Time and dose of irrigation impact Tuber melanosporum *ectomycorrhiza proliferation and growth of* Quercus ilex *seedling hosts in young black truffle orchards*

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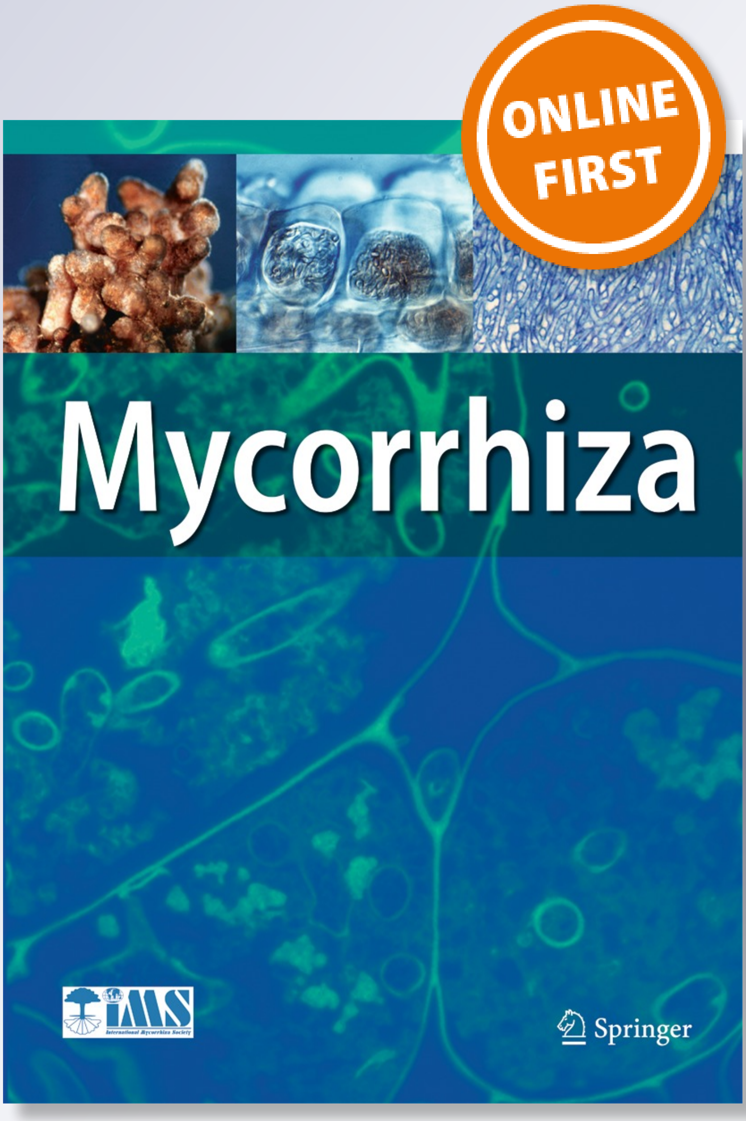

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ORIGINAL PAPER

Time and dose of irrigation impact *Tuber melanosporum* ectomycorrhiza proliferation and growth of *Quercus ilex* seedling hosts in young black truffle orchards

Antoni Olivera · José Antonio Bonet · Daniel Oliach · Carlos Colinas

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Abstract In Mediterranean climate, young truffle-oak orchards are subjected to drought episodes that can compromise the development of *Tuber melanosporum*. We investigated the responses of *T. melanosporum* to water supply in three periods: May to July, August to October, and May to October. In each period, five water doses were established: 0, 25, 50, 75, and 100 % of the reference evapotranspiration (ETo). Five orchards were planted with *Quercus ilex* inoculated with *T. melanosporum*, and in each orchard, we arranged a two-factorial design with irrigation period and irrigation dose as main factors to test their combined effects on the development of both *T. melanosporum* and *Q. ilex* after 3 years in the field. Irrigation period significantly interacted with irrigation doses for the absolute presence per seedling of *T. melanosporum* mycorrhizae. Irrigation in May–July increased significantly *T. melanosporum* colonization in seedlings irrigated with 50 % ETo dose compared to the 0 % ETo dose. A similar pattern with smaller differences in means was observed in August–October period, but the irrigation doses did not change *T. melanosporum* colonization when we watered from May to October. We found ectomycorrhizae different from *T. melanosporum* in 51 % of the seedlings studied, but their presence was marginal. Our results suggest that a moderate irrigation dose promotes seedling growth and number of fine root tips per unit of fine root length, which may be potentially colonized by *T. melanosporum*.

