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Substrate pH mediates growth promotion and resilience to water stress of *Tilia tomentosa* seedlings after Ectomycorrhizal inoculation

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Abstract

Colonization by Ectomycorrhizal (EcM) fungi is key for the health and performance of plants under different stress scenarios, such as those faced by trees in urban environments. Because urban environments can be lacking EcM fungi, we here assessed the benefits of inoculating *Tilia tomentosa* seedlings in a pre-transplantation nursery context with the EcM fungi *Lactarius deliciosus* and *Paxillus involutus*, using substrates of different pH and facing water-stress. *P. involutus* had a more evident positive effect in *T. tomentosa* seedlings and had a good performance in both acidic and alkaline substrate. In acidic substrate the fungus increased the plant height by 0.91-fold, increased the mycorrhization rate by 3.23-fold, expansion rate by 5.03-fold and formation of secondary roots by 0.46-fold, compared to the non-inoculated control. This species also improved the phosphorus content of leaves, which revealed a promotion of nutrient uptake. In alkaline substrate *P. involutus* increased root dry weight by 3.92-fold and the mycorrhization parameters. In contrast, *L. deliciosus* only had a positive effect in the improvement of mycorrhization and expansion rates and phosphorus content in the root, effects visible only in alkaline substrate. When exposed to water-stress the increase of proline content was visible in acidic substrate for both fungi, *L. deliciosus* and *P. involutus*, and in alkaline substrate for the fungus *P. involutus*, a response indicative of the enhancement of defenses in stressing scenarios such as water scarcity. We conclude that fungal inoculation improves the vigour and resilience of *Tilia* seedlings and that it is of utmost importance to select a suitable EcM fungus and to consider the soil pH of the transplanting site. The inoculation approach can be a valuable tool to produce robust seedlings which may have a better performance when transplanted to the challenging urban environment.

Highlights

- Urban trees in urban environments are commonly exposed to stresses, contributing to their health decline;
- Ectomycorrhizal fungi improve the vigour and resilience of *Tilia* seedlings, but their performance is dependent on soil pH.

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- Inoculation promotes growth in height, biomass production, mycorrhization status, nutrient uptake, and proline accumulation, and these effects dependent on fungi species and soil pH;
- Inoculation with EcM can be a strategy to produce healthier saplings that when transplanted to the urban environment will have a better performance.

Keywords Ectomycorrhizal fungi, Inoculation, Urban forest, Soil pH, Water-stress, Nursery management

Introduction

Urban trees provide many ecosystem services to the urban population, including biodiversity protection, carbon sequestration, air quality improvement and mitigation of the *heat-island* phenomenon [1–4]. Urban trees, however, are exposed to adverse environmental conditions that may diminish their health and jeopardize the delivery of ecosystem services [5–8]. These trees are typically limited by a lack of rooting space and low water availability, which can reduce the area and water content of the leaves and expedite foliar scenescence [9]. In fact, the sealing of urban soil by manmade constructions contributes greatly to water-stress in urbanised countries [10]. This implies that the common urban planting system can be by itself a source of stress for urban trees.

Plants have evolved a variety of mechanisms to deal with water scarcity [11], comprising approaches to avoid low water potentials by increasing water uptake through enhancing root growth and formation of deeper taproots, and by limiting water loss through stomatal closure. In a more severe situation, shoot growth can be even restricted. Plants also developed strategies to tolerate dehydration by activation of protection mechanisms, such as the production and accumulation of osmoprotectant solutes like proline [12, 13].

A key factor for a plant to overcome stressful conditions such as drought, is a well-developed healthy root system [14] and trees' individual response to environmental challenges are largely dependent on fungal symbionts [15]. Ectomycorrhizal (EcM) fungi have emerged as a biotechnology tool to improve the health of urban trees in their challenging environments. These microorganisms form a symbiosis with the host plant, and the inoculation of plants with these fungi provide advantages such as an enhanced capacity for water and nutrient uptake, resistance to heavy metal contamination and pathogenic infections [16–19]. The benefits of these fungi rely on the increased absorption surface and on expanding the area of exploration by the external mycelium, through efficient conduction of water by mycelium strands [20]. Other benefits include altering the soil structure for enhanced water retention, increasing the photosynthetic rate, and promoting osmotic adjustment [11, 21, 22].

Under forest nursery context, the inoculation using EcM fungi improved the growth in height, biomass production and nutrition of three important forest species, namely, *Quercus ilex* L., *Pinus pinaster* and *Quercus suber* L. [23–25]. The urban environment, given its stressful nature, can be lacking natural sources of EcM fungi, and for this reason tree seedlings for future plantations in cities can benefit from the inoculation in the nursery. Indeed, Ferrini and Fini [26] have shown that *Tilia* trees inoculated in a nursery had a greater shoot growth when compared to non-inoculated plants, and to plants inoculated only at the moment of transplanting to the urban environment. Yet, such effects are dependent on environmental conditions such as soil pH. Soil pH is known to be a very important factor that modulates the composition, diversity, and richness of the EcM communities in urban trees [27] thereby influencing the capacity of fungi to colonize tree roots and its effect on tree performance. Indeed, Van Geel et al. [27] characterized the soil of three European cities – Porto, Leuven and Strasbourg – and found that the pH varied from acidic in Porto (6.58 ± 0.80) to substantially alkaline in Strasbourg (8.24 ± 0.25), impacting and shaping the EcM fungi communities found in the respective cities. Sillo et al. [28] also showed that the high pH of urban soil as well as high organic matter modulates the microbiome of urban flowerbeds located next to ectomycorrhizal tree-hosts (including *Tilia* sp.) [28]. Other authors also showed that the soil pH explained the variation in EcM communities from European oak forests [29], which reinforces the importance of soil pH in both urban and forest ecosystems.

