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Effect of summer soil moisture and temperature on the vertical distribution of *Tuber magnatum* mycelium in soil

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Abstract - *Tuber magnatum*, the Italian white truffle, is the world's most valuable truffle. Despite the economic importance, its biology and ecology are largely unexplored. This gap of knowledge makes difficult to find reliable methods for its cultivation and to protect and increase the production of the natural productive areas. In this study, the vertical distribution of *Tuber magnatum* mycelium in productive soil patches was evaluated using a quantitative PCR (qPCR) approach. Data were then used to develop for the first time a simulation model to predict the mycelial dynamics of *T. magnatum* at varying soil temperature and moisture. *T. magnatum* mycelium was abundant up to 30 cm depth, while the model determined the optimal temperature (20 °C) and water potential (~0 kPa) for growth of *T. magnatum* mycelium in soil. Such information could be useful to establish proper irrigation scheduling and to enhance the management of *T. magnatum* sites, for increasing mycelial growth and fruiting body production.

Keywords: extra-radical mycelium; quantitative PCR; mycelium dynamics; truffle; mathematical modelling

Introduction

True truffles (*Tuber* spp.) are hypogeous ascomycetes forming ectomycorrhizal (ECM) associations with different forest trees and shrubs in temperate areas (Zambonelli et al. 2016). The genus *Tuber* includes about 200 species worldwide distributed and only few species, mostly European, have a considerable economic value due to the organoleptic proprieties of their ascomata. Among them, *Tuber magnatum* Pico is the world's most expensive truffle. At present it is only found in a few countries including Italy, Croatia, Romania, Serbia, Hungary, France and Slovenia (Bratek et al. 2004; Ceruti et al. 2003; Glamočlija et al. 1997). Attempts to cultivate it are often failed because the ecology and biology of this ECM ascomycete remain largely unexplored (Hall et al. 2003). This knowledge gap depends on the difficulties to obtain adequate pure cultures of this fungus, to grow their ECMs under controlled conditions or to detect it in the field (Bertini et al. 2006; Iotti et al. 2012b; Leonardi et al. 2013; Riccioni et al. 2016; Zambonelli et al. 2015). In natural forests, fruiting body production is scattered and depends on poorly known climate dynamics. Moreover, truffle hunters keep secret any information on the harvested truffles, making difficult to establish the exact amounts, times and sites of fructification (Hall et al. 1998, 2007). Therefore, the extra-radical mycelium is the most reliable target for studying *T. magnatum* in the field also because it can form an extended mycelial network in the productive areas (Zampieri et al. 2010).

The detection of soil mycelium is needed to monitor the development of ECM fungi (Landeweert et al. 2003; Wallander et al. 2013) and the real-time PCR assay can quantify the distribution of extra-radical mycelial biomass in the space or through the soil profile (Uroz et al. 2011) and changes through the seasons (for references see Parlade et al. 2016) or to detect fungal biomass. Quantitative PCR (qPCR) techniques can detect the most valuable *Tuber* species, such as *Tuber melanosporum* Vittad. (the Perigord black truffle; Parladé et al. 2013), *Tuber aestivum* (the burgundy truffle; Gryndler et al. 2013) and *T. magnatum* (Iotti et al. 2012a). Using this technique, Iotti et al. (2012a, 2014) found a positive correlation between the abundance of *T. magnatum*

mycelium in soil and the fruiting body production in four different natural truffle grounds. Salerni et al. (2014) showed that soil tillage increased the mycelial biomass of *T. magnatum*. However, the vertical distribution of the extra-radical mycelium of *T. magnatum* or other ECM fungi is not known.

The influence of climate on truffle production and distribution under field conditions has only been studied in *T. melanosporum* and *Tuber borchii* Vittad. (Büntgen et al. 2012; Le Tacon et al. 2014; Salerni et al. 2014b). In particular, it was showed that temperature and rainfall in the months before the fruiting season are the most important factors affecting the annual fluctuations in truffle productions. It has been hypothesized that the dry season be the most critical period for *T. magnatum* production probably when primordia of the fruiting bodies begin to appear (Zambonelli, personal communication). Only Marjanović et al. (2015) attempted to relate soil type and climate trend to *T. magnatum* productivity **They found that the production might be favoured by waterlogging while a low N availability enhance ECM establishment (during summer).**

Several mathematical models can predict the effects of environmental factors on fungal growth (Boswell et al. 2003), and generally they describe the mycelial growth pattern in heterogeneous matrices such as soil (Boswell et al. 2007; Cazelles et al. 2013; Couteaudier and Steinberg 1990; Hopkins and Boswell 2012) or the fungal growth in response to nutrient concentration (Lamour et al. 2000; Paustian and Schnürer 1987). Modelling of fungal growth in response to temperature and moisture has been proposed for simulating the dynamics of infection due to plant pathogens (Lahouar et al. 2016; Manstretta and Rossi 2015; Palacios et al. 2014; Sharma and Pande 2013) the behaviour of ECM fungi *in vitro* (Zhang et al. 2011).

The aim of this study was to evaluate the vertical distribution of *T. magnatum* extra-radical mycelium in productive soil patches both before and after the dry season. We hypothesized that temperature and moisture can affect the distribution of *T. magnatum* mycelium through the soil

profile. Finally, we have developed a mathematical model simulating the effect of soil temperature and moisture on the mycelial dynamics of *T. magnatum* mycelium.

Materials and Methods

Study site

The study site is located at Saiarino (Argenta municipality, Ferrara, Emilia-Romagna, Italy) (44° 37' 10" N, 11° 48' 55" E, 5 m asl) in the park of the local water reclamation area "Bonifica Renana". This site represents the productive *T. magnatum* areas in the Po Valley. The putative *T. magnatum* host plants are poplar (*Populus nigra* L.) and linden (*Tilia vulgaris* Hayne). The park lies along a canal built during 19th century, with banks reaching 10 m elevation from field level. The banks were built from local loamy alluvisol (USDA-Aquic Ustochrept, coarse loamy, mixed, thermic) with the following characteristics: bulk density 1.1 to 1.60 g/cm³, organic matter 2.4 to 8 %, CaCO₃ 15 to 20 %, active limestone 2.9 to 11.0 % and pH 8 to 8.3. The climate is Humid Subtropical (Koppen-Cfa) with a mean annual air temperature of 14 °C and average annual rainfall of 634 mm in the period 1991-2015. July-August and December-February are the hottest (mean max temperature 23 °C) and coldest (mean min temperature 1 °C) months, respectively (https://www.arpae.it/dettaglio_generale.asp?id=3811&idlivello=1591). The temperature and precipitation monthly records taken from a nearby weather station (Saiarino) are displayed for the experimental period are shown in Figure 1. Detailed descriptions of soil, vegetation and landscape characteristics are given by Iotti et al. (2014).

Since 2008, truffle production has been assessed weekly from September to December by using trained dogs and the spatial distribution of *T. magnatum* extra-radical mycelium has been determined by a qPCR using different soil sampling strategies (Iotti et al. 2012a, 2014).

Plot design and management

A randomized block design was used to evaluate the vertical distribution of *T. magnatum* mycelium. Since the spatial distribution of *T. magnatum* extra-radical mycelium and ascoma production are scattered within the natural truffle grounds, block position was assigned based on previous investigations in the same study site (Iotti et al. 2012a, 2014). A total of four productive soil patches (50 to 100 m²), corresponding to four independent blocks, were selected in different areas of the truffle ground. Block 1 is within a woody area (park), block 2 at the boundary of the main bank, block 3 on the sloped side and block 4 beside the canal. Tree canopy in blocks 1 and 2 is 80% and 20%, respectively, whereas blocks 3 and 4 are not covered by tree canopy (Fig. 2). The putative *T. magnatum* host plants are linden (*Tilia x vulgaris* Hayne) in blocks 1-2-3, oak (*Quercus robur* L.) in block 1, and poplar (*Populus nigra* L.) in blocks 3-4.

