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Alkalinity and structure of soils determine the truffle production in the Pyrenean Regions

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Abstract

Aim of study: The program "Typology of truffle stations in the Pyrenean Regions" aimed to define the ecological conditions and culture practices that favor *Tuber melanosporum* growth and fruiting in this area.

Area of study: Navarra, Catalonia, Midi-Pyrénées and Languedoc-Roussillon.

Material and methods: The program was based on the survey of 212 wild and cultivated truffle beds of evergreen oaks (*Quercus ilex*). The data collected in the field consisted of photographs, samples of soil, roots and mycorrhizae, and information on cultural practices followed by truffle growers.

Main results: (i) truffle soils are alkaline, from neutral, dolomitic, to moderately or very calcareous soils; (ii) truffle soils are light, well-structured and stable to water immersion; (iii) mycelium that colonizes roots survives in suboptimal conditions, but it does not necessarily bear ascocarps. Finally our results suggest that *T. melanosporum* is a relatively ubiquitous fungus able to grow, or at least to persist, in a wide range of physical and chemical soil conditions. We propose a probabilistic model of the environment favorable for fruiting, built around a two-dimensional graph with an axis for the chemical conditions, like soil alkalinity, and another axis for the physical conditions, like soil structure.

Research highlights: Soil alkalinity and structure allow to built a convenient representation of the ecological capacity of a place to be good *T. melanosporum* habitat, and thus of the probability for truffle growers to harvest truffles according to the environmental properties of their truffle orchards.

Key words: dolomite; limestone; mycorrhizae; *Quercus ilex;* field survey; *Tuber melanosporum.*

Introduction

Tuber melanosporum is a hypogeous fungus whose ecology remains poorly known despite of producing the famed Black Truffle. Many authors have tried to specify the environmental properties most favorable for *T. melanosporum* development. A common point of agreement is that a truffle soil must be well-structured, porous and aerated (Delmas and Poitou, 1973, 1974; Poitou, 1988a,b; Raglione and Owczarek, 2005). The soil profile must be such that water can flow freely, and excess water can drain out of the top soil layers where roots and fungi live. Grente *et al.* (1974), Callot and Jaillard (1996), Callot (1999) and Weiller (2000, 2002) have observed that the best truffle soils are developed on stony or gravelly substrates, or highly fractured hard rock that ensure drainage of excess water from the soil profile. Air must be able to circulate freely, preventing hypoxic zones, even if they are lo-

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cal or temporary. From the analytical point of view, soil texture discriminates well soils that produce truffles from those that do not. Soils too sandy are not adequate because of their poor water retention capacity, and neither are heavy clay soils (with > 460 g kg^{-1}) because of elevated compaction (Raglione *et al.,* 2001). However, high levels of clay can support *T. melanosporum* growth depending on stoniness, organic matter and biological activity of soil, all of which help aeration. In fact, wild truffle beds are formed in almost every type of texture (Delmas *et al.,* 1981; Bencivenga and Granetti, 1988). Common soil textures used in the cultivation of black truffle are loam soils: sandy loam, clay loam, silty loam, sandy clay loam (Delmas and Poitou, 1973; Grente and Delmas, 1974; Reyna, 2000), but the preferred textures are loam, sandy loam and sandy clay loam (Colinas *et al.,* 2007). This set of properties favors the hypogeous development of the truffle ascocarps. Truffle fruiting bodies grow and mature for nearly nine months in soil. The surrounding soil must be moist enough for the ascocarp to get the necessary water, as well as well aerated for the ascocarp and mycelial hyphae that surround it to be able to respire and grow.

A second point is that the soil must be calcareous, or at least present a calcareous fraction even though the fine soil may not be carbonated. The pH of truffle beds varies between 7.0 and 8.85 (Bencivenga and Granetti, 1988; Sáez and De Miguel, 1995; García-Montero *et al.,* 2007b). The few authors who have measured active carbonate, frequently report low levels, on the order of only a few tens of grams per kg of soil, with an average of 30 g kg^{-1} of soil (Ourzik, 1999; García-Montero *et al.*, 2007b), but active carbonate can reach more than 80 g kg^{-1} of soil (Delmas and Durand, 1971; Delmas, 1973a,b; Montacchini *et al.*, 1977; Delmas *et al.*, 1981; Bencivenga, 1986; Bencivenga and Granetti, 1988; Bencivenga *et al.*, 1990; Sourzat, 1997; Reyna, 2000; Raglione *et al.,* 2001). In the field, Grente *et al.* (1974), Callot and Jaillard (1996), Callot (1999) and Weiller (2000, 2002) have noted that the most productive truffle beds were leached soils, or at least soils that had lost carbonate. These soils are brown to red, because of the mobilization of iron or manganese (a process called "browning", possibly followed by a "reddening" in Mediterranean climate) (FAO, 2006). Conversely, Sourzat (2001) and Jaillard *et al.* (2008) found truffles grown on acidic soils (on granite or gneiss) whose upper horizons were carbonated by human practices and had less than 20 g active carbonate kg^{-1} of soil. These data led some authors (Jaillard *et al.,* 2007, 2008; García-Montero *et al.*, 2007a, 2009a) to assume that the presence of active carbonate is critical for truffle production, even if it is in small amounts.

A last point would be that the soil of truffle beds must have an important organic component, the organic matter being well decomposed and stabilized (C/N around 10) (Poitou *et al.,* 1983). Yet, we know that the amount of organic matter in truffle bed soils varies considerably ranging from 8 to 174 g kg^{-1} (Delmas *et al.,* 1981; Bencivenga and Granetti, 1988). Callot and Salducci (1997, 1998) showed that *T. melanosporum* grew better on soils with stabilized organic matter. However, soil organic matter management in truffle beds or orchards has not been the subject of many studies.

Data on *T. melanosporum* ecology are abundant in the literature, but many are based on detailed studies conducted on single plots. Quantitative data are not as abundant as desirable, and it is difficult to have a general overview of environmental conditions favorable to truffle growth. Bratek *et al.* (1999) and Raglione *et al.* (2001) conducted large studies on 150 and 350 truffle plots in the Carpathian and Appennine regions, respectively. They showed that the most discriminating variables were pH, carbonate and organic matter contents of soils. Next would be the textural and structural properties of soils, and the mobility of iron and manganese. More recently, Alonso-Ponce *et al.* (2010, 2013) studied 924 truffle beds in Catalonia and Aragon to specify climatic conditions influencing the presence and fruiting of black truffle. These studies suggested that the truffle ecosystem has a relatively narrow optimum. However, that deserves to be confirmed in other truffle regions.

