Mycorrhiza (2009) 19:159–165
DOI 10.1007/s00572-008-0224-z

ORIGINAL PAPER

Calcareous amendments to soils to eradicate *Tuber brumale* from *T. melanosporum* cultivations: a multivariate statistical approach

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Received: 2 October 2008 / Accepted: 22 December 2008 / Published online: 22 January 2009

Abstract Calcareous amendments are being used in *Tuber melanosporum* truffle plantations in attempts to eradicate *Tuber brumale*. However, there are no studies available which provide soil analysis and statistical data on this topic. We studied 77 soil samples to compare the values for carbonates, pH and total organic carbon in *T. brumale* truffières with the values for *T. melanosporum* truffières on contaminated farms and in natural areas. Statistical analyses indicate that the concentrations of active carbonate and total carbonate in the soil are significantly higher in *T. brumale* truffières than in *T. melanosporum* truffières, but that there are no significant differences in pH and total organic carbon. We conclude that liming would not suppress *T. brumale* ectomycorrhizas in contaminated *T. melanosporum* farms, and calcareous amendments do not therefore seem be a means of eradicating *T. brumale* in these farms.

Keywords Ectomycorrhizae · Liming · Truffle culture · *Tuber brumale* · *Tuber melanosporum*

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Introduction

Truffles are a highly profitable cash crop growing in the forests of many Mediterranean regions, and the cultivation of *Tuber melanosporum* Vittad. (Périgord black truffle) has spread throughout many countries in recent decades. The ectomycorrhizas of other species such as *Tuber brumale* Vittad. contaminate *T. melanosporum* brûlés (zones around the host trees free of vegetation) reduce their carpophore production and pose formidable problems for those trying to optimise *T. melanosporum* cultivation (Chevalier and Frochot 1997; Callot 1999; Lefevre and Hall 2001; Riousset et al. 2001; Olivier et al. 2002; Ricard 2003; Sourzat 2005).

It is not known how *T. melanosporum* and *T. brumale* interact with each other in *T. melanosporum* brûlés and the biological and physical–chemical properties of soil. Experiments have been proposed to study this in planted truffières (the truffle-producing area; Mamoun and Olivier 1993a, b) using molecular techniques to detect their mycorrhizas (Rubini et al. 1998; Paolocci et al. 1999; Giomaro et al. 2002; Douet et al. 2004). Other researchers have underlined the importance of conducting studies on the *T. melanosporum* brûlé to explain hydric behaviour and the dissolution and precipitation processes of carbonates (Callot 1999; Ricard 2003; Granetti et al. 2005; Jaillard et al. 2007).

A simultaneous production of carpophores in various *Tuber* species can be observed within the same truffière.

Callot (1999), Ricard (2003) and Granetti et al. (2005) indicate that there is a lack of knowledge as to how the biological and physical–chemical properties of soil influence the development of *T. melanosporum* ectomycorrhizas and their interactions with other *Tuber* species.

These authors and Jaillard et al. (2007) explain that *T. melanosporum* is strictly calcicolous and that their ectomycorrhizas are closely linked to calcium carbonate. They underlined the importance of conducting an overall study of soil brûlé profiles to explain hydric behaviour and the dissolution and precipitation processes of carbonates. Some statistical studies have also shown that a high concentration of active carbonate (calcium carbonate extractable with ammonium oxalate; smaller than 50 µm in size) is responsible for up to 43% of the variance in *T. melanosporum* carpophore production; this accounts for up to 51% of the variance in brûlé sizes and is a major factor in the fruiting and aggressiveness of *T. melanosporum* versus *Tuber aestivum* Vittad., *T. mesentericum* Vittad. and *Tuber rufum* Pico ex Fries (García-Montero et al. 2007a, b, 2008a, b).

Riousset et al. (2001) and García-Montero et al. (submitted) outlined experiments in France and Spain where calcium carbonate was applied to *T. melanosporum* soils in attempts to favour *T. melanosporum* and deter *T. brumale*, but reported that these were unsuccessful. Consequently, we carried out a study to determine if there are differences in pH, carbonate fractions and abundance of total organic carbon (TOC) in soils occupied only by *T. brumale* or *T. melanosporum* or where the two species were present together and from this infer whether calcareous amendments to soils to eradicate *T. brumale* had a sound scientific basis.