Keywords *Quercus ilex* · Black truffle · Evapotranspiration · Preproduction · Ectomycorrhizae

Introduction

Black truffle orchards have been increasing in the last few decades in the Mediterranean area and beyond due to the high prices that black truffles fetch in the market (Mello et al. 2006; Bonito et al. 2011) and to the lack of profitable alternative crops in the rural areas where this fungus grows. The availability of commercial seedlings mycorrhized with the black truffle fungus (*Tuber melanosporum* Vittad.) (Grente and Delmas 1974; Savoie and Largeteau 2011) and the recently gained knowledge about the black truffle life cycle Kües and Martin (2011) have helped reduce the uncertainties of this culture. Nevertheless, several questions remain regarding the management of young black truffle orchards. This is especially important during the pre-productive phase, from planting to the beginning of regular harvests, usually during the eighth or tenth year. Soil cultivation techniques, weed control methods, and irrigation protocols remain open questions for black truffle growers who request concrete protocols from researchers.

Young black truffle orchards are commonly irrigated to avoid the effects of drought events characteristic of the Mediterranean climate where this fungus is endemic (Ricard 2003). The amount of water is a crucial issue because there is not a linear relationship between water input and proliferation of *T. melanosporum* in young orchards (Bonet et al. 2006). Irrigation with high doses did not reach the same positive results obtained with medium doses in a field study (Olivera et al. 2011), and even promoted colonization of root tips by competitor fungi in a glasshouse study (Mamoun and Olivier 1993).

A. Olivera (✉) · J. A. Bonet · C. Colinas
University of Lleida-Agrotecnio (UdL-Agrotecnio), Lleida, Spain
e-mail: antoni.olivera@pvcf.udl.cat

J. A. Bonet · D. Oliach · C. Colinas
Forest Sciences Centre of Catalonia (CTFC), Lleida, Spain

Selecting the best time of year to irrigate remains a concern to black truffle orchard managers. Irrigation in black truffle orchards has been generally applied in the summer (Olivera et al. 2011; Bonet et al. 2006), when hottest and driest conditions occur in the Mediterranean area. However, drought events may also happen in other seasons (Misson et al. 2011). The vegetative activity of most mature Mediterranean trees is higher in spring than in the other seasons, so a drought event in spring could have a larger impact than in summer when the highest temperatures of the year are already limiting the growth of the trees (Coll et al. 2012). A water shortage in spring reduces the photosynthetic activity of the host tree and consequently the allocation of carbohydrates to the ectomycorrhizal partners (Nehls et al. 2010).

Little information exists on the proper methods to estimate the water requirements of truffle orchards. Such methods should conform to the variability of precipitation of the Mediterranean climate, and be easily applied by growers. Methods based on climate data are available now from meteorological networks or microclimate monitoring systems placed in the field for other crops. The reference evapotranspiration (ETo) is a general program based on climatic parameters that was proposed by The Food Agriculture Organization of the United Nations (FAO) (Doorenbos and Pruitt 1975) and refined by Allen et al. (1998), and has been widely adopted in agricultural water management (Villalobos et al. 2013). This methodology also establishes an additional dimensionless crop coefficient calculated to determine the specific water needs of a crop depending on the species and the stage of development. Although FAO has published crop coefficients for many important crops, no coefficient is available for truffle orchards.

The aim of this work is to explore the effects of five irrigation doses, defined as percents of ETo, applied in three seasonal periods on the growth of both *T. melanosporum* and *Q. ilex* in Mediterranean climate conditions. Irrigation was implemented for three consecutive years after planting and the ectomycorrhizal status and the growth of seedlings was evaluated after those 3 years of treatments on excavated seedlings.

