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Effects of Controlled Mycorrhization and Deficit Irrigation in the Nursery on Post-Transplant Growth and Physiology of *Acer campestre* L. and *Tilia cordata* Mill.

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Abstract: The goal of this work was to assess the effects of mycorrhizal inoculation and deficit irrigation applied in the nursery on the post-transplant growth and physiology of *Acer campestre* L. and *Tilia cordata* Mill. For this purpose, 144 preconditioned plants were planted in an experimental plot in northern Italy and were monitored for three growing seasons. Controlled inoculation in the nursery enhanced the root colonization rate three years after transplanting only in *Acer campestre*. Inoculated *Acer campestre* showed higher survival, shoot length, turgor potential and leaf gas exchange than non-inoculated plants throughout the experiment. By contrast, in *Tilia cordata*, no difference in root colonization by mycorrhizal fungi was observed between plants inoculated or not in the nursery three years after transplanting. Indeed, the survival, growth and physiology of *Tilia cordata* after transplanting were little affected by inoculation. Deficit irrigation in the nursery determined higher survival, growth and CO_2 assimilation rate and more favorable water relations in newly transplanted *Acer campestre*. By contrast, *Tilia cordata* exposed to deficit irrigation in the nursery showed lower growth and unaffected survival after transplanting compared to plants which received full irrigation in the nursery. The overall results suggest that nursery preconditioning through mycorrhizal inoculation and deficit irrigation can affect post-transplant performances, although their effectiveness depends on species' mycorrhizal dependency and water use strategy.

Keywords: nursery preconditioning techniques; controlled mycorrhization; deficit irrigation; transplant stress; leaf gas exchange; water potential

Citation: Corsini, D.; Vigevani, I.; Oggioni, S.D.; Frangi, P.; Brunetti, C.; Mori, J.; Viti, C.; Ferrini, F.; Fini, A. Effects of Controlled Mycorrhization and Deficit Irrigation in the Nursery on Post-Transplant Growth and Physiology of *Acer campestre* L. and *Tilia cordata* Mill.. *Forests* **2022**, *13*, 658. https://doi.org/10.3390/f13050658

Academic Editor: Elisabetta Salvatori

Received: 7 February 2022
Accepted: 21 April 2022
Published: 23 April 2022

1. Introduction

It is known that 75% of Europeans live in cities [1]. Poor air quality and the urban heat island effect are just some of the problems that pose significant risks to human health and ecosystems, and they are exacerbated by extreme weather events, increasingly common and impacting urban life [2]. A vast body of literature has described tree benefits in urban areas: they can improve the well-being of urban dwellers through the provisioning of a wide range of ecosystem services, such as mitigation of air pollution [3,4], atmospheric CO_2 uptake and carbon sequestration [5] and microclimate amelioration [6] through shading [7] and transpiration [8,9]. Urban tree planting programs are increasingly being promoted, but an efficient and sustainable long-lasting greening is not achieved in all cases. Heatwaves, drought, diseases, soil compaction, pollution and conflicts with the built environment

are some of the main stressors linked to unsuccessful planting in urban environments. Transplanting, if not properly carried out and managed (e.g., lack of soil volume, deep planting, inadequate staking, lack of irrigation), can become a particular stress in a plant's life and can lead to premature failure of new plantings, with an average annual mortality rate in the first five years after planting of about 4–33% [10], but much higher failure rates can easily occur in harsh environments, or when irrigation is not applied. Altered soil conditions, irradiance exposure and changed plant root-to-shoot ratios are among the primary factors contributing to post-transplant shock. Although container-grown trees do not experience any root loss, their mortality can be even higher than that of balled and burlapped (B&B) plants because of root defects triggered by nursery cultivation and because of the poor suitability of peat as a growing substrate after planting, which hampers root outgrowth [11]. This makes newly planted trees extremely sensitive to drought, a keystone abiotic stress at urban sites [12,13], the impact of which is expected to increase in temperate areas worldwide because of climate change [14]. Research has highlighted that plants have evolved a suite of adaptations to either tolerate or avoid drought through changes occurring at the morpho-anatomical, physiological and biochemical levels [15,16]. During dryness, isohydric species display early reductions in stomatal opening to maintain the leaf water potential and reduce the risk of hydraulic failure. Conversely, anisohydric species display much lower stomatal sensitivity to vapor pressure deficit and soil water availability and sustain photosynthesis despite the reduction in the leaf water potential and the high risk of hydraulic failure [17,18].

Nursery preconditioning techniques are particularly important in this context. They can produce hardy plants with high levels of photosynthetic reserves and adequate morphological characteristics to promote establishment after transplanting [19,20]. Among nursery preconditioning techniques, inoculation with mycorrhizal fungi is widely applied to improve the quality of plant stocks [19,21].

Mycorrhization has been reported to enhance tolerance to sub-optimal environmental conditions in several plant species through different mechanisms, many of which are closely linked [22–24]. They can be summarized as follows: (1) increased hydraulic conductivity of roots [25]; (2) greater water uptake, especially at low soil water availability, due to the absorption activity of extraradical hyphae [22,26]; (3) higher stomatal conductance and transpiration [27] and higher osmotic adjustment [28]; (4) higher antioxidant activities [29]; (5) improved nutritional status [30]. Artificial application of mycorrhizal fungi to trees in urban areas is of increasing interest to mitigate the chronic stresses to which plants are subjected in cities, although results obtained with commercial mycorrhizal inocula are contrasting [31,32]. Controlled inoculation consists in selecting and isolating functional mycorrhizal strains from the planting site to produce an inoculum suitable for inoculating young plants of the same species destined to be transplanted there [33–35]. Controlled inoculation has been proposed as an alternative and sustainable approach to commercial mycorrhizal inocula [36]. Among the overall advantages, this approach enables issues to be overcome which are related to the low specificity of commercial mycorrhizal inoculants and the reduced viability of these products once in the landscape [35,37]. To make controlled mycorrhization applicable to landscaping and urban forestry, a few requirements must be fulfilled: (1) it is possible to find effective native symbionts in the transplanting site; (2) the selected strains are able to survive in a peat-based substrate under nursery conditions; and (3) mycorrhiza can survive transplanting in the landscape. Previous research [35] demonstrated that effective fungal strains can be isolated at urban sites and that inoculated mycorrhizal fungi can persist during the plant cultivation phase in the nursery if no systemic fungicides are applied for the control of pathogens. The tolerance of mycorrhiza to transplanting, however, is still unexplored.

Water stress preconditioning through deficit irrigation consists in applying sub-optimal water regimes to plants during the nursery phase [38] in order to acclimate the plants to a mild and chronic water stress [12,39]. Deficit irrigation has been reported to stimulate multiple physiological, biochemical and morphological responses: (1) an en-

hanced root-to-shoot ratio, an important factor in successful transplanting in the field [40]; (2) a higher leaf trichome density and number of xylem vessels in stems and roots [40,41]; (3) a higher percentage of brown roots due to lignification of the exodermis, which reduces water loss to the soil [42]; (4) higher osmotic adjustment [40,41,43]. Osmotic adjustment is considered one of the crucial processes in plant adaptation to sub-optimal water availability [44,45]. It consists in the net accumulation of osmotically active solutes in cells (soluble sugars, sugar alcohols, proline, glycine betaine, organic acids, calcium, potassium, chloride ions and others) during drought to increase water extraction from the soil and to maintain turgor pressure at a decreasing water potential [46,47]. Water stress preconditioning often reduces plant growth during the nursery stage as a direct consequence of the limited water availability [35,48] but has a positive effect on growth after transplanting in the field [39]. The impact of this technique can, however, be influenced by the water use strategy of different species.

Root mycorrhizal colonization is generally increased by low water availability. It is reasonable, therefore, that mycorrhizal inoculation and deficit irrigation, when applied together in the nursery, may act synergistically in improving the plant response to transplanting, but information about the effects of nursery preconditioning techniques on the post-transplant survival, growth and physiology of trees in the hostile urban environment is quite limited. Indeed, the available scientific evidence about the effects of nursery preconditioning techniques is mostly related to short-term experiments with potted plants and/or in the greenhouse. Conversely, as reviewed by Lehto and Zwiazek [26], Rouphael et al. [49] and Sánchez-Blanco et al. [39], no scientific survey has been carried out to evaluate the long-term effects of mycorrhizal inoculation and water stress preconditioning in the nursery on trees after they have been transplanted in the field.

A previous experiment [35] was carried out to evaluate the effects of selected mycorrhiza obtained in the urban environment and water stress preconditioning on the growth and physiology of potted plants of hedge maples (*Acer campestre* L.) and small-leaf lindens (*Tilia cordata* Mill.) in the nursery. The results demonstrated that native symbionts selected at the transplanting site were able to form a functional symbiosis with *Acer campestre* and *Tilia cordata* plants growing in a peat substrate in a container nursery. Moreover, inoculated plants displayed more favorable physiological traits when exposed to moderate drought [35]. The current experiment represents the continuation of the aforementioned work, with the aim of evaluating the effects of mycorrhizal inoculation and deficit irrigation applied in the nursery on the survival, growth and physiology of *Acer campestre* and *Tilia cordata* during the three growing seasons after the final transplanting into the field. The research questions were: (1) Can controlled inoculation with mycorrhizal fungi in the nursery reduce transplant stress and improve survival, growth, leaf gas exchange and water status during establishment? (2) Do plants hardened by deficit irrigation in the nursery transplant better? (3) Do mycorrhizal inoculation and deficit irrigation have a synergistic effect, or do they determine independent plant responses?

This information may be relevant for the development of best practices for nursery production with the specific focus of improving transplant tolerance. Further, the data provided (e.g., A, g_s, V_{cmax}, J_{max}) here may be relevant for running empiric and process-based models for the estimation of regulation ecosystem services by these two species.

2. Materials and Methods

2.1. Plant Material and Experimental Site

A total of 160 two-year-old potted plants of *Acer campestre* and *Tilia cordata* were either inoculated (+I, 40 plants per species) or not inoculated (−I, 40 plants per species) with species-specific native mycorrhizal strains sampled from healthy mature trees in an urban environment (Milano, Italy). Plants were obtained from a local commercial nursery located near the experimental field and grown in a shade house in 3 L containers filled with a peat/pumice (3:1) substrate [35]. Plants of *Acer campestre* were inoculated only with arbuscular mycorrhizae fungi (AMF), whereas *Tilia cordata* plants were inoculated with both

ectomycorrhizae (ECM) and AMF. During the following two years of nursery cultivation, half of the plants (20 plants per inoculation treatment and species) were well irrigated (WW, 100% of container capacity), and the remaining half (WS, 20 plants per inoculation treatment and species) were exposed to a chronic moderate drought stress (30% of container capacity) (see Fini et al. [35] for further details). At the end of the two-year nursery period, plants had been preconditioned according to a factorial design composed of two levels of inoculation (+I and −I) and two levels of irrigation (WW and WS): +I WW plants (20 per species) and +I WS plants (20 per species) were inoculated with species-specific mycorrhizal fungi and either well-watered or subjected to deficit irrigation, respectively; −I WW plants (20 per species) and −I WS plants (20 per species) were not inoculated and were kept either under well-watered conditions or subjected to deficit irrigation, respectively.