Tilia species occur naturally in Europe. *Tilia tomentosa* is native to Eastern Europe, namely the Balkans, Hungary and Ukraine, and nowadays can be found in most of the Iberian Peninsula [30]. It is one of the most popular ornamental trees for the urban environment, with a wide foliage offering deep shade. They respond to periods of heat and drought by moving its leaves so that its under-leaves face outwards, a strategy to reflect more solar radiation, reducing leaf temperature and water loss thought transpiration [31]. Combined with its resistance to lower light quality, consequence of the shade produced by neighboring buildings [31] and aphid resistance, this makes *T. tomentosa* a popular choice for urban environments [32], being often planted in densely paved areas, hereby suffering from water-stress.

The present study hypothesized that inoculation of seedlings of *Tilia tomentosa* with EcM fungi promotes plant growth and vigour, and that the benefits of inoculation depend on the soil pH. As such, we conducted an

experiment with *Tilia* plants inoculated with two EcM species at varying soil pH, and we assessed plant biometric, biomass, nutritional, photosynthetic and mycorrhization status. As a proof of concept, the protective effect of EcM fungi to drought-stress was assessed by exposing the seedlings to water scarcity and measuring the response of the plant through determination of proline accumulation in the plant.

Because it is not uncommon that trees are acquired at young age and grown in dedicated nurseries until they reach the desired size for transplantation in cities with varying soil pH, our study can inform municipal nurseries on how EcM fungi can be used to improve tree resilience and growth and can give a preliminary insight into the pH conditions that may benefit the EcM fungi used to inoculate the plants.

Materials and methods

Inoculum production

Fungi genera *Paxillus* sp. and *Lactarius* sp. were selected as promising candidates based on previous described associations with *Tilia* species [33, 34]. These fungi had been used previously for growth promotion, enhancement of nutritional status and mycorrhization status in other trees, such as *Pinus pinaster*, *Quercus rubra* and *Betula pubescens* [35–37]. The EcM fungi sporocarps were collected from mixed forests from Northern Portugal. *Lactarius* sp. and *Paxillus* sp. sporocarps were collected based on their morphological characteristics, isolated by placing a piece of sporocarp in a petri dish with Potato Dextrose Agar (PDA) medium and incubated at 23ºC for one month. The mycelium was scraped from the medium and DNA was extracted using DNeasy PowerSoil Kit (Qiagen). The ITS region was amplified using the universal fungi primers ITS1F-ITS4 [38, 39] and sequenced (STAB VIDA Lda., Caparica, Portugal) to confirm the identity of the fungi by comparing the obtained results with annotated sequences of the UNITE database by BLAST [40]. The species identified were *L. deliciosus* and *P. involutus*. The inoculum was produced by inoculating PDA plates with 1 cm diameter disc of mycelium from the two EcM fungi pure cultures and incubating in the dark at 23ºC for one month.

Tilia tomentosa experimental setup

In March 2018, an outdoor experiment was established to test the impact of soil pH in the improvement of the performance of *Tilia tomentosa* seedlings inoculated with EcM fungi.

Tilia tomentosa used in this work had six months of growth and were propagated by cuttings and supplied by a commercial ornamental plant nursery.

The experiment consisted in a 3×2 full factorial design, with the selected factors being "Mycorrhization" (3 levels) and "Substrate pH" (2 levels); the mycorrhization levels consisted of a non-inoculated control, inoculation with *L. deliciosus* and inoculation with *P. involutus*. Based on the earlier reported variation of soil pH in the urban environment [27], substrates with two pH (2 levels) were used in this work: acid pH and alkaline pH. Six months old seedlings of *T. tomentosa* were transplanted to individual pots of 10 L of substrate. The acid substrate (pH ~ 5.8) was obtained by mixing peat moss-perlite-vermiculite (volume ratio of 3:1:1) and the alkaline substrate (pH ~ 7.5) was obtained by adding calcium carbonate to the mix. The pH of the substrate was measured using the method ISO 10390:2005, which consisted in mixing a 1:5 (volume fraction) suspension of substrate in distilled water. The suspension was mixed for 1 h and left to rest without stirring for 30 min until all solids had settled. After this step, the pH was measured with a glass electrode. Additionally, the commercial substrate used in this experiment contained calcium sulphate that acts as a pH buffer, minimizing possible pH variations. At the end of all the experiments the pH was always analyzed, and no significant differences were observed [41]. The substrate was not sterilized, preserving its natural microbial community, including EcM fungi propagules, as usually found in the substrate of forest nurseries. The mycorrhizal treatment was established during April-May 2018. The secondary roots of tilia seedlings were carefully excavated and half petri-dishes of pure mycelium cultures of *L. deliciosus* and *P. involutus* were placed in physical contact with those roots. The roots were covered with the substrate without displacing the culture medium with mycelium from the roots to promote mycorrhization by physical contact. A non-inoculated control was included. Each treatment had 12 replicates, resulting in a total of 72 pots. The plants were watered daily for one hour during the whole experiment using a drip irrigation system.

Water-stress assay

During a four-week experimental period, the effect of water-stress in the plants, by quantification of proline accumulation was evaluated in a 3×2×2 full factorial experiment, with the factors "Mycorrhization" (3 levels), "Substrate pH" (2 levels) and "Water status" (2 levels). Each water-stress assay treatment followed the scheme from Table 1.

Water-stress was imposed to 6 replicates (chosen randomly) from each of the previously mentioned treatments, during September-October 2018. The water-stress consisted in stopping watering until the first drought symptoms like leaf rolling were visible in the plants (the watering system was turned off for 14 days in the water-stressed plants). After resuming the watering, the plants could recover for 14 days from the water-stress.

Table 1 Water-stress assay treatments, each with six randomly chosen replicates. Total pots = 72

Mycorrhization (3 levels)	Substrate pH (2 levels)	Water status (2 levels)
Control	Acidic	Watered
		Water-stress
	Alkaline	Watered
		Water-stress
Inoculation with *L. deliciosus*	Acidic	Watered
		Water-stress
	Alkaline	Watered
		Water-stress
Inoculation with *P. involutus*	Acidic	Watered
		Water-stress
	Alkaline	Watered
		Water-stress

The non-stressed plants were watered daily for one hour using a drip irrigation system, and the same irrigation plan was used during the 14-day recovery period of the water-stressed plants.