-Within each block, two 2 x 2 m permanent plots were established at a distance of 4-6 m from each other. One plot per block was irrigated during summer (July and August) at intervals of 14 days in 2012 and 7 days in 2013 supplying 20 mm of water each time if no rainfall occurred in the previous period.

Soil sampling

Soil was sampled in spring and late summer of two consecutive years, 20 April and 12 September 2012, 23 May and 17 September 2013, and at 3 different depths, 0-10-cm, 10-20-cm and 20-30-cm. In this area the fruiting bodies of *T. magnatum* were only found within the first 30 cm of soil. Nine independent soil cores for each depth along the diagonals of each plot were taken using 1.6-cm diameter disposable polyvinyl chloride tubes. A border area of 30 cm on each side of the plot was excluded from sampling to minimize the edge effects. Soil cores were stored at 4 °C and extracted by breaking the tubes within 24 h since sampling. Soils from the same depth within a plot were thoroughly mixed to obtain three samples per plot for each sampling date. Root fragments, stones or organic debris were carefully removed from each sample. The soil was kept frozen at -80°C until

lyophilization (70 h in a Virtis Benchtop 2 K freeze dryer, SP Industries) and later ground and stirred in a mortar, sieved (1 mm mesh) and then stored at -20°C until DNA extraction.

Soil DNA extraction and qPCR assay

DNA was isolated from 5 g soil aliquots using a CTAB-based buffer (2% CTAB, 2% Polyvinylpyrrolidone, 2 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8) and purified by the Nucleospin Plant II kit (Macherey-Nagel), according to Iotti et al. (2012a). Three biological replicates per soil samples were processed. The yield and quality of the DNA extracts were determined using ad NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Extractions with optical density ratios (260/280 nm and 260/ 230 nm) or DNA concentration lower than 1.4 and 25 ng ml^{-1} , respectively, were repeated. DNA solutions were stored at -20°C until qPCR analysis.

Quantitative PCR was carried out using TaqMan probe and species-specific primers designed by Iotti et al. (2012a) for *T. magnatum*. Each extraction was amplified in duplicate in 25 μL reaction volume containing 1 X Maxima Probe qPCR Master mix (Fermentas), 30 nM ROX, 0.2 mM TaqMan probe (5'-6-FAM reporter dye, 3'-TAMRA quencher dye) (MWG BIOTECH) and 0.5 mM of each primer. Amplifications were performed in a Stratagene Mx3000P QPCR system (Stratagene) with the following profile: 10'' at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s.

Samples from plots of the same block collected in the same year were processed in the same plate for a total of 72 reactions [3 sampling depths (0-10, 10-20 and 20-30 cm) x 2 plots (irrigated and control) x 2 sampling dates x 3 biological replicates x 2 technical replicates].

A specific calibration curve was generated for each soil layer following the procedure described by Iotti et al. (2014). *T. magnatum* mycelium-free soils from the same truffle ground were used as negative controls and for preparing the calibration curve samples. A total of 5 ten-fold serial dilutions of *T. magnatum* sterile tissue per g of soil (from 1 mg to 1×10^{-4} mg) were processed in triplicate for each soil layer.

Statistical analyses

The values of *T. magnatum* mycelial abundance were analysed with a two-way ANOVA considering sampling depth (0-10, 10-20 and 20-30 cm), sampling year (2012 and 2013), sampling season (May and September) and treatment (watered and non-watered plots) as independent variables. Fisher's post-hoc test was used to separate means ($p > 0.05$). Data were log-transformed after Bartlett's test to meet the ANOVA requirements of homogeneity of variance. Statistical analyses were performed using XLSTAT software version 7.5.2 (Addinsoft).

Soil observation and mycelium model

Soil water content (θ) and soil temperature (T_s) at 10, 20 and 30 cm of depth were measured using six DM400 multi-sensor probes (DFM software, ZA). As only blocks 1 and 3 were split in watered and non-watered plots (while 2 and 4 only have watered plots) 6 probes were used to monitor θ and T_s . θ values were formerly used to obtain hydrological parametrisation of soils (Vitali et al. 2018) and used to obtain the values of water potential (ψ_s , Fig. S5).

Water potential reflects the effort an organism has to do to extract water from soil matrix. Therefore, a model aimed at simulating the response of extra-radical mycelial density (y) of *T. magnatum* should account for ψ_s together with other variables as T_s . In particular, our model describes mycelium growth y by a simple response to the above-mentioned variables. The model also considers the possible bi-directional flux of nutrients between the mycelium networks in the different soil layers. Because of a lack of information on surface water flows (water balance could not be appreciated with sufficient detail at 0-cm depth), only the lower layers (10-20 and 20-30 cm) have been investigated. The mycelium growth is described by the following equation:

$$dy_i / dt = g \cdot w(Ts_i, \psi_{Si}) \cdot y_i + \phi_{i,i+1} - \phi_{i,i-1} \quad (1)$$

where dy/dt is the mycelial growth rate in soil layers i , g is the maximum mycelial growth rate; w is the local response function to ψ_S and T_S , and ϕ_{ij} is the exchange rate of nutrients between the layers i and j . The response function is given by the product of two asymmetric Gaussian response functions (R, see Figure S1 in the supplementary material), respectively focused on temperature T_o and water potential ψ_o , corresponding to the optimal to mycelial growth

$$w = R(\psi_i; \psi_o, \psi_d) \cdot R(Ts; T_o, T_d) \quad i = 1, 2 \quad (2)$$

where T_d and ψ_d represent the spread and the sensitivity, respectively. The exchange rate is given by the function:

$$\phi_{i,j} = b \cdot \text{sign}(w_i - w_j) \cdot \min(y_i, y_j) \quad (3)$$

where b is maximum rate, $\text{sign}(w_i - w_j)$ is the direction of nutrient flux, given by the differences of w at the two depths; $\min(y_i, y_j)$ is the minimum value of mycelial density, when there is not a flow with the biomass of donor or recipient.

The model has been used within a non-linear least-square based optimisation procedure aimed at identifying the characteristic points of response functions, and it has been run with Matlab and Simulink (The Mathworks, Inc.).

Results and discussion

A total of 288 DNA extracts (96 soil samples per 3 biological replicates) were processed in four sampling seasons. A mean amount of 9.13 μg of total DNA per g of soil with $\text{OD}_{260/280}$ nm and $\text{OD}_{260/230}$ nm ratios of 1.83 and 1.80, respectively, was obtained. These mean values are similar to those previously reported on the same soil by Iotti et al. (2014). However, the different sampling strategy (vertical vs spatial) revealed strong differences among soil samples collected at different depths. In fact, DNA yields significantly decreased with depth (Fig. S2), probably due to the

decrease in microbial biomass by increasing depth (Baldrian et al. 2012; Fall et al. 2012) although the soil composition can also affect DNA extraction (Wallander et al. 2013). In this study, the effect of soil composition can be clearly inferred by the calibration curves generated for each soil depth after qPCRs (Fig.3).

Similar curves were obtained from soil samples collected at 10-20 and 20-30 cm (mineral horizon) of depth whereas the slope of the curve obtained from 0-10 cm soil samples was markedly higher. This difference was mainly due to the organic matter content which was 6-7 times higher in the upper sampling layer whereas the other soil component are quite similar through the soil profile (http://umi.unibo.it/magnatum/Relazione_pedologica%201.pdf). Organic matter is known to be one of the most important bias in assessing soil microbial diversity by molecular approaches (Feinstein et al. 2009; Schöler et al. 2017; Vestergaard et al. 2017).