Material and methods

Study area

Five truffle-oak orchards located in Northeastern Spain were established between January and March of 2001 in four sites, at altitudes ranging from 251 to 1,205 m above sea level (Table 1). The two orchards sharing the same site were on different properties and aspects. The soils had loamy soil textures with moderate–high levels of calcium carbonate and a soil pH in water that ranged from 7.8 to 8.5. Mean monthly precipitation and evapotranspiration of the four sites in the 3 years of this study showed a Mediterranean climate type with high reference ETo year-round and low precipitation in the summer season. In the 3-year study, the average annual precipitation and ETo of the four sites were 690 and 924 mm, respectively. The precipitation and temperature data were obtained from installed probes (Hobo Onset Computer Corp., Bourn, Massachusetts, USA) in each site, and the ETo was obtained from a nearby automatic meteorological weather station (Agro-climatology Network of Catalonia, Generalitat of Catalonia). Soil and climate characteristics of the sites were adequate for truffle production according to the recommendations provided by Colinas et al. (2007). Seedlings were planted in rows separated 6 m, and within each row, the distance between seedlings was 5 m. Before planting, the soil was subsoiled to a depth of 60 cm to break-up hardpans and to promote deep soil aeration, and then it was tilled superficially several times to provide a planting zone free of herbaceous vegetation. Weeds around the seedlings were manually removed with a hand hoe as needed to provide a competition-free zone. No other treatments were applied to the orchards.

Table 1 Position and soil characteristics of the black truffle orchards

	Orchards 1 and 2	Orchard 3	Orchard 4	Orchard 5
Latitude	42°00′N	42°01′N	41°54′N	41°18′N
Longitude	0°59′E	1°57′E	1°35′E	1°14′E
Altitude (m)	1,205	666	678	251
Slope (%)	9	2	2	0
Soil pH (H2O)	7.8	8.1	8.3	8.5
Organic material (%)	5.4	4.6	4.4	2.1
Calcium carbonate (%)	28	27	38	38
Soil texture class (USDA)	Sandy clay loam	Sandy loam	Loam	Loam

Plant material

All seedlings were 1-year old Holm oak (*Quercus ilex* L.) inoculated with *T. melanosporum* Vittad. purchased from a commercial nursery, where they were grown in 5×5×20 cm pots filled with peat-perlite-vermiculite substrate. Prior to outplanting, the seedlings had a mean of 210 *T. melanosporum* mycorrhizae per seedling (CI_{95} (95 % confidence interval) 165–254), which accounted for 48 % (CI_{95} 44–51) of their total amount of root tips. These mycorrhizal characteristics are considered good-quality status for outplanted truffle inoculated seedlings (Alvarado and Manjon 2013). No ectomycorrhizae formed by other species of fungi were found in the seedlings sampled.

Experimental design

We used a factorial treatment structure with two factors as follows: (a) irrigation with five levels: doses of 0, 25, 50, 75, and 100 % of ETo, and (b) period of irrigation with three levels: watering from May 1st to July 31st, from August 1st to October 31st, and from May 1st to October 31st. We selected the end of July as the time to change the irrigation according to changes on vegetative growth in wet or irrigated holm oak trees observed previously (Montserrat-Martí et al. 2009; Sanz-Perez et al. 2009). In each period, the frequency of irrigation was 2 or 3 weeks according to the evaporative demand. This design resulted in 15 treatment combinations replicated in 5 orchards considered as blocks with a total of 75 experimental units. The experimental unit consisted in an area of 6×10 m with six planted seedlings in two rows plus the addition of three seedlings planted at the same time between both rows to be extracted for destructive root sampling. To prevent a border effect of the irrigation treatments, we left a row of seedlings between experimental units that were not irrigated.

Irrigation treatments

During the 3 years of the study, the averages of the amount of water supplied throughout May–July, August–October, and May–October periods for the 100 % ETo dose were 254, 211, and 359 mm, respectively. Precipitation and ETo for the irrigation periods are shown in Table 2. The remaining doses of irrigation were applied in the same days as the maximum dose (100 % ETo) and followed the proportional volumes established in the irrigation plan. Although we planned to supply the water doses referring to the accumulated ETo since the previous irrigation, occasional precipitation on days following irrigation hindered the optimization of the irrigation frequency as planned. In these cases, the frequency of irrigation varied between 3 and 4 weeks depending on the natural precipitation. The irrigation doses at each irrigation period were calculated to achieve the target % of ETo taking into consideration any precipitation above 15 mm that may have occurred after the last watering. The water was supplied in a circle with a radius of 20 cm around the seedling stem during the first and second year, and in a 40-cm circle the third year. At early stages of development, as in our case, *Q. ilex* seedlings develop a deep taproot and only short lateral roots. From previous work in similar sites (Olivera et al. 2011), we found that the root system of *Q. ilex* seedlings does not spread beyond 30–40 cm from the stem after 4 years in the field.