After removing the biggest and the smallest plants per treatment (8 plants per species), in March, 72 plants of *Acer campestre* and 72 plants of *Tilia cordata* (18 per species and per preconditioning treatment) were transplanted into the field and were monitored for the three growing seasons following transplanting (Figure A1). Treatments were arranged following a randomized block design with 6 blocks and 3 plants per species and treatment in each block (24 plants in each block; 144 plants in total). Groups of three plants for each inoculation and irrigation treatment were planted by separating +I from −I plants with plants that were not measured and not inoculated to avoid mycorrhizal fungi migration. Before planting, the soil was plowed to 40 cm and plants were planted into hand-dug planting holes (as deep as the rootball and 1.5 times the width of the rootball) following a 3 × 3 m planting design. Trees were planted in an experimental orchard (45°43′41.7″ N 9°04′55.3″ E) at Fondazione Minoprio (Como, Italy). The site is characterized by a warm temperate climate (Cfb, according to Köppen and Geiger), with an average annual temperature and rainfall, recorded over the last 30 years, of 13.3 °C and 1106 mm, respectively. The daily temperature and rainfall were recorded using a weather station (Vantage Pro 2, Davis, San Francisco, CA, USA) throughout the experimental period (monthly average temperature and total rainfall are reported in Figure 1).

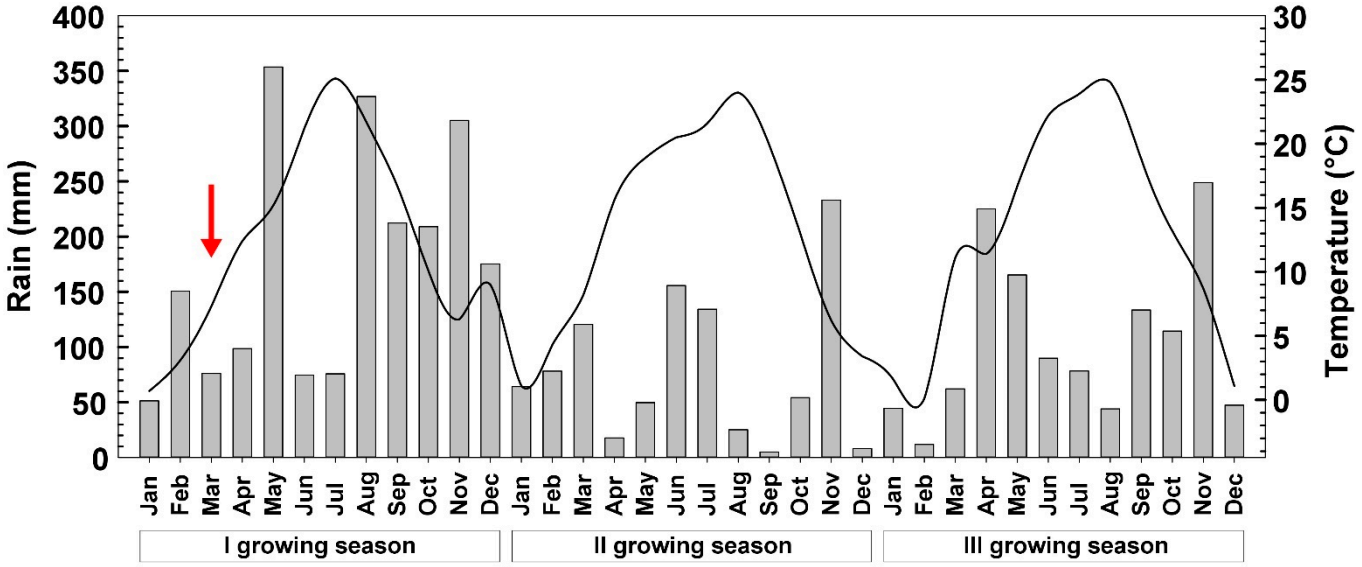


Figure 1. Air temperatures and precipitation at the experimental area during the research (first, second and third growing seasons after transplanting plants from the nursery to the field). The red arrow indicates the month of transplanting in the field.

According to national legislation (DM 13/09/99), the soil of the experimental site was classified as loam (51% sand, 40% silt and 9% clay) and sub-acid (pH 6.41), with high levels of gravel (16%) and available phosphorus (317 mg kg^{-1}), a normal content of organic matter (18 g kg^{-1}) and total N (1.30 g kg^{-1}), medium base saturation (66%),

exchangeable K (0.26 meq 100 g^{-1}) and low levels of CEC (5 meq 100 g^{-1}) and exchangeable Ca (2.68 meq 100 g^{-1}). Field capacity occurs at a soil moisture (v/v) around 13.2% and the wilting point at 2.8%. Soil physical and chemical traits were similar to those of the site where the mycorrhizal inocula were selected (see Fini et al. [35] for further details). After transplanting, and for the rest of the experiment, plants were neither irrigated nor fertilized except for a single irrigation event carried out at planting. All plants were grown with no supplemental management except for chemical weeding on the row and only differed in the preconditioning imposed during the nursery period.

2.2. Measurements

The mycorrhizal colonization, plant survival and growth, leaf gas exchange, chlorophyll fluorescence, water relations and leaf concentrations of soluble carbohydrates were monitored for three growing seasons after transplanting.

2.2.1. Root Colonization

To investigate the percentage of root colonization, 2 samples of 500 g of root material mixed with soil were harvested at a depth of 10–35 cm from the central plant of each block per species and treatment. Root + soil samples were collected at about 50–70 cm from the root flare by manual excavations conducted along the row with an orientation of 180 degrees from each other. Samples were collected from 4 blocks (64 samples from 32 plants in total) at 30 months after transplanting (54 months after inoculation). To assess AMF colonization, roots were stained using Trypan Blue [50], and mycorrhizal colonization was measured following the method proposed by Phillips and Hayman [51]. The staining protocol was slightly modified to adapt it to woody species, since the original timing was not sufficient for the disintegration of the cell wall and subsequent staining of the fungal hyphae. Root samples were chopped into 1 cm portions, washed under running water in a sieve and then cleared in 10% KOH for 48 h at room temperature. After clearing, roots were carefully washed under running water and acidified in a 2% HCl solution for 20 min in a water bath at 100 °C. Washed again, roots were stained with a solution containing water/glycerol/lactic acid (1:1:1 by volume) and 0.05% Trypan Blue for 5 min at 100 °C. The samples were rinsed with 50% lactic acid to remove excess dye. Finally, roots were mounted in 50% lactic acid on microscope slides, aligned parallel to the long axis of the slides and observed at 400× magnification (Nikon eclipse E400 microscope). The magnified intersection method was used to estimate the percentage of mycorrhizal colonization [52], measuring the symbiotic colonization of AMF in *Acer campestre* and both AMF and ECM in *Tilia cordata* (Figure A2).

2.2.2. Plant Mortality and Growth

Plant mortality was assessed by counting dead plants in each block at the end of the experiment. Stem diameter was measured at 5 cm above the root flare during the dormant season 11, 23 and 34 months after transplanting. At the same time, shoot length was measured on 5 shoots per plant. It was recorded starting from the apical bud to the end of in-year growth. Thirty-four months after transplanting, 1 plant per block, treatment and species (48 plants in total) was harvested for biomass measurements. Plants were cut at the root flare, and the above-ground portion of the tree was divided into leaves and stems. Roots were manually dug taking care to preserve all structural roots. After extraction from the soil, roots were cleaned from the medium with an air flush. To determine dry weight (DW), leaves, stems and roots were oven dried at 70 °C until a constant weight was reached (≈72 h).

2.2.3. Leaf Gas Exchange and Chlorophyll Fluorescence

Leaf gas exchange was measured 4, 5, 14, 16, 18, 27, 28 and 30 months after transplanting using an infrared gas analyzer (Ciras-2, PP-System, Hertfordshire, UK) on 3 leaves per block, treatment and species (144 leaves in total). Measurements were carried out on

sunny days on the first fully expanded leaf of the shoot, at a saturating light intensity (1300 µmol m^{-2} s^{-1}) and 400 ppm of CO_2. Volumetric soil moisture, detected with a frequency domain reflectometry (FDR) probe (SM100 Watermark, Spectrum Technologies, 3600 Thayer Court, Aurora, IL, USA), was in the range 5–10% when leaf gas exchange was measured. Measured parameters were: net CO_2 assimilation (A), transpiration (E), stomatal conductance (g_s) and CO_2 concentration in the intercellular airspace (C_i). Water use efficiency (WUE) was calculated as the A-to-E ratio [53,54].

Response curves of carbon assimilation (A) to the internal CO_2 concentration (C_i) were drawn 4 and 27 months after transplanting, as described by Fini et al. [55]. To avoid stomatal closure, measurements were carried out by decreasing the external CO_2 concentration (C_a) from 400 ppm to 30 ppm, then a C_a of 400 was restored and, finally, C_a was increased stepwise to 1800 ppm. The stomatal (L_s) and non-stomatal (L_{ns}) limitations to photosynthesis were calculated from A/C_i curves as described in previous works [56,57]. Non-stomatal limitation includes both diffusive (i.e., decrease in mesophyll conductance) and biochemical components [58]. The apparent maximum rate of carboxylation by Rubisco (V_{cmax}) and the apparent maximum electron transport rate contributing to ribulose 1,5-BP regeneration (J_{max}), two of the main biochemical processes driving the Calvin cycle, were estimated from A/C_i curves as reported by Sharkey et al. [59]. In addition, 27 months after transplanting, the actual quantum yield of PSII (φPSII) was measured simultaneously to leaf gas exchange using the integrated chlorophyll fluorescence module (CFM, PP-system, Amesbury, MA, USA). This allows the calculation of mesophyll conductance to CO_2 diffusion (g_m) using the variable J method [60].

