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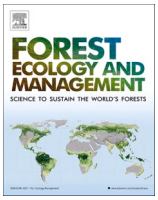

Modelling environmental drivers of *Tuber melanosporum* extraradical mycelium in productive holm oak plantations and forests

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ABSTRACT

The black truffle (*Tuber melanosporum* Vittad.) is a highly appreciated edible ectomycorrhizal fungus. It grows belowground and the mycelium and sporocarp production greatly depends on the abiotic environmental conditions. Although there is some evidence about the variables influencing the truffle mycelium at local scale, there is still a lack of knowledge on the soil and climatic patterns driving mycelium growth at broader scales. We aimed to decipher the potential environmental drivers of *T. melanosporum* mycelium across its westernmost natural distribution area. A paired-design experiment with truffle productive plantations and forests was set up across 10 sites. Mycelium biomass was qPCR-quantified, physicochemical soil analyses were done, and climatic data were collected to perform generalised additive modelling. Mycelium dynamics was driven by a combination of soil and climatic variables that accounted for 65.7% of mycelium variance in plantations and 53.4% in forests. Longitude, $CaCO_3$, Na and Zn were related, either positively or negatively, with soil mycelium distribution in forests, while elevation, organic matter, P, Mg and B, were the main potential drivers of truffle mycelium biomass in plantations. These results strengthen our knowledge on black truffle ecology and can be applied to design management strategies for optimization of truffle mycelial biomass and sporocarp production.

1. Introduction

Among the edible fungi trade, truffles exemplify a multimillion business around the world (Oliach et al., 2021), and their cultivation represents a particularly important economic resource in marginal areas (Donnini et al., 2013). Notably, truffle species such as *Tuber melanosporum* Vittad., *Tuber aestivum* Vittad. and *Tuber borchii* Vittad. contribute to agroforestry ecosystems, where inoculated seedlings have been successfully managed to produce truffle sporocarps both within and out of the respective natural distribution ranges of the associated truffle species (Donnini et al., 2013; Reyna & Garcia-Barreda, 2014; Chen et al., 2016). Because of its high profitability, truffle production has promoted the use of agricultural land in low-productive areas through private forestation, as well as important associated ecosystems services such as carbon fixation and soil quality improvement (Leonardi et al., 2021).

The life cycle of black truffle initiates in late winter or early spring, when the released spores germinate and recent hyphae establish ectomycorrhizal symbiosis with the roots of a range of potential host plants, including the representative Mediterranean holm oak tree species (*Quercus ilex* L.). Subsequently, the primordia of truffle ascocarps are formed in May or June, and they mature from November to March of the following year (Payen et al., 2014). Throughout this extended life cycle, soil properties and climatic conditions are considered to be critical for the development of black truffle sporocarps (Jaillard et al., 2016; Le Tacon et al., 2016; Garcia-Barreda et al., 2019). Castrignanò et al. (2000) showed a positive relationship between the growth and production of *T. melanosporum* with soil aggregates and Fe and Mn contents in the brûlé (the area around the tree with little or no vegetation due to the allelopathic effect of truffle mycelium), in a plantation in central Italy. Other soil characteristics such as active carbonate and exchangeable Ca^{2+} have also been found to contribute to truffle sporocarp production and mycelium development in a naturally black truffle-producing forest in central Spain (García-Montero et al., 2007,

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2008). Although these studies have provided insights into how soil variables affect the fructification and brûlé size of black truffle at the plot scale, a broader scale view including different land uses (i.e., forest and plantations) is necessary to drawing generalisable conclusions beyond local contexts.

Climatic conditions also play a fundamental role in the persistence and proliferation of ectomycorrhizal fungi, both epigeous (Egli, 2011; Martínez-Peña et al., 2012; De la Varga et al., 2013) and hypogeous like truffles (Garcia-Barreda et al., 2019). Pacioni et al. (2014) associated the increased soil metabolic activity (i.e., CO_2 emissions) targeted by variations in soil moisture and temperature in spring and summer with an intense mycelial growth and fruiting initiation of *T. melanosporum*. Numerous studies at medium-large time scales (Queralt et al., 2017; Baragatti et al., 2019; Garcia-Barreda et al., 2019; Büntgen et al., 2019) support the idea that truffle production is mainly affected by summer temperature and precipitation. In this line, Le Tacon et al., (2014) showed that water balance in late spring and summer, along with the number of extremely cold days in winter, were the main predictors of annual fluctuations in black truffle production during a 25-yr period in France. In fact, truffle sporocarps develop during winter, and their small and fragile nature makes them susceptible to growth constrains caused by former late-spring-summer water deficit (Baragatti et al., 2019).

A validated method to track the persistence of a given fungus throughout its biological cycle, besides sporocarp´ production, is to detect its extraradical mycelium in the soil using specific DNA probes (Parladé et al., 2016). Tracking inter-seasonal changes in soil mycelium biomass may help to understand the complex interactions between truffle´ life cycle and the surrounding environment, facilitating the optimisation of management of truffle-producing systems. To the best of our knowledge, such a study investigating the relationship between black truffle mycelial biomass and soil properties and/or recent climatic data in orchards and forests at a large spatial scale, has never been conducted before.

Given the tight relationships between the truffle life cycle and the variations in the abiotic environment, firstly, we hypothesised that (i) the truffle mycelium biomass would be dependent on the type of productive system (forests *vs.* plantations) and the season, and that these patterns would be consistent at the regional scale. We expected an increase in truffle mycelium biomass in soils of truffle plantations because of their managed-controlled environmental conditions compared with those in wild forests. Additionally, given the heterothallic nature of truffle mycelium (Riccioni et al., 2008) and the need for compatible hyphae matching for sporocarps formation, greater mycelial biomass was expected in spring than autumn, independently of the production system. Secondly, we hypothesised that (ii) truffle mycelium biomass would respond to different environmental abiotic variables in wild-productive forests and plantations. Given that irrigation is a common practice in truffle orchards, a greater dependency of truffle mycelium on climatic conditions was envisaged in wild-productive forests, as well as a differential response to soil fertility.

To test these hypotheses, we analysed the response of black truffle mycelium biomass to a range of environmental variables in wild and managed production systems, across the westernmost natural distribution area of *T. melanosporum* in Spain. The main objective of our study was to identify general variability patterns of black truffle mycelium in relation to soil physicochemical characteristics and climate in both productive plantations and truffle-producing forests.