Vertical distribution of mycelium

Statistical analyses revealed significant differences for all variables considered in the analyses, except for the treatment (irrigation). Differences were found for experimental block ($p < 0.0001$) as well as for year ($p = 0.0005$), depth ($p = 0.036$) and season ($p = 0.037$) of sampling (see statistical reports in Appendix A). However, the couples of variables “season / block” and “treatment / block” showed significant interactions. On the contrary, no significant interactions were found between the year or depth of sampling and the other variables considered in this analysis. While, the higher abundance of *T. magnatum* mycelium detected in 2013 was most likely due to the higher summer rainfall than that registered during summer 2012 (Fig. 1), the differences in the distribution of mycelium through the soil profile are more questionable. In this case, mycelial density was significantly higher in soil samples collected at 0-10 and 20-30 cm than those at 10-20 cm of depth (Fig. 4).

Generally, the abundance and richness of fungal species decrease from litter to mineral horizons (Baldrian et al. 2012; Jumpponen et al. 2010; Lindahal et al. 2007; Uroz et al. 2013) but

some ECM fungal species does not show the vertical trend. Landeweert et al. (2003) reported that 16 out of 25 ECM mycelia of basidiomycetes were found exclusively in mineral soil and Rosling et al. (2003) found that at least 50% of the mycorrhizal fungal taxa in a podzol soil were exclusively associated with the mineral soil horizons. For many fungal species, the vertical distribution of extra-radical mycelia does not match with the distribution of ECMs (Anderson and Cairney 2007; Genney et al. 2006; Landeweert et al. 2003), whose abundance decreases together with root density with soil depth (Courty et al. 2008; Genney et al. 2006; Rosling et al. 2003). In the case of *T. magnatum*, this mismatch is much more evident because its ECMs were not found in the orchard under investigation (Leonardi et al. 2013).

Our results showed that *T. magnatum* mycelium did not decrease with depth but probably, it may extend to a greater depth since its fruiting bodies were found up to 80 cm (Ceruti et al. 2003). The ability of *T. magnatum* to develop a conspicuous extra-radical mycelial network in deeper soil horizons may represent an advantage in competing for nutrients, and forming symbiosis or being protected against environmental stresses, such as high temperatures and drought. Indeed *T. magnatum* is sensitive to environmental stresses occurring during hard winters and dry summers (Le Tacon 2016) and this suggests that the summer drought is particularly detrimental for mycelial growth of this truffle species.

Modelling results

A major advantage of a simulation approach with respect to the statistical one concerns the possibility to fully account for organism dynamics, and its relation to environmental factors. The soil temperatures during the study period differed slightly through the soil profile but varied considerably between seasons (Fig. S3). Lower temperatures ($\sim 5^{\circ}\text{C}$ in all plots) were reached during winter (December-February) whereas summer maximum values varied among blocks due to presence/absence of the canopy: a difference of $3\text{-}5^{\circ}\text{C}$ was registered between shady (1 and 2) and sunny (3 and 4) blocks.

The differences in soil temperature between irrigated and rain-fed plots of blocks 1 and 3 (Fig. 5a) were very small, especially in the woody area (block 1) and at different depths. It is noteworthy, however, that irrigation increased soil temperature up to 3°C in the unshaded block 3, likely because of the water warming during infiltration through the upper warmer soil layer.

When considering the water content, greater differences between soil depths and plots were found with respect to differences in the temperature values (Fig. 5b; details in Fig S4, S5). The water content at 10-cm depth was generally greater than that at 20 cm and 30 cm through the year. The peaks occurring after water supply were higher than those after the natural precipitation events.

However, water volumes supplied during the two investigated summers were not sufficient to restore an adequate level of water content. In fact, comparison between irrigated and rain-fed plots of blocks 1 and 3 showed evident differences in water content at 20 cm of depth, with residual effects in the winter season, but not in the surface (10 cm) and deeper (30 cm) layers (Fig. 6). Such a limited effects of irrigation may explain the lack of statistically significant differences in mycelial abundance between irrigated and rain-fed plots.

Since the mycelial abundance was often always close to zero in rain-fed plots, model simulations may be better appreciated in irrigated plots (see Figure 7).

The adopted inversion technique identified the following optimal values of growth and biomass exchange rates: $g = 0.057$ and $b = 0.43$, respectively; optimal conditions of soil water potential and temperature were: $\Psi_o = 0$ cm (saturation) and $T_o = 19.6$ °C, respectively; and values at which mycelial growth was closes to zero: $\Psi_d = 31$ cm and $T_d = 1.5$ °C, respectively.

In the plots having the highest benefit from irrigation (Fig.7a, 7b) the mycelial abundance was higher in the layer 20-30 cm than in the w layer10-20 cm, probably because of a more stable moisture trend. In addition, the mycelial growth was the highest in the late spring, when temperatures and water availability increased after the beginning of the host plant activity after winter (Ekblad et al. 2013).

The method also proves to be able to characterise species-dependent parameters as the optimal temperature close to 20 °C and an water content near saturation confirming what already observed the environmental conditions promoting high productive *T. magnatum* areas in Serbia (Marjanović et al. 2015).

The comparison between simulated and measured mycelium abundances (qPCR values) is reported in Figure 8; the simulation model underestimates values, probably due to biotic and abiotic components not considered in this preliminary analysis. The model developed in this work is based on several assumptions reducing the number of required parameters, but trying to simulate mycelium dynamics properly. Simulation outputs confirm the hypothesis that mycelium is an interconnected network, where the performance of one layer can be related to others. The mycelium of ECM fungi “takes up irregularly dispersed nutrients in soil” (Read 1992) and supplies them to the host plant, whereas plants release the photosynthates to all the soil mycelial net.

Conclusions

For the first time we have monitored the vertical distribution of an ECM fungal species by applying the qPCR technique. In addition we have developed a model to simulate the dynamics of the extra-radical mycelium in soil as a function of soil water potential and temperature. To date, vertical distribution of ECM mycelia of single species has been only assayed by applying other molecular techniques (e.g. T-RFLP in Dikie et al. 2002 and Genney et al. 2006) and no mathematical models have simulated this distribution in the field. The studies on the distribution and dynamic of *T. magnatum* mycelium in soil are fundamental to improve the knowledge on this truffle and to find reliable methods for its cultivation. Future researches should address how far down *T. magnatum* mycelium can develop in different soil types, but other sampling strategies should be applied to collect soil samples below 30 cm depth. It could be also interesting to extend this sampling strategy

to other ECM species or to the whole ECM communities, since most studies ignore the distribution of ECM mycelia in the deeper mineral soil layers.

The model developed in this work predicted *T. magnatum* dynamic in summer, the most critical season because of high soil temperatures and water scarcity. Through this model the optimal temperature (20 °C) and water content of soil was determined, and these values may be useful for irrigating sites with growing *T. magnatum*, in order to increase mycelium development and, consequently, fruiting body production (Iotti et al. 2012a). However, the validity of the model needs to be verified in other soils and other climate conditions.

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Table & Figure captions

Fig. 1. Temperature and rainfall during the period of study. On the left scale are indicated min (dotted line), max (dashed line) and mean (solid line) monthly temperatures; on the right scale the monthly precipitations (columns).

Fig. 2. Scheme of experimental block localization with respect to the surrounding canal and arable fields.

Fig. 3. Calibration curves for the quantification of *T. magnatum* mycelial biomass at 0-10 (dashed line), 10-20 (dotted line) and 20-30 cm (solid line) of soil depth. Linear curves were obtained by plotting the log of dry weight of fungal biomass ($\text{mg} \times \text{g}^{-1}$ of dried soil) against the *T. magnatum* DNA concentration ($\text{pg} \times 200 \text{ ng}^{-1}$ of total DNA). A total of 5 ten-fold serial dilutions of fungal tissue per g of soil (from 1 mg to 1×10^{-4} mg) were processed in triplicate for each soil layer.