The maximum quantum yield of PSII (F_v/F_m) was measured on the same dates and on the same twigs as leaf gas exchange by using a HandyPEA portable fluorescence spectrometer (Hansatech Instruments Ltd., King's Lynn, UK). Fluorescence values were obtained after adapting leaves to darkness for 40 min by attaching light exclusion clips to the leaf lamina in different positions in the canopy. Then, a saturating flash of actinic light (3000 µmol m^{-2} s^{-1}) was applied for 1 s, and fluorescence rose from the ground-state value (F_o) to its maximum value (F_m) [61].

2.2.4. Water Relations and Soluble Carbohydrates

Pre-dawn water potential (Ψ_W) was measured between 02:30 a.m. and 04:30 a.m. on the night immediately before gas exchange 5, 14 and 28 months after transplanting. Measurements were conducted on 2 leaves per species, treatment and block (96 leaves in total on each date) using a pressure bomb (PMS Instruments, Albany, OR, USA; [62]). Immediately after Ψ_W measurements, leaves were frozen in liquid nitrogen and stored at −30 °C for osmotic potential (Ψ_Π) measurements. Samples for Ψ_Π were thawed, and leaf sap was extracted and immediately analyzed by a vapor pressure osmometer (Wescor 5520, Wescor Inc., Logan, UT, USA) [48,63]. Turgor potential (Ψ_P) was calculated as follows: $\Psi_P = \Psi_W - \Psi_\Pi$ [46]. At the end of the trial, leaf osmotic potential at full turgor ($\Psi_{\Pi FT}$) was calculated using the following formula [64]: $\Psi_{\Pi FT} = \Psi_\Pi$ [(RWC − AWF)/(100 − AWF)], where RWC is the relative water content, and AWF is the apoplastic water fraction estimated at 6%. RWC was measured as follows [64]: RWC = 100 × (FW − DW)/(TW − DW), where FW and DW are fresh and dry weight, respectively, while TW is turgid weight, measured after fully hydrating fresh leaves for 24 h at 4 °C in darkness. The contribution of dehydration (D) to osmotic adjustment (i.e., passive osmotic adjustment) was calculated with the formula: $D = \Delta\Psi_\Pi - \Delta\Psi_{\Pi FT}$, where $\Delta\Psi_\Pi$ and $\Delta\Psi_{\Pi FT}$ are the differences between treated plants (+I and WS) and control plants (−I and WW) in total leaf osmotic potential (i.e., total osmotic adjustment) and leaf osmotic potential at full turgor (i.e., active osmotic adjustment), respectively [65].

Soluble carbohydrates were analyzed during each growing season, specifically 5, 14 and 28 months after transplanting on 6 leaf samples per species and treatment, as previously described by Tattini et al. [66]. In detail, 200 mg of fresh leaf tissue was extracted with three consecutive applications of 5 mL of a solution of EtOH/H_2O (75/25, v/v). The ethanol

solution was reduced to dryness under vacuum at 30 °C using a Büchi P12 Multivapor unit equipped with a Büchi V-855 vacuum controller (Buchi, Flawil, Switzerland). The resulting pellet was rinsed with 2 mL of milli-Q water and then purified by solid–liquid extraction through -CHX and -SAX pre-packed Bond-Elute cartridges (Varian, Harbor City, CA, USA). The eluted solution was reduced to dryness under vacuum. Samples were rinsed with ultrapure water and injected in a Series 250 LC binary pump equipped with an LC 30-RI detector (all from Perkin Elmer, Bradford, CT, USA). Soluble carbohydrates were separated on an 8 × 300 mm SC1011 column maintained at 85 ± 1 °C and equipped with a 6 × 50 mm SC1011 pre-column (all from Showa Denko, Tokyo, Japan). The eluent was ultrapure water at a flow rate of 0.8 mL min^{-1} during a 25 min run. Individual carbohydrates were identified by comparing the retention times of each peak with those of authentic standards (Sigma-Aldrich, Milano, Italy). The content of each carbohydrate was calculated per dry weight, using the fresh weight/dry weight ratio measured for each sample.

2.3. Statistical Analysis

In the present experiment, species were independently analyzed for all the measured parameters because of the different tested inoculants applied to species. All dendrometric and physiological parameters were analyzed on each individual sampling date using General Linear Model (GLM; SPSS 16.0, SPSS Inc., Chicago, IL, USA), where mycorrhizal inoculation and nursery irrigation were considered as fixed factors. Significant differences among means were estimated at $p < 0.05$, using the Sidak test. Because no significant interactions between inoculation and water regime were found for most parameters (with the exception of leaf photosynthetic enzyme activity and limitations to photosynthesis), only the results for the main effects are presented. Before ANOVA, outliers were identified and removed from the dataset using scatter plots and residual plots in order to respect the assumption of the normality distribution.

3. Results

During the experiment, the average annual temperature (12.75 °C) was close to the 30-year average of 13.30 °C, with the highest monthly means in July and August (23.50 °C). Mean yearly rainfall was above the 30-year average (1106.00 mm), except for the second year of the research (946.10 mm) (Figure 1).

3.1. Root Colonization

Three growing seasons after transplanting, mycorrhizal colonization occurred on both inoculated and non-inoculated plants of *Acer* and *Tilia* species. Controlled inoculation increased AMF root mycorrhization by 7.6% in *Acer campestre*, while in *Tilia cordata*, no significant differences were found between +I and −I plants for both AMF and ECM fungi. The water regime imposed during the nursery period did not affect the mycorrhization percentage after transplanting in either species (Table 1).

3.2. Plant Mortality and Growth

In *Acer campestre*, no mortality occurred in +I plants, regardless of irrigation management in the nursery. However, −I plants suffered a mortality of 5.6% to 11.1% for WS and WW trees, respectively. In *Tilia cordata*, mortality was 5.6% for both +I WW and −I WS plants, whereas none of the +I WS and −I WW plants died (data not shown).

Mycorrhizal inoculation in the nursery enhanced shoot length in *Acer campestre* at 23 and 34 months after transplanting. No effects of mycorrhizal inoculation in the nursery were found on stem diameter and biomass throughout the experiment (Table 2). Mycorrhizal inoculation increased shoot elongation in *Tilia cordata* when measured 23 months after transplanting but significantly reduced stem diameter growth in the first growing season (11 months after transplanting).

Table 1. Effect of nursery preconditioning techniques, i.e., mycorrhizal inoculation (with arbuscular mycorrhizae fungi (AMF) and/or ectomycorrhizae (ECM)) and water regime, and their interaction on mycorrhizal colonization of *Acer campestre* L. and *Tilia cordata* Mill. measured 34 months after transplanting (i.e., 54 months after inoculation).

Species	Inoculation (I)		Water Regime (W)		Significance		
	+I (%)	−I (%)	WW (%)	WS (%)	I	W	I × W
Acer campestre (AMF)	70.6 a	63.0 b	66.0 a	67.6 a	*	n.s.	n.s.
Tilia cordata (AMF)	38.6 a	33.5 a	33.8 a	38.3 a	n.s.	n.s.	n.s.
Tilia cordata (ECM)	87.7 a	91.7 a	90.0 a	89.3 a	n.s.	n.s.	n.s.

Different letters within the same line and factor indicate significant differences between +I and −I plants and between WW and WS plants. +I, inoculated plants; −I, non-inoculated plants; WW, well-watered plants; WS, water-stressed plants. Within the same line and factor, different letters indicate significant differences between +I and −I plants and between WW and WS plants at $p \leq 0.05$ (*); n.s. indicates no significant differences.

Table 2. Effect of nursery preconditioning techniques, i.e., mycorrhizal inoculation and water regime, and their interaction on total plant, leaf, shoot and root dry weight (DW), root:shoot, shoot length and diameter measured 11, 23 and 34 months after transplanting in *Acer campestre* L. and *Tilia cordata* Mill.

Species	Parameter	Months after Transplanting	Inoculation (I)		Water Regime (W)		Significance		
			+I (%)	−I (%)	WW (%)	WS (%)	I	W	I × W
A. campestre	Plant DW (g)	34	3519 a	3360 a	3168 b	3711 a	n.s.	*	n.s.
	Leaf DW (g)	34	535.5 a	574.1 a	515.3 a	594.2 a	n.s.	n.s.	n.s.
	Shoot DW (g)	34	2115 a	2017 a	1897 b	2235 a	n.s.	*	n.s.
	Root DW (g)	34	867.9 a	768.6 a	881.5 a	755.0 a	n.s.	n.s.	n.s.
	Root:shoot (%)	34	0.41 a	0.38 a	0.46 a	0.34 a	n.s.	n.s.	n.s.
	Shoot length (cm)	11	16.2 a	19.1 a	14.4 b	20.9 a	n.s.	**	n.s.
	Shoot length (cm)	23	64.8 a	56.8 b	52.4 b	69.3 a	*	**	*
	Shoot length (cm)	34	54.4 a	45.6 b	43.0 b	56.9 a	*	**	n.s.
	Stem diameter (cm)	11	2.27 a	2.24 a	2.30 a	2.20 a	n.s.	n.s.	n.s.
	Stem diameter (cm)	23	3.64 a	3.70 a	3.61 a	3.73 a	n.s.	n.s.	n.s.
	Stem diameter (cm)	34	5.75 a	5.68 a	5.43 a	6.00 a	n.s.	n.s.	n.s.
T. cordata	Plant DW (g)	34	2684 a	3077 a	3394 a	2367 b	n.s.	**	n.s.
	Leaf DW (g)	34	385.4 a	486.8 a	488.8 a	383.4 a	n.s.	n.s.	n.s.
	Shoot DW (g)	34	1702 a	1962 a	2170 a	1495 b	n.s.	*	n.s.
	Root DW (g)	34	596.9 a	627.7 a	735.7 a	488.8 b	n.s.	**	n.s.
	Root:shoot (%)	34	0.35 a	0.32 a	0.34 a	0.33 a	n.s.	n.s.	n.s.
	Shoot length (cm)	11	16.4 a	15.8 a	19.1 a	13.1 b	n.s.	**	n.s.
	Shoot length (cm)	23	78.5 a	69.4 b	78.4 a	69.5 b	*	*	n.s.
	Shoot length (cm)	34	64.3 a	65.5 a	64.6 a	63.2 a	n.s.	n.s.	n.s.
	Stem diameter (cm)	11	2.15 b	2.32 a	2.35 a	2.12 b	*	**	n.s.
	Stem diameter (cm)	23	4.01 a	4.03 a	4.27 a	3.78 b	n.s.	**	n.s.
	Stem diameter (cm)	34	6.01 a	6.04 a	6.47 a	5.58 b	n.s.	*	n.s.