2. Materials and methods

2.1. Study sites and sampling

Sampling sites were selected covering the geoclimatic diversity of the native distribution area of the black truffle in Northeastern Spain. For each site, a paired-design was set to compare two types of truffle production geographically close to each other: a) planted black truffle orchards and b) nearby wild black truffle woodlands (Fig. 1), with a total of 10 sites surveyed. All the sampled plantations are productive holm oak monocultures over 10 years, with standard truffle culture management (i.e., soil tillage, irrigation, pruning). In the forest sites, we selected productive *Q. ilex* trees (according to local collectors) in areas dominated by this tree species with an understory composed of different combinations of *Quercus faginea* Lam., *Juniperus* spp., *Crataegus monogyna* Jacq., *Thymus* spp., *Cistus albidus* L. and *Rosmarinus officinalis* L. Concerning the landform of the study sites, for the most part they were flat or, in the case of some natural forests, they had a slight local slope.

The whole study area is characterised by a continental, Mediterranean-type climate with seasonal hydroclimatic variations (Table S1). Mean annual temperature and precipitation across sites are 12.3 ± 1°C and 538 ± 136 mm, respectively. Mean altitude of sites is 976 ± 253 m (a.s.l.). To account for plot heterogeneity, we selected at least four productive *Q. ilex* trees for sampling in each plot, with a total of 88 holm oaks monitored that had been previously verified as truffle productive by the orchard owners or the truffle hunters. To account for seasonal variability, soil samplings were conducted at two times in each site, in autumn 2019 and in spring 2020.

A metallic probe was used to obtain soil cylinders of 4 cm diameter and 20 cm deep within the brûlé area of each tree. Four samples per tree were collected from a randomly oriented rectangular area within the brûlé (approximately at the middle point between the trunk and the limit of the brûlé, or at a 50 cm distance from the tree trunk when the brûlé edge was fuzzy) and then pooled in a unique sample per tree. In compacted and rocky soils, found in some forest areas, we excavated 10 ×10×20 cm soil holes with a small shovel to take the sample. All the soil samples were stored in plastic bags at 4°C until lab processing.

2.2. Soil analyses

All the soil samples taken along the two sampling periods were manually homogenised and separated in different aliquots according to the type of analysis. To estimate soil stoniness, a fraction of soil was separated, weighted, and sieved (2 mm) after air-drying. Soil texture (sand, clay and silt %) was determined by the Bouyoucos method (Bouyoucos, 1962). The gravimetric soil moisture (GSM) of soil samples was determined by drying at 105°C for 48 h. Sieved air-dried soils were measured for pH (2 g of soil in 10 ml of H_2O, 1:5, w:v), electrical conductivity (EC) (1:5, w:v in H_2O), organic matter (OM) (Walkley & Black, 1934) and total N (Dumas method; Food and Agriculture Organization of the United Nations (FAO), 2021). Extractable P was determined by the Olsen method, after extraction in 0.5 N sodium bicarbonate solution. Extractable potassium (K), calcium (Ca), sodium (Na) and magnesium (Mg) were determined after ammonium acetate (1 M, pH 7) digestion, and extractable iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni) and boron (B) were determined after DTPA (0.005 M, pH 7.3) digestion. All elements were measured by inductively coupled plasma spectrometry (ICP, Optima 4300 DV, PerkinElmer Inc., Massachusetts, USA). The calcium carbonate ($CaCO_3$) of soils was estimated by gasometric determination of CO_2 with a Chittick apparatus. The active carbonate content was determined by extraction in ammonium oxalate and subsequent potassium permanganate reaction (NF X31-106, 1982). Soil variables were measured for all samples except texture variables and the content of P and calcium carbonate that were just determined in autumn 2019.

2.3. Climatic data

Climatic data were derived from WorldClim v2.0 (Fick & Hijmans, 2017) with 30 seconds (~1 km^2) spatial resolution. We obtained the data of the average minimum and maximum temperature (°C) of every month and the annual rainfall precipitation (mm) from the historical monthly weather database for the period 2010–2018 (Table S1). The Emberger pluvial-thermic index (Emberger, 1932) was additionally

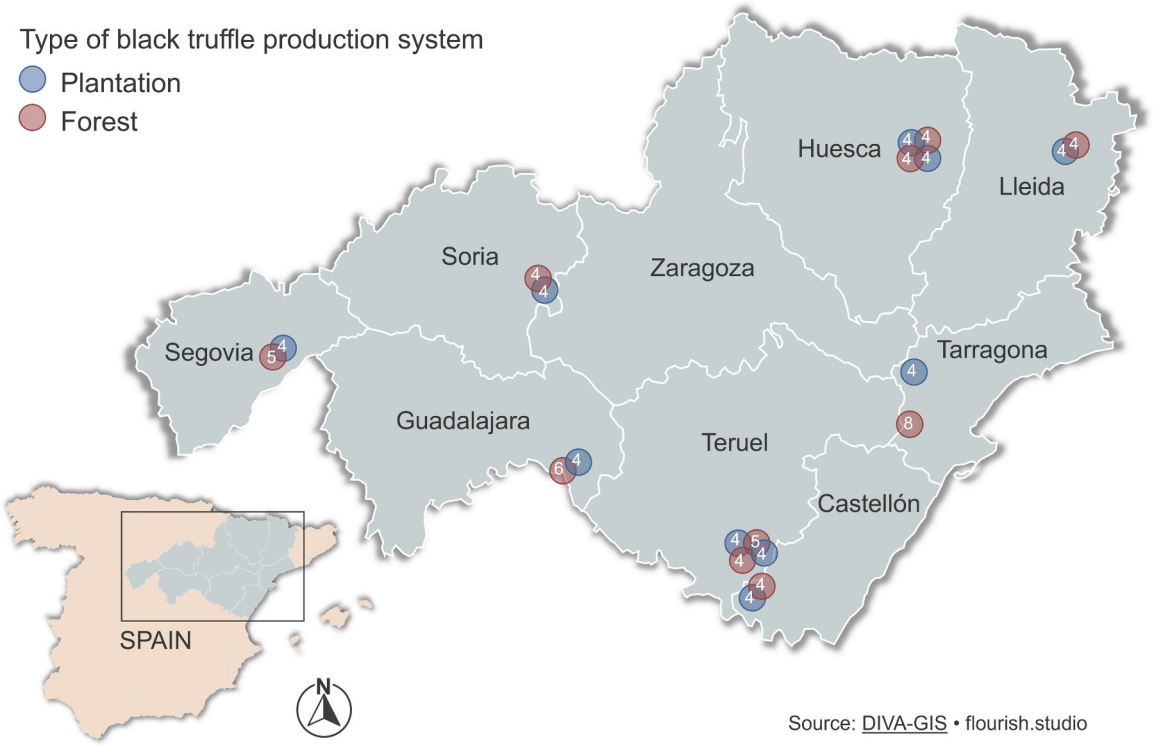

Fig. 1. Spatial distribution of the sites selected for this study in nine provinces of Spain, with two different truffle production systems per site: plantation (blue) or forest (red). The number of holm oak trees sampled in each site is noted within the corresponding symbol. Map created with flourish.studio (https://flourish.studio) and adapted by the authors.

