium-sized root systems whereas the other symbionts (*Scleroderma* sp., *Cenococcum* sp., *Pulvinula globifera*) developed in parallel to the root volume. The practical potential of this system is discussed.

Key words Clone · Competition · Mycorrhizal infection · *Tuber melanosporum*

Introduction

The black truffle *Tuber melanosporum* (Vitt.) is an ectomycorrhizal fungus which has been harvested in the wild for a very long time (Chatin 1869). In recent decades, the culture of *T. melanosporum* has improved by using artificially infested host-plants (Mannozi-Torini 1970; Chevalier and Grente 1979). At the present time, the heterogeneity of truffle production is due partly to the fact that neither the host-plant nor the *Tuber* inoculum are genetically well characterized. The cloning of both fungus and host-plant should increase our knowledge of these organisms and provide improved material for planting truffle orchards.

A clone of hazel (*Corylus avellana* L.) is now routinely produced by the INRA – Agri-Obtention Laboratory (Dijon, France) as the result of work carried out at the INRA – Bordeaux (Al Kaï et al. 1984; Guinberteau et al. 1990)

Our objective was to observe the receptivity of the cloned hazels to artificial infection with *T. melanosporum* and the effect of their residual phenotypic variability. Colonization by naturally occurring symbionts in the glasshouse and climatic chamber, competing with *T. melanosporum* for mycorrhizal infection sites as previously observed on hazel seedlings (Mamoun and Olivier 1990, 1993) was taken into consideration.

Materials and methods

Plants

The clone H219 of *C. avellana* L. was obtained at the Fruit Breeding Station, INRA, Bordeaux from the progeny of a cross between the cultivated varieties Segorbe 350 and Butler 372. In vitro multiplied shoots of clone H219 were provided by the INRA Agri-Obtention Laboratory (Dijon, France). Post in vitro rooting was performed at INRA, Bordeaux. The shoots were individually planted in pasteurized (100 °C, 1 h) peat:sand:perlite (1:1:1, volume), and grown for 3 months under mist in the glasshouse for rooting. The 146 plants obtained were transplanted in a peat:siliceous gravel (1:1, volume) substrate and grown for 1 year under glasshouse conditions without mist.

Soil

The clay-calcareous soil came from the INRA truffle orchard (Dordogne, France). Its physical and chemical properties have been described previously (Mamoun and Olivier 1993). The sieved fraction below 30 mm was steam disinfected twice for 1 h at 100 °C.

M. Mamoun (✉) · J.-M. Olivier
Station de Recherches sur les Champignons, INRA – B.P. 81,
F-33883 Villenave d'Ornon Cédex, France
Tel.: 33–56–843171; Fax: 33–56–843178;
e-mail: Michele.Mamoun@Bordeaux.inra.fr

Experimental design

Fifteen-month-old cloned hazels were removed from their substrate and the roots washed with demineralized water. All the plants were transferred individually to plastic pots filled with 800 g of disinfected truffle-orchard soil mixed with 20 ml inoculum of *T. melanosporum* (5×10^6 spores). After inoculation, the plants were grown for 1 year in a climate chamber (day and night temperature 20–22 °C, air moisture 50–70% day, 70–90% night, photoperiod 14-h day, 10-h night, light intensity 35 ± 10 μmol m^{-2} s^{-1}). Soil moisture (–0.022 MPa) was maintained by watering the pots to a predetermined weight.

Data assessment

Plant growth and mycorrhizal infection were assessed after a 1-year culture period in a climate chamber.

Plant growth

The total shoot length was recorded for each plant. After removal from the soil, each root system was washed under tap water and introduced up to the collar into a graduated cylinder filled with water to assess the root volume.