Our study aimed to define more specifically the ecological conditions and culture practices that favor *T. melanosporum* growth and fruiting in the Pyrenean regions, with a particular focus on the parameters related to soil alkalinity. The Pyrenees is a mountain range consisting of ancient sediments, limestone, dolomite and schist, alternating in layers of moderate thickness. This alternating acid and alkaline sediments create a mosaic in which alkalinity varies substantially, from an acidic end represented by granites and schist to a highly alkaline end represented by marly limestone and marl. The specificity of the Pyrenees is thus a wide range of soil conditions that we have sought to include in the collection of truffle beds surveyed.

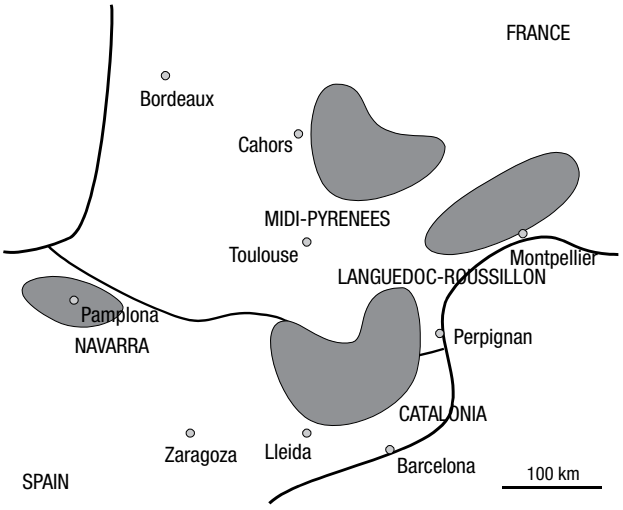

Figure 1. Geolocation of truffle beds surveyed. The study was based on the survey of 212 wild and cultivated evergreen-oak truffle beds located in the Pyrenean Regions: 15, 30, 29 and 138 beds in Navarra and Catalonia (Spain), Midi-Pyrénées and Languedoc-Roussillon (France), respectively.

Material and methods

Truffle beds survey

The study was done in the framework of an inter-regional research program (Jaillard *et al.*, 2013; Oliach *et al.*, 2013). It was based on the survey of 212 wild and cultivated *Quercus ilex* truffle beds located in the Pyrenean Regions: 15, 30, 29 and 138 beds in Navarra, Catalonia, Midi-Pyrénées and Languedoc-Roussillon, respectively (Fig. 1). The climate of Pyrenean Regions is Atlantic in the West to Mediterranean in the East with rainfall ranging from 1,000 to 600 mm respectively. The truffle orchards studied were larger than 2,500 m^2 and had been planted for 5 years at least. Some, but not all, were or had been productive. The survey included also 24 wild truffle beds. In the survey, we collected data on the environment and history of the sites, the seedling source and mycorrhization of plants, the planting techniques, and the cultural practices conducted by the growers including soil improving and tillage, mulching and irrigation, tree pruning and truffle harvest. Samples of soil, roots and mycorrhizas were collected in the field. The survey began in winter 2009 and ended in late spring 2010. The protocols remained the same throughout the survey, except those concerning the analyses of soil physical properties because they were not initially planned: the soil structure measurement was decided during the survey when it became clear that it was likely to be one of the most important ecological parameters.

Soil sampling and physical analysis

Soil was sampled between rows of the truffle beds. Each soil sample was a composite of five elemental samples collected in the top 5-15 cm of the soil profile. Soil samples were air-dried, then sieved on 4 mm and 2 mm meshes. The ratios of the soil fraction larger than 4 mm (4 mm < x), comprised between 2 and 4 mm (2 < x < 4 mm) and smaller than 2 mm (x < 2 mm) were visually estimated (at ± 5%). The 4 mm < x fraction was divided into stone fraction (4 mm < stones) and aggregated soil fraction (4 mm < aggregates). The structure stability of soil immersed in water was measured in the 2 < x < 4 mm soil fraction according to the method of Le Bissonnais (1996) (ISO, 2011). A subsample of 5-10 g of 2 < x < 4 mm aggregates was weighted, then placed for 10 min in a beaker filled with 50 ml of water without stirring. The water was then removed using a pipette, and the broken aggregates were transferred to ethyl alcohol to stop the disaggregation process. The broken aggregates were air dried, then sieved on 2, 1, 0.5, 0.2, 0.1 and 0.05 mm meshes. The mean diameter of aggregates stable in water was calculated as the geometrical mean size of aggregates broken by the immersion in water, *i.e.* the sum of different size classes, weighted by the mass ratio of broken aggregates within each size class.

Soil chemical analysis

Soils were analyzed for their chemical properties with a particular focus on the parameters related to soil alkalinity: pH, contents of limestone and contents of reactive calcium and magnesium extracted with different extractive solutions. The analyzed soil properties were therefore pH in water (1:5) and pH in KCl 1 M (1:5, NF ISO 10390), contents of total limestone (total-$CaCO_3$, extracted by HCl, NF ISO 10693) and active limestone (active-$CaCO_3$, extracted by NH_4-oxalate, NF X31-106), cation exchange capacity and contents of cations extracted by NH_4-acetate at pH 7.0 (Metson-CEC, -K, -Mg and -Ca, NF X31-130 and NF X31-108) and contents of metals extracted by EDTA (EDTA-Cu, -Zn, -Mn, -Fe, -Mg and -Ca, NF X31-120). The x < 2 mm fraction was chemically analyzed by the

Laboratoire Centre-Atlantique (LCA, 1 rue Samuel Champlain, 17974 La Rochelle Cedex, France).

Roots and mycorrhizas sampling and preparation

In each truffle bed, a holm oak if possible with a visible burn, was randomly chosen. The soil and root sample from the burn resulted from the mixture of four elemental samples, collected in the 5-15 cm top soil. Four 25 × 25 cm holes located on two orthogonal axes were dug in the interior side of the edge of burn. The sample of soil and all the roots were collected, placed in a plastic bag and stored at 4°C before analysis. In the laboratory, the roots were first sorted and washed with tap water. Fine roots were observed under a stereo-microscope and four ectomycorrhizas showing *Tuber* characteristics (color, morphology and size) were randomly collected and placed in microtubes for analysis. If no *Tuber* ectomycorrhizas were observed, then four root tips were randomly collected for molecular analysis. At the end, the roots were placed on a plate and classified according to their mean diameter visually estimated under a stereo-microscope.