calculated for each plot as it summarises adequately the local climatic conditions. This index is used to estimate desertification risks and explain plant species distribution Vessella et al., 2015; Mitsopoulos & Xanthopoulos, 2016; Jiménez-González et al., 2020). Based on the WorldClim data, the coefficient of Emberger (Q) was calculated as follows: $Q = 100P/(M^2 \quad -m^2)$ (Rubio-Ríos et al., 2022), where P is the annual rainfall, M is the average maximum temperature of the warmest month, and m is the average minimum temperature of the coldest month. Coordinates (latitude, longitude) are omitted from Table S1 to maintain the privacy of the studied sites, according to the collaborating stakeholders.

2.4. Molecular analyses

The DNA from all the soil samples was extracted from 0.25 g of sieved soil using the DNeasy Power Soil HTP 96 kit (Qiagen, Hilden, Germany), and its concentration was determined by a NanoDrop™ 3300 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Extracted DNA was stored at −20°C until further analysis.

Mycelium biomass of *T. melanosporum* in each soil sample was quantified by real-time Taqman® PCR (qPCR) with the StepOnePlus™ Real-Time PCR System (Applied Biosystems™ by Thermo Fisher Scientific, Wilmington, USA). The method to create the standard curve for absolute quantification was adapted from Parladé et al. (2007), using the DNA from *T. melanosporum* mycelium instead of sporocarp. For this purpose, we prepared mycelium of *T. melanosporum* in Petri dishes of BAF medium (DSMZ, GmbH, Germany) covered with a cellophane sheet (Fig. S1). The plates were inoculated with an active-growing colony of *T. melanosporum* and incubated at 25°C for two months until an active growth of the mycelium was obtained. Then, the mycelium was removed from the cellophane using a scalpel, pooled into an Eppendorf tube, and dried at 50°C for 1 h in a heating chamber (ED-S 115, ©BINDER GmbH, USA). We extracted the DNA from a known weight (7 mg) of dried mycelium using the Soil DNA Isolation Plus Kit (Norgen Biotek Corp., Canada). The standard curve was created based on the C_T values of a ten-fold dilution series of the mycelium DNA, from 10^0 to 10^{-4}. The reaction mix and cycling conditions of qPCR were as described in Parladé et al., (2013). Three technical replicates per soil sample were included in the reaction plates. The C_T values of soil samples were then interpolated into the standard curve to calculate the mycelium quantity of *T. melanosporum* (Parladé et al., 2007; De la Varga et al., 2013), expressed in µg of mycelium per g of soil. To perform and interpret the qPCR assays, the MIQE (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines, proposed by Bustin et al. (2009) were considered.

2.5. Statistical analyses

Truffle mycelium biomass and environmental variables were checked for normality by the Shapiro-Wilk test and the error distribution of data was checked and considered for model building.

2.5.1. Effect of the type of production and the season on truffle mycelium biomass

To test the effect of the production type (forest *vs.* plantation), the season of sampling (autumn *vs.* spring) and their interaction on truffle mycelium variance (hypothesis 1), considering spatial-temporal random effects, we first fitted linear mixed models to the paired-design dataset ($N = 176$). However, the non-normal distribution of the residuals indicated the use of generalised linear mixed models (GLMMs). Furthermore, as an extension of GLMMs, generalized additive mixed models (GAMMs) allowed to fit both linear (similarly to GLMM) and non-linear terms, without assuming a priori a specific type of relationship between the dependent variable and the predictors. In this step, a null generalised additive mixed model (hereafter 'null GAM') was set to describe the effect of the linear fixed terms 'type' and 'season' on truffle mycelium variance. The model formula for the null GAM was set as:

$$mycelium \sim type * season + s(site, bs = \text{'re'}) + s(site, by = season, bs = \text{'re'})$$

where 'mycelium' is the response variable, 'type' of production system and 'season' are the fix factors included as linear predictors, 'site' and 'site' nested in 'season' (accounting for spatial variation and repeated measures, respectively) are the random effects ('re') included as non-linear predictors by applying the spline smooth function *s*() to fit non-parametric regression, and setting a Gamma error distribution. GAMs were run with the *gam* function of the 'mgcv' R package (Wood, 2008), and the distribution of residuals was checked for normality and homogeneity by the *gam.check* function before model validation.

2.5.2. Response of truffle mycelium to abiotic environmental factors

Next, we analysed the environmental factors affecting the truffle mycelium biomass distribution in productive plantations and forests (hypothesis 2). In a first step, the collinearity among all environmental variables (26 in total) was analysed by Pearson correlation, using the *rcorr* function in the 'Hmisc' R package (Harrell, 2019) and plotted by the *corrplot.mixed* function of the 'corrplot' R package (Wei & Simko, 2021). In a second step, variables were grouped as 'geoclimatic factors' (G), 'soil fertility' (F) and 'other edaphic properties' (E) (Table 1) to examine whether a specific group of variables or any combination of them would show high relative contribution in explaining truffle mycelium variation.

Finally, we fitted models with all possible variable combinations, independently of their previous assignation in Table 1. For this, a 'full subsets generalised additive modelling (GAM)' approach was applied by using the FSSgam R package (Fisher *et al.*, 2018). This procedure consists of fitting models of all possible variable combinations (subsets) with up to a maximum of three predictor variables per model (to ensure that the models will remain ecologically interpretable), comparing all resulting GAMs and identifying the best-fitted ones through stepwise selection based on the Akaike's information criterion (AIC) and parsimony principle. The continuous variables were specified as smooth terms and the degrees of freedom (*df*) were restricted to 4 for each smoother ($k = 4$) to avoid models over fitting, as recommended by Fisher *et al.* (2018). The factors 'type' and 'season' were included as linear predictors and the random effects were added to all model construction, as previously described in null GAM. All models were fitted to a Gamma distribution and log-link function. To tackle with the high collinearity among continuous variables, the Pearson's correlation cut-off in the FSSgam package was limited to 0.28.