The proline content was quantified at three time points: before the stress was applied ("before-stress"), after the 14 days of water-stress application ("stress") and after the plants recovered from the water-stress during the following 14 days ("recovery"). Primary leaves from the top, middle and bottom of all plants were collected from the before-stress, stress and recovery points and preserved at -80ºC until further analysis.

Response variables

Biometric parameters

After nine months, during November-December 2018, the seedlings were removed from the pots for height and diameter measurement. The remaining leaves on the seedlings were separated from the stem to determine the foliar fresh/dry weight and fresh/dry weight per leaf. The root system was also separated from the shoot and washed to remove adhered substrate and root fresh/dry weight was determined.

Mycorrhization status

Before drying the root system, the mycorrhization status was determined using a stereomicroscope (SZ30, Olympus, Japan) using the gridline intersect method [16]. Summarily, the roots were carefully washed and placed in a petri dish superimposed in a petri dish lid with a grid of $1cm^2$. The intersections of the roots with the lines of the grid were counted, both horizontally and vertically. The number of intersections that consist in a root tip and of these root tips, the number of which were mycorrhized was also registered. Several morphotypes of mycorrhizas were observed but were neither described nor identified or sequenced. The total length of the root was determined by the number of intersections between the roots and the grid lines. For the determination of mycorrhization status these parameters were quantified: percentage of mycorrhization (percentage of root tips that were mycorrhized), secondary roots (number of intersections of the roots with the grid lines which translates in the growth of the root) and expansion rate (number of ectomycorrhiza tips per area of root).

Nutritional parameters – Nitrogen and Phosphorus determination

Leaf and root material were dried and ground, and 0.2 g were digested according to the protocol of Novozamsky et al. [42]. Subsequently, total nitrogen and phosphorus concentration in the leaves and roots was determined by colorimetry [43] using a 96-well plate reader spectrophotometer (Multiskan GO spectrophotometer using the SkanIt™ 3.2 software, Thermo Fisher Scientific Corporation). The results were presented in milligrams of N or P per gram of leaf/root dry weight.

Photosynthetic pigments

The quantification of the photosynthetic pigments was performed following Nayek et al. [44]. In short, circles of fresh leaves (~0.5 g) were homogenized with liquid nitrogen and 80% acetone was added for extraction. The samples were centrifuged for the separation of the supernatant from the pellet. The absorbance was measured using a 96-well plate reader spectrophotometer (Multiskan GO spectrophotometer using the SkanIt™ 3.2 software, Thermo Fisher Scientific Corporation) on three wave lengths: 663 nm for chlorophyll A, 647 nm for chlorophyll B and 470 nm for carotenoids. The results were presented in milligrams of photosynthetic pigments per gram of leaf dry weight.

Proline concentration

Under water-stress conditions, plants accumulate molecules like proline [12, 13, 45] in an attempt to maintain their osmotic status. The protocol of Bates et al. [46] was used to measure this amino acid. In short, ~0.25 g of fresh leaf was macerated using liquid nitrogen and homogenized with 5 mL of 3% aqueous sulfosalicylic acid. The homogenized part was filtered using filter paper. 1 mL of the filtrate, 1 mL of acid-ninhydrin and 1 mL glacial acetic acid were added to a test tube and incubated for one hour at 100ºC. The mixture was cooled in an ice bath. The reaction mixture was extracted using 2 mL of toluene and vortexed for 20 s. The colored toluene was pipetted from the aqueous phase and the absorbance was measured at 520 nm using toluene as blank in a 96-well plate reader spectrophotometer (Multiskan GO spectrophotometer using the SkanIt™ 3.2 software, Thermo Fisher Scientific Corporation). Proline concentration

was calculated according to a proline standard curve prepared using solutions of proline at 0, 5, 10, 20 and 30 µg mL^{-1}. The results were presented as micrograms of proline per gram of leaf dry weight.

Statistical analysis

Two-way ANOVA and one-way ANOVA with Tukey's HSD tests ($p<0.05$) for multiple comparisons were applied to evaluate treatment effects (inoculation and substrate pH) on the response variables after normality of the response variables and homogeneity of variance of data were verified. In the analysis of the photosynthetic pigments' results, homogeneity of variance was not observed and as such a non-parametric test – Kruskal-Wallis – was used; statistically significant results are those with an adjusted (Bonferroni) p-value <0.05.

The Pearson correlation coefficient was used to investigate the correlation between the responses measured during this study, with a level of confidence of 95%.

For the water-stress assay, repeated measurements ANOVA was applied on the proline content measured at three time points (before-stress, stress and recovery). The Mauchly's sphericity test was violated in one analysis (analysis of data of samples inoculated with *L. deliciosus*, in acid substrate and exposed to water-stress) and was corrected using the Greenhouse-Geisser correction. The pairwise comparisons were performed with a level of confidence of 95%, being the mean difference significant at the 0.05 level.

All statistical analysis was performed using the software SPSS 26.0.

Results

Effects of inoculation in the development of *T. tomentosa* seedlings under different pH levels

Biometric and biomass parameters of non-inoculated and inoculated plants

In the acid substrate, the inoculation with EcM fungi promoted the growth of the seedlings, with *P. involutus* significantly increasing the growth in height when compared to the control group (0.91-fold) and the group inoculated with *L. deliciosus* (0.55-fold). In the alkaline substrate, the inoculation did not benefit plant growth (Fig. 1a). The diameter of the plants was not affected by inoculation with EcM fungi in both substrates (Fig. 1b).

The leaf biomass of the seedlings in both substrates was not significantly affected by inoculation, although in the acid substrate the results were close to significant ($p=0.07$) (Fig. 1c). In contrast, plants growing in the alkaline substrate inoculated with *P. involutus* had a significant increase of root dry weight compared to the control treatment (3.92-fold) and the *L. deliciosus* treatment (2.65-fold) (Fig. 1d).