Materials and methods

Case study areas

The study areas were located in the mountainous regions of Central Spain in supra-Mediterranean bioclimatic belts, with predominantly Jurassic and Cretaceous limestones and dolomites, and the soils lithic and rendzic leptosols. Seventy-seven soil samples were collected from *T. brumale* and *T. melanosporum* truffières from two planted truffières and two natural truffieres in regions with and without a natural presence of *T. brumale*.

Table 1 summarises the environmental characteristics of study areas. In Sarrión (Teruel), *T. melanosporum* plantation is 15 years old and has 50% productive *T. melanosporum* truffières (>500 g/year) and 20% contaminated truffières that produce more than 500 g/year of *T. brumale* carpophores. In Campezo (Alava), *T. melanosporum* plantation is 14 years old and has 60% productive *T. melanosporum* truffières (>500 g/year) and 20% contami-

Table 1 Study area characteristics

Species	Truffière type	Soil samples	Symbiotic plant	Village	Province	Altitude	P	T	Vegetation
Tuber brumale	Natural	17	*Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	Iruña de Oca	Alava	498	700	10	*Spiraeo hispanicae-querceto rotundifoliae sigmetum*
Tuber melanosporum	Natural	20	*Quercus faginea* Lam. and *Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	Peralejos de las Truchas	Guadalajara	1,150	797	10	*Cephalanthero longifoliae-Querceto fagineae sigmetum* and *Junipero thuriferae-Querceto rotundifoliae sigmetum*
Tuber melanosporum	Planted	15	*Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	Sarrión	Teruel	991	500	11	*Junipero thuriferae-Querceto rotundifoliae sigmetum*
Tuber brumale	Contaminated	15	*Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	Sarrión	Teruel	991	500	11	*Junipero thuriferae-Querceto rotundifoliae sigmetum*
Tuber melanosporum	Planted	5	*Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	Campezo	Alava	578	732	12	*Spiraeo hispanicae-Querceto rotundifoliae sigmetum*
Tuber brumale	Contaminated	5	*Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	Campezo	Alava	578	732	12	*Spiraeo hispanicae-Querceto rotundifoliae sigmetum*

Altitude in metres. Vegetation = geobotanical classification type according to Rivas-Martínez (1987)
P average annual precipitation (in mm), *T* average annual temperature (in °C)

nated truffières that produce more that 300 g/year of *T. brumale* carpophores.

Soil analysis

The soil samples were taken according to FAO (1990) recommendations from inside the holes that the collectors made to extract the carpophores in truffières with a significant production (>300 g/year). Only the first 30 cm of each soil profile was studied because *T. melanosporum* usually bears fruit in this range (Verlhac et al. 1990). The soils were sampled always using the same spade (20-cm blade) and a rule. These samples included a mixture of 1,000 g of the soil surface horizons that have not been differentiated, as truffières contain a constant mixture of horizons due to the continuous digging that the harvesters, their dogs and wildlife engage in to extract the carpophores.

The following soil determinations were made: pH in water, total organic carbon and total carbonate (equivalent calcium carbonate: Marañes et al. 1994; ISRIC 1995) following the methods of the ISRIC (1995); active carbonate (calcium carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982).

We have also used some additional data from 20 *T. melanosporum* soil samples from two previous works (García-Montero et al. 2006, 2008a).

Statistical analysis

Statistical analysis of the data was carried out using the Statistica Program v. 6 (StatSoft, Tulsa, OK, USA, 1999). Before the analysis, the distributions of the variables were adjusted to comply with the prerequisites of the parametric statistical analysis. These transformations were selected using the Box and Cox (1964) tests. Normality was checked using the Shapiro–Wilks and Kolmogorov–Smirnov tests, and homogeneity of variances was verified by the Levene test. The soil data obtained in natural areas did not follow a normal distribution, and therefore, non-parametric tests were applied to these variables.

