

## Article

# Induced Drought Stress Response of European Beech Seedlings Treated with Hydrogel and Ectomycorrhizal Inoculum

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**Abstract:** Bareroot European beech (*Fagus sylvatica* L.) seedlings are frequently used in reforestation programs in Central Europe. However, beech outplanting is often unsuccessful due to drought stress. In this study, the effects of a simulated water deficit and of a hydrogel and ectomycorrhizal fungi (EMF) application were estimated on the development of 1 + 1 beech seedlings. The roots of 1-year-old bareroot seedlings were treated with the additives at the time of transplanting to pots in spring and then exposed to the whole growing season under different watering regimes: (i) full watering (FW; volumetric water content 70%), (ii) reduced watering (RW; 40%), (iii) periodic watering (PW; substrate rewetted to 70% after drying), and (iv) no watering (NW). Almost all FW seedlings survived the whole growing season, whereas all NW seedlings died after 17 weeks of desiccation. The survival and growth of FW and NW seedlings were significantly higher and lower, respectively, than those under both PW and RW treatments. The additives significantly increased survival in RW seedlings only but had no effect on growth. A promoting effect of FW on the chlorophyll *a* fluorescence parameters was found. The number and EMF colonization of the short roots were significantly lower in FW seedlings compared to the other watering treatments. The additives did not affect these parameters. The occurrence of neither treatment-specific EMF root morphotype nor fungi applied in the inoculum and traced by molecular analysis indicated the formation of ectomycorrhizas with native EMF in all treatments. The seedlings in the water-deficient treatments exhibited higher foliar nutrient concentrations than FW seedlings. The additives increased the concentration of nutrients in PW and phosphorus in NW seedlings. The results suggest the significant effect of the water supply, but a weaker effect of the additives tested on beech seedling development under experimental conditions.

**Keywords:** water deficit; drought stress; beech; potted seedlings; hydrogel; ectomycorrhizal inoculation



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## 1. Introduction

In Central Europe, old, even-aged monoculture Norway spruce (*Picea abies* [L.] Karst.) forests are threatened and frequently disrupted by detrimental agents induced by climate change, such as windstorms, heat, droughts, bark beetle outbreaks, and their synergistic effects [1,2]. Subsequently, the generated salvage-felled areas and established plantations are continuously exposed to heatwaves and drought periods [3,4]. The basic approach in the effort to enhance the static stability, overall resistance, and sustainability of threatened spruce forests involves a partial exchange of spruce with other tree species, to a great extent with European beech (*Fagus sylvatica* L.) (beech) [1,5,6]. This is because beech had previously colonized a large part of the recent spruce area prior to being replaced with spruce in order to achieve better commercial timber exploitation of forest land [7,8].

Beech is the prevalent tree species (32% of forest area) in Slovakia [9]. In Slovakia, approximately 10 million beech seedlings had been planted each year by the 2010s, with

approximately 95% being bareroot seedlings [9]. Such a stocktype has to interact with the environment of the planting site immediately after planting to have a chance of survival [10]. However, bareroot beech seedlings typically form a distinctive taproot, with a small number of fine lateral roots providing low capability for the rapid colonization of the soil to ensure sufficient water and nutrient supply. Another difficulty is that beech is not sufficiently evolutionarily adapted to the adverse conditions of large clear sites [11]. Consequently, the outplanting performance of bareroot beech seedlings is often rather poor in these environments [5,6]. Experiments carried out in a controlled environment have revealed the variable resistance and resilience of beech seedlings to water deficits depending on the drought intensity, seedling and soil origin, soil biota, and other circumstances [12–14].

An efficient way to overcome plant drought stress involves the use of hydrophilic polymers (hydrogels) that are able to absorb, retain, and provide a great deal of water to the roots [15,16]. A predominant number of commercially available hydrogel products are in the form of granules, mostly in the 0.1–4.0 mm particle size range. The granules consist of crosslinked copolymers of acrylic acid and acrylamide, partially neutralized with either sodium- or potassium-based salts, showing high water adsorption capacities of up to 100 g g<sup>-1</sup> [15]. Mixing a hydrogel with a growth medium and dipping the bareroot systems or container root plugs into the hydrogel slurry before planting can provide extra moisture to the roots and promote seedling vitality and performance under a wide range of drought stress intensities and even under sufficient water supply [16–18]. However, a longer-lasting severe drought can, in certain circumstances, cause disruption to the hydrogel structure and thus impair the water supply to the roots [19,20].