Dry weight per leaf was similar in plants growing in either substrate, irrespective of inoculation, with a tendency of an increase in plants inoculated with *P. involutus* (Fig. 1e).

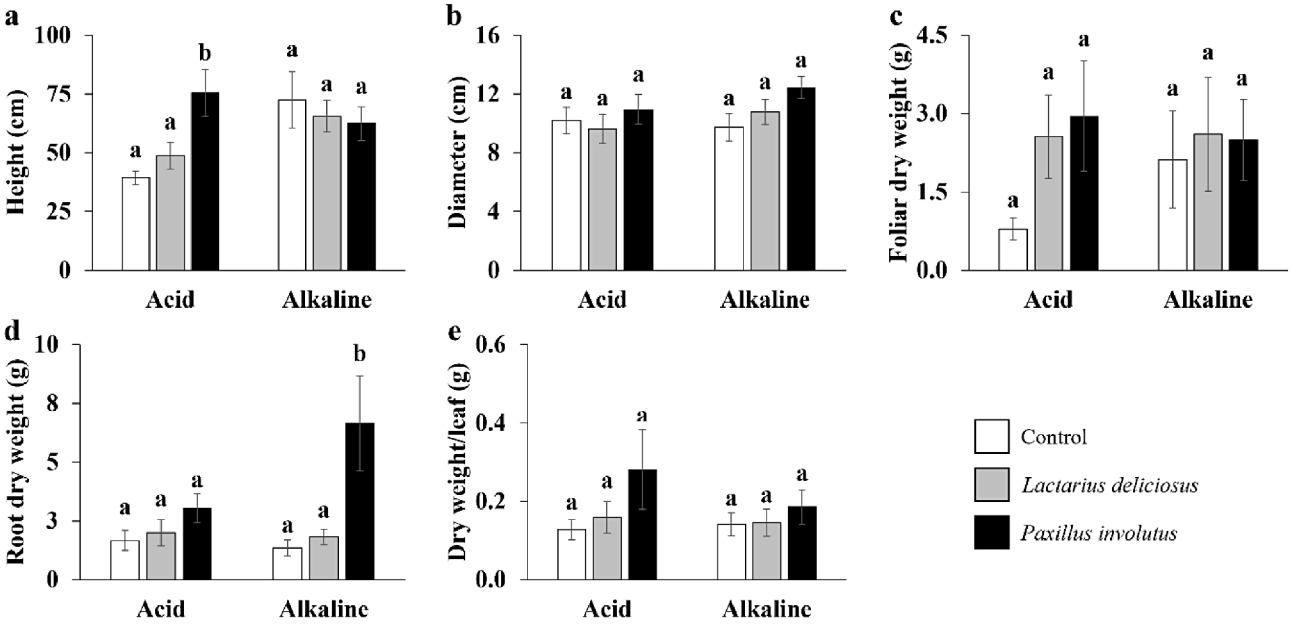

Fig. 1 Biometric (height (**a**) and diameter (**b**)) and biomass (foliar dry weight (**c**), root dry weight (**d**) and dry weight per leaf (**e**)) parameters of the control group, and plants inoculated with *L. deliciosus* and *P. involutus* on acid and alkaline substrates. Each bar represents the mean ± SE and different letters represent statistically significant results ($p<0.05$) according to Tukey's HSD test. No statistical analysis was performed for differences between substrates. The analysis was performed using 12 replicates for each treatment ($n=12$)

Mycorrhization status

The mycorrhization rate increased with inoculation. The plants growing in acid substrate and inoculated with *P. involutus* had a statistically significant higher mycorrhization rate of 49.3% compared to the mycorrhization rate of control (11.6%) and *L. deliciosus* (19.7%), representing an increase of 3.23 and 1.51-fold, respectively. The inoculation with *L. deliciosus* and *P. involutus* in the alkaline substrate significantly increased the mycorrhization rate, with values of 58.02% (3.45-fold) and 55.79% (3.28-fold), respectively, compared to the mycorrhization rate of 13.03% for the control group (Fig. 2a).

The expansion rate also increased with inoculation. In the acid substrate, there was a significant 5.03-fold increase of *P. involutus* inoculated plants compared to the control, and a 1.80-fold increase compared to *L. deliciosus* inoculated plants. In the alkaline substrate, the plants inoculated with *L. deliciosus* and *P. involutus* had a significant increase of 3.23 and 4.34-fold, respectively, compared to the control group (Fig. 2b).

The plants inoculated with *P. involutus* had longer secondary roots on both substrates, with a statistically significant increase of 0.46-fold and 0.65-fold in the acid substrate compared to the control and *L. deliciosus*, respectively, and 1.05-fold in alkaline substrate compared to the control group (Fig. 2c).

Nutritional parameters – Nitrogen and Phosphorus in leaves and roots

The nitrogen content of leaves and roots of plants growing in acid substrate were not affected by inoculation with EcM fungi. In contrast, the content of phosphorus increased in both roots and leaves, and the increase was statistically significant for plants inoculated with *L. deliciosus* for roots (0.33-fold increase compared to control), and with *P. involutus* for leaves (increase of 0.42-fold compared to control). For the plants growing in the alkaline substrate, the foliar nitrogen and phosphorous content was not affected by inoculation. The root nitrogen content in the inoculated groups was low compared to the control which was significantly higher compared to both treatments (0.17-fold higher than the *L. deliciosus* group and 0.19-fold higher than *the P. involutus* group), and in contrast, the root phosphorus content increased significantly in the *L. deliciosus* inoculated plant group, with a 0.30-fold increase compared to the control and 0.29-fold compared to *P. involutus* treatment (Fig. 3a).