Table 2 Mean precipitation and ETo of the four stations near the study sites and the water supplied in the 3 years of the study

	Precipitation (mm)	ETo (mm)	Irrigation (mm)
May–July	170	452	254
August–October	266	286	211
May–October	436	738	359
November–April	248	187	0

Seedling extraction and fine root tips analysis

Between December 2003 and January 2004, one seedling per experimental unit was randomly selected and extracted by digging a 2-m deep circular trench 2 m from the seedling stem with a small backhoe and manually loosening the soil towards the plant until it could be released from the soil without damaging the roots. Excavated whole seedlings were taken to the laboratory, where adhering soil particles were carefully washed from the roots. Roots were cut in pieces of 3 to 15 cm and observed under a stereomicroscope. Subsequently, we sorted the roots into fine, with diameters <2 mm, and large otherwise. Afterwards, we estimated the root length of each fraction using the grid intercept method (Marsh 1971). When it was necessary, we cut the roots again. The roots were then dried at 60 °C for 72 h to determine the dry weight of the fine roots.

Seedling stem diameter and height were measured before cutting the root system. We considered root tips to be ectomycorrhizal if (a) root hairs were absent, (b) they appeared swollen, and (c) had a hyphal sheath. All root tips were counted and classified as uncolonized, *T. melanosporum* morphotype, or other morphotypes, which were considered competitors. The total number of ectomycorrhizae of *T. melanosporum* per seedling was recorded, and its relative abundance was calculated as the number of root tips colonized by this morphotype divided by the total number of ectomycorrhizal root tips. In this process, we used a dissecting microscope (×50) and a compound microscope (×100–1,000). *T. melanosporum* ectomycorrhizae were identified following the description of Rauscher and Chevalier (1995).

Statistical analysis

We used the mixed model procedure in SAS (SAS 2008, Cary, NC, USA) to analyze our data, so that we could specify the random effects of blocks and the interaction between the two main factors, calculate type III F-test for effects of irrigation period and dose, and the interaction between them. The homogeneity of the group variances was checked by the F and Levene tests. When the test for homogeneity of variances indicated that the variances were not equal, a model with unequal variances, residual variances in this case, was specified with the repeat statement with the group option (Littell et al. 2006). The means were estimated by least-squares. As a rule, we tested for differences among levels using t tests for the statistical significance of the difference between the means in each pair, alpha <0.05, and when the variances were unequal, the estimated standard errors of the differences were combinations of the variances, and thus the degrees of freedom were approximated.

Results

T. melanosporum and competitor fungi mycorrhizal colonization

The amount of *T. melanosporum* mycorrhizae varied significantly across irrigation doses ($P<0.0003$) and irrigation periods ($P<0.0024$). Moreover, the interaction of dose by period was also significant ($P<0.0012$). For individual treatments, irrigation only produced significant effects on *T. melanosporum* colonization when applied on the May–July and August–October periods (Fig. 1). Seedlings irrigated with 50 % ETo in May–July had significantly more *T. melanosporum* mycorrhizae than all other treatments, while the August–October period had the same tendency in 25 and 50 % ETo irrigation doses. The May–October period irrigation doses did not produce significant effects on the number of *T. melanosporum* mycorrhizae.

T. melanosporum relative abundance did not vary with irrigation treatments. We observed an average of 80 % (CI_{95} 74–87) for all seedlings. Likewise, we did not observe irrigation effects on the amount of competitor mycorrhizal colonization per seedling. There was an average of 75 (CI_{95} 46–103) mycorrhizae of competitor morphotypes per seedling. In 20 % of the seedlings with competitors, the relative abundance of *T. melanosporum* was lower than 50 %, and 49 % of all seedlings were completely free of competitors.

Ectomycorrhizal colonization

The amount of uncolonized root tips per seedling was significantly ($P<0.0081$) affected by the irrigation doses, as was the total amount of root tips per seedling ($P<0.0007$), but neither of these parameters were affected by irrigation period. The number of uncolonized root tips was higher in seedlings with 50, 75, and 100 % ETo doses than in seedlings not irrigated, while the amount of total root tips was higher in the 50 % ETo dose than in all other treatments (Fig. 2). The relative abundance of ectomycorrhizae (number of root tips colonized by ectomycorrhizal fungi divided by total number of root tips) was only significant at $P<0.085$ for the irrigation dose and decreased with increasing water availability.