Another approach to seedling drought stress prevention is the promotion of the colonization of root tips with ectomycorrhizal fungi (EMF), which provides increased nutrient and water uptake to plants [21,22]. The external EMF hyphal network functions as an extension of the roots and transports water and nutrients from the soil to the seedlings with much higher efficiency than nonmycorrhizal roots [22]. The functioning EMF-seedling root associations reduce the need for bulk water transport to supply mineral nutrition, and seedlings can obtain similar amounts of nutrients at a reduced water demand [23]. EMF, whether applied in the field or in the nursery, inevitably competes in the colonization of the roots with indigenous EMF adapted to the site conditions, potentially resulting in a decrease in effectivity or even the failure of inoculation [24–26]. However, compared to field EMF inoculation, nursery inoculation appears to be a simpler, cheaper, more reliable, and efficient means of controlled ectomycorrhiza formation targeting the successful outplanting seedling performance [27,28].

The soil water deficiency of planting sites caused by rising temperatures due to climate change has incited the testing of water-holding amendment applications and EMF inoculation to potentially support seedling water uptake under drought stress in the temperate zone of Central Europe [4–6,18,26,29]. Although a number of experiments have focused on the effect of hydrogel application on seedling performance, only a few have investigated beech [5,18,30]. The role of EMF in determining the drought resistance of beech seedlings was studied by growing seedlings in forest soil containing EMF propagules, and the results showed that ectomycorrhizas can increase the drought resistance of beech [13,31,32]. However, the inoculation of beech seedlings exposed to drought with spore or mycelial EMF inoculum has not, to the best of our knowledge, been addressed in the literature. Experiments conducted under controlled conditions simulating adverse environmental scenarios, including water deficiency, appear to be a beneficial tool by which to predict the impacts of various effects on seedling development in the real environments of planting sites [31,33,34]. Accordingly, the objective of this study was to assess the effects of commercial additives (hydrogel and spore–mycelial EMF inoculum) applied into the seedling root zone on the development of European beech seedlings exposed to simulated water deficits of different intensities.

## 2. Materials and Methods

### 2.1. Experimental Design

One-year-old bareroot beech seedlings were transplanted in spring to three-liter pots filled with peat growth substrate and grown under polyethylene sheeting to avoid precipitation to the end of the growing season. Four watering treatments were conducted throughout the growing season:

- (i). Full watering (FW) (volumetric water content of the substrate 70%, easily available water).
- (ii). Reduced watering (RW) (volumetric water content 40%, poorly available water).
- (iii). Periodic watering (PW) (drought periods; substrate rewetted to 70% when volumetric water content decreased to wilting point (21%).
- (iv). No watering (NW).

Within each of the three repetitions (blocks) of each watering regime, 20 seedlings were treated either with the EMF product (inoculum) Ectovit (Symbiom s.r.o., Lanškroun, Czech Republic) or with the hydrogel Agrisorb (Evonik Nutrition & Care GmbH, Essen, Germany), or remained untreated (control). The experiment was arranged in a completely randomized block design containing 12 treatment combinations (4 watering levels  $\times$  3 additives, including control) repeated three times (3 blocks), involving 720 seedlings in total (Figure 1).

	FW	RW	PW	NW
BLOCK 1	Ectovit	Agrisorb	Control	Agrisorb
	Agrisorb	Control	Ectovit	Ectovit
	Control	Ectovit	Agrisorb	Control
BLOCK 2	Agrisorb	Control	Ectovit	Control
	Control	Ectovit	Agrisorb	Agrisorb
	Ectovit	Agrisorb	Control	Ectovit
BLOCK 3	Control	Ectovit	Agrisorb	Ectovit
	Ectovit	Agrisorb	Control	Agrisorb
	Agrisorb	Control	Ectovit	Control

**Figure 1.** Schematic treatment arrangement in experiment testing effects of watering regime (FW—full watering, RW—reduced watering, PW—periodic watering, NW—no watering) and additive application (hydrogel Agrisorb, ectomycorrhizal inoculum Ectovit, untreated control) on development of 1 + 1 potted European beech seedlings. A total of 20 seedlings were established in each watering  $\times$  additive  $\times$  block combination, for 720 in total.

### 2.2. Seeds, Seedlings, and Cultural Practices

The experiment was conducted in the forest nursery (Lokca, altitude 620 m, 49°22'20" N, 19°24'18" E, northern Slovakia) of the state forest enterprise of the Slovak Republic. Beech seeds were collected from a certified source (mature stand localized in eastern Slovakia, altitude 735 m, 48°47' N, 22°17' E). Seeds were broadcast sown for operational use in an open-soil seedbed in spring (end of April) after being pregerminated for 10 weeks (air temperature 3–5 °C, seed water content 30%). The basic analytical parameters of the seedbed soil obtained by the National Forest Centre (Zvolen, Slovakia) were as follows: dry matter 98.04%, pH<sub>H2O</sub> 7.27, C 5.56%, N 0.35%, C/N 15.9, P 70 mg kg<sup>-1</sup>, K 255 mg kg<sup>-1</sup>, Ca 4001 mg kg<sup>-1</sup>, Mg 154 mg kg<sup>-1</sup>, bulk density 0.839 g cm<sup>-3</sup>, and electrical conductivity 0.127 ms cm<sup>-1</sup>. The soil was fumigated with the fungicide Basamide (200 g m<sup>-3</sup>, 5 days of fumigation, 14 days of aeration). The granule fertilizer Cererit (NPK 8-13-11% + 2% Mg+microelements, 30 g m<sup>-2</sup>) was thoroughly mixed with approximately 10 cm of the upper layer of the soil before sowing. The seedbed was sheltered with wooden sunshades, enabling the soaking of water and providing mechanical protection to