+I, inoculated plants; −I, non-inoculated plants; WW, well-watered plants; WS, water-stressed plants. Within the same line and factor, different letters indicate significant differences between +I and −I plants and between WW and WS plants at $p \leq 0.05$ (*) and $p \leq 0.01$ (**), respectively; n.s. indicates no significant differences.

In *Acer campestre*, deficit irrigation in the nursery resulted in increased shoot length in all three growing seasons after transplanting, as well as increased plant and shoot dry weight measured 34 months after transplanting, compared to plants well irrigated in the nursery. Conversely, in *Tilia cordata*, deficit irrigation in the nursery reduced plant, stem and root dry weight (34 months after planting), shoot length (11 and 23 months after planting) and stem diameter (11, 23 and 34 months after planting), compared to plants which were well-watered before transplanting.

The root-to-shoot ratio was affected neither by mycorrhizal inoculation nor by deficit irrigation in both species.

3.3. Leaf Gas Exchange

The effects of mycorrhizal inoculation (Figure 2) and water regime (Figure 3) in the nursery on post-transplant stomatal conductance (g_s), net CO_2 assimilation (A) and water use efficiency (WUE) were species-specific. No significant interaction between the two treatments was found (Table S1).

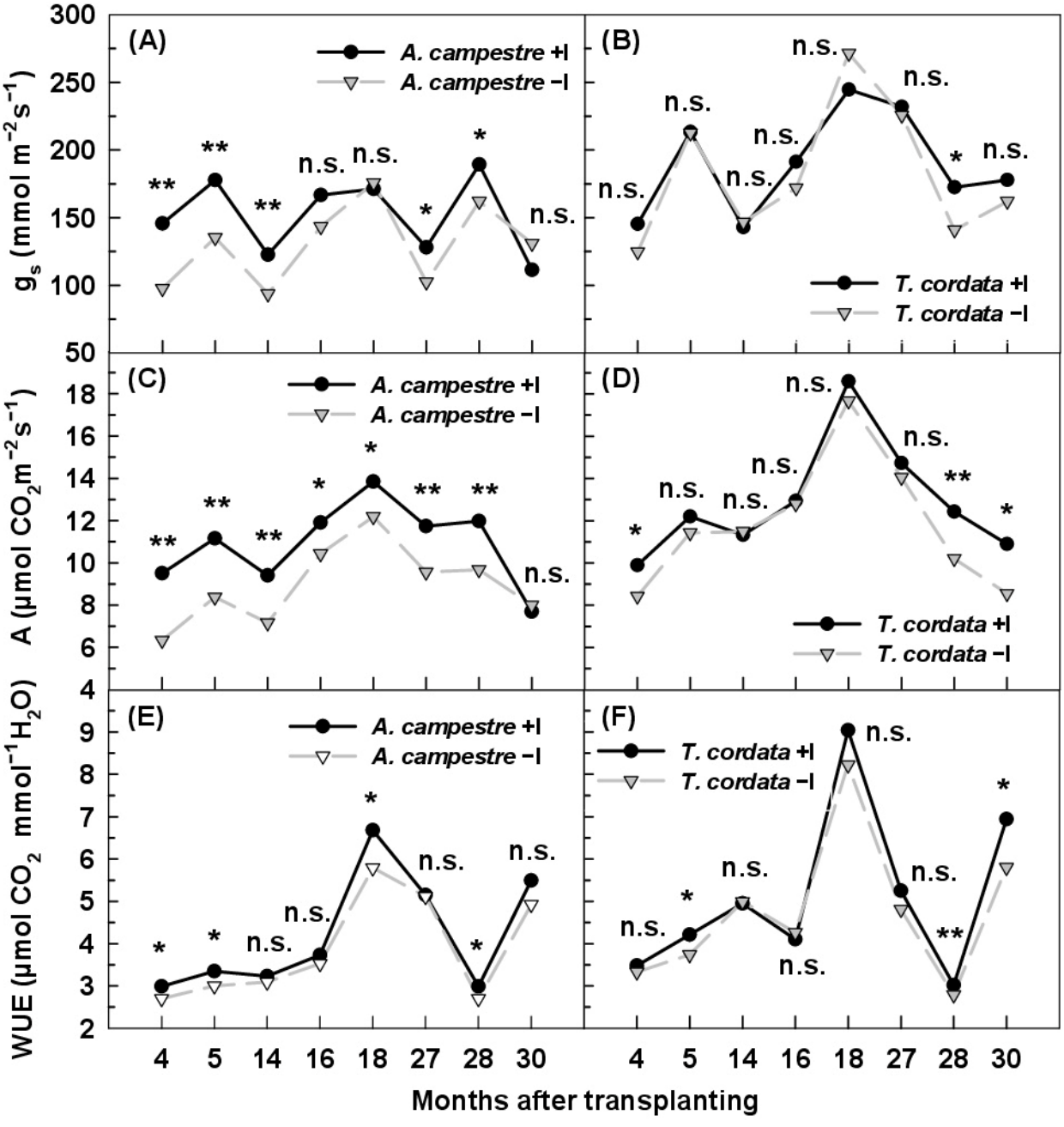


Figure 2. Effect of mycorrhizal inoculation in the nursery on stomatal conductance (g_s, mmol m^{-2} s^{-1}), carbon dioxide assimilation (A, µmol CO_2 m^{-2} s^{-1}) and water use efficiency (WUE, µmol CO_2 $mmol^{-1}$ H_2O) of *Acer campestre* L. (**A**,**C**,**E**) and *Tilia cordata* Mill. (**B**,**D**,**F**) transplanted in the field. Within each panel and sampling date, * and ** indicate significant differences between +I (inoculated) and −I (non-inoculated) plants at $p \leq 0.05$ and $p \leq 0.01$, respectively; n.s. indicates no significant differences.

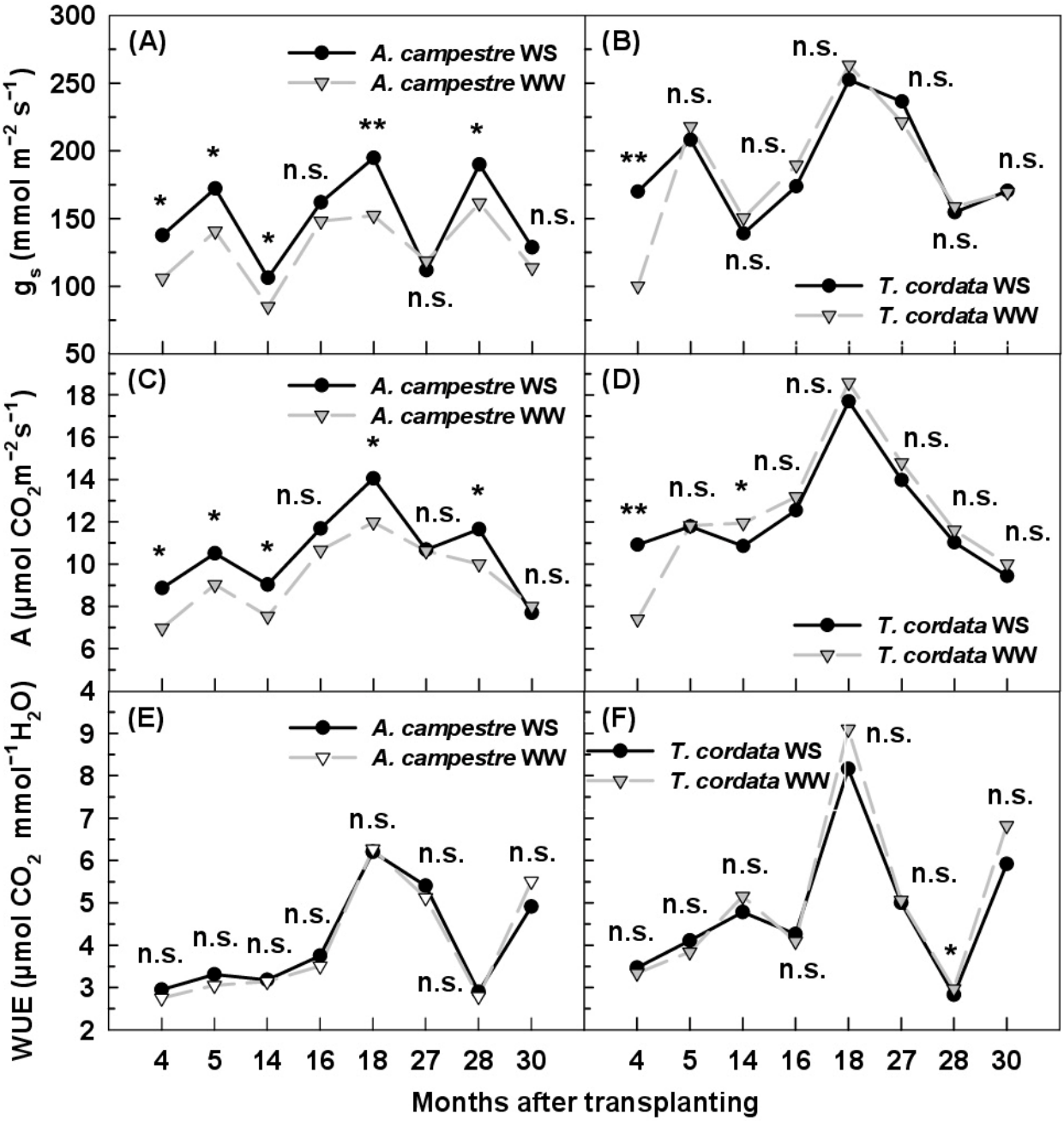


Figure 3. Effect of water regime in the nursery on stomatal conductance (g_s, mmol m^{-2} s^{-1}), carbon dioxide assimilation (A, µmol CO_2 m^{-2} s^{-1}) and water use efficiency (WUE, µmol CO_2 $mmol^{-1}$ H_2O) of *Acer campestre* L. (**A**,**C**,**E**) and *Tilia cordata* Mill. (**B**,**D**,**F**) transplanted in the field. Within each panel and sampling date, * and ** indicate significant differences between WW (well-watered) and WS (water-stressed) plants at $p \leq 0.05$ and $p \leq 0.01$, respectively; n.s. indicates no significant differences.

In +I plants of *Acer campestre* (Figure 2), g_s was higher than that of −I plants, with exceptions only occurring 16, 18 and 30 months after planting. +I *Acer campestre* showed higher A compared to −I plants throughout all of the experiment. WUE was higher in +I *Acer campestre* 4, 5, 18 and 28 months after transplanting compared to −I plants. Conversely, in *Tilia cordata* (Figure 2), the effects of mycorrhization were less clear. g_s was higher in +I plants compared to −I plants only 28 months after planting. +I plants displayed higher A only soon after transplanting (4 months) and at the end of the experiment (28 and 30 months after transplanting). Similarly, 5, 28 and 30 months after transplanting, +I plants of *Tilia cordata* displayed higher WUE than −I plants.