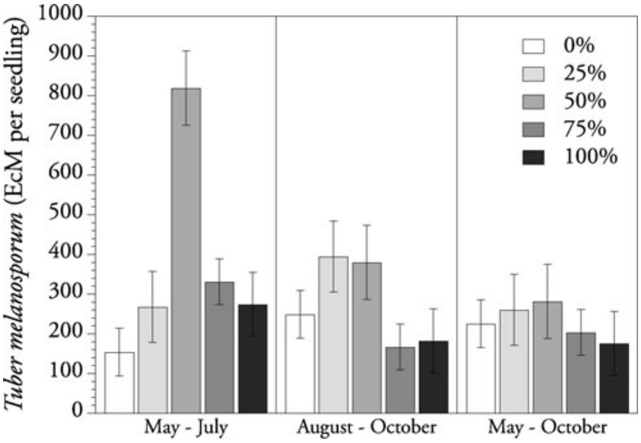


Fig. 1 Root tips colonized by *T. melanosporum* in response to five irrigation doses based in the replacement of 0, 25, 50, 75, and 100 % of the estimated evapotranspiration applied in three irrigation periods. In each irrigation period, *vertical bars* represent ± 1 SE

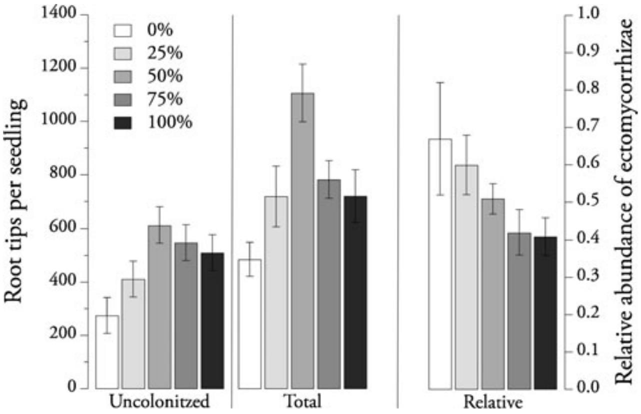


Fig. 2 Uncolonized root tips, total root tips (colonized plus uncolonized), and relative abundance of ectomycorrhizae (total ectomycorrhizal root tips per seedling divided by total root tips) in response to five irrigation doses based in the replacement of 0, 25, 50, 75, and 100 % of the estimated evapotranspiration. In each parameter, *vertical bars* represent ± 1 SE

Seedling growth

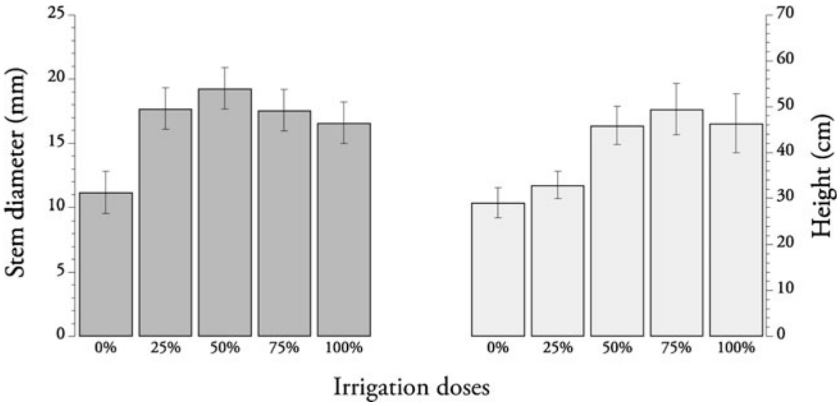
Irrigation doses had significant effects on both shoot height ($P<0.0004$) and stem diameter ($P<0.0001$). Irrigated seedlings were taller in 50, 75, and 100 % ETo treatments than in the lowest dose or the control (Fig. 3). The stem diameter of nonirrigated seedlings was smaller than all irrigated seedlings. Fine root weight and fine root length were not affected by any treatment.