Molecular analysis: DNA extraction and PCR amplification conditions

For each ectomycorrhiza or root tip sample, total genomic mycorrhizas DNA was extracted according to the method of Paolocci *et al*. (1999). PCR-multiplex amplification was carried out on a Thermocycler Electron (Thermal cycler Px2) in mixture containing 1 µL DNA, 2.5 mM $MgCl_2$, 200 µM for each dNTP, 0.8 µM of each primer and 1 U of Taq polymerase (Thermo-start Taq), supplemented to 20 µL with BSA (0.5%). The species-specific primers used were MELF/MELR for *T. melanosporum* and SYLV1/SYLV2 for *T. brumale* (Douet *et al*., 2004), and BTTA-F/BTAemb-Rev for *T. aestivum* (Schiaffino *et al*., 2006). The cycling conditions were: 15 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 59°C, 1 min at 72°C and 10 min at 72°C. We amplified the extracted DNA with the universal primers ITS1f/ITS4 (White *et al.,* 1990) to verify that there is no PCR reaction inhibition when the PCR-multiplex was negative. The PCR-amplification was carried out in a mixture containing 1 µL DNA, 1.5 mM $MgCl_2$, 100 µM for each dNTP, 2.5 µM of each primer and 1 U of Taq polymerase (Thermo-start Taq) supplemented to 20 µL with BSA (0.5%).

Statistical analysis

The statistical analysis was performed using the R software (R Development Core Team, 2008). The agglomerative hierarchical clustering was performed using the "cluster" package with the "Manhattan" metric on standardized values of raw analytical data. The mean comparisons were performed using Kruskall-Wallis test when the variable distribution was not normal or the sample size was too small to determine the variable distribution. The comparisons of proportions were performed using a binomial test.

Results

Soil classification and location of wild and cultivated truffle beds

We classified the soil types studied during the survey by means of hierarchical clustering on chemical soil properties. A partitioning in four clusters put in evidence the structure of the Pyrenean truffle soils (Fig. 2). A first cluster, noted A, corresponded to slightly acidic soils. The lime content was negligible or null (total-$CaCO_3$ = 2 ± 5 g kg^{-1}), likely in form of calcareous sands since active carbonate is null (active-$CaCO_3$ = 0 ± 0 g kg^{-1}). Water-pH ranged form 5.5 to 8.0 (water-pH = 6.3 ± 0.8), and the exchange complex was saturated by the divalent Mg and Ca cations (Metson-(Mg+Ca)/CEC ratio = 1.0 ± 0.3 $mol_c\ mol_c^{-1}$). The slightly acidic soils had developed mainly on granite and schist in the Eastern Pyrenees and on sandstone in Gard, France. They represented less than 8% of the surveyed truffle beds.

A second cluster, noted B, corresponded to dolomitic soils. The lime content was low (total-$CaCO_3$ = 55 ± 75 g kg^{-1}) and active carbonate was lower than 1% (active-$CaCO_3$ = 4 ± 7 g kg^{-1}). Water-pH was close to 8 (water-pH = 7.9 ± 0.3). The exchange complex was saturated by the divalent Mg and Ca cations, but the Metson-(Mg+Ca)/CEC ratio was higher than one (Metson-(Mg+Ca)/CEC ratio = 1.7 ± 0.5 $mol_c\ mol_c^{-1}$), indicating that NH_4-acetate mobilized a part of the reactive Mg and Ca of the soil (Ciesielski and Sterckeman, 1997). Another specific feature of this cluster was the relative high content of Mg extractable by NH_4-acetate as well by EDTA (Metson-Mg/Ca ratio = 0.18 ± 0.11 $mol_c\ mol_c^{-1}$