Fig. 4. Mean amount of extra-radical soil mycelium of *T. magnatum* in the different soil layers (0-10, 10-20 and 20-30 cm). Error bars represent standard error ($n = 32$). ANOVA was carried out on log-transformed values [$y = \log(x + 1)$]. Different letters indicate significant differences between soil layers ($p < 0.036$).

Fig. 5. Temperature differences (ΔT_s) between rain-fed and irrigated plots (blocks B1 and B3) at 10, 20, and 30 cm of soil depth.

Fig. 6. Water content differences ($\Delta \theta$) between rain-fed and irrigated plots in block B1 (a) and B3 (b) at 10, 20, and 30 cm of soil depth. Raw records (θ^*) are used.

Fig. 7. Simulated and observed values of mycelial abundance in the 6 plots and overall comparison at observation times.

Fig. 8. Simulated vs observed values for all observed plots

Appendix A. Supplementary material

Fig. S1. Response functions obtained by the adaptation procedure

Fig. S2. Mean amount of total genomic DNA isolated from different soil layers (0-10, 10-20 and 20-30 cm). Error bars represent standard error ($n = 32$, values obtained from the mean of three biological replicates). Different letters indicate significant differences between soil layers ($p < 0.0001$).

Fig. S3. Soil temperature records (from DFM probes) during the study period.

Fig. S4. Soil water content records (from DFM probes) during the study period.

Fig. S5. Soil water potential computed from estimated soil parameters (retention curve) for the study period.