This analytical procedure was applied to the complete dataset ($N = 176$), and separately to the truffle plantations ($N = 80$) or forests ($N = 96$) datasets (in these cases the term 'type' was excluded from models). The explanatory variables were sequentially added in models to get seven model's clusters: 1) using only geoclimatic characteristics (model 'G'), 2) only soil fertility (model 'F'), 3), only other edaphic properties (model 'E'), 4) both geoclimatic factors and soil fertility (model 'GF'), 5) both geoclimatic factors and edaphic properties (model 'GE'), 6) both other edaphic properties and soil fertility (model 'EF') and 7) altogether geoclimatic factors, other edaphic properties and soil fertility (model 'GEF') (Table S4). After obtaining all possible models, the top-ranked ones of each cluster were selected among those that showed less than two AICc units of difference respect to the most parsimonious model (lowest AICc) (i.e., $\Delta AICc < 2$) (Burnham & Anderson, 2004). The normal distribution of residuals and the absence of autocorrelation effects were checked for each model.

Once the top-ranked models were selected, the relative contribution of each group of variables in explaining truffle mycelium variation was determined based on the GAMs deviances explained (DEs) (Table S4), according to Zhou et al. (2021). For this, the unique contribution (the deviance exclusively explained by a single group of variables, i.e., model scenarios 'G', 'E' and 'F') and the common contribution (the deviance explained by multiple groups of variables, i.e., model scenarios 'GE', 'GF', 'EF' and 'GEF') of variables, as well as their respective differences were quantified as indicated in Table S4. Finally, for the model scenario with the highest relative contribution on truffle mycelium variance, the relative effect of each individual explanatory variable was calculated. For that, the importance of each explanatory variable across all candidate models was estimated by the summed AICc weights (second order information criterion, taking into account the sample size) of all the models where the explanatory variable appeared, ranking as more important those predictors with higher summed values. The relationships among the most relevant individual explanatory environmental variables with the truffle mycelium biomass were plotted with the *plot smooth* function of the 'itsadug' R package (van Rij et al., 2022).

All statistical analyses of this study were performed with the R software v.4.2.1 (R Core Team, 2022).

Table 1
Groups of explanatory variables used in this study: geoclimatic, soil fertility and other edaphic properties.

Geoclimatic (G)	Fertility (F)	Other edaphic properties (E)
Emberger coefficient (Q)	N (%)	Sand (%)
Elevation (m.a.s.)	P (mg kg^{-1})	Clay (%)
Latitude (°)	K (mg kg^{-1})	Silt (%)
Longitude (°)	Ca (mg kg^{-1})	Stoniness (%)
	Na (mg kg^{-1})	pH
	Mg (mg kg^{-1})	Gravimetric soil moisture (%)
	Fe (mg kg^{-1})	Electric conductivity (μs cm^{-1})
	Zn (mg kg^{-1})	Organic matter (%)
	Mn (mg kg^{-1})	Active carbonate (%)
	Cu (mg kg^{-1})	Calcium carbonate (%)
	Ni (mg kg^{-1})	
	B (mg kg^{-1})	

3. Results

3.1. Effect of the production type and the season on soil truffle mycelium biomass

Overall, forests had higher average mycelium biomass of *T. melanosporum* than plantations in both seasons, and in autumn the average mycelium showed higher values than in spring in both production types (Fig. 2). However, when the site was taken into account in the analysis, the null model (GAM) showed that just the season and the random effects (i.e., spatial-temporal factors), but not the type of production, were significant predictors of truffle mycelium (42.2% deviance explained; AIC = 1676.73) (Table S2).

3.2. Relative contribution of edaphoclimatic factors to the truffle mycelium biomass variance

Mean values and variation of the climatic and soil variables analysed in this study are shown in Table S3. These environmental variables showed high collinearity and, except for soil sand and pH, most of the correlations observed were positive (Fig. S2).

When the entire dataset was modelled as a function of general groups of explanatory variables (geoclimatic factors G, soil fertility F, and other edaphic properties E), the best-fitted models showed values of truffle mycelium deviance explained between 41.1 – 45% (Table S4). Compared with the whole dataset, the separate analysis of truffle plantations and forests datasets gave better yields for all model parameters e.g., mycelium deviance explained: 67.5 – 70.5% for plantations and 43.6 – 57.4% for forests (Table S4). Within each dataset, the seven best-fitted models gave quite similar results. However, compared with forests, all the best-fitted models in truffle plantations had the highest deviance explained and the lowest AIC, indicating that the environmental variables tested were better predictors of the mycelium biomass variability in plantations than forests.

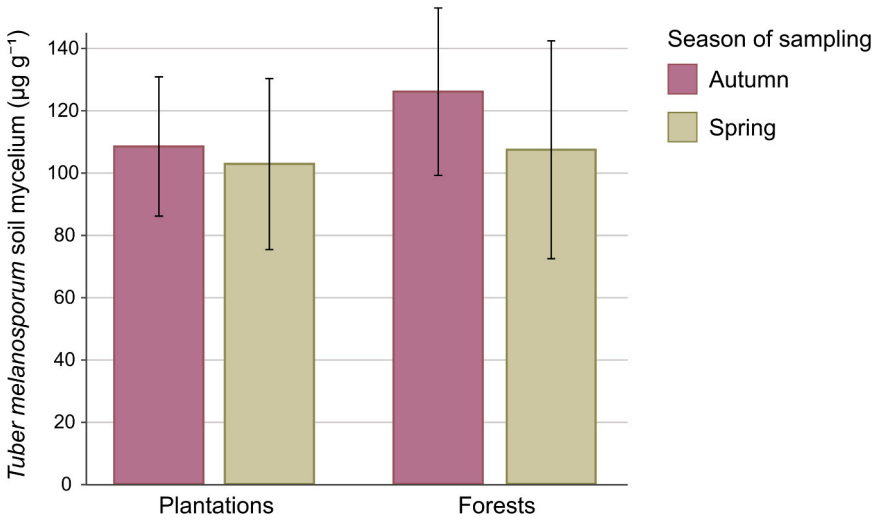

Fig. 2. Mean mycelium biomass of *Tuber melanosporum* in soils of two black truffle production systems: plantations and forests, at two seasons: autumn and spring. Error bars denote 1SE.

When the relative contribution of each group of environmental variables was calculated, it was showed that none group i.e. geoclimatic G, other edaphic properties E, soil fertility F, had a predominant separate weight explaining truffle mycelium variation when they were considered by pairs or alone, but they did have when considered together (rates of mycelium variance: 42.6% total dataset, 65.7% plantations, 53.4% forests) (Fig. 3; Table S4).