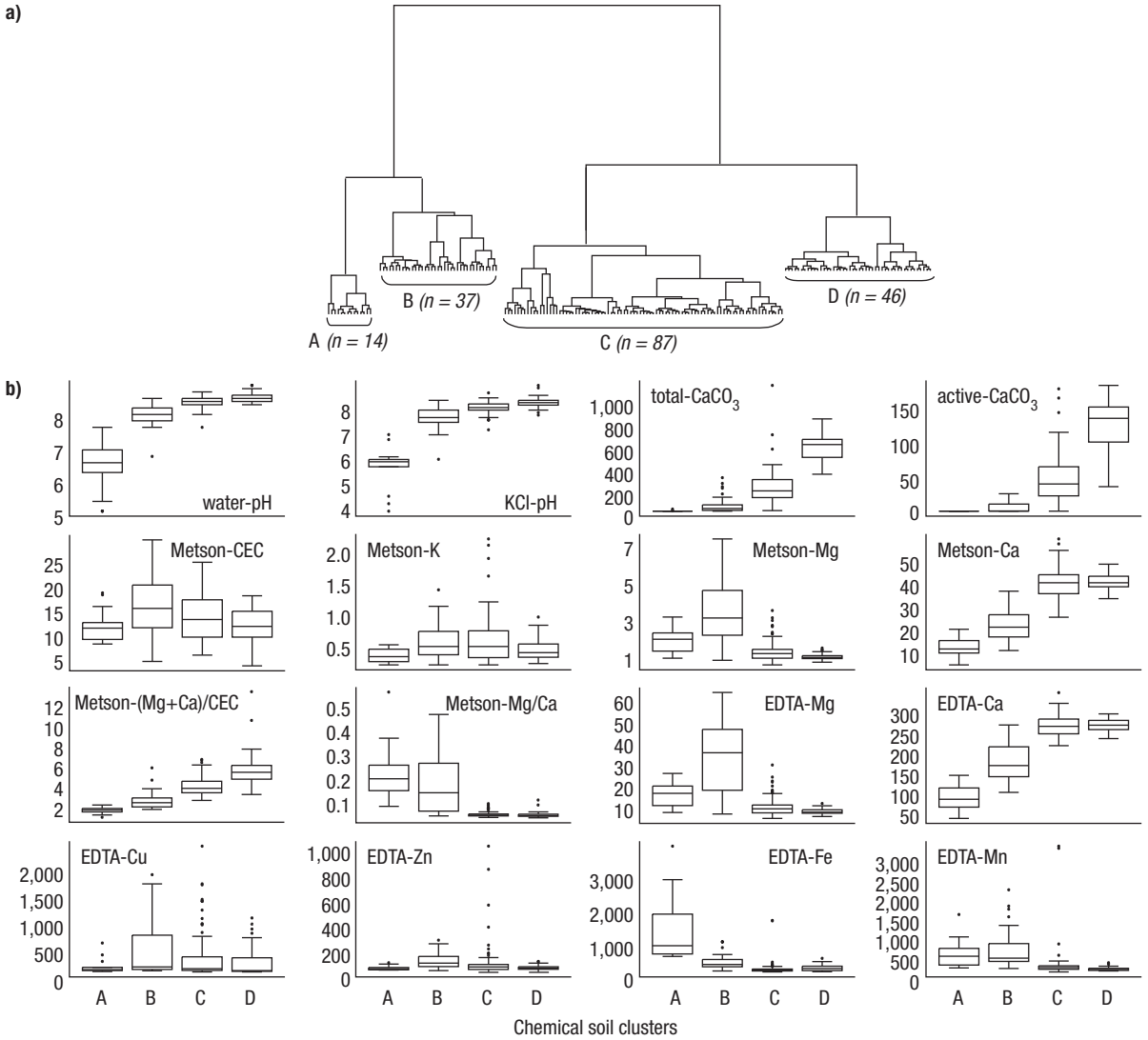

Figure 2. Agglomerative hierarchical clustering of truffle bed soils based on their chemical properties: dendrogram (a) and boxplots of soil properties by cluster (b). Soils were analyzed for their chemical properties with a particular focus on the parameters related to soil alkalinity: pH in water (water-pH) and in KCl (KCl-pH), contents of total lime (total-$CaCO_3$, g kg^{-1}) and active carbonate (active-$CaCO_3$ extracted by NH4-oxalate, g kg^{-1}), soil cation exchange capacity (Metson-CEC, mol_c kg^{-1}) and contents of cations extracted by NH_4-acetate (Metson-K, -Mg and -Ca, mol_c mol_c^{-1}), contents of metals extracted by EDTA (EDTA-Mg, -Ca, -Cu, -Zn, -Fe and -Mn, mg kg^{-1}). The agglomerative hierarchical clustering was performed on the standard scores of these raw data, using "Manhattan" metric. The four A, B, C and D chemical soil clusters corresponded to slightly acidic soils (8%), dolomitic soils (20%), moderately calcareous soils (47%) and very calcareous soils (25%), respectively. Both Metson-(Mg+Ca)/CEC and Metson-Mg/Ca (in mol_c mol_c^{-1}) parameters were calculated *a posteriori* for their ability to discriminate the soil clusters well. They were not used for clustering.

and EDTA-Mg/Ca ratio = 0.21 ± 0.15 mol_c mol_c^{-1}). The dolomitic soils were lying mainly on dolomitic limestone of the Eastern Pyrenees and Herault, France. They represented 20% of surveyed truffle beds.

The third and fourth clusters corresponded to calcareous soils that represented more than 72% of all surveyed soils. The soil water-pH was higher than 8.0 and Metson-(Mg+Ca)/CEC ratio was higher than 2 mol_c mol_c^{-1}. These calcareous soils were split in moderately calcareous soils (cluster C, with water-pH = 8.3 ± 0.2, total-$CaCO_3$ = 210 ± 150 g kg^{-1} and active-$CaCO_3$ = 46 ± 35 g kg^{-1}) and very calcareous soils (cluster D, with water-pH = 8.4 ± 0.2, total-$CaCO_3$ = 570 ± 120 g kg^{-1} and active-$CaCO_3$ = 125 ± 36 g kg^{-1}). If water-pH were equal, the divalent Mg and Ca cations Metson-(Mg+Ca)/CEC ratio increased from 3.4 ± 1.2

$mol_c\ mol_c^{-1}$ in moderately calcareous soils to 4.1 ± 1.8 $mol_c\ mol_c^{-1}$ in very calcareous soils, as increasing active limestone content (Ciesielski and Sterckeman, 1997). Calcium was the main divalent cation and the Mg/Ca ratio was consequently low (Metson-Mg/Ca = 0.02 ± 0.01 $mol_c\ mol_c^{-1}$ and EDTA-Mg/Ca = 0.03 ± 0.02 $mol_c\ mol_c^{-1}$). The moderately calcareous soils represented 47% of surveyed truffle beds although very calcareus soils represented less than 25% of surveyed truffle beds.

Fig. 3 shows the proportions of wild, productive and non-productive planted truffle beds according to the soil clusters. In the whole collection, wild truffle beds represented 9% of surveyed truffles beds, productive truffle beds were 69% and non-productive truffle beds less that 22% of all the surveyed truffles beds. But these proportions were different according to chemical soil clusters. On dolomitic soils (cluster B), the proportion of wild truffle beds was much higher (46%) than the mean value (9%) since the proportion of non-productive planted beds was much lower (9%) than the mean value (22%). At the opposite, on very calcareous soils (cluster D), the proportion of wild truffle beds was lower (6%) than the mean value (9%) since the proportion of non-productive planted beds was much higher (45%) than the mean value (22%). Consequently, the ratio of all productive, wild and planted, truffle beds was higher than 91% on dolomitic soils (cluster B), while it was lesser than 55% on very calcareous soils (cluster D). In regard to planted truffle beds only, of highest interest for truffle growers, the ratio of planted productive truffle beds were 83% and 46% on dolomitic and very calcareous soils, respectively. These observations suggest that, in the Pyrenean Regions, dolomitic soils were the most favorable and that very calcareous soils were the least favorable for truffle production.

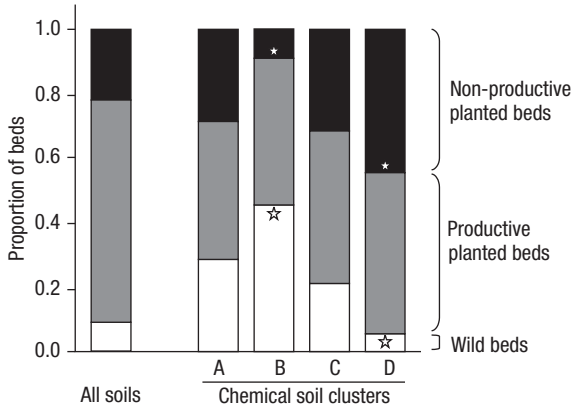

Figure 3. Productivity of truffle beds *versus* chemical soil clusters. Truffle beds were classed as (non-planted) wild beds, non-productive planted beds and productive planted beds based on data supplied by the truffle growers. The proportion of wild truffle beds was highest (46%) on dolomitic soils (B chemical soil cluster) and lowest (6%) on very calcareous soils (D chemical soil cluster). On the contrary, the proportion of non-productive planted beds was highest (45%) on very calcareous soils (D chemical soil cluster) and lowest (9%) on dolomitic soils (B chemical soil cluster). The proportions were compared to the mean values using the non-parametric Kruskall-Wallis test: a star indicated a proportion significantly different from the mean value (p-value < 0.05): white star for wild beds, and black star for non-productive planted beds.