Photosynthetic pigments

In acid substrate, no significant variation of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) was observed between treatments. In contrast, the levels of the pigments of plants growing in alkaline

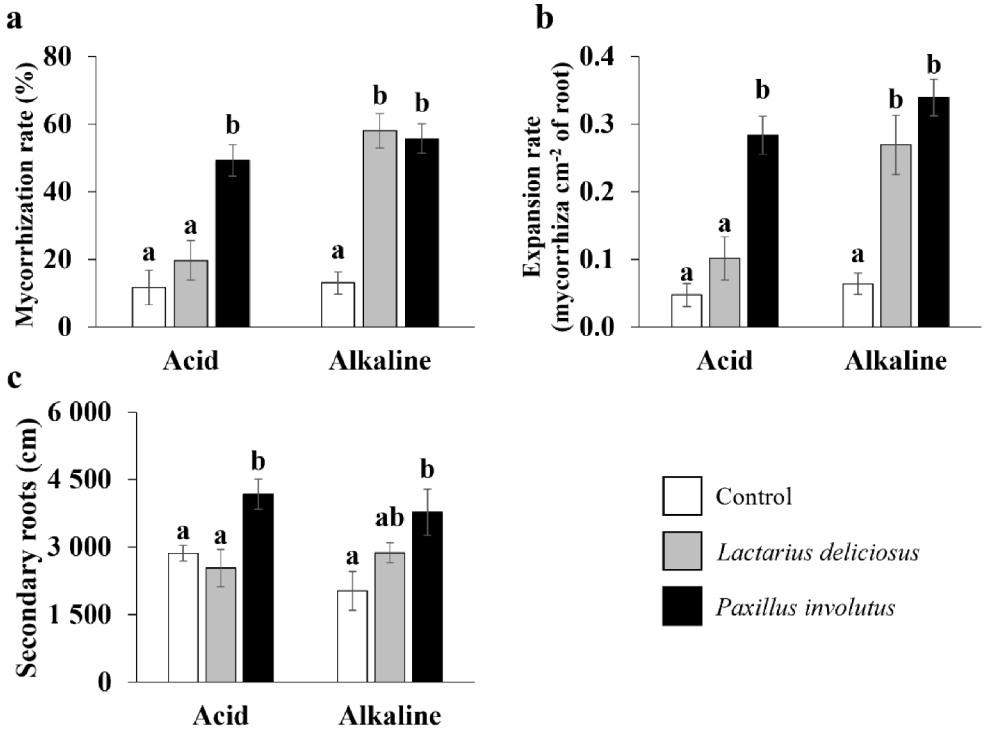

Fig. 2 Mycorrhization status (mycorrhization rate (**a**), expansion rate (**b**) and secondary roots (**c**)) of the control group, plants inoculated with *L. deliciosus* and *P. involutus*, on acid and alkaline substrates. Each bar represents the mean ± SE and different letters represent statistically significant results ($p < 0.05$) according to Tukey's HSD test. No statistical analysis was performed for differences between substrates. The analysis was performed using 12 replicates for each treatment ($n = 12$)

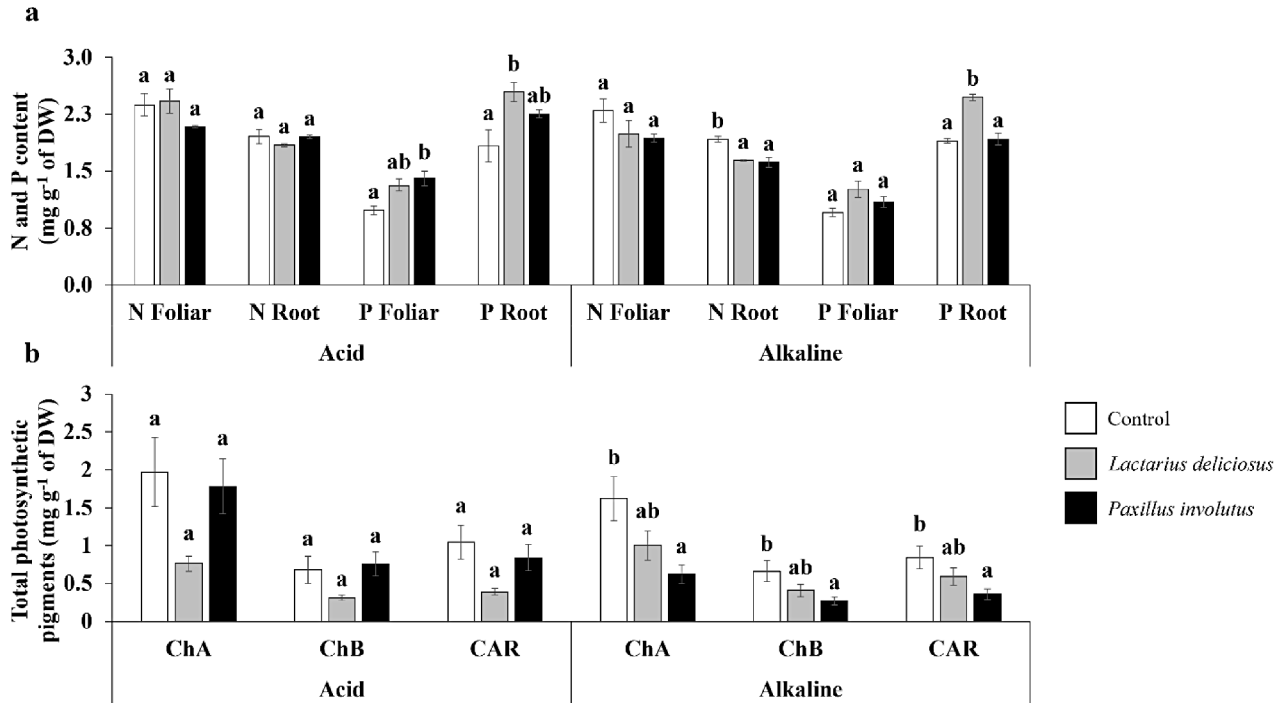

Fig. 3 N and P content of leaves and roots (**a**) and photosynthetic pigments (**b**) of the control group, plants inoculated with *L. deliciosus* and *P. involutus* on acid and alkaline substrates. Each bar represents the mean ± SE and different letters represent statistically significant results according to Tukey's HSD test (for N and P content; $p < 0.05$) and Kruskal-Wallis test followed by pairwise comparisons (for total photosynthetic pigments; adjusted *p*-value < 0.05). No statistical analysis was performed for differences between substrates. For the determination of N and P content, 3 replicates were used for each treatment ($n = 3$), and the results are presented in milligrams of N or P per grams of foliar and root dry weight; for the determination of total photosynthetic pigments, 12 replicates were used ($n = 12$), and the results are presented in milligrams of total photosynthetic pigments per gram of leaf dry weight