WS plants of *Acer campestre* (Figure 3) showed higher g_s and A compared to WW plants during the whole experiment, although the differences were not significant 16, 27 and 30 months after transplanting. Conversely, after transplanting, g_s and A of *Tilia cordata* leaves (Figure 3) were little and inconsistently affected by the water regime in the nursery. Deficit irrigation in the nursery did not affect WUE in either species throughout the experiment, except for 28 months after transplanting when WS *Tilia cordata* showed lower WUE than WW plants.

Four months after transplanting, +I *Acer campestre* had higher stomatal limitations (L_s) compared to −I plants, regardless of the irrigation regime in the nursery. WW plants had lower L_s compared to WS plants belonging to the same inoculation treatment (Table 3). Differences in L_s among treatments were no longer significant after 27 months. Non-stomatal limitations (L_{ns}) were higher in −I than in +I plants only in WW *Acer campestre*, four months after transplanting. Twenty-seven months after transplanting, +I plants still displayed lower L_{ns} than −I plants, although the differences were significant only in WW plants. Consistently, 4 months after transplanting, +I plants of *Acer campestre* had a significantly higher apparent maximum rate of carboxylation by Rubisco (V_{cmax}) and apparent maximum electron transport rate contributing to ribulose 1,5-BP regeneration (J_{max}) compared to −I plants, although the differences in J_{max} between +I and −I plants were only significant in WW plants. WS plants had higher V_{cmax} and J_{max} than WW plants, 4 months after transplanting. Twenty-seven months after transplanting, +I plants still displayed higher V_{cmax} and J_{max} compared to −I plants, while the effect of the irrigation treatment was no longer significant. +I *Acer campestre* displayed a lower stomatal-to-mesophyll conductance ratio (g_s/g_m) than −I plants, because +I plants displayed higher mesophyll conductance (g_m) than −I plants.

In *Tilia cordata*, significant interactions between treatments were found for L_s and L_{ns} (4 months), V_{cmax} (4 and 27 months), J_{max} (27 months) and g_m (27 months) (Table 3). Four months after transplanting, +I WS *Tilia cordata* displayed lower L_s and L_{ns} compared to the other treatments. Consistently, only +I WS plants displayed higher V_{cmax} and J_{max} compared to WW plants regardless of inoculation, 4 months after transplanting. Twenty-seven months after transplanting, L_s were no longer affected by treatments, while L_{ns} were higher in both −I WW and −I WS plants compared to −I WW plants. Twenty-seven months after transplanting, +I WW plants had higher V_{cmax} and J_{max} than −I WW plants, while inoculation failed to increase V_{cmax} and J_{max} in WS plants of *Tilia cordata*. Mesophyll conductance was higher in +I WW than in −I WW plants, while +I WS plants displayed lower g_m than −I WS plants.

3.4. Chlorophyll Fluorescence

The maximum quantum yield of PSII (F_v/F_m) was affected by mycorrhizal inoculation and deficit irrigation in the nursery in both species (Figure 4), but no significant interaction between the two treatments was found (Table S1). Mycorrhizal inoculation in the nursery increased F_v/F_m in both *Acer campestre* and *Tilia cordata* at 4, 5 and 14 months after transplanting (short term). F_v/F_m did not differ between +I and −I plants for the rest of the establishment period (long term), with the only exception in *Tilia cordata* 27 months after transplanting. Similarly, WS *Acer campestre* and *Tilia cordata* had, in general, higher F_v/F_m in the first year after transplanting, but the differences did not persist in the long run.

Table 3. Effect of mycorrhizal inoculation and water regime in the nursery on stomatal (L_s, %) and relative mesophyll limitations (L_{ns}, %), apparent maximum rate of carboxylation by Rubisco (V_{cmax}, μmol m^{-2} s^{-1}), apparent maximum electron transport rate for ribulose 1,5-BP regeneration (J_{max}, μmol m^{-2} s^{-1}), mesophyll conductance (g_m, μmol CO_2 m^{-2} s^{-1}) and ratio between stomatal and mesophyll conductance (g_s/g_m, %) of *Acer campestre* L. and *Tilia cordata* Mill. 4 and 27 months after transplanting.

Species	Parameter	Months after Transplanting	−I WW	+I WW	−I WS	+I WS	Significance		
							I	W	I × W
A. campestre	V_{cmax} (μmol m^{-2} s^{-1})	4	10.7 d	27.0 c	46.8 b	72.7 a	**	**	n.s.
	V_{cmax} (μmol m^{-2} s^{-1})	27	70.3 b	80.2 ab	70.3 b	87.6 a	*	n.s.	n.s.
	J_{max} (μmol m^{-2} s^{-1})	4	27.8 c	47.6 b	79.5 a	84.9 a	*	**	*
	J_{max} (μmol m^{-2} s^{-1})	27	114.2 b	133.9 a	120.9 b	140.1 a	**	n.s.	n.s.
	L_s (%)	4	0.06 c	0.12 b	0.12 b	0.22 a	*	*	n.s.
	L_s (%)	27	0.25 a	0.26 a	0.19 a	0.25 a	n.s.	n.s.	n.s.
	L_{ns} (%)	4	0.67 a	0.53 b	0.01 c	0.00 c	*	*	*
	L_{ns} (%)	27	0.19 a	0.01 b	0.07 ab	0.00 b	**	n.s.	n.s.
	g_m (mmol m^{-2} s^{-1})	27	62.0 b	78.0 ab	65.0 b	92.0 a	*	n.s.	n.s.
	g_s/g_m	27	2.29 a	1.81 b	2.23 a	1.69 b	**	n.s.	n.s.
T. cordata	V_{cmax} (μmol m^{-2} s^{-1})	4	39.4 b	40.9 b	45.7 b	81.1 a	**	**	**
	V_{cmax} (μmol m^{-2} s^{-1})	27	77.2 b	89.9 a	97.9 a	79.5 b	n.s.	n.s.	**
	J_{max} (μmol m^{-2} s^{-1})	4	58.1 b	57.9 b	80.9 ab	121.9 a	*	**	n.s.
	J_{max} (μmol m^{-2} s^{-1})	27	109.9 c	154.2 a	131.4 b	125.0 b	**	n.s.	**
	L_s (%)	4	0.19 a	0.17 a	0.19 a	0.07 b	*	*	*
	L_s (%)	27	0.24 a	0.22 a	0.24 a	0.24 a	n.s.	n.s.	n.s.
	L_{ns} (%)	4	0.59 a	0.55 a	0.43 a	0.00 b	*	*	*
	L_{ns} (%)	27	0.30 a	0.00 b	0.32 a	0.12 ab	*	*	n.s.
	g_m (mmol m^{-2} s^{-1})	27	75.0 c	111.0 a	98.0 b	65.0 c	n.s.	*	**
	g_s/g_m	27	1.75 a	1.20 a	0.87 a	2.16 a	n.s.	n.s.	n.s.

V_{cmax} and J_{max} were calculated from A/C_i curves as described by Sharkey et al. [59]. L_s and L_{ns} were calculated from A/C_i curves as described by Lawlor [56]. L_{ns} is zero by definition in plants with the highest A values at the time of A/Ci curve measurements [56]. g_m was calculated from combined data of gas exchange and leaf fluorescence [60]. −I WW, non-inoculated well-watered plants; +I WW, inoculated well-watered plants; −I WS, non-inoculated water-stressed plants; +I WS, inoculated water-stressed plants. Within the same line and factor, different letters indicate significant differences among treatments at $p \leq 0.05$ (*) and $p \leq 0.01$ (**); n.s. indicates no significant differences.

3.5. Water Relations

The pre-dawn leaf water potential (Ψ_W) was affected by inoculation and deficit irrigation in the nursery both in *Acer campestre* and *Tilia cordata* (Table 4). +I promoted a less negative Ψ_W in both species compared to their −I counterparts throughout the experiment (the mean increments in Ψ_W were 31% in *Acer campestre* and 32% in *Tilia cordata*). WS *Acer campestre* had a less negative Ψ_W than WW plants during the whole experiment (mean increment of 24%). On the contrary, the effect of deficit irrigation on Ψ_W in *Tilia cordata* was not consistent.

Fourteen months after transplanting, +I plants of *Acer campestre* and *Tilia cordata* had a more negative osmotic potential (Ψ_Π) compared to −I plants, whereas Ψ_Π was similar in +I and −I plants when measured 5 and 28 months after transplanting (Table 4). Five months after transplanting, a significant interaction between inoculation and water regime in the nursery was found for Ψ_Π in *Tilia cordata*. The Ψ_Π of +I *Tilia cordata* was unaffected by the nursery water regime, whereas Ψ_Π was more negative in −I WS than in −I WW plants of this species (data not shown). Deficit irrigation in the nursery did not affect the Ψ_Π of both species until 28 months after planting, when contrasting effects were observed in the two species. WS *Acer campestre* had a less negative Ψ_Π and $\Psi_{\Pi FT}$ than WW plants. WS *Tilia cordata* had a more negative Ψ_Π than WW plants, while $\Psi_{\Pi FT}$ was unaffected.