Mycorrhizal infection

Ten small pieces (2–3 cm long) of secondary roots were taken from each plant. Two root segments were cut near the collar, four from the inner part of the root system, and four from the less melanized part. The mean number of mycorrhizae observed per plant was 46, 71, 77, 92, 106 and 127 for root systems with volumes of <5, 5–9, 10–14, 15–19, 20–24 and ≥ 25 cm^3, respectively. The regression coefficient obtained with the 146 pairs of data ($r = 0.564$, significant at $P < 0.01$) showed that the number of mycorrhizae observed is proportional to the root volume. The various types of mycorrhizae (i.e. *T. melanosporum* and other symbionts naturally infecting the plants under glasshouse and climate chamber conditions) were identified under a light microscope. *T. melanosporum* was identified by its spinulae (particularly short hyphae growing out of the mantle and differing from external mycelium) and sheath ornamentation (Palenzona 1969; Giraud 1988). Mycorrhizae of *Pulvinula globifera* were previously observed on hazel roots and described by Olivier and Mamoun (1994). *Cenococcum* sp. and *Scleroderma* sp. were identified according to external hyphae and sheath ornamentation as described by Garbaye (1990). For each plant, the percentage of root infected by each ectomycorrhizal type was assessed and the sum of the percentages used to indicate the overall infection.

Graphic representation

The mycorrhizal infection (Figs. 2–4) is shown using a box-plot representation (Chambers et al. 1983) generated by the S-Plus computer program (Statistical Sciences Inc., Seattle, Wash.). The median 50% of the data is represented as a box. The horizontal line segment inside the box corresponds to the median itself. The higher and lower fractions are represented by dashed line segments extending from the top and bottom of the box. Extra lines above and below the box show extreme results.

Results

At the end of the experiment, the 27-month-old cloned hazels showed a wide diversity of shoot development. Measurements of total shoot length fell into a pattern close to a binomial distribution, with 6 cm as the minimal and 97.5 cm as the maximal value. The 30–40 cm class was represented the most (26% of the plants) and the 20–50 cm classes included 66.4% of the plants. Very extensive shoot development, above 70 cm, rarely occurred (Fig. 1a).

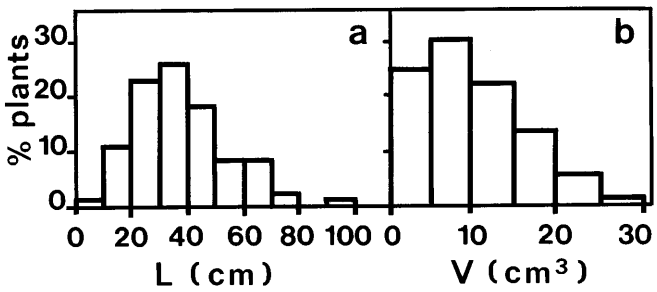


Fig. 1 Distribution of cloned hazels according to **a** total shoot length (L), and **b** root volume (V).

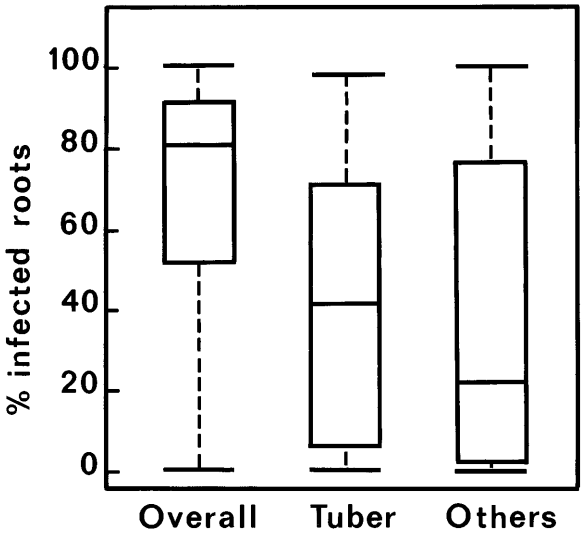


Fig. 2 Distribution of the ectomycorrhizal infection observed among cloned hazels

The histogram of the root system volumes shows an asymmetrical distribution, ranging between 1 and 28 cm^3. The most frequently observed class, 5–10 cm^3, included 30.1% of the plants. Only 8.3% of the hazels developed extensive root systems, above 20 cm^3 (Fig. 1b). Small root systems, 1–5 cm^3, were very poorly branched while those of 10–28 cm^3 were well branched, with numerous short roots. A weak correlation was observed between the total shoot length and the root volume. Although low, the determination coefficient ($r^2 = 0.31$) was significant ($t = 8.1$; $d.f. = 145$).