3.3. Effect of individual variables in truffle mycelium biomass variance

Since the combination of geoclimatic factors G, soil fertility F, and other edaphic properties E had the highest explicative potential of *T. melanosporum* mycelium variation, more attention was drawn to the best-fitted GAMs considering all variables (i.e., independently of their previous grouping). The full subsets analysis considering all possible variable combinations (subsets) with up to a maximum of three predictor variables yielded a total of 761 and 848 GAMs in the datasets of plantations and forests, respectively. The top-ranked models of this 'full subsets analysis' for plantations and forests reported only two with $\Delta AICc < 2$ (Table 2) for each type of production system. When the importance of each predictor within the top-ranked models was estimated (i.e., by the summed AICc weights in Table 2), in general, scores were lower in plantations than in forests, and the variables that were respectively most influencing in each truffle-producing system were different (Fig. 4). The best model ($\Delta AICc = 0$) for predicting truffle mycelium biomass in plantations (Table 2) showed significant contributions of elevation, organic matter and magnesium (Fig. 4**a**), whereas for forests it included longitude, calcium carbonate and sodium (Fig. 4**b**).

In plantations, truffle mycelium biomass slightly decreased between 400 and 800 m, but increased at higher elevations (Fig. 5**a**). Soil organic matter and magnesium content had a positive linear relationship with truffle mycelium biomass (Fig. 5**b,c**). Regarding the forests, truffle mycelium first increased at approximately −4° to 0° longitude and then decreased with increasing values (i.e., in eastern sites) (Fig. 5**f**). Mycelium biomass showed a nonlinear relationship with calcium carbonate content in soil, with a negative trend at rates up to 20% and above 60% and a positive trend at intermediate rates (Fig. 5**g**). Truffle mycelium biomass in forests showed a negative relationship with the sodium content in soil (Fig. 5**h**).

As regards the second best-fitted GAMs (plantations: elevation + P + B; forests: longitude + Na + Zn; Table 2), in truffle plantations, the relationship with phosphorous content was slightly positive at low levels, and then it became negative at rates over 20 mg kg^{-1}, whereas boron levels had a positive influence on mycelium biomass with an asymptotic relationship, with a limit of the positive trend over 0.10 mg kg^{-1} soil (Fig. 5**d,e**). In forests, the truffle mycelium showed a negative relationship with the content of zinc (Fig. 5**i**).

4. Discussion

The relative importance of environmental variables as potential drivers of the extraradical mycelium of *T. melanosporum* was assessed along two seasons, in truffle orchards and forests in Spain. Studying truffle mycelium dynamics is relevant since fruiting body production has been positively related to mycelium in local studies involving truffles (Suz et al., 2008; Zampieri et al., 2012; Queralt et al., 2017). This relationship has also been observed in the common practice consisting of placing fungal material into small pit dugs under truffle host trees (truffle traps), where the stimulation of the vegetative development of maternal mycelium is achieved and the fruitbody production is favoured (Taschen et al., 2022).

Contrary to hypothesis 1, we found that the amount of truffle mycelium was independent of the type of production, and overall mycelium biomass was higher in autumn than in spring. However, while the sampling season was a significant predictor for mycelium variability with the entire dataset (i.e., null GAM), it was not the case for the datasets of truffle plantations and forests separately. Previous studies have related intra- and inter- annual variability of black truffle production with climatic factors (Büntgen et al., 2015; Garcia-Barreda et al., 2020) Nevertheless, no information is available on the spatial and temporal patterns of soil mycelium of this fungus at a large scale. Castaño et al. (2017) also observed peaks of extraradical mycelium of *Lactarius vinosus* (Quél.) Bataille in spring and autumn in a Mediterranean forest. However, a similar study in a *T. melanosporum* plantation (Queralt et al., 2017) showed a different seasonal variation pattern of mycelium biomass in two consecutive years, and a significant correlation with the accumulated precipitation of the month previous to sampling throughout the year. Thereby, it seems that the mating events of truffle mycelium in spring do not involve an increase of soil mycelium biomass in *T. melanosporum* and that the temporal pattern is rather related to local soil-climatic factors.

Regarding the type of production, it is known that *T. melanosporum* outcompetes other ectomycorrhizal fungi in the brûlés (Napoli et al., 2010). In fact, Oliach et al. (2020) did also find that holm oaks inoculated with *T. melanosporum* and planted in areas surrounded by forests