Principal components analyses (PCA) were done with the soil variables studied in contaminated plantations in order to analyse the statistical patterns of the overall soil properties regarding the fruiting of *T. brumale* versus *T. melanosporum* carpophores in truffières of contaminated plantations. Analysis of variance (ANOVA) test was performed to determine whether there were any significant differences between the variables for the soils of *T. brumale* versus *T. melanosporum* in truffières of contaminated plantations. The non-parametric Mann–Whitney *U* test was applied to analyse the statistical patterns of the soil properties of *T. brumale* truffières versus *T. melanosporum* truffières in natural areas.

Table 2 Analytical results of 20 soils located inside burns with *T. brumale* production in contaminated *T. melanosporum* plantations (active carbonate, total carbonate and TOC expressed in g kg^{-1})

No.	Province	pH_{H2O}	Active carbonate	Total carbonate	TOC
1	Teruel	8.80	134.53	342.50	20.66
2	Teruel	8.14	182.66	502.50	18.28
3	Teruel	8.23	92.66	227.50	23.87
4	Teruel	8.23	141.67	447.50	19.72
5	Teruel	8.24	128.54	472.50	16.45
6	Teruel	8.32	127.66	307.50	24.03
7	Teruel	8.24	173.91	442.50	21.19
8	Teruel	8.21	172.66	422.50	18.83
9	Teruel	8.31	158.91	397.50	20.00
10	Teruel	7.85	162.50	412.59	23.80
11	Teruel	7.87	173.13	457.19	17.30
12	Teruel	7.93	150.63	393.10	22.08
13	Teruel	8.18	155.63	395.57	21.87
14	Teruel	8.17	137.50	371.13	19.04
15	Teruel	7.90	78.75	273.27	33.08
16	Alava	8.06	160.16	422.50	19.97
17	Alava	7.92	126.22	472.50	19.60
18	Alava	8.16	87.16	312.50	20.37
19	Alava	8.15	133.91	537.50	8.39
20	Alava	8.24	132.66	492.50	10.54

Table 3 Analytical results of 20 soils located inside burns with *T. melanosporum* production in contaminated *T. melanosporum* plantations (active carbonate, total carbonate and TOC expressed in g kg^{-1})

No.	Province	pH_{H2O}	Active carbonate	Total carbonate	TOC
21	Teruel	8.34	126.41	362.50	21.83
22	Teruel	8.18	156.41	492.50	21.20
23	Teruel	7.99	153.75	311.07	21.46
24	Teruel	7.85	68.75	226.33	33.99
25	Teruel	7.98	78.75	207.76	16.29
26	Teruel	8.06	65.00	241.43	14.83
27	Teruel	8.10	66.88	251.38	14.96
28	Teruel	8.09	47.50	55.31	15.11
29	Teruel	8.05	105.00	162.63	15.14
30	Teruel	8.12	135.00	320.04	22.07
31	Teruel	8.01	123.13	323.78	28.39
32	Teruel	8.12	150.63	391.49	25.93
33	Teruel	8.05	140.00	453.83	24.68
34	Teruel	7.69	61.25	253.00	37.63
35	Teruel	7.71	68.13	266.16	40.41
36	Alava	8.26	118.54	427.50	3.46
37	Alava	8.31	123.90	422.50	11.14
38	Alava	8.08	91.40	357.50	16.93
39	Alava	8.28	112.00	340.00	12.46
40	Alava	8.32	90.20	437.50	10.84

Table 4 Analytical results of 17 soils located inside burns with *T. brumale* production in natural areas (active carbonate, total carbonate and TOC expressed in g kg^{-1})

No.	Province	pH_{H2O}	Active carbonate	Total carbonate	TOC
41	Alava	7.79	129.38	637.22	17.33
42	Alava	8.20	148.96	705.33	14.97
43	Alava	7.73	134.38	629.84	17.99
44	Alava	8.00	131.88	616.37	13.18
45	Alava	7.74	131.88	756.63	21.23
46	Alava	7.70	121.88	660.37	20.79
47	Alava	7.73	128.75	682.93	17.42
48	Alava	7.75	134.38	656.69	19.03
49	Alava	7.86	101.88	674.24	14.89
50	Alava	7.63	125.63	651.02	23.61
51	Alava	7.95	139.38	676.76	14.40
52	Alava	7.62	130.00	664.29	18.19
53	Alava	8.18	113.91	530.00	21.54
54	Alava	8.22	115.78	472.50	21.74
55	Alava	8.19	107.16	537.50	17.73
56	Alava	8.17	100.91	457.50	19.32
57	Alava	8.16	107.66	497.50	19.31