One year after inoculation, the cloned hazels were generally extensively colonized by ectomycorrhizal fungi (Fig. 2). The box plot representation showed that 75% of the plants bore 50–100% infected roots. Infection by *T. melanosporum* appeared to be well developed, reaching above 40% of the roots for half of the

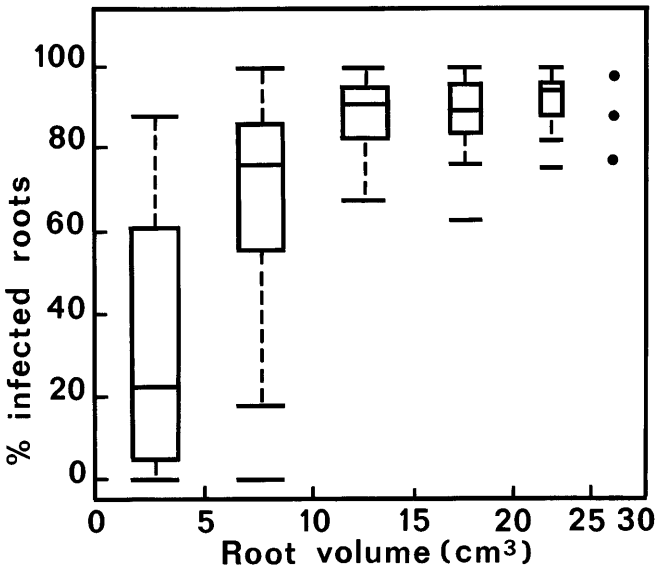


Fig. 3 Variation of the overall infection related to the volume of the root system. Each box width is proportional to the number of observations. ● Data from poorly represented classes

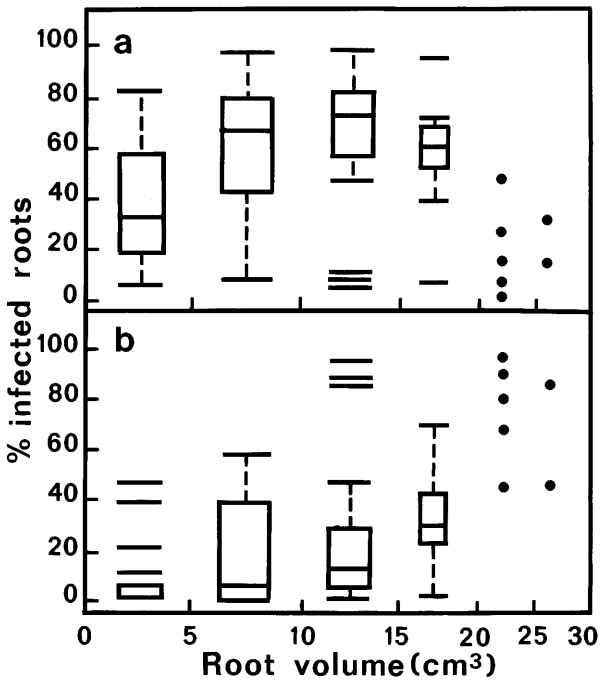


Fig. 4a,b Variation of infection related to the volume of the root system. Infection by **a** *Tuber melanosporum* and **b** other symbionts observed on the same plants. Each box width is proportional to the number of observations. ● Data from poorly represented classes