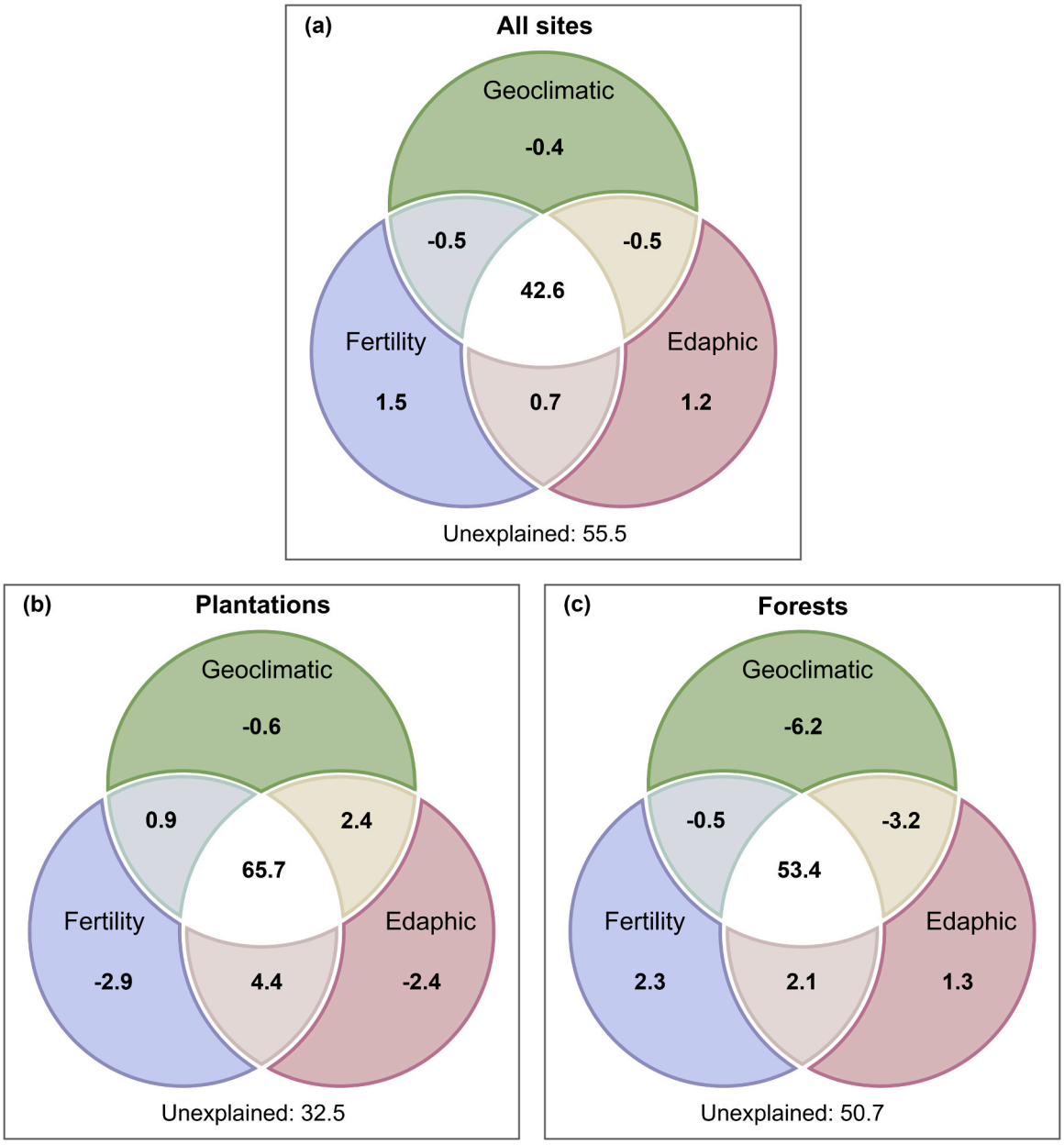

Fig. 3. The unique and common contributions (%) of three environmental scenarios: geoclimatic factors (G), soil fertility (F), other edaphic properties (E) and their combinations (GE, GF, EF, GEF) in mycelium biomass variance of *Tuber melanosporum* across **(a)** all sampled sites, **(b)** truffle plantations and **(c)** forests. The relative contribution of each scenario was quantified by the deviances explained (DE) of the best-fitted generalised additive models (GAMs) calculated as indicated in Table S4. Negative rates resulted when the combination of a single group or two groups of variables (GE, GF, or EF) explained more deviance than the combination of the three groups of variables (GEF) (see Table S4).

were colonised by other ectomycorrhizal fungi, but even so, the abundance of *T. melanosporum* mycelium was not reduced in the proximity of the forest. Our data also support that ectomycorrhizal competition in productive forests does not affect the soil density of *T. melanosporum* mycelium, as compared to monospecific plantations.

According to our second hypothesis, truffle mycelium biomass did respond to different abiotic variables in wild-productive forests and plantations. Modelling the geographic characteristics, edaphic properties and soil fertility variables as potential drivers of black truffle mycelium biomass showed that, in every model scenario, the best-fitted model explained 11–25% more deviance in plantations than in forests. Similarly, the common contribution of the environmental variables to mycelium biomass was 12% higher in plantations than in forests. This result may be related to the fact that truffle plantations have been subject of long-term cultivation practices, such as tillage, simplifying, this way, the soil environment and decreasing the background noise. Besides the underlying differences in the soil-climatic properties of the two productive systems, these results indicate that other variables (e.g., biological interactions and soil structure) not taken into account in this study are likely to be relevant explaining *T. melanosporum* mycelial variance, particularly in unmanaged forest systems. For example, different biological interactions with the soil microbial community and/or different soil structure that may affect the nutrient availability to soil microorganisms (Erktan et al., 2020) could have accounted for the unexplained part of our models. In addition, the relative importance of variables was clearly different in both production types, as well as the key variables explaining truffle mycelium variation in each system. The differences found could be explained in terms of different soil

Table 2
Summary results from the 'full subsets analysis' showing generalised additive models (GAMs) with ΔAICc < 2 respect to the most parsimonious model (that with lowest AICc) for black truffle mycelium prediction, in truffle plantations and forests. AIC corrected for small sample size (AICc), difference from lowest reported AICc (ΔAICc), AICc model weights (wi.AICc), deviance explained (DE) and estimated degrees of freedom (edf) of the respective GAMs are reported.

Dataset	Model	AICc	ΔAICc	wi. AICc	DE	edf
Plantations	Elevation + organic matter + Mg	788.45	0	0.12	0.68	16.38
	Elevation + P + B	789.83	1.37	0.06	0.70	17.08
Forests	Longitude + CO_3Ca + Na	843.18	0	0.26	0.49	19.04
	Longitude + Na + Zn	844.41	1.23	0.14	0.48	18.18

development and biology of plantations *vs.* forests, and/or previous land use in plantations (Thervi̇lle et al., 2013; Flores-Rentería et al., 2016).

Among the specific variables identified by the 'full subsets analysis' to explain mycelium variation, the elevation of sites had a significant positive relationship with truffle mycelium in plantations, but not in forests. This could be related to the drastic reduction during Mediterranean summer season, highlighting the importance of soil moisture for black truffle production (Garcia-Barreda et al., 2019). This altitude effect would be less evident under the canopies of the sampled forests with soils richer in organic matter, and with higher water holding capacity (Hudson, 1994).

Truffle mycelium biomass in forests showed a bell curve shaped relationship with longitude, compatible with the agro-climatic zones of black truffle productive forests proposed by Garcia-Barreda et al. (2019) in the Iberian Peninsula. The distribution pattern of fungal mycelium in our samples showed a longitudinal zonation with the highest mycelium abundance in Soria and Guadalajara provinces, corresponding to the western areas of our study.

Regarding soil factors, the mycelium biomass had a positive and linear relationship with soil organic matter in truffle plantations. Different authors have shown the association between soil fungal mycelium and organic matter content and fractions (Soukupová et al., 2008; Hu et al., 2023; Raza et al., 2023). Compared to forests, long-term cultivation of agricultural land can result in decreased content of soil organic carbon (Beheshti et al., 2012). Indeed, lower organic matter content was observed in plantations than forests and this could possibly indicate a higher dependence of truffle mycelium biomass on organic matter quantity and quality, in soils with less availability.

Calcium carbonate was a relatively important variable for *T. melanosporum* mycelium in both forests and plantations, even if in the latter case the variable was not present in the best-fitted models. This agrees with the results of Gryndler et al. (2017) regarding the positive effect of calcium carbonate on *Tuber aestivum* mycelium and with those of García-Montero et al. (2007, 2008) about the contribution of active carbonate and exchangeable Ca^{2+} to sporocarp production and burn size of *T. melanosporum*. Moreover, García-Montero et al. (2024) have recently shown the ongoing formation of active carbonates inside the brûlé, suggesting the active habitat modification and niche construction by the black truffle.