Table 5 Analytical results of 20 soils located inside burns with *T. melanosporum* production in natural areas (active carbonate, total carbonate and TOC expressed in g kg^{-1}; García-Montero et al. 2006, 2008a)

No.	Province	pH_{H2O}	Active carbonate	Total carbonate	TOC
58	Guadalajara	8.35	19.53	25.00	4.89
59	Guadalajara	7.73	27.50	423.69	125.60
60	Guadalajara	7.54	65.00	399.34	116.90
61	Guadalajara	7.90	11.25	628.23	68.10
62	Guadalajara	7.90	10.63	662.32	79.00
63	Guadalajara	8.03	6.88	684.24	39.70
64	Guadalajara	8.08	0.00	501.61	38.50
65	Guadalajara	7.97	11.88	14.61	16.40
66	Guadalajara	7.90	22.50	31.17	34.03
67	Guadalajara	7.88	70.10	78.80	35.62
68	Guadalajara	7.50	16.25	127.88	23.02
69	Guadalajara	7.90	3.75	91.82	31.52
70	Guadalajara	7.80	28.75	71.90	75.24
71	Guadalajara	8.15	26.25	41.76	11.58
72	Guadalajara	7.90	81.40	115.00	36.07
73	Guadalajara	8.15	23.75	45.27	10.80
74	Guadalajara	7.90	33.75	102.09	25.51
75	Guadalajara	8.07	3.75	62.77	5.25
76	Guadalajara	7.95	28.75	78.14	31.10
77	Guadalajara	7.10	15.00	145.63	1.00

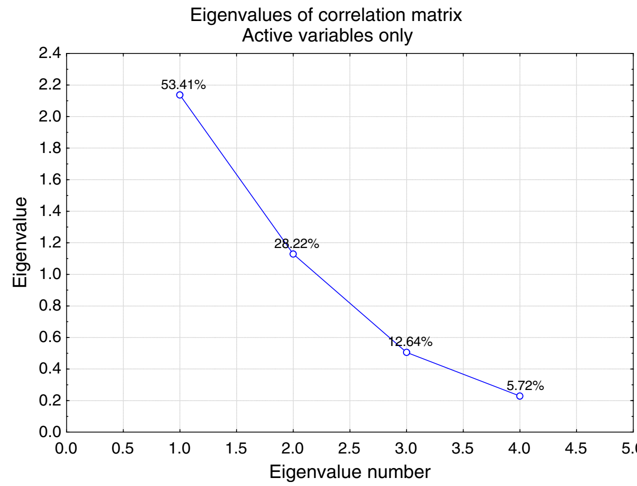

Fig. 1 Scree plot of principal components analysis

Identification of the harvested truffles

Macroscopic features of the carpophores and microscopic studies of the morphological characteristics of the ascospores permitted a clear identification of the harvested *T. brumale* and *T. melanosporum* truffles following the descriptions and indications proposed by Riousset et al. (2001).

Results

Tables 2, 3, 4 and 5 show the physical–chemical properties of soil samples collected in the natural areas and contaminated *T. melanosporum* plantations. Many of the soil samples have a moderately basic pH and a very variable percentage of total carbonate and active carbonate concentration. Levels of TOC are moderate.