Fig1

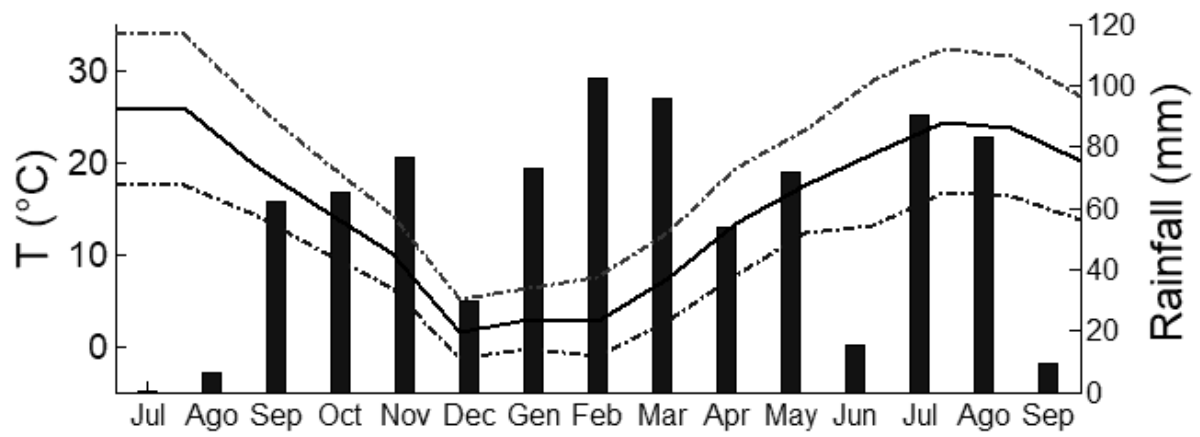


Fig2

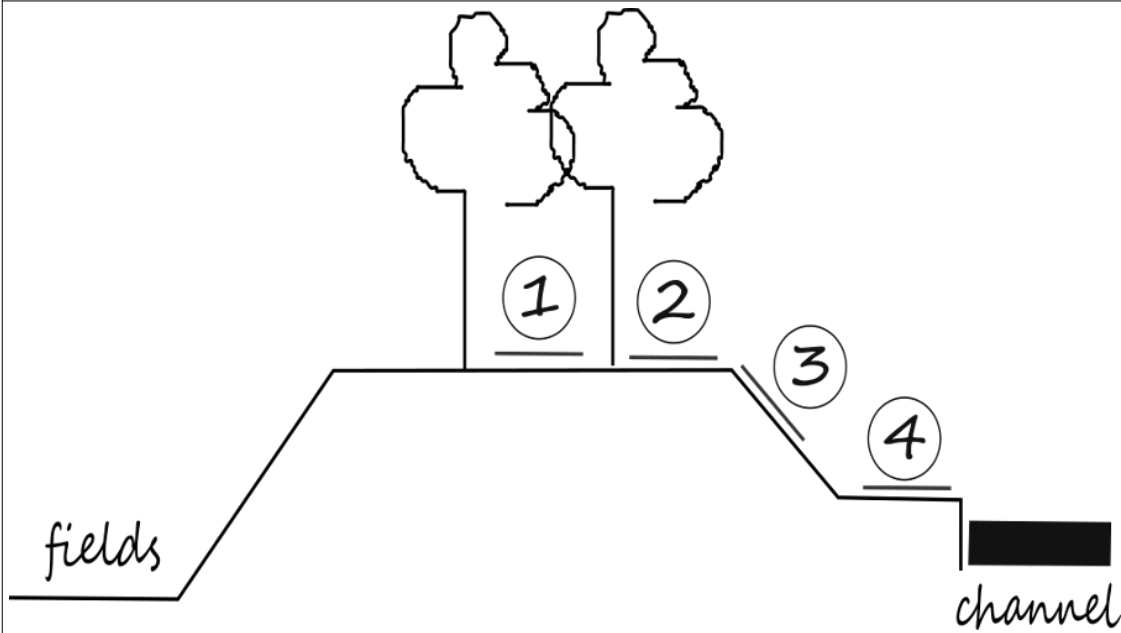


Fig.3

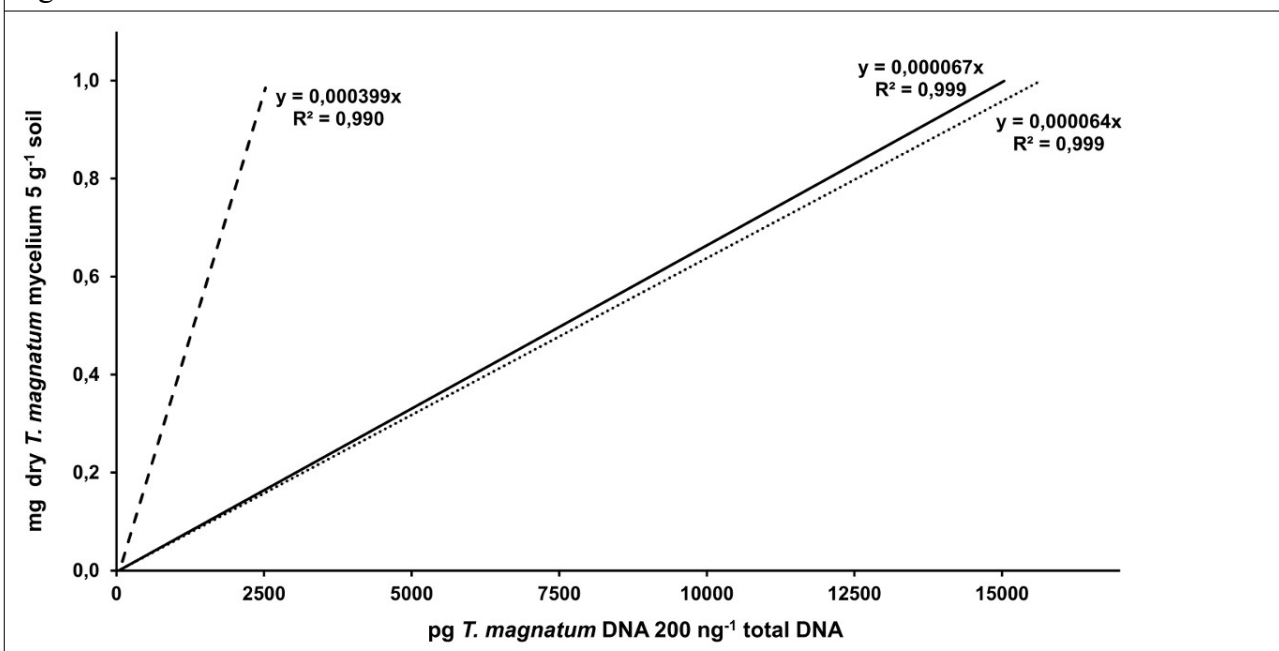


Fig.4

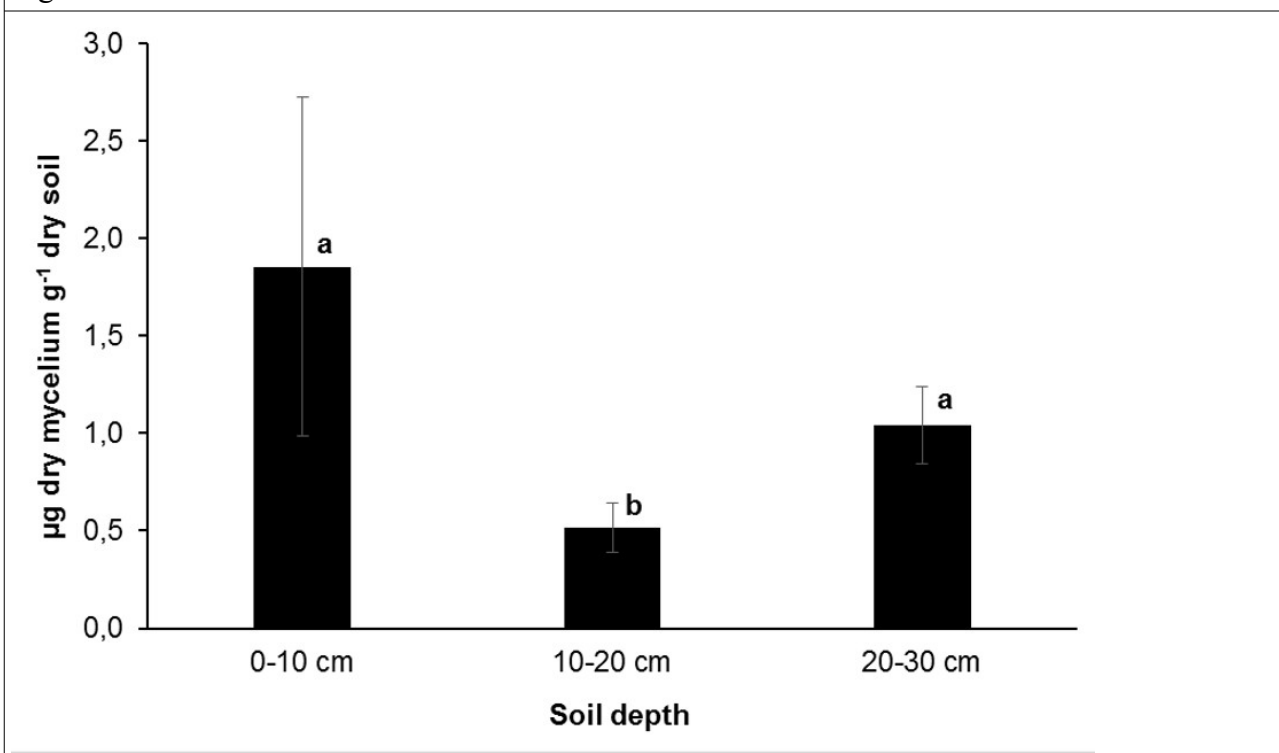