Regarding soil fertility, the magnesium content had a positive linear relationship with mycelium biomass in truffle plantations (generally lower than in forests). Magnesium is an essential element, the deficiency of which affects photosynthesis and carbohydrate partitioning in crops (Farhat et al., 2016; Wang et al., 2020). Soil carbon allocation depends on nutrients such as Mg (Ericsson, 1995) that are closely related to photosynthesis, and any change in C allocation can affect mycelium growth of ectomycorrhizal fungi (Bahr et al., 2013). We suggest that the positive effect of Mg on truffle mycelium observed in our study might be related to the improvement of the photosynthetic activity of the host plant and thus the higher carbon deliver for fungal mycelial growth.

We also found a positive trend of boron on mycelium biomass in plantations. B can affect the soil microbiome (Vera et al., 2021) and play a role in plant cytoskeleton formation (Bassil et al., 2004), which may be also related to the mycorrhizal establishment (Lehto et al., 2010). The availability of B increases in alkaline soils, such as those appropriate for truffle production as in our study. Our plantations were slightly more alkaline than the forest sites, which possibly enhanced B content in soil, favouring also the mycorrhizal association with plants.

Truffle mycelium biomass was negatively related with sodium in forests. Although all the studied sites were within the distribution area of saline and sodic soils in the European Union (Tóth et al., 2008), their Na^+ values were within the range measured in productive black truffle plantations in the province of Teruel, NE Spain (Alonso Ponce et al., 2014). Surprisingly, this variable did not appear as relatively important to explain the variation of mycelium biomass in plantations, where the mean Na^+ amount in soil was higher than in forest areas probably because of the secondary soil salinization occurring in agricultural drylands through water logging or improper irrigation systems (Stavi et al., 2021).

The relationship of phosphorous with truffle mycelium in plantations at low levels was positive, but after a certain threshold, it displayed a negative trend. Zhang et al. (2019) found both positive and negative relationships of exogenous P and N on *Tuber indicum* Cooke & Massee colonisation depending on the application dosage, while it is well known that high P concentrations in soil can inhibit mycorrhiza establishment and root colonization (Smith & Read, 2008). Forests sites showed less bioavailable P than plantations, possibly related to indirect negative effects of drought in Mediterranean forests (Sardans & Peñuelas, 2004) compared with well-irrigated plantations. Besides, truffle plantations were probably established in previously cultivated fields where fertilisation may have been applied, increasing the P content in the soil.

Soil Zn was negatively related to mycelium biomass in forests. Zinc is an important micronutrient for plants, whose deficiency in alkaline soils creates hurdles in achieving optimum crop growth (Saboor et al., 2021). Zinc can become an environmental concern at toxicity levels, although our sampled soils are well below the limits considered toxic for plants (Noulas et al., 2018; Kaur & Garg, 2021).

In addition to abiotic environmental factors that interact with black truffle, a recent study has identified some fungal and bacterial species that co-occur with *T. melanosporum* and may drive its dynamics in soils of truffle plantations (Herrero de Aza et al., 2022). Other authors have pointed that the capacity of *T. melanosporum* to expand in the soil and uptake nutrients might be facilitated but also interfered by the surrounding microbial community (Rubini et al., 2014). While in natural forests the *Tuber* spp. colonisation depends on fungal competition, in truffle orchards (i.e., agriculture soils depleted of ectomycorrhizal propagules), the inoculated plants are advantageous for the development of the introduced fungus provided the absence of competition with other ectomycorrhizal species (Leonardi et al., 2021). Čejka et al. (2023) suggested that the identification of common fungal taxa across *Tuber magnatum* Pico sites and their attribution to functional guilds would help to distinguish productive sites from unproductive ones. Further studies on microbial communities that coexist with *T. melanosporum* will help to evaluate the ecology and successful establishment of this fungus in the soil.

5. Conclusion

The dynamics of black truffle mycelium in our study is driven by a combination of climatic and soil factors, rather than by a particular effect of either climatic or soil properties. The combination of the studied environmental factors accounted for almost two thirds of the mycelium variance in plantations and half of the mycelium variance in forests, whereas the groups of variables alone or in dual combinations explained