substrate presented the same dynamics for the three pigments in study, with the control presenting the highest values of pigment content per gram of dry weight: for chlorophyll a the control presented an increase of 1.62-fold, for chlorophyll b the increase was 1.48-fold, and for carotenoids the increase was 1.36-fold, compared to the content of pigments of plants inoculated with *P. involutus*. The content of pigments of the plants inoculated with *L. deliciosus* did not differ from either control or *P. involutus* (Fig. 3b).

Response of the plants to water-stress

The proline content of leaves varied during the drought-stress experiment. In the acid substrate, the plants irrigated during the experiment did not exhibit a significant variation in proline content for both the control plants and those inoculated with *L. deliciosus* and *P. involutus* (Fig. 4a). The plants exposed to water deficit responded differently, with the control plants exhibiting statistically significant lower levels of proline in the stress (0.97-fold decrease) and recovery (0.83-fold decrease) points compared to the before-stress point; the plants inoculated with *L. deliciosus* presented an increase of proline content in the stress point as a response to water scarcity (increase of proline levels by 2.56-fold compared to the levels of before-stress point) followed by a decrease in the recovery point (decrease of 3.06-fold); the plants inoculated with *P. involutus* had a behavior similar to those inoculated with *L. deliciosus* with an increase of proline levels in the stress point compared to the before-stress point (increase of 1.11-fold) and subsequent decrease to the recovery point (decrease of 0.67-fold) (Fig. 4b).

In the alkaline substrate with normal irrigation, the non-inoculated plants (control) and the plants inoculated with *L. deliciosus* did not alter the production of proline in the three analyzed time points; in contrast, the plants inoculated with *P. involutus* exhibited higher proline content in the stress point (increase of 1.32-fold) compared to the before-stress point, suffering a decrease of proline content (1.53-fold) in the subsequent two weeks (recovery point) (Fig. 4c). In the plants exposed to water-stress, the levels of proline did not vary in the control plants and the dynamics of proline production by the inoculated plants was different; in the plants inoculated with *L. deliciosus* the levels of proline decreased from the before-stress to the stress point (0.61-fold) and from the latter to the recovery point (1.05-fold), contrasting with the dynamic of plants inoculated with *P. involutus* which increased significantly the proline content in the stress point (increase of 2.03-fold) as a response to the

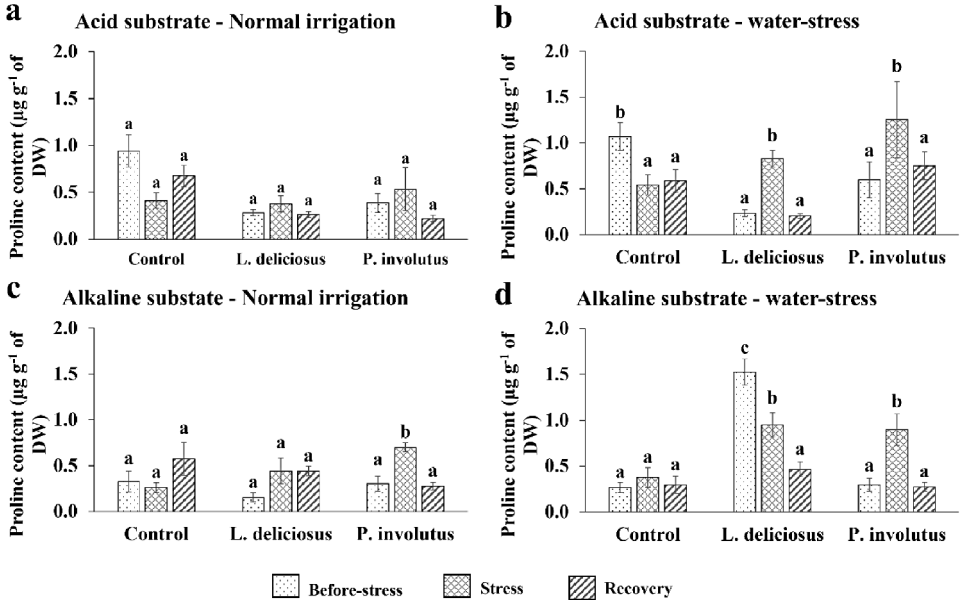

Fig. 4 Proline content of *T. tomentosa* seedlings exposed to normal irrigation and water scarcity in the before-stress, stress and recovery points. (**a**) Control and inoculated plants in acid substrate under normal irrigation, (**b**) control and inoculated plants in acid substrate under water-stress, (**c**) control and inoculated plants in alkaline substrate under normal irrigation, (**d**) control and inoculated plants in alkaline substrate under water-stress. Each treatment had 5 replicates selected randomly ($n = 5$). Each bar corresponds to a time point representing the mean ± SE response and different letters represent statistically significant differences ($p < 0.05$) according to the pairwise comparison between time points. No statistical analysis was performed for differences between inoculation treatments. The results are presented in micrograms of proline per gram of dry weight of leaf

water-stress, with a decrease of the levels of proline (2.31-fold) as a result of the recovery from the stress, exhibiting levels similar to those observed before the stress was induced (Fig. 4d).

General effects of fungal inoculation, substrate pH and the interaction of both factors on plant and fungal responses and correlation between responses (Annex 1 – Supplementary Tables 1 and 2)

The general significant effects of the factors Inoculation and pH are shown in Supplementary Table 1, Annex 1, and are synthesized here: the pH had a significant effect in the mycorrhization rate and nitrogen content of the root; the inoculation of the plants had a significant effect in the mycorrhization rate, root dry weight, nitrogen and phosphorus content of the roots and leaves ; the interaction of the factors inoculation and pH had a significant effect in the mycorrhization rate, height of the plants and nitrogen content of the root.