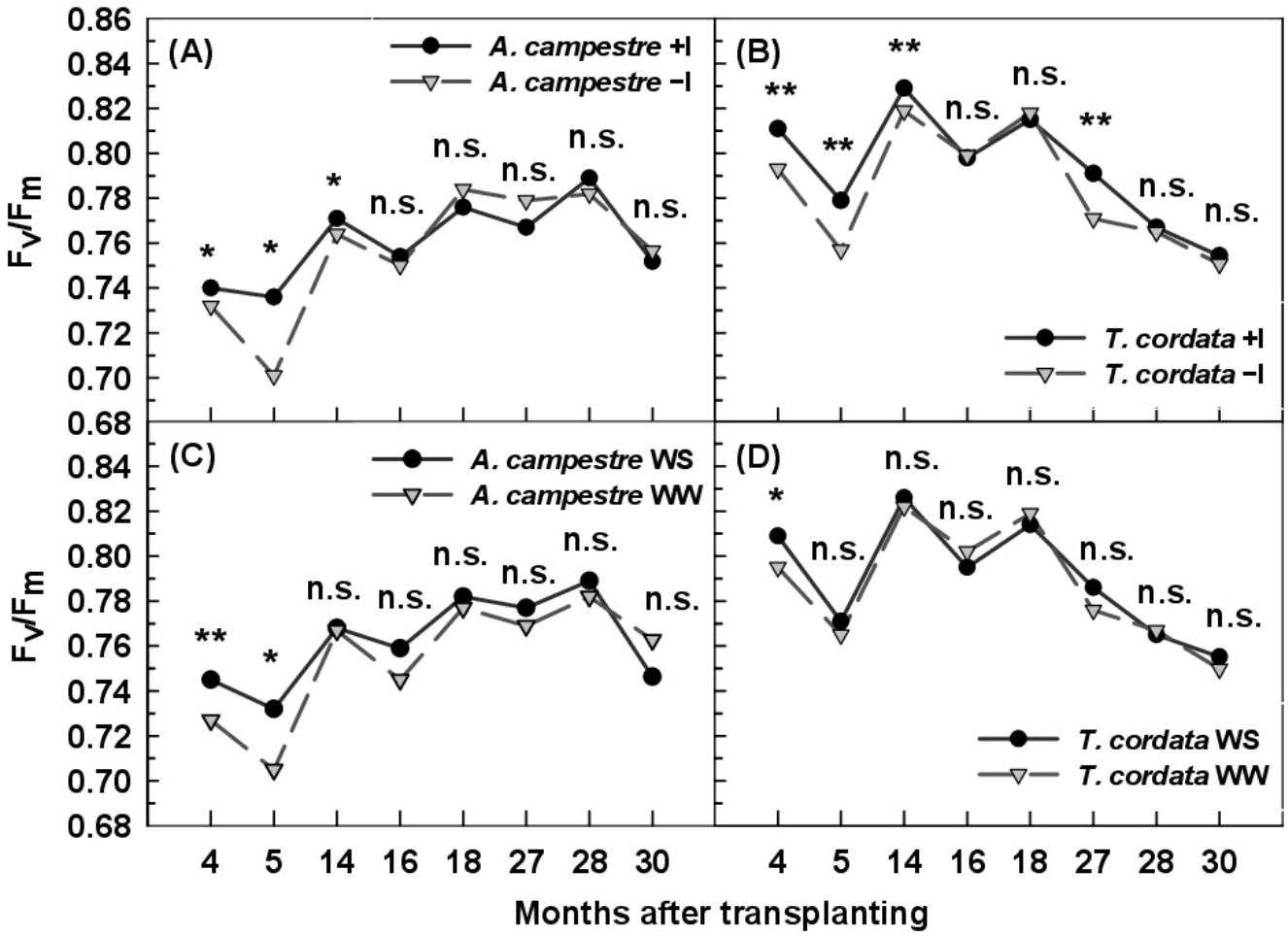


Figure 4. Effect of mycorrhizal inoculation and water regime in the nursery on maximal quantum yield of PSII (F_v/F_m) of *Acer campestre* L. (**A**,**C**, respectively) and *Tilia cordata* Mill. (**B**,**D**, respectively) transplanted in the field. Within each panel and sampling date, * and ** indicate significant differences between +I (inoculated) and −I (non-inoculated) plants and between WW (well-watered) and WS (water-stressed) plants at $p \leq 0.05$ and $p \leq 0.01$, respectively; n.s. indicates no significant differences.

The turgor potential (Ψ_P) was higher in +I than in −I *Acer campestre* throughout the experiment (Table 4). Five months after planting, a significant interaction between controlled inoculation and deficit irrigation in the nursery was found for Ψ_P in *Tilia cordata*. The Ψ_P of +I *Tilia cordata* was unaffected by the nursery water regime, whereas Ψ_P was higher in −I WS than in −I WW plants. Later on, +I (14 months) and WS (28 months) *Tilia cordata* displayed a higher Ψ_P than −I and WW plants, respectively.

3.6. Soluble Carbohydrates

In *Acer campestre*, inoculation did not affect the total soluble sugars (TSS), but +I plants had higher sucrose and fructose than −I plants 14 months after transplanting. Mannitol first increased (5 months) because of inoculation, but 28 months after transplanting, +I *Acer campestre* had lower leaf mannitol compared to −I plants (Figure 5). Five months after transplanting, +I *Tilia cordata* showed higher TSS than −I plants, but the opposite was found 14 months after transplanting. In *Tilia cordata*, inoculation reduced sucrose and mannitol (5 months after transplanting) and glucose and galactose (14 months) compared to non-inoculated plants. Conversely, galactose was higher in +I than in −I plants (5 months after transplanting), similar to sucrose (14 months).

Table 4. Effect of mycorrhizal inoculation and water regime in the nursery on leaf pre-dawn water potential (Ψ_W, MPa), leaf osmotic potential (Ψ_Π, MPa) and leaf turgor potential (Ψ_P, MPa) of *Acer campestre* L. and *Tilia cordata* Mill. measured 5, 14 and 28 months after transplanting.

Species	Parameter	Months after Transplanting	Inoculation (I)		Water Regime (W)		Significance		
			+I (%)	−I (%)	WW (%)	WS (%)	I	W	I × W
A. campestre	Ψ_W	5	−0.30 a	−0.44 b	−0.42 b	−0.32 a	**	**	n.s.
	Ψ_W	14	−0.35 a	−0.40 b	−0.41 b	−0.34 a	*	**	n.s.
	Ψ_W	28	−0.30 a	−0.40 b	−0.38 b	−0.32 a	**	**	n.s.
	Ψ_Π	5	−1.74 a	−1.71 a	−1.76 a	−1.69 a	n.s.	n.s.	n.s.
	Ψ_Π	14	−1.77 b	−1.66 a	−1.71 a	−1.73 a	*	n.s.	n.s.
	Ψ_Π	28	−1.91 a	−1.90 a	−1.94 b	−1.87 a	n.s.	*	n.s.
	Ψ_P	5	1.44 a	1.27 b	1.34 a	1.37 a	**	n.s.	n.s.
	Ψ_P	14	1.42 a	1.26 b	1.30 a	1.39 a	*	n.s.	n.s.
	Ψ_P	28	1.61 a	1.50 b	1.56 a	1.55 a	**	n.s.	n.s.
T. cordata	Ψ_W	5	−0.25 a	−0.36 b	−0.33 b	−0.28 a	**	*	n.s.
	Ψ_W	14	−0.22 a	−0.28 b	−0.25 a	−0.25 a	**	n.s.	n.s.
	Ψ_W	28	−0.25 a	−0.31 b	−0.27 a	−0.29 a	**	n.s.	n.s.
	Ψ_Π	5	−1.47 a	−1.46 a	−1.43 a	−1.50 a	n.s.	n.s.	*
	Ψ_Π	14	−1.74 b	−1.59 a	−1.64 a	−1.69 a	*	n.s.	n.s.
	Ψ_Π	28	−1.76 a	−1.79 a	−1.69 a	−1.86 b	n.s.	**	n.s.
	Ψ_P	5	1.22 a	1.10 a	1.10 a	1.22 a	n.s.	n.s.	*
	Ψ_P	14	1.52 a	1.31 b	1.39 a	1.44 a	**	n.s.	n.s.
	Ψ_P	28	1.51 a	1.48 a	1.42 b	1.57 a	n.s.	**	n.s.

+I, inoculated plants; −I, non-inoculated plants; WW, well-watered plants; WS, water-stressed plants. Within the same line and factor, different letters indicate significant differences between +I and −I plants and between WW and WS plants at $p \leq 0.05$ (*) and $p \leq 0.01$ (**), respectively; n.s. indicates no significant differences.

In *Acer campestre*, deficit irrigation in the nursery first increased TSS, compared to WW plants (5 months after transplant). Twenty-eight months after transplanting, however, TSS were lower in −I than in +I *Acer campestre.* WS *Acer campestre* showed lower sucrose and higher glucose leaf concentrations early after transplanting (5 months). Twenty-eight months after transplanting, both sucrose and glucose were lower in WS *Acer campestre* than in WW plants. In *Tilia cordata*, no effect of the water regime on TSS was detected. However, WS plants showed a lower concentration of sucrose and glucose than WW plants when measured 5 months after transplanting, whereas 28 months after transplanting, WS *Tilia cordata* displayed a higher concentration of sucrose than WW plants.

The lack of significant interaction between mycorrhizal inoculation and water regime is reported in Table S2.