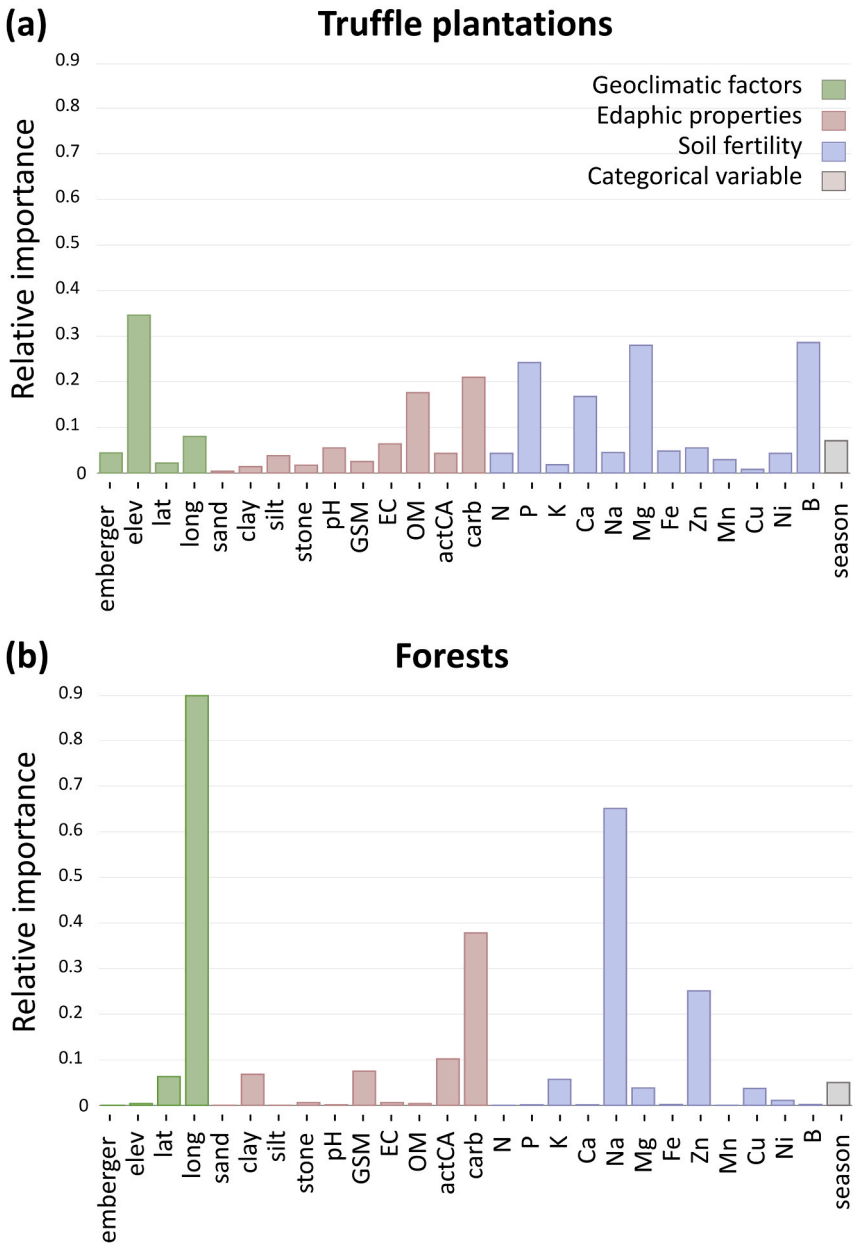

Fig. 4. Variable importance scores from a full subsets analyses exploring the effect of geoclimatic factors (green), edaphic properties (pink) and soil fertility (purple) on *Tuber melanosporum* mycelium biomass in **(a)** truffle plantations and **(b)** forests. Variable importance was calculated by the summed AICc weights of all the models that each predictor appeared through the model construction process. Emberger = Q index = 100 *P* (annual rainfall)/M^2 (average max. temperature of the warmest month) – m^2 (average min. temperature of the coldest month), lat = latitude, long = longitude, elev = elevation, GSM = gravimetric soil moisture, EC = electric conductivity, OM = organic matter, actCA = active carbonate, carb = calcium carbonate.

a negligible part (< 5%) of the mycelium variation. At the regional scale (i.e., independently of the site), the relative contribution of explanatory variables showed that longitude, carbonates, Na and Zn were related, either positively or negatively, to soil mycelium distribution in forests. In plantations, the elevation, content of organic matter, P, Mg and B were related to the regional distribution of soil mycelium. Our results give important clues on black truffle ecology and are highly valuable for applied purposes e.g., the design of tailored fertilisation schedules to maximise fungal biomass, assuming that this will improve truffle production. Further studies on truffle ecology are needed to fully understand the environmental drivers of black truffle mycelium. In any case, accurate data on truffle production would notably help to design fruiting models to be compared with those developed for soil mycelial biomass in this study.

CRediT authorship contribution statement

Vasiliki Barou: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Javier Parladé:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ana Rincón:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

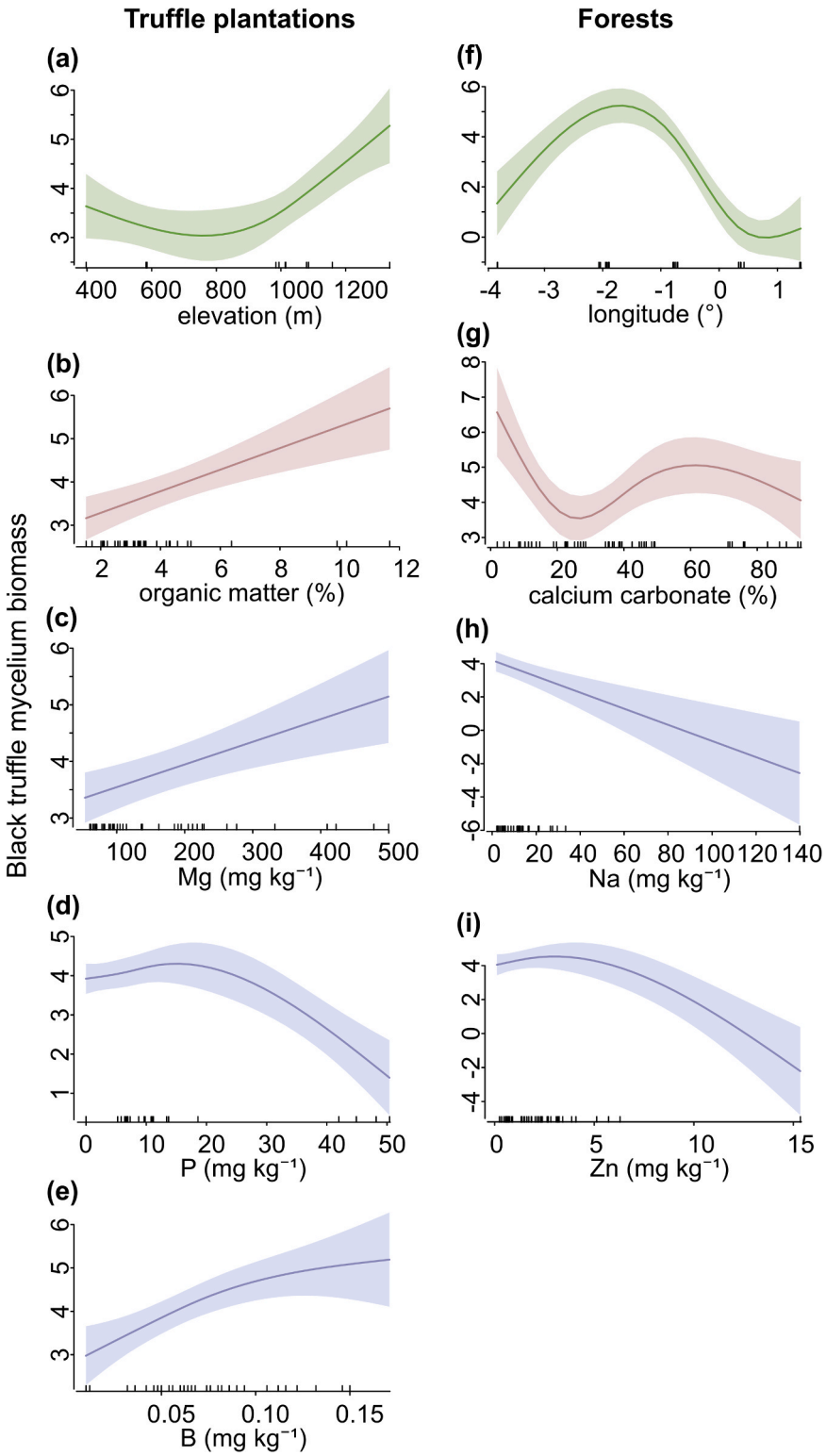

Fig. 5. Partial effect of the most important environmental variables explaining black truffle mycelium biomass in plantations **(a, b, c, d, e)** and forests **(f, g, h, i)**, based on the AIC of the most parsimonious generalised additive models (GAMs) (a, b, c, f, g, h) and the second best-fitting GAMs (d, e, i). The solid line indicates the estimated smoothing curve and the shaded areas indicate the 95% confidence interval of the regression line. Green represents geoclimatic factors (G), pink represents edaphic properties (E) and purple represents soil fertility (F) variables.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.foreco.2024.121988.

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