Content of available metals extracted by EDTA were measured to check possible toxicity and verify a possible relation with truffle production (Fig. 2). The contents of Cu, Zn and Mn were not significantly different between soil clusters. The content of Fe is higher in slightly acidic soils of cluster A. There was no relation with truffle production. On the other hand, the contents of Mg and Ca extracted by EDTA were measured for comparison with NH_4-acetate extraction (Fig. 2): EDTA and NH_4-acetate extractions gave similar qualitative results.

Root morphology and mycorrhizas ratios

The results of roots and mycorrhizas sampling are showed in Figs. 4 and 5, respectively. In general, roots

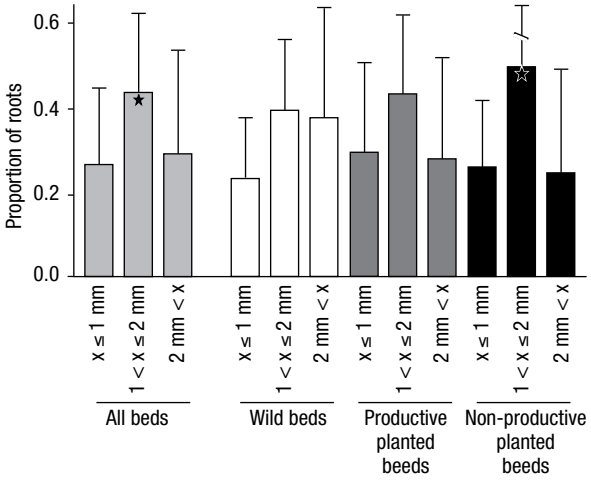

Figure 4. Proportion of roots of evergreen-oak beds in soil bends samples collected inside burns. Samples collected inside the burn of evergreen-oak had a mean volume of 25 liters. All roots were sorted in three classes ($x \leq 1$ mm, $1 < x \leq 2$ mm, $2\ mm < x$) according to their mean diameter visually estimated under a stereo-microscope. The proportions of roots of different diameters were compared using the non-parametric Kruskall-Wallis test: a star indicated a proportion significantly different from the mean value (p-value < 0.05).

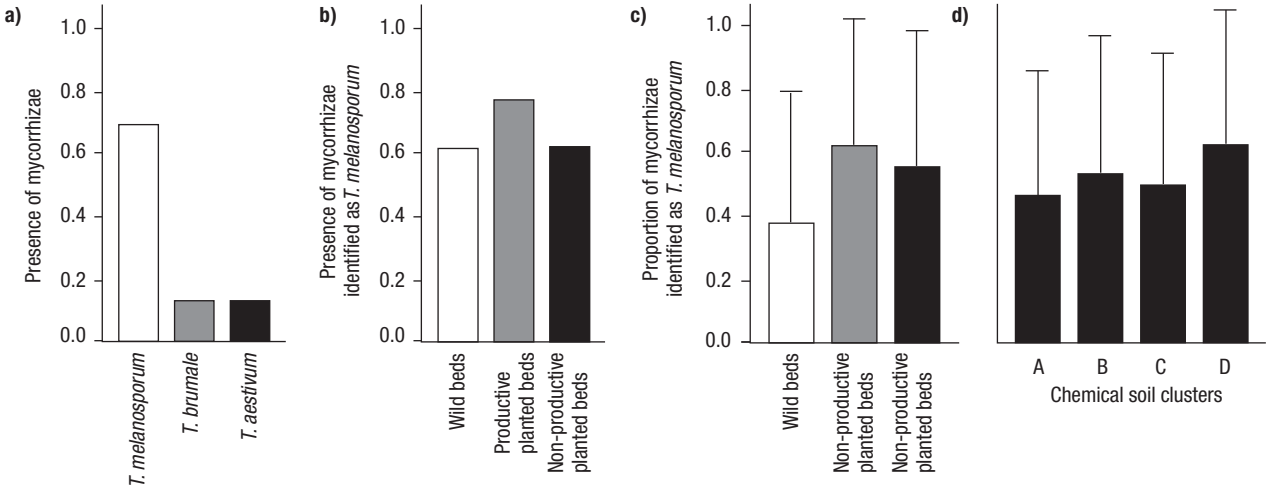

Figure 5. Presence and proportion of mycorrhizas identified as *Tuber melanosporum, T. brumale* and *T. aestivum.* Presence of at least (a) a mycorrhiza of *T. melanosporum, T. brumale* or *T. aestivum* in truffle beds, or (b) a mycorrhiza of *T. melanosporum* according to the productivity of truffle beds. Proportion of mycorrhizas of *T. melanosporum* according to the productivity of truffle beds (c), or the soil clusters (d). The proportions of presence were compared using the binomial test, the proportions of mycorrhizas using the non-parametric Kruskall-Wallis test: neither presence nor proportion of mycorrhizas of *T. melanosporum* were significantly different from the mean value (p-value < 0.05).

1-2 mm in diameter were more abundant than roots either less than 1 mm or more than 2 mm in diameter (Fig. 4). On the other hand, the presence (as a binary variable) or the ratios of mycorrhizas of *T. melanosporum* were not related to the productivity of truffle beds (Fig. 5): *T. melanosporum* was identified in 69% of sampled oaks (Fig. 5a), and the mean proportion of sampled mycorrhizas identified as *T. melanosporum* was 53 ± 42% (Fig. 5c). The presence ratios and the proportions of sampled mycorrhizas identified as *T. melanosporum* did not vary with either the productivity of truffle beds (Fig. 5b and c) or the soil cluster (Fig. 5d). *T. brumale* and *T. aestivum* were mainly detected in moderately calcareous soils, but the number of observations is too small to be significant.

Soil structure and stability

During the survey, soil structure progressively appeared as a likely important determinant of truffle productivity. A first idea was to determine the structure stability of soil. This determination needs the sieving of soil samples for obtaining aggregates of size comprised between 2 and 4 mm. All the 212 surveyed soils were so sieved and their structural stability was determined. However, a second idea resulting from the observation was to estimate during the sieving the proportions of soil passing through the 4 and the 2 mm meshes. These physical soil properties were only measured on a subset of the whole soil collection of the survey. The subset was composed of 43 soils, 11, 13, 9 and 10 from chemical soil clusters A, B, C and D, respectively. Statistical pairwise t-tests showed that the mean properties of the subset and the initial set were not different. Fig. 6 shows that more than 60% of soil samples passed through the 2 mm mesh and 20% in average are structured in 2-4 mm aggregates. That suggested that, in general, soils had a fine sandy or