In the contaminated *T. melanosporum* plantations, the PCA shows that the first three factors (PC_1, PC_2 and PC_3) accounted for 94.27% of the variance contained in the original matrix (Fig. 1). PC_1 and PC_2 are the components that best explain the interactions between the variables. PC_1 accounts for 53.41% of the variance. This highlights the differences between soils with a greater quantity of total carbonate and active carbonate versus TOC (Table 6). The

Table 6 Principal components analysis of 40 soils located inside burns with *T. brumale* and *T. melanosporum* production in contaminated *T. melanosporum* plantations: factor loadings

Variables	Factor 1 (PC_1)	Factor 2 (PC_2)	Factor 3 (PC_3)
% Active carbonate	−0.788498	−0.508883	0.131959
% Total carbonate	−0.827594	−0.426789	−0.161942
pH	−0.654151	0.564755	0.496169
% TOC	0.634011	−0.607285	0.464654

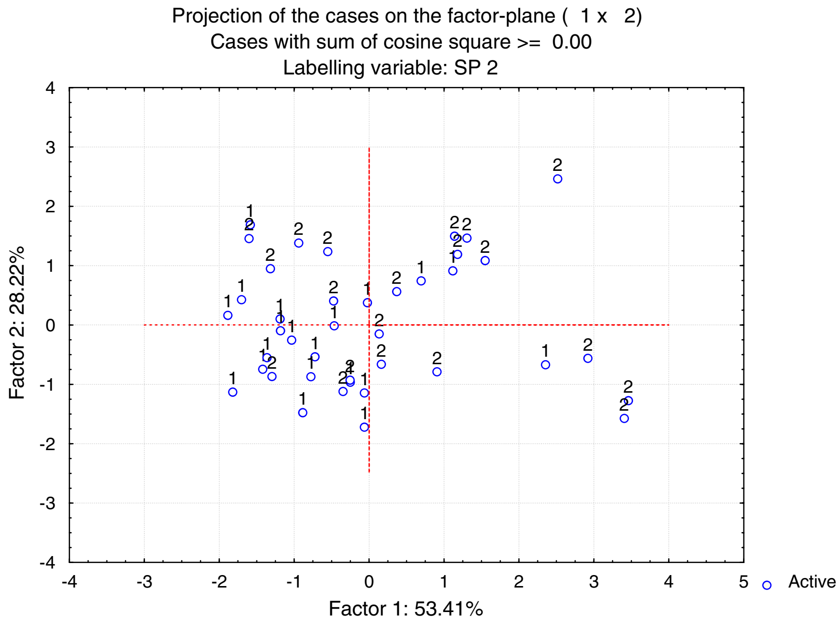

Fig. 2 Relationship between the presence/absence of *T. brumale* and *T. melanosporum* production in contaminated *T. melanosporum* plantations and PCA factors. Samples coded with *1* are soil samples taken at points with production of *T. brumale* ascocarps inside the burns, and samples coded with *2* are soil samples taken at points with production of *T. melanosporum* ascocarps inside the burns

second factor (PC_2) represents 28.22% of the variance. PC_2 indicates that it opposes pH and the other variables studied (Table 6). Figure 2 shows different soils according to PC_1 and PC_2. This graph shows how soils from inside the *T. brumale* truffières have an important correspondence with left semi-axis PC_1, related to high active carbonate and total carbonate contents, and soils from inside the *T. melanosporum* truffières have a significant correspondence with right semi-axis PC_1, related to low active carbonate and total carbonate contents.

The ANOVAs indicate that the mean concentration of active carbonate ($F_{1,38}=13.02$; $p<0.001$) and total carbonate ($F_{1,38}=8.81$; $p=0.005$) differ significantly depending on the location of the soil. In the soils from *T. brumale* truffières, the content of active and total carbonates is significantly higher than in *T. melanosporum* truffières (Table 7). Nevertheless, the ANOVAs of the pH and TOC indicate that there are no significant differences in the average values of these variables between the soils inside *T. brumale* truffières versus *T. melanosporum* truffières in contaminated plantations (Table 7).

The Mann–Whitney *U* test shows that significantly higher active carbonate content ($p<0.001$) is found in soils from *T. brumale* truffières than in *T. melanosporum* truffières (Table 7). The abundance of total carbonate ($p<0.001$) shows the same pattern (Table 7); however, the data distribution of total carbonate does not support the assumption of homocedasticity. There are no significant differences in the values of the pH and TOC between the soils inside *T. brumale* truffières versus *T. melanosporum* truffières.