The most biologically relevant correlations between responses can be described as follows (Supplementary Table 2, Annex 1): the mycorrhization rate is positively correlated with the expansion rate, with the height and diameter of the plants and root dry weight; the height of the plants is positively correlated to the plant diameter, foliar fresh and dry weight, and root fresh and dry weight; the foliar dry weight is positively correlated to the root dry weight; the content of nitrogen of the root is positively correlated to its content of phosphorus.

Discussion

The inoculation of *Tilia* seedlings with the EcM fungi *L. deliciosus* and *P. involutus* improved several plant parameters. The fungi also conferred the plant with mechanisms to mitigate the effects of water scarcity by provoking the accumulation of proline as a water-stress response. In this study a biotechnological approach was followed, focusing on the impact of EcM fungi inoculation in the plant, rather than the impact in the microbial community of the plant root and rhizosphere. The observed results in the plants are attributed to the application of the inoculum and to the specific fungus that was introduced in the native microbiota of the plant.

The fungus *P. involutus* had a growth promotion effect in the plants, both in acidic (height) and alkaline substrate (root dry weight). In contrast, the fungus *L. deliciosus* did not exhibit any significant growth promotion effect in the plant. *Tilia tomentosa* is known to prefer neutral to alkaline soils [47], suggesting that in acidic substrate, the plant should be under stress, which facilitates the positive effects of EcM fungi in the inoculated plants. Indeed, the effects of EcM fungi are known to be more pronounced in less favorable situations, conferring protection against biotic and abiotic stresses to the plants [35, 48]. The root dry weight was augmented by *P. involutus* in alkaline substrate, which suggests that the plant had a radicular system clearly more developed, compared to the other plants, with an almost 4-fold increase compared to the non-inoculated control; even though the

alkaline substrate pH should not be a stress factor for the plant, the fungus had a positive effect nevertheless, reinforcing the importance of EcM fungi to the resilience of the plant during transplantation to field with varying soil pH, conferring a survival advantage. The two-way ANOVA analysis showed that the promotion of root biomass relates to inoculation, supporting the effect of EcM fungi in the improvement of this parameter. Indeed, Egerton-Warburton [49], demonstrated that inoculation of *Eucaliptus rudis* with three different isolates of the EcM fungus *Pisolithus* greatly increased the root biomass by alleviating aluminum toxicity and increasing nutrient uptake, and Sebastiana et al. [50] reported a statistically significant increase in the root biomass of *Quercus suber* inoculated with *Pisolithus tinctorius*. The potential of EcM fungi to improve the radicular system of the plant is important since it confers an advantage to the plant in the moment of transplantation to the field.

P. involutus increased the mycorrhization parameters, such as percentage of mycorrhization, expansion rate and formation of secondary roots in both substrates, in contrast to *L. deliciosus* that improved the mycorrhization and expansion rates in alkaline substrate. *L. deliciosus* is widely distributed and tolerates a wide range of soil pH. It can be frequently found in pine tree forests, rich in organic matter and acidic pH but *L. deliciosus* prefers a soil pH close to neutral and calcareous soils [51, 52]; in our work this fungus had a marked preference for alkaline soil, which reveals its polyvalency in different ecosystems. In urban context Zhang et al. [53] described a decrease in the root: shoot ratio of *Tilia cordata* under drought stress, but interestingly, the development of deep fine roots within the root system was enhanced for the tree to be able to obtain water from profound layers of ground. In this study, the authors considered that *Tilia cordata* Greenspire did not have the means to tolerate severe drought stress [53]. In our study, the inoculation with EcM fungi not only increased the root biomass, but also mycorrhization, formation of secondary roots and expansion of root, hence the symbiosis of EcM with this *Tilia* species and other urban trees may be a way to improve its drought tolerance; EcM fungi have root system-enhancement properties, and its mycelium strands have the ability to explore a larger area of soil and reach deeper in the soil [22–24] to absorb water in severe drought situations, alleviating this condition. Interestingly, the photosynthetic pigment content per dry mass of leaf was higher in the control non-inoculated group, with the plants inoculated with *P. involutus* presenting the lowest values. Abdel-Salam et al. [54] reported higher content of chlorophyll in mycorrhized plants, increasing the levels of the photosynthetic pigments and therefore improving photosynthesis, but contrarily, in our work, this effect was not observed [23, 24, 37, 55].

Mycorrhization with EcM fungi is known to increase the uptake of nutrients including phosphorus and nitrogen [23, 24, 37, 55]. In our work, inoculation did not favor the accumulation of nitrogen in both leaves and roots, for all treatments and in both substrates but the accumulation of phosphorus was improved. *L. delicious* had a significant effect in promoting the accumulation of phosphorus in roots in both substrates, and *P. involutus* promoted the accumulation of phosphorus in leaves in the acid substrate. Sousa et al. [37] observed the same increase of phosphorus shoot content in *Betula pubescens* as a result of inoculation with *P. involutus*. Since urban environments are characterized by harsh conditions for the plants, including nutrient scarcity [5, 6], inoculation can enhance the capacity of the plant to extract nutrients from a nutrient deficient soil such as the urban soil. Indeed, Authier et al. [56] explains that the selection of plants for urban establishment still underestimates the benefit of the enhanced capacity for nutrient uptake conferred by EcM to urban trees [56].

Several studies have demonstrated that mycorrhization improves the performance of plants during drought-stress [57–61]. The accumulation of the amino acid proline, among other substances, is crucial for the plant osmotic adjustment to keep a gradient of water potential favorable to water entrance into the plant [62]. It also works as an osmoprotectant as it prevents enzymes from denaturing, oxidative stress, and contributes to the maintenance of cell osmotic status to mitigate the drought-stress effects [63]. Proline is a useful indicator of tolerance to water-stress in plants, as described by Dien et al. [64] that verified that drought-tolerant variants of rice had the ability to accumulate more proline when compared to drought-susceptible variants, when exposed to water scarcity in field [64].