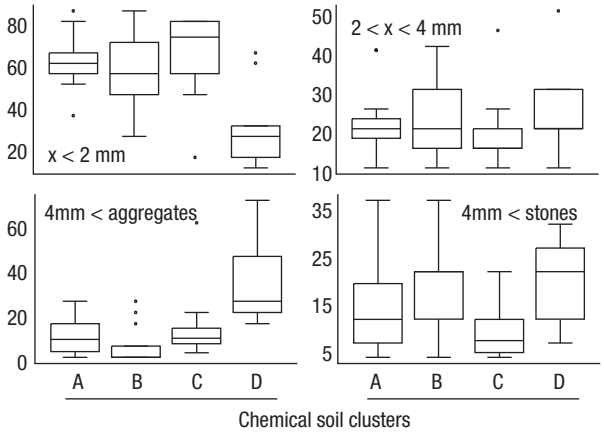

Figure 6. Coarse granulometry of soil clusters. Soil samples were sieved at 4 and 2 mm to collect soil aggregates. The ratios of each fraction were evaluated visually: x < 2 mm, 2 < x < 4 mm and 4 < x mm. The last oversize fractions were separated in 4 mm < aggregates and 4 mm < stones.

pseudo-sandy structure. However, the soil samples of cluster D, *i.e.* very calcareous soils and the least productive truffle beds, had a contrasting behavior: less than 30% in average passed through the 2 mm mesh. Soil cluster D contained a higher ratio of coarse soil aggregates of size over 4 mm than other clusters: it was higher than 20% in average and reached up to 60% of soil samples.

Fig. 7a shows the results of the structure stability of 2-4 mm soil aggregates immersed in water for 10 minutes. A hierarchical classification of the results made it possible to divide the truffle bed soils in three clusters (Fig. 7b). A first cluster, noted α corresponded to soils very stable in water, according to the scale of Le Bissonnais (1996). An examination of washed 2-4 mm aggregates revealed they were mainly gravel soils from the Eastern Pyrenees: measured stable aggregates corresponded indeed to stone gravels. Only 41% of these soils are truffle productive, less than the mean value of 60% in the sub-sample. A second cluster, noted β, corresponded to soils that were stable to immersion in water. The coarse 2-4 and gave smaller aggregates of size 1-2 mm. These soils had coarse pseudo-sandy structure. More than 73% of these soils corresponded to productive truffle beds. A third cluster, noted γ, corresponded to soils that were moderately stable to unstable in water. The coarse 2-4 mm aggregates were completely collapsed by water and gave fine soil pseudo-particles of size comprised between 0.5 and 0.05 mm. Less than 50% of these soils were truffle productive. Fig. 7a shows that the soils corresponding to the three clusters differed mainly by the proportion of 2-4 mm aggregates stable in water: more than 60%, between 30 and 60%, and less than 30% for clusters α, β and γ respectively.

Discussion

Enough data for statistical approach

Bratek *et al.* (1999) and Raglione *et al.* (2001) had studied 150 and 350 truffle beds, respectively. The number of truffle beds surveyed here is of the same order: it was large enough for sound statistical analyses with a good accuracy. The statistical methods based on regression analysis gave no significant results with our data: this led us to prefer an approach based on agglomerative hierarchical classification for identifying more homogenous groups that can then be compared between them. Regression analysis is generally used when the response variables and explanatory variables vary monotonously: its failure suggested that the development and the fruiting of *T. melanosporum* are multi-factorial processes, and that the measured variables were not the most discriminant in regard to

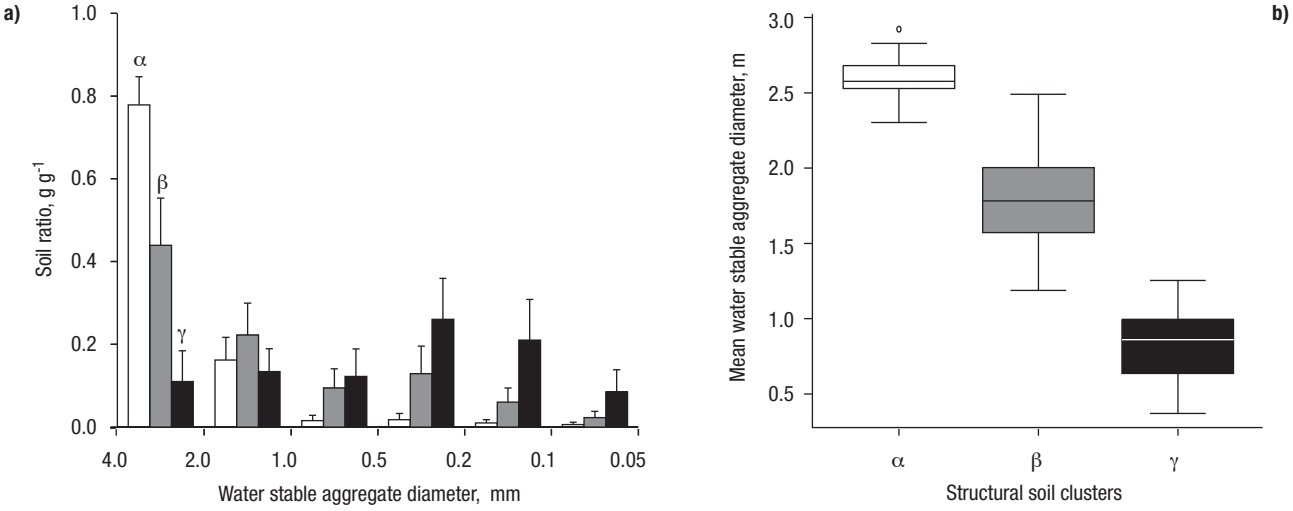

Figure 7. Soil structure stable in water of truffle beds. (a) Granulometry of aggregates after immersion in water (fast wetting without stirring) of 2-4 mm soil aggregates. (b) Mean weighted diameter of water stable aggregates of the three α, β and γ soil clusters determined by hierarchical clustering of aggregates granulometry after immersion in water. The structure stability was measured on 172 soil samples. The α, β and γ soil clusters corresponded to water very stable, stable and unstable soils, respectively. In our sample, very stable soils were mainly gravel soils. Stable soils give pseudo-sandy soils after immersion in water. Unstable soils disaggregated in water. The proportions of productive truffle beds in α, β and γ soil clusters were 41, 73 and 51%, respectively.

the studied processes. This fact implies that several hundreds of truffle beds is a number just sufficient to draw general conclusions because the whole sample collection should be split in smaller sub-samples to allow a pertinent analysis.