Fig.5a

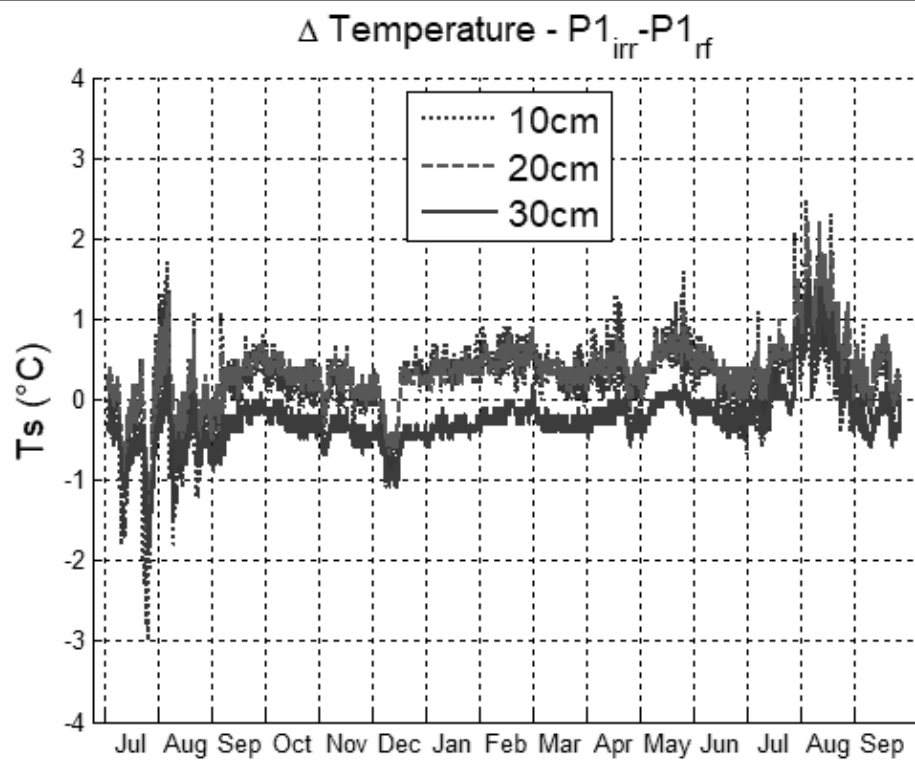


Fig.5b

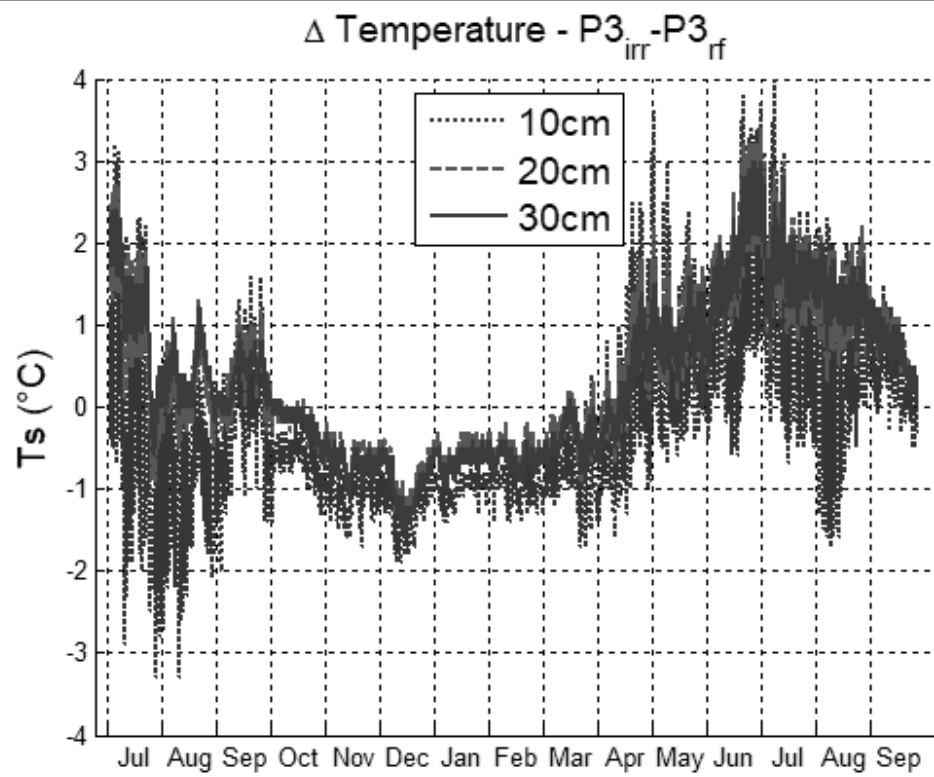


Fig.6a

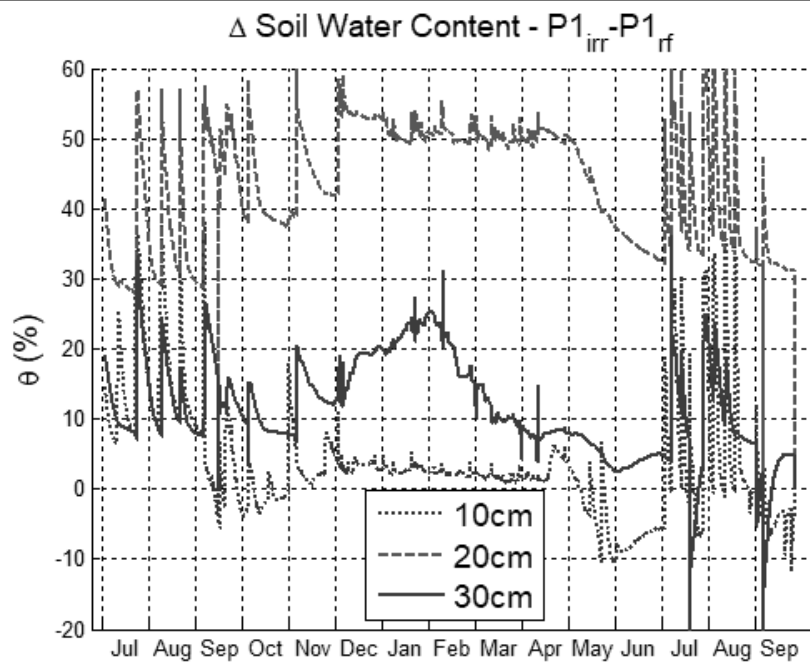


Fig.6b