seeds. The sunshades were raised up to 30 cm to shade the seedlings after germination started and were removed after approximately half of the growing season (the end of July). After the beginning of the leading shoot growth, the seedlings were fertilized once weekly with a 0.1% solution of the fertilizer Kristalon (NPK 19-6-20% + 3% Mg + microelements) for the next 5 weeks. The seedlings were irrigated with a sprinkler irrigation system to balance the deficit of atmospheric precipitation and weeded manually or with herbicides if needed. One-year-old seedlings were overwintered in the seedbed, lifted in the dormant stage in the second half of April, and stored at 2 °C until being planted.

Seedlings of conformable morphological quality were selected from this operational lot for use in the experiment. Seedlings with a root collar diameter of  $3.4 \pm 0.2$  mm, stem height of  $19.5 \pm 2.3$  cm, root system dry weight of  $0.41 \pm 0.11$  g, aboveground part dry weight of  $0.46 \pm 0.13$  g, and root-to-shoot dry weight ratio of  $0.89 \pm 0.26$ , corresponding to standard size and quality for reforestation, were used. The number of short roots per 1 cm of lateral roots and EMF colonization rate of the seedlings were  $7.5 \pm 1.2$  and  $80 \pm 5\%$ , respectively. The introduced attributes of the 1 + 0 seedlings were assessed on 20 randomly selected seedlings from those intended for the experiment. The root systems of all seedlings were shortened to the same length of 13 cm before seedlings were transplanted to pots at the beginning of May. After transplanting and simultaneous application of the additives tested, the seedlings were placed outdoors on a wooden bench 15 cm above the soil surface and grown for one growing season under simulated watering regimes. Transparent polyethylene sheeting of 17  $\mu\text{m}$  thickness was installed approximately 2 m above the seedlings to eliminate atmospheric precipitation. Potted seedlings were treated once with acaricide and insecticide as preventive measures against mites and insects, respectively, and manually weeded if needed. No other pesticides or fertilizers were applied. Both bareroot 1 + 0 and potted 1 + 1 seedlings were cultivated without regulation of temperature, air humidity, and light regimes, which were influenced by outdoor environmental conditions. The long-term (1991–2020) mean annual/growing season (April–September) air temperature and precipitation extrapolated from data recorded by four meteorological stations of the Slovak Hydrometeorological Institute [35] situated close to the nursery were 4.5/11.0 °C and 1196/670 mm, respectively. The mean annual/growing season air temperatures in the nursery recorded using a data logger (EMS, Brno, Czech Republic) were 4.6/11.2 °C and 4.9/11.9 °C in the years of the 1 + 0 and 1 + 1 seedling cultivation, respectively.

### 2.3. Pots, Growth Substrate, and Additives

PVC pots with a 3-liter volume (13.0  $\times$  13.0 cm top, 11.5  $\times$  11.5 cm bottom, 20 cm height) were filled with growth substrate (VermiVital s.r.o., Záhorce, Slovakia) consisting of white peat with a fiber length of 0–20 mm (80%), black peat with a fiber length of 0–10 mm (20%), powder fertilizer PG Mix macro- and microelements at  $1.9 \text{ kg m}^{-3}$ , the wetting agent Fibazorb at  $0.1 \text{ L m}^{-3}$ , and the root growth stimulator Bioroot at  $200 \text{ mL m}^{-3}$ . The basic analytical parameters of the substrate were as follows: dry matter 83.38%,  $\text{pH}_{\text{H}_2\text{O}}$  5.69, C 31.1%, N 0.983%, C/N 31.6, P 250  $\text{mg kg}^{-1}$ , K 640  $\text{mg kg}^{-1}$ , Ca 8322  $\text{mg kg}^{-1}$ , Mg 921  $\text{mg kg}^{-1}$ , bulk density  $0.137 \text{ g cm}^{-3}$ , and electrical conductivity  $0.162 \text{ ms cm}^{-1}$ .