Alkaline rather than calcareous soils

Hierarchical classification based on soil chemical properties of truffle beds can distinguish four groups of soils: slightly acidic soils that are rather rare in the context of this study, dolomitic soils that represent a fifth of soils studied, and a large majority of more or less calcareous soils. However, 28% of the surveyed truffle beds were not calcareous, including soils developed from acidic rock and dolomitic limestone. Our results show that, in the Pyrenean Regions, the most calcareous soils bear the least productive truffle beds, while dolomitic soils, that do not contain reactive or cold soluble carbonate, have the most productive truffle beds. Finally, more than half of soils considered as acidic are truffle productive. We believe that the traditional perception of limestone soils as good truffle soils should be revised in the light of these results.

This observation has several consequences. First, it implies that the contents of total or active carbonate are not relevant descriptors of truffle habitat as assumed by many authors (Sourzat, 1997, 2008; García-Montero *et al*., 2007a; Callot, 1999; Jaillard *et al*., 2007, 2008). The analysis of our data shows that other descriptors would be more relevant. The pH is often used, but it has the disadvantage of an asymptotic increase in the alkaline range of interest: it reaches the ceiling of 8.25 that is the water-pH value of a soil solution in equilibrium with lime. The ratio between the sum of divalent cations and cation exchange capacity, *i.e.* (Mg+Ca)/CEC, has the advantage of a gradual variation from acid soils whose exchange complex is not or just barely saturated, up to highly calcareous that contain high level of reactive carbonates of magnesium or calcium (Ciesielski and Sterckeman, 1997). Dolomitic soils fit easily in this scale because their exchange complex is saturated and may contain a fraction of magnesium or calcium that are soluble and reactive. This index seems a good estimator of the total alkalinity of soil, better than pH. In addition, it avoids the nature of the soil minerals that support the alkalinity, and takes into account the difference of cation exchange capacity between soils. Finally, Metson "exchangeable" cations and cation exchange capacity are commonly and inexpensively measured in routine soil analysis. In truffle ecology, the (Mg+Ca)/CEC ratio as indicator of alkalinity should consequently be preferred to other chemical descriptors of soil.

This observation implies also that, contrary to the opinion of several authors (Callot, 1999; García-Montero *et al*., 2009a,b; Demerson, 2012), the limestone of truffle soils may not play a very direct role in the development of the mycelium or fruiting of ascocarps of *T. melanosporum*. If this was the case, all truffle soils should be exclusively calcareous. Yet, the lime content of soil is a major determinant of its alkalinity, and our results show that this last parameter is likely the best indicator of soil chemical conditions favorable for truffle fruiting. That truffle soils are not necessarily calcareous implies that acidic soils can become truffle beds by natural or artificial carbonatation. *T. melanosporum* harvest in acidic environments has already been described in the literature (Sourzat, 2008, 2012; Jaillard *et al.*, 2008). The development of truffle culture in Australia, New-Zeland, Chile and the United-States (Sourzat, 2012), has been largely based on this principle. As part of our investigation, some of the wild truffle beds observed were grown on soils whose exchange complex was just close to saturation. Valverde-Asenjo *et al*. (2009) underlined that active carbonate in soil is a factor easy to manage, and some Pyrenean truffle growers are thinking of mimicking this natural process by reasonable liming of their truffle beds.

Finally, our study highlights that very calcareous soils may not be the best for truffle production, mostly when compared to dolomitic soils with an active carbonate content of 4 ± 7 g kg^{-1}. This result reinforces the observations reported by García-Montero *et al*. (2007a,b, 2009a,b) or Jaillard *et al*. (2007) that suggested that best truffle beds frequently had low active carbonate, on the order of only a few tens of grams per kg of soil. Interestingly, Valverde-Asenjo *et al*. (2009) recently analyzed in Aragon 77 truffle beds colonized by *T. melanosporum* and *T. brumale:* they showed that the active carbonate content of *T. melanosporum* beds was lower in wild than in cultivated soils, *i.e.* lower than 30 g kg^{-1} and higher than 100 g kg^{-1} in average, respectively. This suggests that truffle growers plant truffle trees in soils even more calcareous than wild truffle soils. On the other hand, Raglione *et al.* (2001) showed that the levels of iron and manganese extracted by EDTA discriminated soils producing

T. melanosporum from soils producing *T. brumale* or *T. aestivum*. Our study shows that iron and manganese contents were not determinant. However, our results should be considered in the context of the Pyrenean Regions, that did not include all types of truffle soils, for instance only a few cambisols (as terra rossa) or leptosols (as rendzinas) (FAO, 2006) were sampled in our survey.

Soils with a water stable structure

Visiting more than 200 truffle beds led us to visually observe that the places identified by the truffle growers as the most productive were usually characterized by a particular pseudo-sandy structure, an organic-origin lumpy structure or a subangular blocky structure generated by a significant clay or oxyhydroxide content. Moreover, even in dry conditions, the soils could yet be dug easily by hand or with a small pocket-knife. This observation has led us to perform a complementary analysis of our soils to determine the cohesion or structural water stability of soil aggregates: unfortunately we could only perform this test on a subset of our samples.

The results obtained were less contrasted than expected, but they confirmed our field observations. Our sampling of truffle beds included a large group of gravelly soils, of which the fraction between 2 and 4 mm is mostly gravels glued with few other inorganic or organic particles: gravels are not soil aggregates, then water test says nothing about the stability of fine soil aggregates. In the future, these soil samples grouped in cluster α could be separated by measuring the gravel content in the coarsest 2-4 mm and 1-2 mm aggregate fractions. Our results show that other soil samples can be divided into clusters β and γ. Cluster β corresponds to very stable aggregates in water, of a size between 1 and 2 mm, while cluster γ corresponds to unstable aggregates that break down in water into soil particles of size between 50 and 500 µm. Cluster β is very productive, while cluster γ is only moderately productive. This property confirms the field observations that soils favorable to truffle production have a rather granular structure, which makes them light, soft and easy to dig by hand. Finally our results show that the most productive soils were those whose structure is sandy or pseudo-sandy with aggregates smaller than 4 mm that remain stable during fast immersion in water. On the contrary, soils whose structure is blocky and with aggregates larger than 4 mm that collapse in water, were the least productive. Indeed, unstable soils had the annoying property to solidify during drying and to collapse during wetting, while stable and well-structured soils stay granular and thus soft and uncohesive regardless of the moisture conditions.