Several studies have demonstrated that mycorrhizal fungi mitigate the effect of abiotic stresses such as drought [57–61]. The association of arbuscular mycorrhizal fungi and drought resistance mediated by proline accumulation might be contradictory, with some studies describing the accumulation of proline [65–67] and other studies reporting reduced content of this amino acid in mycorrhized plants compared to the non-mycorrhized [68–70] but, in general, proline accumulation correlates in a positive way with water-stress tolerance/resistance of mycorrhized plants [71]. The accumulation of proline in plants mycorrhized with EcM has also been described by Kebert et al. [72] who verified a greater response of accumulation of proline in mycorrhized *Quercus robur* L., when exposed to severe drought [72].

In this study, in acid substrate, the plants under normal irrigation did not alter its proline content since there was no external stimulus to produce or degrade this amino acid. The plants that were exposed to water scarcity

had different dynamics depending on the treatment. In the non-inoculated plants (control) the levels of proline decreased significantly when exposed to water-stress, suggesting an increase in the catabolism of this amino acid, compared to its production/accumulation. In fact, the degradation of proline during abiotic stresses is prevented during de-hydration [73, 74] however our non-inoculated control plants may have not had the capacity to regulate the catabolism of proline when exposed to this level of water-stress, in acid pH, which was already considered less favorable to tilia seedlings, making them more susceptible. Additionally, the levels of proline were maintained, and did not lower as expected in the "recovery" phase, reinforcing that the plants may not be able to regulate the transcription of proline dehydrogenase, an enzyme responsible for the catabolism of this amino acid, that is known to be activated by re-hydration [73–75], which happened during the resuming of watering of the plants.

In contrast, the plants inoculated with *L. deliciosus* and *P. involutus* increased the levels of proline in the stress point seemingly to mitigate the effects of water-stress, normalizing after the recovery period. This dynamic seems to show the capacity of these EcM fungi to mitigate the effect of water-stress in tilia seedlings in a non-favorable pH for the host tree. Indeed Chitarra et al. and Fan & Liu described an increase of proline as a consequence of water-stress [66, 67] with its degradation occurring after re-hydration of the plants [73]. In our study, the duration of water-stress treatment was determined by the visualization of some stress signs in the plant such as leaf rolling. The leaf rolling is considered a drought avoidance mechanism of plants (reviewed in [76]), where the plant loses the ability to maintain the leaf turgidity. To counteract this state, the plants facing water-stress enhance its hydraulic conductivity [77] by accumulating solutes such as proline, which degrades when the plant recovers from the stress. The results observed seem to indicate this kind of protection.

For the alkaline substrate, the plants exposed to normal irrigation from the control and *L. deliciosus* treatments did not suffer a variation on their proline levels. An increase of proline is visible at the stress point of plants inoculated with *P. involutus* even though there was no cessation of irrigation, which cannot be explained by the present data. The non-inoculated plants exposed to the water scarcity did not show a variation in their proline levels, revealing that a more favorable soil pH for the plant might be helpful for the activation of the defense strategies of healthier plants. During water-stress, the dynamics of proline production of the EcM fungi were different. The plants inoculated with *P. involutus* presented the same dynamic observed in the acid substrate exposed to water scarcity, with levels of proline rising after stress and decreasing after re-hydration. Differently, the plants inoculated with *L. deliciosus* suffered a decrease of proline from the before-stress point until recovery. This dynamic may indicate that the plant was using proline in the period of water scarcity to maintain its osmotic status, and that after re-hydration proline catabolism pathways were activated as described by Kiyosue et al. [73], decreasing even more the levels of this amino acid.

The proline variation observed in this study reveals the important role of EcM fungi to promote the absorption of water to maintain the plants' normal water status under stress. The production of the osmolyte proline caused by the association with EcM fungi contributes to the enhancement of the osmotic adjustment of the plants, which could be important not only as a response to a punctual water-stress scenario as it happened during this study, but also in an environment characterized by water scarcity such as the urban environments.

The introduction of EcM fungi in the young stages of *Tilia* plantlets in a nursery context improved the growth and vigour of the plants and enhanced defenses in scenarios of different soil pH and water-stress induction. Overall, the inoculation improved the biometric parameters, nutritional status and water-stress resilience of the plants, and those results were both fungi and substrate pH-dependent. Overall *P. involutus* had a better performance than *L. deliciosus* in both substrates, indicating that this fungus might be more appropriate for soils with variability in pH and *L. deliciosus* might be more suitable for soils with neutral to alkaline pH.

This work contributes to the understanding of the dynamic between *T. tomentosa* seedlings and two different EcM fungi. The pH was used as the factor that modulates the behaviour and effectiveness of the symbiosis plant-fungi. The results obtained give an insight into this response and may be helpful to predict outcomes in a non-controlled environment with abiotic stresses.

The inoculation approach can help nurseries in the improvement of their techniques for enhancing the growth and resilience of seedlings, but it is of utmost importance to select a suitable EcM fungus and to take into account the soil characteristics of the transplanting site.

Abbreviations

%	Percentage
µg	Microgram
ANOVA	Analysis of variance
Car	Carotenoids
Ch A	Chlorophyll A
Ch B	Chlorophyll B
cm	Centimeter
cm^2	Square centimeter
DW	Dry weight
ExpRate	Expansion Rate
FW	Fresh weight

g	Gram
L. deliciosus	Lactarius deliciosus
mL	Milliliter
Myco	Mycorrhiza
N	Nitrogen
nm	Nanometers
ºC	Celsius
P	Phosphorus
P. involutus	Paxillus involutus
PDA	Potato Dextrose Agar
SE	Standard error
SecRoots	Secondary Roots
sp	Species
T. tomentosa	Tilia tomentosa
TxMyco	Mycorrhization rate

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Data availability
No datasets were generated or analysed during the current study.

Declarations

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The authors declare no competing interests.

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