Table 7 Analytical results of soils located inside brûlés with *T. melanosporum* or *T. brumale* carpophore production in three contaminated plantations without and with calcareous amendments (2,500 kg/ha) and three uncontaminated control plantations

N°	Data Table	Province	Study area type	Carpophore production	pH_{H2O}	Active carbonate	Total carbonate	TOC
Mean	Table 2	Alava/Teruel	Contaminated Plantations	*Tuber brumale*	8.16	140.57	405.14	19.95
SD					0.21	29.15	80.36	5.01
Mean	Table 3	Alava/Teruel	Contaminated Plantations	*Tuber melanosporum*	8.08	104.13	315.21	20.44
SD					0.18	34.51	109.15	9.39
Mean	Table 4	Alava	Natural areas	*Tuber brumale*	7.92	123.75	618.04	18.39
SD					0.23	13.77	86.74	2.89
Mean	Table 5	Guadalajara	Natural areas	*Tuber melanosporum*	7.89	25.33	216.56	40.49
SD					0.27	22.44	235.17	35.31

We have monitored the carpophore production of these 4 plantations. After 1 year, we analysed 46 soil samples from *T. melanosporum* and *T. brumale* brûlés (active carbonate, total carbonate and TOC expressed in g kg^{-1})

Discussion

Several authors report that the simultaneous production of the carpophores of various truffle species is distributed within the space of the truffières in a clearly defined manner: *T. brumale*, *T. rufum*, *T. aestivum* and *T. mesentericum* carpophores are frequently collected outside *T. melanosporum* truffières or in their innermost part. Inside the truffières, a time succession of the various truffle species can also be observed: *T. rufum* carpophores are the first to be collected, then *T. melanosporum* and finally *T. brumale* (Montacchini et al. 1972; Falini and Granetti 1998; Callot 1999; Riousset et al. 2001; Ricard 2003; Granetti et al. 2005).

The disappearance of grasses in the brûlés causes modifications in soil surface layers which affect soil organic matter (Lulli et al. 1999; Castrignano et al. 2000). Callot (1999), Ricard (2003) and Granetti et al. (2005) propose that the time succession of *T. brumale* in *T. melanosporum* truffières may be due to the evolution of both the quantity and quality of the organic matter. Our results indicate that in contaminated plantations and natural areas, the soils of *T. brumale* truffières show lower concentrations of TOC than *T. melanosporum* soils (Table 7). However, the statistical tests indicate that these differences in the TOC content are not statistically significant. Therefore, as proposed by Callot (1999) and Ricard (2003), new studies are required to determine the characteristics and properties of soil organic matter in relation with the competition of *T. melanosporum* and *T. brumale* ectomycorrhizas.

The statistical analyses show that the abundance of active carbonate and total carbonate are significantly higher in soils from *T. brumale* truffières compared to *T. melanosporum* both in contaminated plantations and natural areas (Figs. 3 and 4). From these results, it could be deduced that liming would not negatively affect the *T. brumale* ectomycorrhizas in contaminated *T. melanosporum* cultivations.

Calcareous amendments could otherwise play a significant role in boosting production of both *Tuber* species. This proposal is in agreement with the first results of an experimental design that we are developing in two *T. melanosporum* cultures contaminated by *T. brumale* over a period of 4 years. We are administering calcareous amendments, monitoring the production of carpophores and comparing them with two nearby control cultivations which are uncontaminated by *T. brumale* and were given no calcareous amendments. The results obtained in the first year show that *T. melanosporum* production increased by 30–32% and *T. brumale* production increased by 69–274% in the two cultures with calcareous amendments. However, the two control cultivations show that *T. melanosporum* production decreased by 18–26% due to the climatic conditions (García-Montero et al., submitted).