Discussion

Moderate irrigation doses can promote *T. melanosporum* development in short irrigation periods

Seedlings treated with moderate doses of irrigation both in May–July and August–October periods had more *T. melanosporum* ectomycorrhizae compared to nonirrigated or heavily irrigated seedlings. Our observations on the effects of irrigating at the end of the growing season are consistent with previous work by Bonet et al. (2006) and Olivera et al. (2011) who found that August and September irrigation in moderate doses promoted the development of *T. melanosporum*. Yet, in our study, *T. melanosporum* performed even better when irrigation took place at the beginning rather than at the end

Fig. 3 Means of stem seedling diameter and height for each irrigation dose after 3 years in the field. In each parameter, *vertical bars* represent ± 1 SE



of the growing season. This fact could be related to the differences in the distribution of precipitation and evaporative demand in both periods. The evaporative demand was higher in May–July than in August–October, while precipitation was lower in May–July than in August–October. Therefore, irrigation in May–July reduced water stress periods more than in August–October.

Our finding that *T. melanosporum* grows more in plots moderately irrigated in May–July is supported by the seasonal pattern of carbon fixation of the host plant. The net photosynthesis of *Q. ilex* has its highest peak in spring (Gratani et al. 2008), when *T. melanosporum* accumulates carbon in mycorrhizae (Le Tacon et al. 2013). The coincidence in time between the spring photosynthate demands of the fungal partner and the photosynthetic activity of the vascular partner explains why photosynthesis-limiting water stress can be more damaging to the fungus earlier than later in the growing season.

Long period of irrigation did not affect colonization of *T. melanosporum*

T. melanosporum colonization did not benefit when irrigation was applied throughout the growing season. A possible explanation for this would be the seasonal pattern of root tip production and longevity in Holm oak. Root tip longevity in Mediterranean field conditions is lowest in spring and highest in the summer (López et al. 2001). By prolonging the spring high soil moisture conditions through the summer and into fall, we may have forced a high turnover of short-lived root tips through the summer. This shorter lifespan of individual short roots may limit the capacity of *T. melanosporum* to form mycorrhizae.

Ectomycorrhizal colonization and competition

The relative abundance of *T. melanosporum* was not related to irrigation but was quite high, with an average of 80 % of the mycorrhizae belonging to the truffle morphotype. This surprisingly high proportion of *T. melanosporum* morphotype was greater than the 67 % observed by Olivera et al. (2011) in similar young orchards, the 44 % found by Martínez de Aragón et al. (2012) in a plantation on a burnt forest, and the range of 44–69 % observed by Águeda et al. (2010) in mature truffle orchards. A possible explanation could be the progressive colonization of the root systems by other ectomycorrhizal fungi as the orchards grow older. Regardless of the timing of the irrigation, the highest amount of root tips observed per seedling was obtained when we compensated 50 % of the ETo. Taking into account that we did not find differences in fine root length, the 50 % ETo dose would have promoted the formation of root tips, which could be due to the formation of ectomycorrhizae in clusters. The decrease in the total amount of root tips in seedlings heavily irrigated did not occur for uncolonized tips, suggesting that most of the reduction of root tips caused by excess irrigation took place in ectomycorrhizal root tips.

Irrigation can modify stem diameter and height of seedlings

Seedlings irrigated with medium-high doses for 3 years gained more stem diameter and height than those not irrigated. In xeric sites, water availability is a clear growth-limiting factor for *Q. ilex* (Terradas and Savé 1992; Ogaya and Peñuelas 2007) and, consequently, higher water availability promotes seedling growth. Growth increases related to extra water supply in the summer were also observed by Rey Benayas (1998) in a field study of *Q. ilex* seedlings planted in a semiarid continental Mediterranean region. Our seedling results are consistent with the 66 % diameter growth increase resulting from summer irrigation in a high-density mature holm oak coppice (Mayor and Rodà 1994). However, the fact that higher irrigation doses did not affect the growth of seedlings in our study suggests that water may not be limiting beyond a certain threshold as was observed by Olivera et al. (2011) in a similar study under less arid conditions.

Concluding remarks

This study provides information on the effects of different irrigation regimes, based on evapotranspiration demand, on the proliferation of *T. melanosporum* mycorrhizae. The results suggest that the "comfort zone" of the black truffle regarding water availability is quite narrow, both in terms of amount and timing. Maybe that is the reason why its natural habitat is so reduced. A recommended watering regime for the establishment phase of *T. melanosporum*–*Q. ilex* orchards in continental Mediterranean conditions would consist of complementing natural precipitation up to 50 % of the ETo during the first half of the growing season and allowing for the onset of a mild drought before the autumn rains.

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