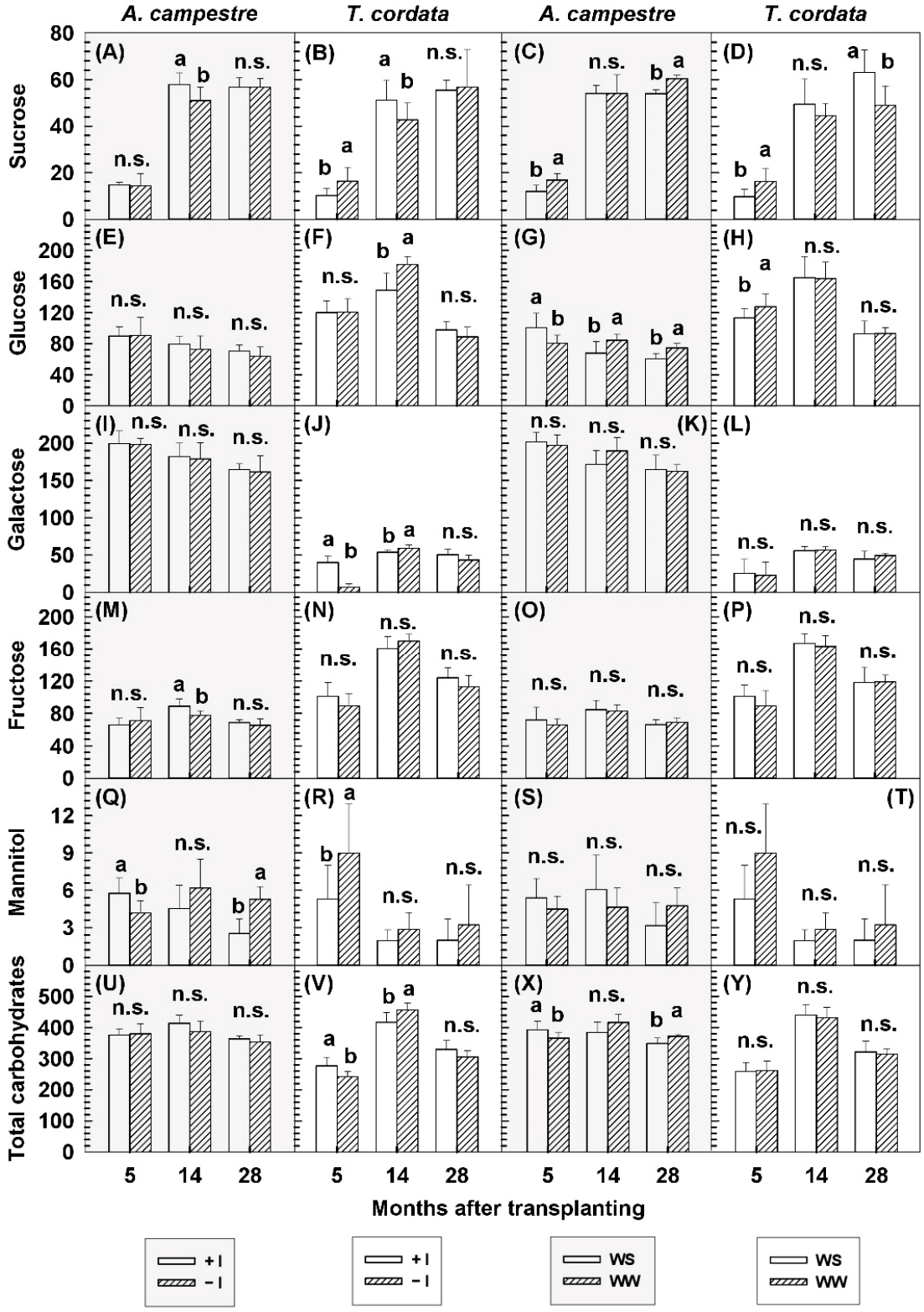


Figure 5. Effect of mycorrhizal inoculation and water regime in the nursery on leaf soluble carbohydrate content (expressed as μmol g^{-1} of dry weight) of *Acer campestre* L. and *Tilia cordata* Mill. measured 5, 14 and 28 months after transplanting. +I, inoculated plants; −I, non-inoculated plants; WW, well-watered plants; WS, water-stressed plants. Plots in gray represent carbohydrate values of *Acer campestre*, whereas plots in white represent values of *Tilia cordata*. (**A**–**V**,**X**,**Y**) Within each panel and sampling date, different letters indicate significant differences between +I and −I plants and between WW and WS plants at $p \leq 0.05$ and $p \leq 0.01$, respectively; n.s. indicates no significant differences.

4. Discussion

To mitigate transplant stress in newly planted trees and increase the effectiveness of tree planting programs, we tested the hypothesis that two nursery preconditioning techniques (controlled inoculation with mycorrhizal fungi and deficit irrigation) can yield sturdy plants capable of better establishment after transplanting. This information may be crucial under the current climate change scenario, which exposes newly planted trees to a high transpirational demand by the atmosphere while the root water uptake capacity is still reduced because of transplanting. After demonstrating that it was possible to isolate functional mycorrhizal strains on urban *Tilia cordata* and *Acer campestre*, and the mycorrhiza persisted for two years of cultivation in containers in the nursery [35], here, we explored whether the symbiosis can persist and be functional after transplanting. In the current experiment, a separate discussion of the results for controlled mycorrhization and deficit irrigation was carried out, because the interactions among treatments were not significant for most of the investigated parameters, denoting independent effects, rather than synergistic or antagonistic effects, of the two preconditioning techniques evaluated.

4.1. Effects of Mycorrhizal Inoculation in the Nursery on Post-Transplant Growth and Physiology

The effects of mycorrhizal inoculation in the nursery on growth and eco-physiology over three years after transplanting were species-specific. In *Acer campestre*, the root colonization rate by mycorrhizae-forming fungi detected three years after planting in the field was positively stimulated by the inoculation carried out in the nursery phase. The higher level of mycorrhizal symbiosis after inoculation is coherent with the results found in the nursery phase [35] and with studies carried out on different *Acer campestre* varieties [32] and other forest tree and shrub species transplanted in the field [67–70]. A higher percentage of root colonization resulted, in *Acer campestre,* in a higher survival rate after transplanting. Our findings support and extend to a cultivated landscape the previous finding that transplant success is positively correlated with the rate of mycorrhizal symbiosis [19,32,68,70]. The ability to maintain a more positive turgor pressure during the three years following transplanting was likely related to the higher transplant success observed in inoculated *Acer campestre* than in non-inoculated plants. Both the less negative pre-dawn water potential, which reflects water uptake by mycorrhizal tree roots [22,26], and the more negative osmotic potential, which was observed the second year after transplanting, explain the capacity of inoculated *Acer campestre* to maintain more favorable turgor. This was not the case when the osmotic potential in inoculated *Acer campestre* was more negative during the second year, when the annual precipitations were below the 30-year average. However, precipitation which occurred in the experimental area during the three years of the research was generally favorable, and this could have hidden more marked effects. Inoculation promoted the net increase in sucrose and fructose in *Acer campestre* leaves, at the expense of mannitol, which may explain the change in osmotic potential, although total soluble carbohydrates did not vary between inoculated and non-inoculated plants [71]. The better tissue hydration of leaves allowed inoculated *Acer campestre* to preserve the functionality of the photosynthetic apparatus during the first year after transplanting, unlike non-inoculated plants which experienced a steep reduction in the maximum quantum yield of PSII photochemistry (F_v/F_m). Moreover, the higher apparent carboxylation rate by Rubisco, the higher contribution of electron transport to ribulose regeneration and the higher mesophyll conductance to CO_2 diffusion allowed inoculated *Acer campestre* to minimize non-stomatal limitations to photosynthesis. Consistently, inoculated *Acer campestre* maintained higher net CO_2 assimilation than non-inoculated plants after transplanting. Keeping non-stomatal limitations to photosynthesis low can be advantageous to plants because it helps in maximizing CO_2 assimilation at a given degree of stomatal opening [72]. Therefore, it was not surprising to observe higher CO_2 assimilation per unit water transpired (i.e., higher water use efficiency) in inoculated *Acer campestre* than in non-inoculated plants. Higher water use efficiency had been previously observed in mycorrhizal plants in greenhouse studies [49,73]. However, this research shows that the impact of nursery

inoculation on leaf gas exchange persisted in *Acer campestre* after transplanting in the field. Growth, on the contrary, was little affected by inoculation in *Acer campestre*. Although a higher turgor pressure likely triggered the longer shoot extension observed in inoculated plants, neither above-ground nor below-ground dry biomass differed between inoculated and non-inoculated plants three years after transplanting, consistent with previous work conducted on container-grown plants [35,74]. Other leaf and root traits which were not directly measured in this research, including specific leaf weight, thickness and nitrogen concentration, root thickening and root branching, might have affected the photosynthetic performances of the inoculated plants [75,76].

Although, at planting, inoculated *Tilia cordata* had higher root colonization than non-inoculated plants [35], such a difference was not confirmed three years after transplanting in the field. The lack of differences in the colonization rate in *Tilia cordata* three years after transplanting may be due to: (1) the high capacity of this species to establish generalist mycorrhizal symbioses in the open field [77] compared to other forest tree species [78,79]; (2) the percentage of root colonization by mycorrhizal symbiosis being quantified without sequencing the specific fungal communities. Thus, although mycorrhizal colonization was not increased by inoculation, fungal symbionts associated with roots might differ between inoculated and non-inoculated plants [80]. Although inoculation promoted a less negative pre-dawn water potential in *Tilia cordata*, its effects on turgor pressure and osmotic potential were no longer significant after the second growing season after transplanting. The decline in glucose and galactose observed in inoculated *Tilia cordata* exceeded the increase in sucrose, resulting in a decline in total soluble carbohydrates compared to non-inoculated plants and suggesting that the changes observed in the osmotic potential might be due to dehydration rather than to net solute accumulation [64]. Similar findings on *Tilia cordata* were obtained by Porcel and Ruiz-Lozano [28], who suggested that the lower total soluble sugar accumulation in leaves of inoculated plants during sub-optimal water conditions could be due to a lower availability of photosynthates for storage in these tissues as a consequence of mycorrhizal fungi, which can be a strong competitor for root-allocated carbon under conditions limiting photosynthesis [81]. Small differences were found for net CO_2 assimilation between inoculated and non-inoculated *Tilia cordata*. Inoculation did not improve stomatal regulation and did not reduce stomatal limitations to photosynthesis in the long run. Even for non-stomatal limitations, which were reduced by inoculation throughout the experiment, the effects of inoculation on V_{cmax}, J_{max} and g_m were inconsistent across years, also being affected by the nursery irrigation regime. It can be noted, however, that a synergistic effect of mycorrhizal inoculation and deficit irrigation in the nursery minimized non-stomatal limitations to photosynthesis early after planting by improving the apparent carboxylation efficiency by Rubisco. These findings are confirmed by the maximal efficiency of PSII (F_v/F_m), which was higher in inoculated plants compared to control ones plants (in accordance with Nowak [82] and Ortuño et al. [83]) in the first period. The growth and survival of *Tilia cordata* were not consistently increased by inoculation (as also reported by Sýkorová et al. [79]), corroborating the idea, suggested by eco-physiological measurements, that inoculated fungal taxa might have been outcompeted by native microorganisms within three years from planting.

4.2. Effects of Deficit Irrigation in the Nursery on Post-Transplant Growth and Physiology

As with mycorrhizal inoculation, the effect of deficit irrigation was species-specific. In *Acer campestre*, plants preconditioned to water shortage in the nursery displayed higher CO_2 assimilation and a better water status than their well-watered counterparts after transplanting in the field. Early after transplanting, a higher apparent rate of carboxylation by Rubisco and a higher contribution of electron transport to ribulose regeneration resulted in lower non-stomatal limitations to photosynthesis in plants acclimated to deficit irrigation than in those which received full irrigation in the nursery. Consistently, the maximum quantum yield of PSII (F_v/F_m) was higher in preconditioned plants in the short-term period. Although such changes were transient, they helped preconditioned plants to

maintain higher CO_2 assimilation throughout the experiment [17]. This is consistent with the better capacity of preconditioned plants to maintain more favorable water relations after transplanting, although the mechanisms leading to a less negative leaf pre-dawn water potential are controversial. Deficit irrigation promoted the leaf accumulation of soluble sugars and the breakdown of sucrose to glucose, but such changes were too small to induce a significant active osmotic adjustment [39,40,84]. This suggests that there is no memory effect for osmotic adjustment in plants and that the trait is only affected by the current growing conditions rather than by those experienced during the current and the previous growing seasons. This may be due to the massive relocation of carbohydrates from leaves [85] and may hamper the opportunity to boost osmotic adjustment through deficit irrigation in the long run, different from previous reports [86,87]. On the other hand, no changes in the root-to-shoot ratio were observed between preconditioned and well-watered plants. This was surprising, since increases in the absorbing root surface over the transpiring leaf area have been widely reported following deficit irrigation [39,88]. It must be considered, however, that changes in root morphology which can hardly be detected by weighing, such as root thickening and root branching, may have occurred in plants subjected to deficit irrigation [86]. This is corroborated by the higher stomatal conductance displayed by preconditioned plants, which suggests a better water uptake capacity. It must be considered that, at planting, plant dry weights of preconditioned *Acer campestre* were about 33% lower than those of well-watered plants [35] and then, after three growing seasons from transplanting, became about 17% higher. Moreover, a higher shoot length and shoot dry weight were found in preconditioned plants, once in the field. As expected, the reductions in growth during the nursery period triggered by the exposure of plants to sub-optimal water availability [35] were overcompensated by increased growth after transplanting. This behavior was also demonstrated in previous studies on the water stress acclimation in many forest tree species, such as in *Acer pseudoplatanus*, where a reduction in growth in the nursery phase was followed by faster growth after transplanting in the open field [19]. This reveals how deficit irrigation is an efficient practice to strengthen *Acer campestre* plants to stress conditions in the field, improving the ability to extract water from the soil [88,89] and enhancing the post-transplanting survival [20,39], as occurred in this experiment.