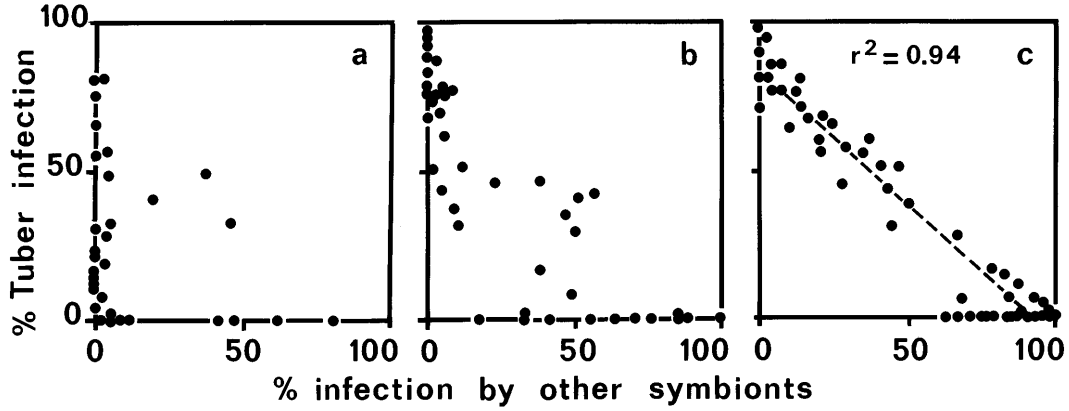
plants. A *Tuber* colonization of 70–98% was observed on 25% of the hazels. Half of the plants showed less than 20% of their roots infected by other symbionts, and only 25% of the plants were highly infected (80–100%) by these fungi. *Scleroderma* sp. was responsible for the major part of infection by contaminant symbionts, and *Cenococcum* sp. and *Pulvinula globifera* Boud. were also found. The overall infection, *T. melanosporum* plus other symbionts, increased with increase in the volume of the root system from 1 to 9 cm^3, but was consistently extensive (68–100% of roots infected) on 10- to 28-cm^3 root systems (Fig. 3).

On hazels bearing *T. melanosporum*, the infection by *Tuber* developed with a bell-shaped distribution centered on medium-sized root systems (Fig. 4a). A different pattern was observed with other symbionts where infection increased with the development of the roots (Fig. 4b). On plants without *T. melanosporum*, infection by the other symbionts spread as shown in Fig. 3.

The relationship between infection by *T. melanosporum* and colonization by other symbionts depended on the volume of the root system. There was close to an exclusion phenomenon on root systems smaller than 5 cm^3, a linear relationship ($r^2 = 0.94$) on 10–28 cm^3 root systems and an intermediate relationship with 5–9 cm^3 root volumes (Fig. 5).

Considering other symbionts individually, a linear correlation was observed between *T. melanosporum* and *Scleroderma* sp. ($r^2 = 0.72$) on 10–28 cm^3 root systems. No constant relationship was detected on smaller

Fig. 5 Relationships between ectomycorrhizal infection by *T. melanosporum* and by other symbionts for three classes of root system: **a** <5 cm^3, **b** 5–9 cm^3 and **c** ≥ 10 cm^3



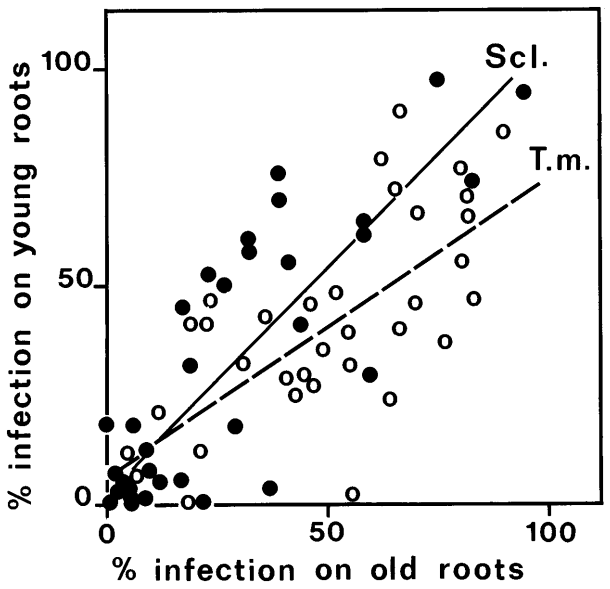


Fig. 6 Distribution of ectomycorrhizal infection on the root systems. ● *Scleroderma* sp. (*Scl.*), ○ *T. melanosporum* (*T.m.*)

plants. There was a slight ($r^2 = 0.30$) but significant ($P < 0.05$) linear correlation between *T. melanosporum* and *Cenococcum* sp. on 10–28 cm^3 root systems (data not shown).