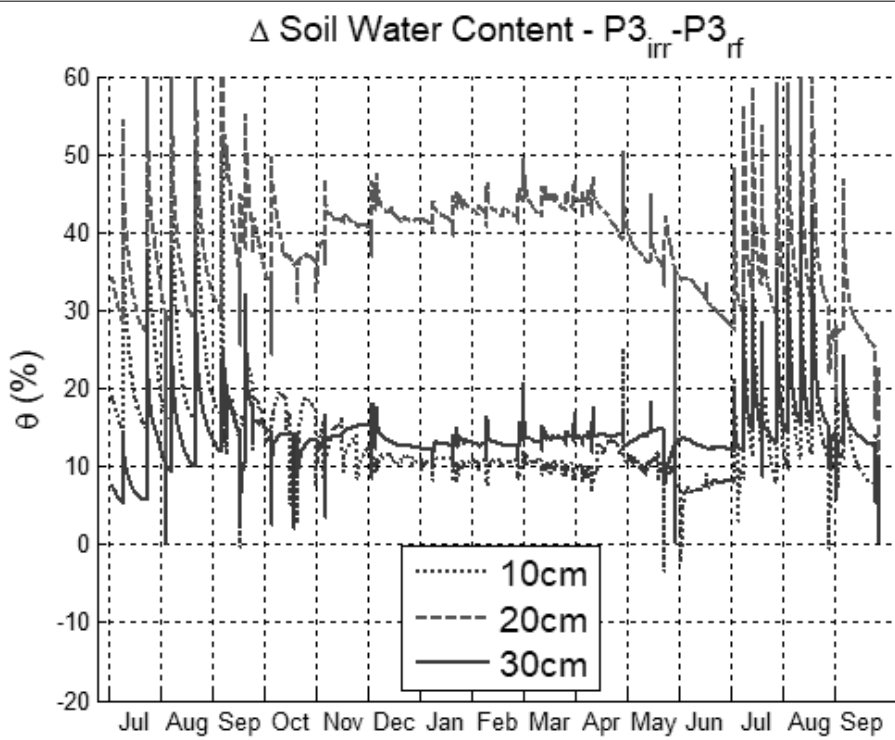


Fig.7

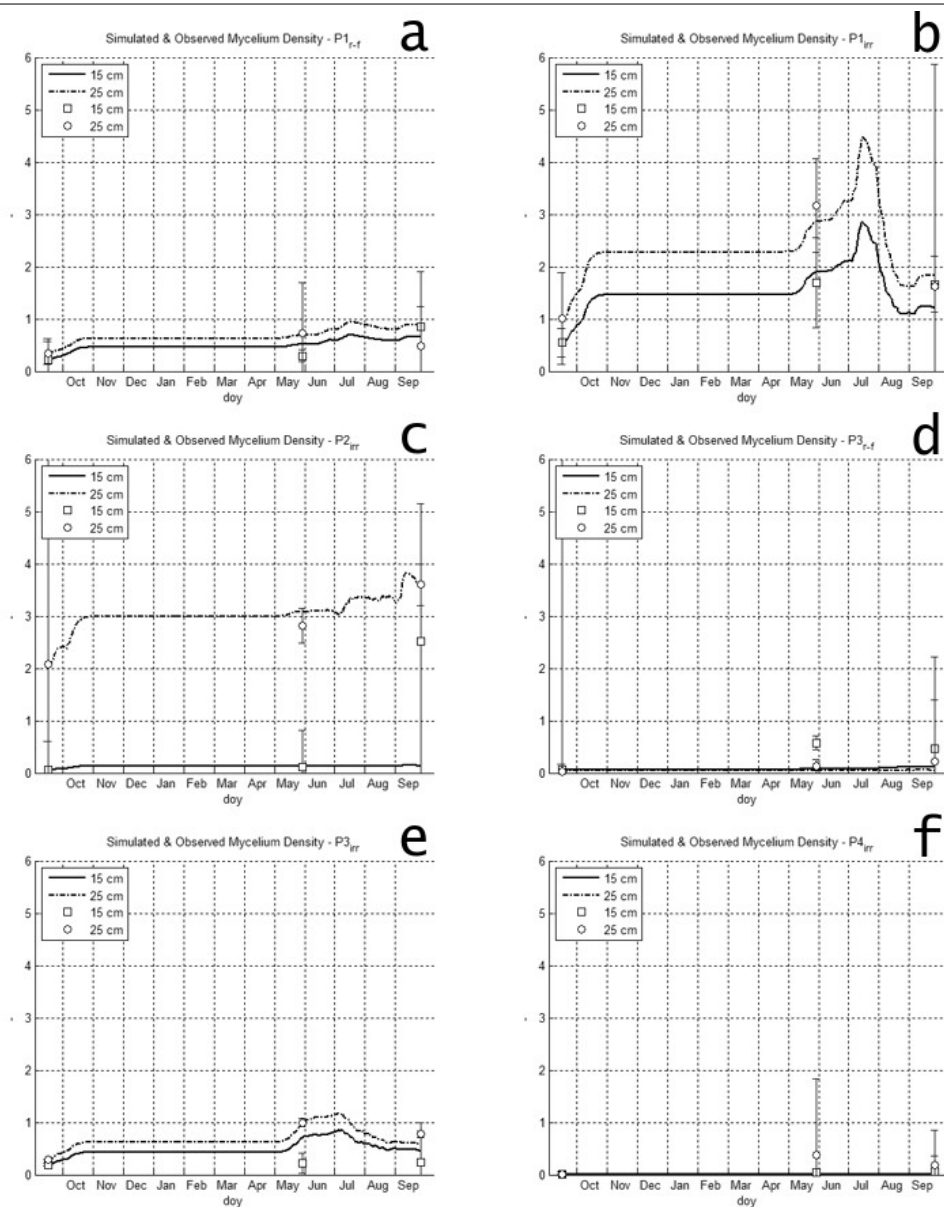


Fig.8

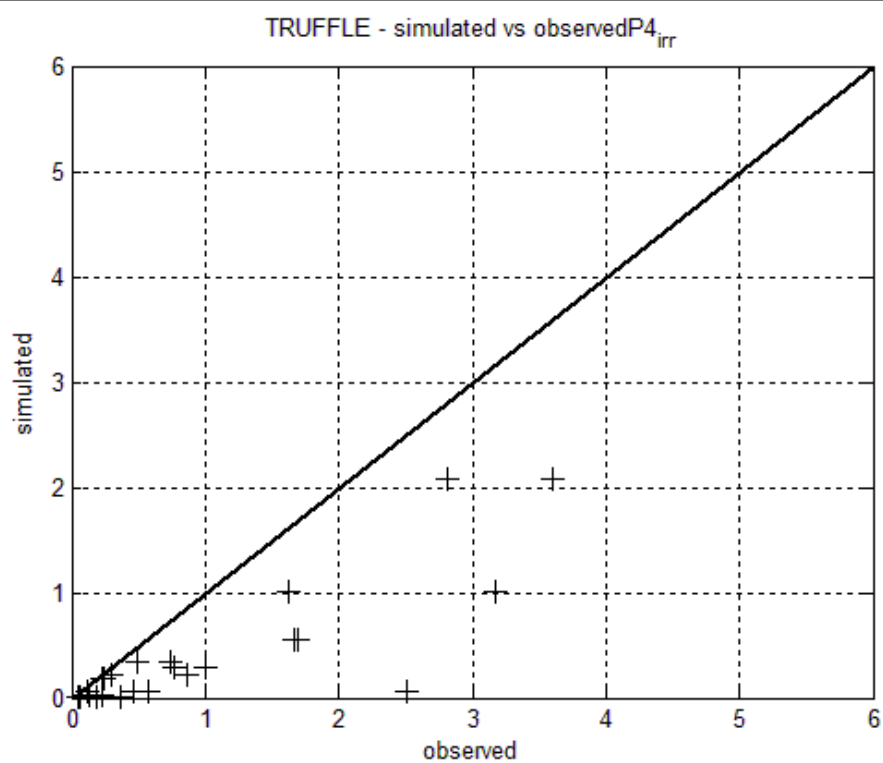


Fig.S1

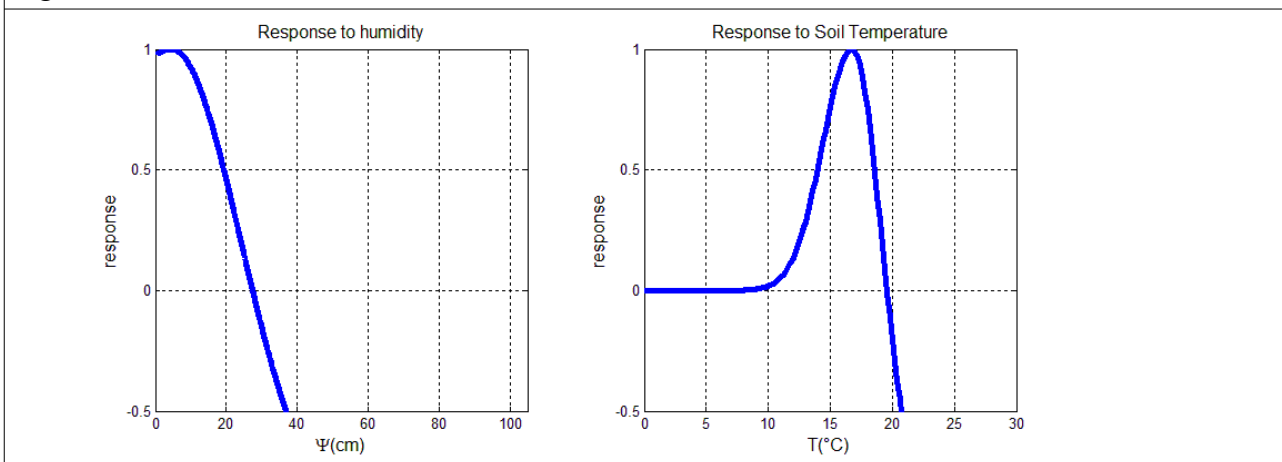