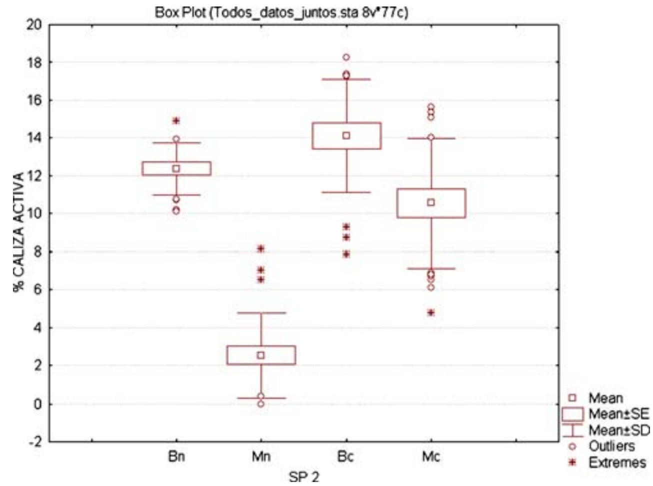

Fig. 3 Average of active carbonate (%) values for comparison of *T. brumale*/*T. melanosporum* soils from burns in natural areas and contaminated *T. melanosporum* plantations. Samples coded with *Bn* are soil samples taken at points with production of *T. brumale* ascocarps in natural areas; samples coded with *Mn* are soil samples taken at points with production of *T. melanosporum* ascocarps in natural areas; samples coded with *Bc* are soil samples taken at points with production of *T. brumale* ascocarps in contaminated plantations; samples coded with *Mc* are soil samples taken at points with production of *T. melanosporum* ascocarps in contaminated plantations

In summary, the statistical patterns of soil carbonates obtained in the study areas show that *T. brumale* ectomycorrhizas appear to be closely linked to calcium carbonate, as is *T. melanosporum*. Therefore, there is no scientific basis to

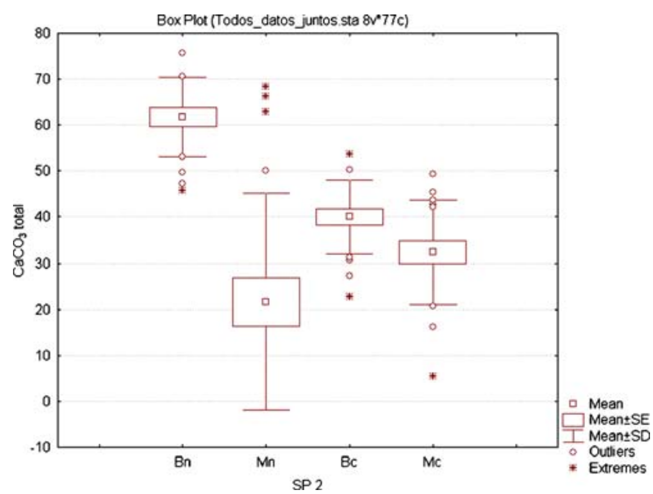

Fig. 4 Average of total carbonate (%) values for comparison of *T. brumale*/*T. melanosporum* soils from burns in natural areas and contaminated *T. melanosporum* plantations. Samples coded with *Bn* are soil samples taken at points with production of *T. brumale* ascocarps in natural areas; samples coded with *Mn* are soil samples taken at points with production of *T. melanosporum* ascocarps in natural areas; samples coded with *Bc* are soil samples taken at points with production of *T. brumale* ascocarps in contaminated plantations; samples coded with *Mc* are soil samples taken at points with production of *T. melanosporum* ascocarps in contaminated plantations

propose calcareous amendments as a means of eradicating *T. brumale* in contaminated *T. melanosporum* orchards.

However, calcareous amendments could increase the production of *T. brumale* and *T. melanosporum* carpophores, thereby increasing the profitability of contaminated orchards. Ricard (2003) also suggests the use of calcareous amendments of fine limestone in truffle culture, although he recommends that they should be used with care and in moderation. New studies are required to look into the effects of fine limestone on the biology of *T. brumale* and *T. melanosporum* ectomycorrhizas.

Acknowledgements We would like to thank Manuel Doñate and Juan María Estrada (Inotruf S.L., Sarrión, Teruel) and Luis María Ibáñez (Alava) for their fieldwork; Paloma Díaz, Cristina Pascual, Margarita, Luis, Miriam and Pablo for their support and Prudence Brooke-Turner for her linguistic assistance. We also thank the Department of Soil Science of the Complutense University in Madrid.

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