The structure of truffle soils is a parameter often mentioned. Raglione *et al.* (2001) also identified a sandy texture and a structure parameter to discriminate soils favorable to *T. melanosporum*. Sourzat (1997, 2002, 2012) also recommend a regular tillage in surface to keep the soil soft and well-structured. However, this parameter is seldom measured and we lack quantitative references on the subject. Bragato (1997), Lulli *et al.* (1999), Castrignano *et al.* (2000) and Bragato *et al.* (2001) have measured the soil structure at the scale of a truffle bed, according to the method set up by Oades and Waters (1991), *i.e.* prewetting then gentle wet sieving of aggregates. Lulli *et al.* (1999) showed that soil structure was granular and aggregate size was 1-2 mm near the soil surface. Castrignano *et al.* (2000) studied the same truffle bed by using geostatistical analysis: they showed that the spatial pattern of the probability of finding a productive burn was related to the soil structure, suggesting that *T. melanosporum* may prefer a soft and well-aerated soil environment to grow and fruit. Bragato *et al.* (2001) show that, inside the burn, soil aggregate size decreased and the conditions were more oxidative than outside: this pattern was related to a 50% decrease of total organic C and microbial biomass C, assuming that the disappearance of herbaceous cover in the burn and the increase in soil macroporosity might increase air flow in soil surface layers (Bragato, 1997). Our data on structure stability in water of soil aggregates of truffle beds confirm the results reported by these authors. However, according to our data, this is only a trend, a higher probability, but it is not a strict requirement for the production of truffles.

Tuber melanosporum, a relatively ubiquitous fungus

All ecological data show that the best habitat of *T. melanosporum* is still poorly defined. Bratek *et al.* (1999) reported that the water-pH of soil in which *T. melanosporum* grows is between 6.8 and 9.0, with an average of 7.9. Raglione *et al.* (2001) reported equally broad ranges of the various parameters measured.

Sourzat (2002, 2012) described *T. melanosporum* across diverse environments. Our own results show that the fungus can persist as mycorrhizas in slightly acidic soils as well as in highly calcareous and alkaline soils. Taken together, these data suggest that ultimately *T. melanosporum* is a relatively ubiquitous fungus able to grow, or at least to survive, in a wide range of physical and chemical conditions whose optimum is difficult to identify. This difficulty is enhanced by the meteorological (Alonso Ponce *et al.,* 2010, 2013) and biological conditions that are favorable to it, and that we do not know in depth. Moreover, the reasons why *T. melanosporum* needs alkaline and water stable soils to grow and fruit are not known. This lack of knowledge has led us to propose a probabilistic approach of the favorable conditions for fruiting *T. melanosporum*.

Fig. 8 summarizes our two-dimensional model that defines areas of probability that a truffle bed will or will not be productive. The *x*-axis represents the physical soil properties. We know that truffle soils should be well-drained, with no water excess. We note here that soil must be well-structured, and this macrostructure must be stable in water. The structure stability of soil can be represented by the mean diameter of water stable aggregates, the optimum soils for truffle production would have a mean diameter between 1 and 2 mm, with a sandy or pseudo-sandy structure, like "in coffee grounds". This property can be more simply represented by the proportion of 2-4 mm aggregates stable in water. Gravel soils and unstable soil have a low probability of being suitable for fruiting of *T. melanosporum*, even though its mycelium and mycorrhizas may persist. The *y*-axis represents the chemical soil properties. We have known for a long time that calcareous soils are favorable to *T. melanosporum*. We have observed that other soils can also be favorable, such as slightly acidic soils carbonated by nature or human action, and dolomitic soils. By representing this property by the soil alkalinity, then the moderately alkaline soils are the most favorable to the fruiting of *T. melanosporum*.

This probabilistic view is an image of the environmental flexibility of truffle. The probability of production should be considered as the chance for a truffle grower to obtain truffles on his or her truffle beds. However the properties of the environment are never static: they change over time and with cultivation practices. Soil alkalinity can be easily and successfully managed (Valverde-Asenjo *et al.*, 2009): it increases with the addition of lime or calcium or magnesium carbonate, and may decrease slightly with the addition of clay or carbon-rich organic matter. Soil structure can also be easily modified (Le Bissonnais, 1996). Soil tillage is the main practice used: it is often recommended as a truffle culture practice (Sourzat, 1997, 2012). Liming is another common way, but it is effective only in desaturated acidic soils. Finally the most commonly used practice is the addition of well-decomposed organic matter that can tremendously increase the structure stability of soils (Le Bissonnais, 1996; Lulli *et al.,* 1999). This culture practice is well known in horticulture but it is seldom practiced in truffle cultivation and should be encouraged as a means to keep high the stability of soil structure in truffle beds. Fig. 9 illustrates well this: it is a photograph of two soil lumps collected only a few meters apart: one was removed from a grassy woodland whi-

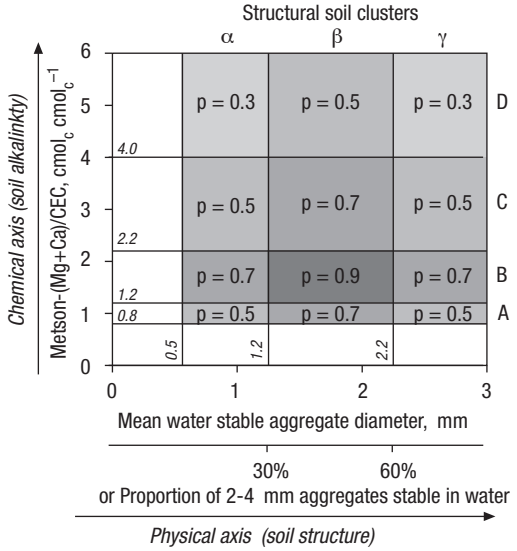

Figure 8. Schematic representation of soil parameters that determine the truffle production. The productivity of truffle beds depends on chemical and physical properties of soil. The chemical properties (*y*-axis) correspond to soil alkalinity. This soil property is the best estimated by the ratio between divalent cations extracted by NH_4-acetate and cation exchange capacity (Metson-(Mg+Ca)/CEC), all parameters measured in the same extraction. The physical properties (*x*-axis) correspond to a sandy or pseudo-sandy soil structure. This soil property can be estimated by soil sieving at 2 mm, then measuring the mean water stable aggregate diameter of 2-4 mm soil aggregates fast immersed in water. It can also be more easily estimated by the proportion of remaining 2 mm < aggregates after fast immersion in water. Both the *y*- and *x*-axis allow to determine the proportion of, thus the probability *p* to observe, productive truffle beds. A probability is necessarily between 0 and 1. The highest probability *p* to observe productive truffle beds was in soils with a moderate alkalinity and sandy or pseudo-sandy water stable structure.

Figure 9. Photography of a simple of soil taken up under a wild grassy woodland (on the right) and a cultivated field. Both samples were only a few meters apart. The woodland was truffle productive. The difference in soil structure resulted mainly from a difference in soil organic matter contents and culture practices.

le the other was taken from a close cultivated field, a little clayey and likely with no organic matter input for many years. The forest was wild truffle habitat, and our opinion is that the cultivated field would need huge efforts now to become truffle habitat again.

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