Fig.S2

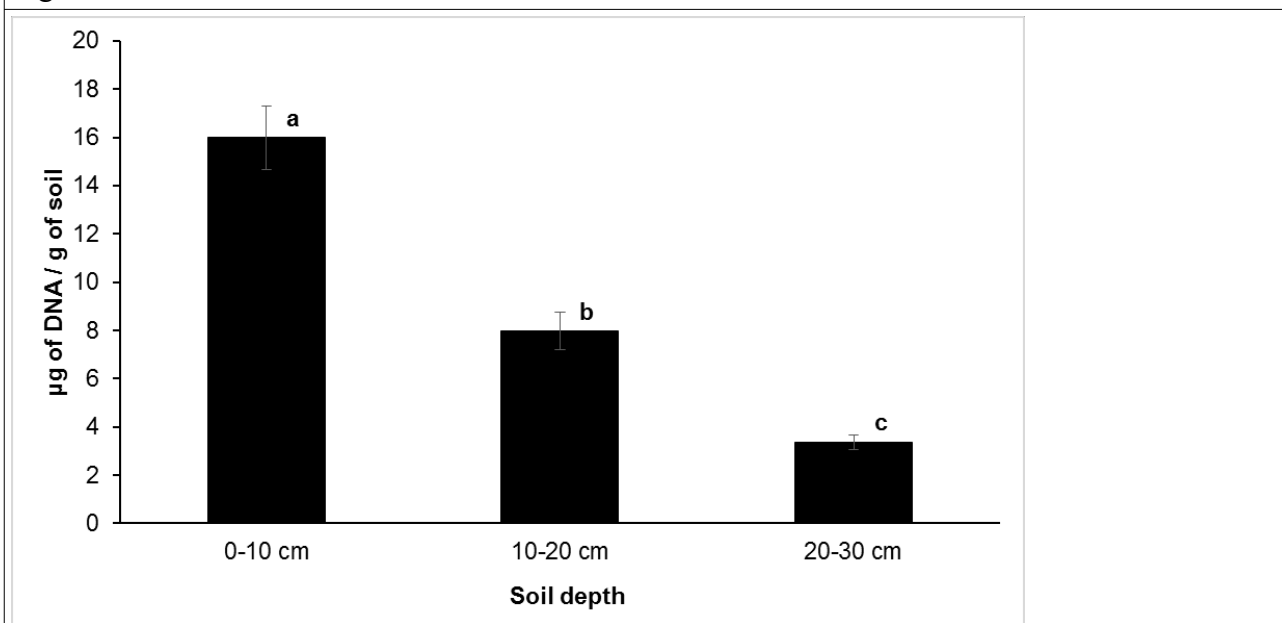


Fig.S3

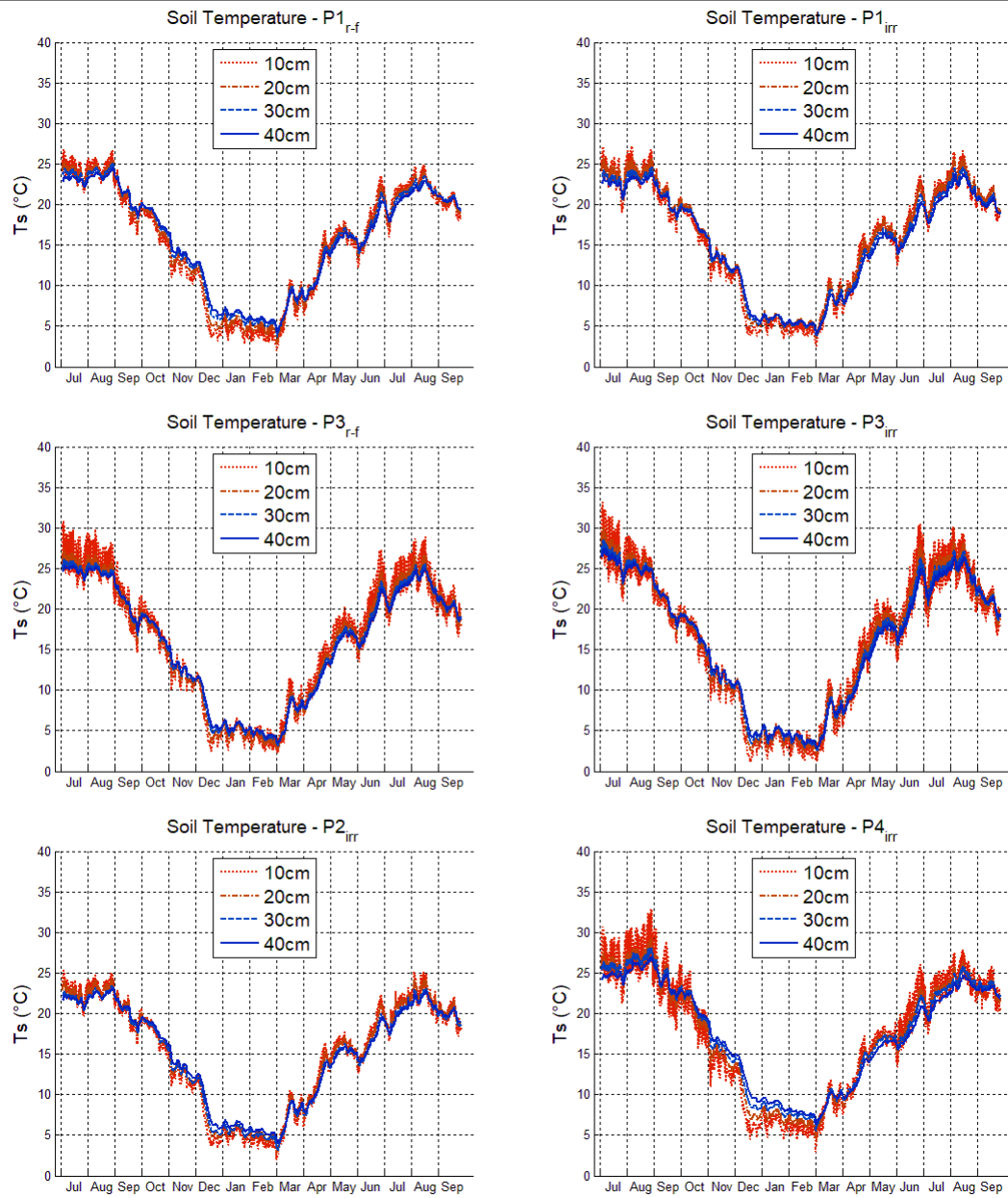


Fig.S4

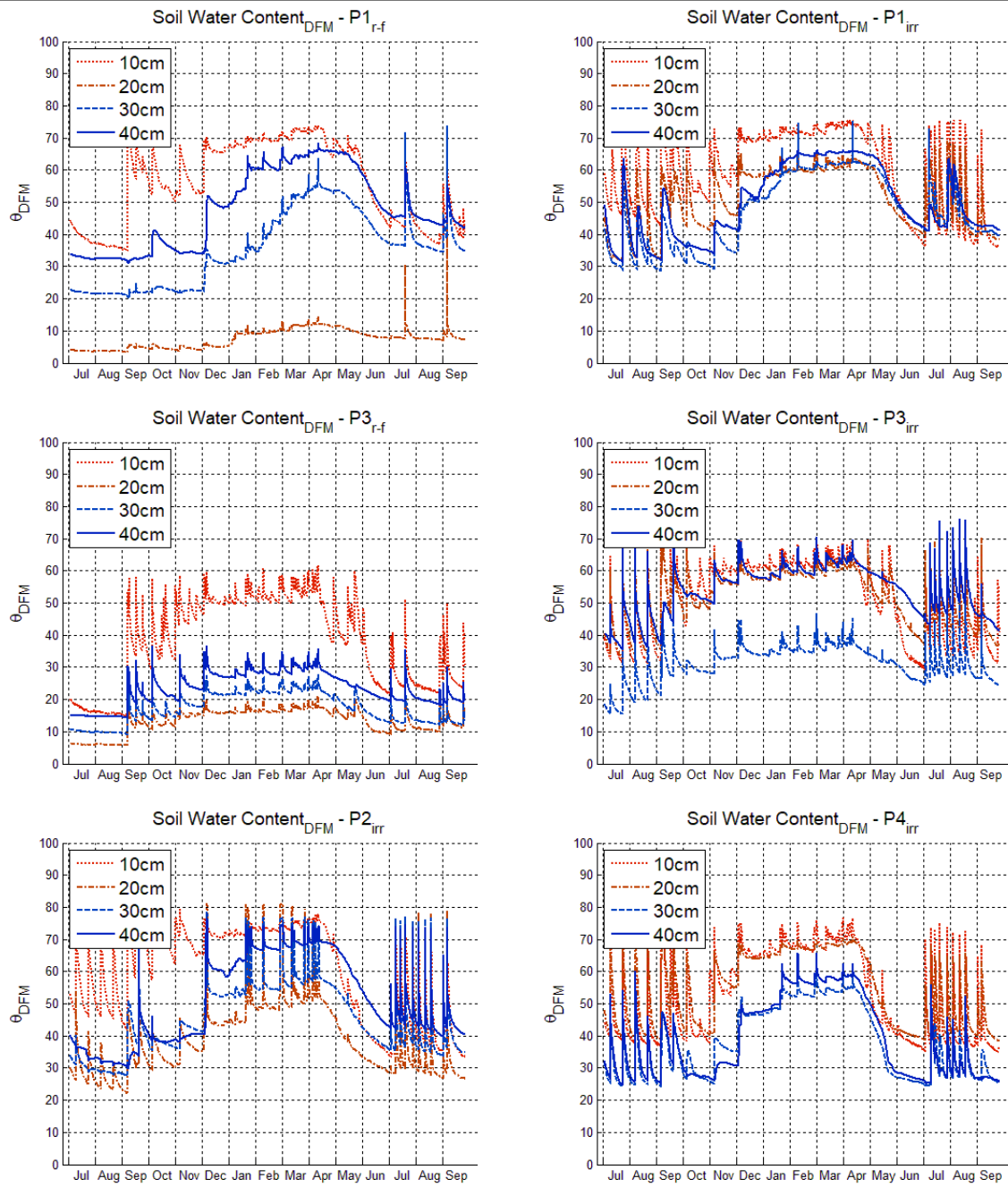


Fig.S5