In *Tilia cordata*, although deficit irrigation positively affected physiological parameters in the short run after transplanting, it failed to promote growth in the long run. The more favorable pre-dawn water potential shown by preconditioned plants in the first year after planting was not likely due to a better capacity of preconditioned plants to adjust osmotically. Consistently, sucrose and glucose were lower during the first growing season after transplanting in plants subjected to deficit irrigation in the nursery, even if total soluble sugars did not differ among irrigation treatments. Three years after transplanting, the more negative osmotic potential found in WS than in WW *Tilia cordata* may be due to sucrose accumulation, caused by the lower growth rate of preconditioned plants and the lower sink strength of the vegetative organs. However, it is worth noting that soluble sugars are not the only compounds responsible for osmotic adjustment. Indeed, other compatible solutes could be involved in this process. For instance, an important role of proline in active osmotic adjustment in *Tilia cordata* was observed in container-grown seedlings subjected to slow-developing drought [90]. The CO_2 assimilation of *Tilia cordata* was only transiently promoted by deficit irrigation in the nursery. This was mostly due to the short-term improvement in biochemical factors related to the Calvin–Benson cycle observed in preconditioned plants, although a possible synergic role of mycorrhizal inoculation cannot be excluded in this case. Instead, in the long-term period, deficit irrigation failed to improve leaf gas exchange because of higher mesophyll diffusion limitations, particularly in plants acclimated to deficit irrigation and inoculated with mycorrhizal fungi [58]. Consistently, the maximal efficiency of PSII (F_v/F_m) was higher in acclimatized plants only immediately after planting. In *Tilia cordata*, the small physiological improvement observed in the first stage of the trial did not enhance survival and growth over the whole experiment. In particular,

preconditioned plants showed lower growth in the analyzed components, confirming the trend shown in the nursery phase [35].

The differential impact of deficit irrigation preconditioning on post-transplant growth may be partly explained by the different water use strategies adopted by the two species [91]. According to Li et al. [92], *Acer campestre* displays a near-anisohydric behavior, while, as reported by Leuschner et al. [93], *Tilia cordata* shows a near-isohydric behavior. Anisohydric species display lower stomatal control in order to sustain CO_2 assimilation at a decreasing water potential, despite the risk of hydraulic failure [18]. In these species, deficit irrigation may promote a suite of adjustments (including a higher efficiency of carboxylation enzymes, as shown in this research) that results in a higher photosynthetic rate and growth after transplanting. Conversely, isohydric species display early regulation of stomatal opening to prevent an excessive decline in the leaf water potential, and thus hydraulic failure [16]. The chronic limitations to CO_2 assimilation experienced by isohydric plants when grown under deficit irrigation in the nursery may hamper growth after transplanting.

4.3. Effects of Deficit Irrigation on Post-Transplant Root Mycorrhization

In *Acer campestre,* preconditioning to water stress in the nursery did not enhance mycorrhizae root colonization three years after transplanting, different from the findings obtained during the nursery phase [35]. Although other studies found that high levels of drought can promote greater root colonization in the field [22], the less severe drought conditions occurring in the field compared to the nursery probably smoothed out the initial differences among preconditioned and well-watered plants of *Acer campestre* in this experiment.

Even in *Tilia cordata*, deficit irrigation did not produce significant effects on root colonization by mycorrhizal fungi three years after transplanting, coherent with what had been observed in the previous experiment in the nursery [35]. There is evidence that shows the absence of significant differences in the rate of mycorrhization in plants exposed and not exposed to early water stress [48], confirming that deficit irrigation, even in situations of transplanting in anthropized environments, is not always functional to mycorrhizal colonization.

5. Conclusions

Mycorrhizal inoculation and deficit irrigation applied in the nursery phase did not lead to a synergistic effect after transplanting, providing independent responses for most of the investigated parameters. This research extends the available scientific knowledge, mainly focused on short-term period effects on potted plants and/or in the greenhouse, to a cultivated landscape over three years after field transplanting.

Plants inoculated in the nursery with specific symbionts selected at the planting site sustained higher CO_2 assimilation and more favorable water relations after transplanting. The effectiveness of inoculation may be lower in species such as *Tilia cordata* which commonly establish generalist mycorrhizal symbioses in the open field. The results indicate that controlled inoculation in the nursery is technically feasible and more cost-effective than inoculation at transplanting because smaller quantities of the inoculum are needed per plant. However, the results also underline the need for a better understanding of plant species–fungal strain interactions, which might be achieved by sequencing the specific fungal communities in nurseries and at urban sites.

Imposing deficit irrigation (water supply at 30% container water holding capacity) on plants in the nursery may help trees to overcome transplanting shock, even if with a different effectiveness depending on species and their water use strategies. In general, deficit irrigation was more effective in near-anisohydric species, such as *Acer campestre*, than in near-isohydric species, such as *Tilia cordata*. A deeper understanding of plant water use strategies, which is currently limited to a few widely used species and genera, is needed to extensively apply deficit irrigation practices in nurseries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13050658/s1, Table S1: Two-way ANOVA results for stomatal conductance, CO_2 assimilation, water use efficiency and maximal quantum yield of PSII for *Acer campestre* and *Tilia cordata* 4, 5, 14, 16, 18, 27, 28, 30 months after transplant; Table S2: Two-way ANOVA results for sucrose, glucose, galactose, fructose, mannitol and total carbohydrates leaf content for *Acer campestre* and *Tilia cordata* 5, 14, 28 months after transplant.

Author Contributions: Conceptualization, F.F., P.F. and A.F.; methodology, S.D.O., C.B., C.V. and A.F.; validation, D.C., I.V., S.D.O. and A.F.; formal analysis, D.C., I.V., S.D.O., J.M. and A.F.; investigation, S.D.O., P.F., C.B., J.M., C.V. and A.F.; resources, P.F., C.V., F.F. and A.F.; data curation, D.C., I.V., S.D.O., P.F., C.B., J.M., C.V. and A.F.; writing—original draft preparation, D.C., I.V., S.D.O. and J.M.; writing—review and editing, D.C., I.V., S.D.O., P.F., C.B., C.V., F.F. and A.F.; visualization, D.C., I.V., S.D.O. and J.M.; supervision, C.V., F.F. and A.F.; project administration, F.F., P.F. and A.F.; funding acquisition, P.F., C.V., F.F. and A.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Regione Lombardia—Agricultural Department (Research Project no. 1749-dgr 1180/2010), according to the Plan of Research and Development, as a part of the research project "Metodologie produttive e gestionali per migliorare la qualità del verde ornamentale—METAVERDE".

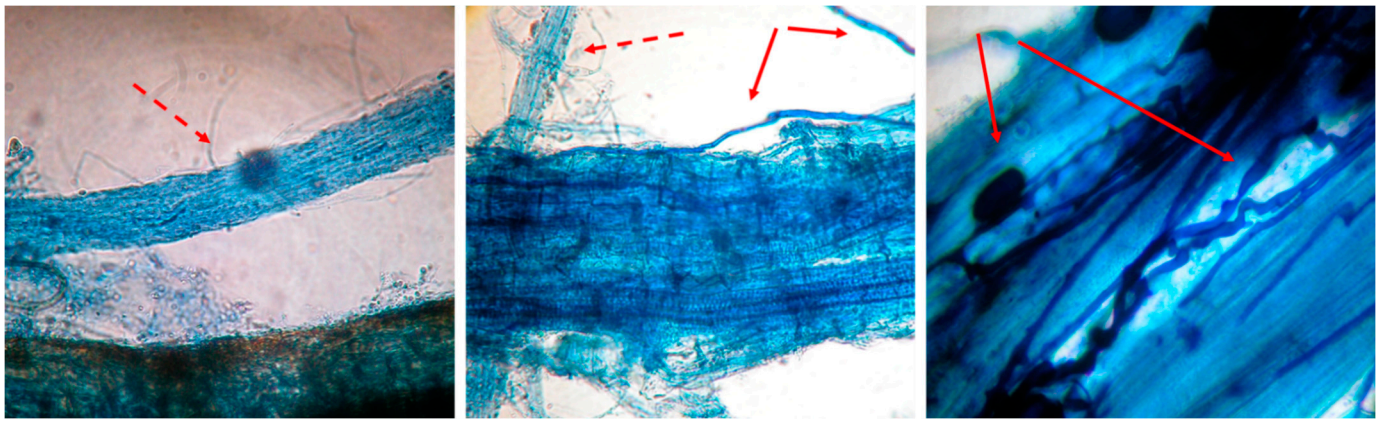
Acknowledgments: The authors are grateful to Gabriele Amoroso, Riccardo Piatti and Marco Faoro and Floricoltura San Donato-Grandi Trapianti Italiani (S. Donato Milanese, Milan, Italy) for the technical assistance provided during this research.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Appendix A

(**a**) (**b**)

Figure A1. Experimental field: (**a**) immediately after transplanting; (**b**) three growing seasons after transplanting.



(**a**) (**b**) (**c**)

Figure A2. Microscope images of mycorrhizal symbioses: (**a**) fungal mantle of ECM in *Tilia cordata* Mill. observed at 400× magnification (dashed line); (**b**) ECM (dashed line) and AMF (solid line) in *Tilia cordata* observed at 100× magnification; (**c**) arbuscular structure (solid line, left) and fungal hyphae (solid line, right) of AMF in *Acer campestre* L. observed at 1000× magnification.

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