For plants bearing both *T. melanosporum* and *Scleroderma* sp., linear correlations between young, whitish roots and old melanized roots were obtained, with $r^2 = 0.57$ for the former symbiont and 0.71 for the latter (Fig. 6). Comparison of data for *T. melanosporum* and *Scleroderma* sp. using ANCOVA revealed a slight but significant difference ($P < 0.05$) between the two fungi in the distribution of the infection on the root system. Infection by *T. melanosporum* was more developed on old roots ($b = 0.70$), while colonization by *Scleroderma* was similar on old and young roots ($b = 1.03$). The two regression coefficients were significantly different at $P < 0.05$.

Discussion

The heterogeneity of root system volume previously observed between 10 and 35 cm^3 for uncloned hazel seedlings (Olivier and Mamoun 1994) was not completely suppressed by cloning, but very extensive growth unfavorable to *Tuber* colonisation rarely occurred (8.3%). However, the aerial growth of the cloned hazels was heterogeneous. Although significant, the poor correlation between aerial and root development ($r^2 = 0.31$) on the same plant does not allow a reliable estimation of the root system without removing the plants from the substrate. Consequently, a rapid choice of hazels highly receptive to *Tuber* infection (=with medium-sized root systems) will not be possible from measurement of the aerial system.

Despite the genotypic homogeneity of the host-plants, different overall levels of infection were reached. This seems partly related to the development and morphology of the root system. On small plants, the low number of potentially infectable small roots probably explains the low overall infection observed. When sufficiently developed (≥ 10 cm^3), the root system was highly receptive to ectomycorrhizal symbionts, and similar levels of overall infection occurred whatever the root volume. The significant colonization by *Tuber* compared with other symbionts observed in our experiment in theory makes the clone interesting for artificial infection with *T. melanosporum*. The different pattern observed for *T. melanosporum* and other fungi for the percentage infection on root systems of ≥ 10 cm^3 implies that colonization by other symbionts could be minimized using particular methods, such as irrigation (Mamoun and Olivier 1990, 1992, 1993) adapted to the different soil compositions.

As reported for uncloned hazel seedlings (Olivier and Mamoun 1994), we observed a decrease in *T. melanosporum* infection on extensive root systems, which may be related to the slow growth rate of the *Tuber* mycelium compared with the spread of *Scleroderma* sp. and *P. globifera*. The lower percentage of cloned plants showing an extensive root system (8.3%) than uncloned seedlings (17.8%, unpublished results) gives the clone an advantage for artificial infection with *T. melanosporum*. Moreover, *Tuber* infection above 87% was never observed with uncloned hazels using the same experimental time course and inoculation procedure, but some cloned plants were infected 100%.

Under the experimental conditions used here, not only the overall infection as defined above, but also the relationship between *T. melanosporum* and other symbionts appears to depend on the morphology of the root system, in particular the number of short roots available for infection. Mixed mycorrhizae, as observed with other fungi (Marks and Foster 1967; Villeneuve et al. 1991), exist for *T. melanosporum* and the other symbionts and raise the question of the involvement of such mixed mycorrhizae in the colonization of the root system. The results involving *T. melanosporum* and *Scleroderma* sp. may be related to field observations reporting that *Scleroderma* mycorrhizae are found at the edge of the *brûlé* and may modify the environment for the *Tuber* (Sourzat 1993).

Cloning hazels seems a reliable means of producing host-plants favorable to artificial infection by *T. melanosporum*. Despite the morphological heterogeneity observed, medium-sized cloned plants were highly infected by *T. melanosporum*. In addition, cloned plants may be chosen for interesting agronomic characteristics such as resistance to frost or to bacterial canker.

For experimental purposes, using cloned plants provided the material with an interesting homogeneity. Investigations on competition after controlled inoculation with *Tuber* and other symbionts, e.g. *Scleroderma* or *P. globifera*, may be improved by minimizing the effects of

plant variability. Intra- and interclone studies will be carried out in the future on the receptivity of the host-plant to infection with *T. melanosporum*. Initial results on intraspecific variation among *T. melanosporum* have recently been obtained in our laboratory (Cameleyre and Olivier 1993) and further studies are in progress. Consequently, the ability of genetically identified isolates of *T. melanosporum* to infect roots could soon be tested on cloned hazels without effects related to genetic heterogeneity of the host-plant. Ability to fruit will be later investigated in experimental orchards and particularly efficient combinations of cloned hazel-*T. melanosporum* strains may be anticipated for planting new generations of truffle orchards.

Acknowledgements This work was supported by a grant from the European Community (PIM–URTA project). We are indebted to Dr. P. Gasqui (Biometry Laboratory, INRA, Jouy en Josas, France) for graphic representation advice, and to C. Boussion (INRA, Bordeaux, France) for utilization of the computer program S-Plus. We are grateful to E. Germain and G. Salesses (Fruit Breeding Station, INRA, Bordeaux) and P. Mussillon (Agri-obtention Laboratory, Dijon, France) for providing *Corylus avellana* clone H219.

References

Al Kaï H, Salesses G, Mouras A (1984) Multiplication in vitro du noisetier (*Corylus avellana* L.). Agronomie 4:399–402

Cameleyre I, Olivier JM (1993) Evidence for intraspecific isozyme variation among French isolates of *Tuber melanosporum* (Vitt.). FEMS Lett 110:159–162

Chambers JM, Cleveland WS, Kleiner B, Tukey PA (1983) Graphical methods for data analysis. Duxbury Press, Boston

Chatin A (1869) La Truffe. Etude des conditions générales de la production truffière. Bouchard-Hazard, Paris

Chevalier G, Grente J (1979) Application pratique de la symbiose ectomycorhizienne: production à grande échelle de plants mycorhizés par la truffe. Mushroom Sci 10:483–505

Garbaye J (1990) Pourquoi et comment observer l'état mycorhizien des plants forestiers. Rev For Fr 42:35–47

Giraud M (1988) Prélèvement et analyse de mycorhizes. Bull FNPT 10:49–63

Guinberteau J, Salesses G, Olivier JM, Poitou N (1990) Mycorhization de vitroplants de noisetiers clonés. In: Bencivenga M, Granetti B (eds) Atti del Secondo Congresso Internazzionale sul Tartufo. Communità Montana dei Monti Martini e del Serano, Spoleto, Italy

Mamoun M, Olivier JM (1990) Dynamique des populations fongiques et bactériennes de la rhizosphère des noisetiers truffiers. III. Effet du régime hydrique sur la mycorhization et la microflore associée. Agronomie 10:77–84

Mamoun M, Olivier JM (1992) Effect of soil *Pseudomonads* on colonization of hazel roots by the ectomycorrhizal species *Tuber melanosporum* and its competitors. Plant Soil 139:265–273

Mamoun M, Olivier JM (1993) Competition between *Tuber melanosporum* and other ectomycorrhizal fungi under two irrigation regimes. I. Competition with *Tuber brumale*. Plant Soil 149:211–218

Mannozi-Torini L (1970) Manuale di Tartuficola. Edagricole, Bologna, Italy

Marks GC, Foster RC (1967) Succession of mycorrhizal associations on individual roots of radiata pine. Aust For Res 31:193–201

Olivier JM, Mamoun M (1994) Competitions entre *Tuber* et autres symbiotes sur de jeunes noisetiers truffiers. Acta Bot Gall 141:559–563

Palenzona M (1969) Sintesi micorrizica tra *Tuber aestivum* Vitt., *Tuber brumale* Vitt., *Tuber melanosporum* Vitt. e semenzali di *Corylus avellana* L. Allionia 15:121–132

Sourzat P (1993) Trufficulture. Résultats techniques d'expérimentations. LEPA, Cahors, France

Villeneuve N, Le Tacon F, Bouchard D (1991) Survival of inoculated *Laccaria bicolor* in competition with native ectomycorrhizal fungi and effects on the growth of outplanted Douglas-fir seedlings. Plant Soil 135:95–107