The commercial EMF inoculum Ectovit contained mycelium of three species of EMF (*Amanita rubescens* (Pers. ex Fr.) Gray, *Pisolithus arrhizus* (Scop.) Rauschert, and *Paxillus involutus* (Batsch) Fr.), basidiospores of two species of EMF (*Pisolithus arrhizus* and *Scleroderma citrinum* Pers.) and natural ingredients (humates, ground minerals, and extracts from sea algae) incorporated into a finely decomposed peat carrier. EMF inoculum was mixed with the growth substrate at a ratio of 1:6 (v:v) and placed tightly around the root system (100 mL per seedling) at the time of transplanting the seedlings to pots. The inoculum-substrate mixture applied to one seedling contained 17 mL of mycelium. The number of spores in Ectovit was not known. Hydrogel Agrisorb is an acrylic acid copolymer partially potassium-neutralized in the form of free-flowing white granules (particle size 0.2–1.0 mm). The absorption rate of Agrisorb in distilled water is 250–300  $\text{mL g}^{-1}$ . Agrisorb was applied by dipping the root systems into a slurry (gel) prepared by mixing

the hydrogel granules with water at a ratio of 1:100 (*v:v*) immediately before transplanting seedlings to pots.

#### 2.4. Setting and Maintenance of Watering Regimes

Volumetric water content (VWC) is the percentage of the volume of water from the volume of growth medium (total pore space plus volume of the medium solid phase). The VWC values introduced in Section 2.1 were derived from conventional levels of soil water potential. The bottom bounds of the ranges of soil water potentials corresponding to easily (−1 to −5 kPa) and poorly available water (−10 to −1500 kPa) and permanent wilting point (−1500 kPa) [36–38] were chosen as the levels of the watering regime. Poorly available water can be released from a medium but not necessarily absorbed by plants, especially at lower soil water potentials. At the wilting point, soil particles hold water so strongly that it becomes difficult for plant roots to extract it. To determine the VWC of the substrate equivalent to these water potentials and to obtain reference weights for configuration of the watering treatments, three small cylinders (diameter 5.3 cm, height 4.6 cm) were filled with growth substrate and wetted from the bottom for 24 h. Fully saturated substrate in the cylinders was weighed, then placed in a pressure container and weighed repeatedly at water potentials of −1, −10, and −1500 kPa, and finally after oven drying at 105 °C for 48 h. The VWC for each water potential was calculated (and reasonably rounded up for FW and RW) using the gravimetric method [36,38].

For regulation of the watering regimes in the pots, the weight of the substrate in the pots was calculated for each watering treatment from the substrate weight in the small cylinders determined as described in the paragraph above. At the start of the experiment, a substrate VWC of 70% (FW) in the pots was setup for all treatments. To maintain the VWC assigned to the watering regimes, three extra pots were installed within the seedlings of each watering treatment and weighed under FW daily from the beginning of the experiment, under RW daily when the VWC fell to 40%, and under PW weekly during the experiment. When the pots' weight dropped below the reference weights related to the defined levels of VWC, an amount of water equivalent to the weight loss was added to all pots in each watering regime. Water was added almost every day under FW and RW, and under PW, water was added about 2 months after the beginning of the experiment and then three times over a one-month interval.

#### 2.5. Sampling and Measurements

##### 2.5.1. Survival and Growth

Potted 1 + 1 seedlings were assessed after the second growing season (end of October), i.e., one growing season after exposure of seedlings to the simulated drought and application of the additives. Seedling mortality was recorded continually during the growing season. Seedlings were regarded as dead when all their leaves turned brown and started falling off [30]. Survival was determined as the percentage of the number of surviving seedlings from the number of seedlings transplanted to the pots. For each watering regime × additive × block combination, 10 seedlings were randomly excavated (every other seedling from 20 established per combination) for estimation of stem height, root collar diameter, root system (root), and aboveground (shoot) dry weight (48 h at 80 °C), and frequency of short roots and ectomycorrhizas. The root-to-shoot dry weight ratio (RDW/SDW) was calculated. Fewer than 10 out of 20 seedlings in any repetition of the additive-untreated PW and RW treatments survived; all the viable seedlings were assessed. Dead NW seedlings with developed buds (19% of those 180 established) were also assessed.

##### 2.5.2. Frequency of Short Roots and Ectomycorrhizas

Three sections of approximately 3 cm long of the fine lateral roots (diameter < 1 mm) bearing short roots were sampled from each of the top, middle, and basal parts of the washed root system of seedlings used for the growth assessment (approximately 27 cm of fine

lateral roots in total per 1 seedling) [39]. Short roots were counted at 10–40× magnification using a dissecting microscope (Zeiss, Stemi, Göttingen, Germany). Short-root frequency was determined as the number of short roots per 1 cm of the lateral roots. Short roots were categorized as ectomycorrhizal or nonmycorrhizal short roots. Based on gross morphological characteristics such as ramification, shape, color, outer mantle characteristics, and the presence of hyphae and rhizomorphs, the short roots were classified into EMF morphological types (morphotypes) [40]. No quantification of EMF morphotypes was performed. Ten short roots for each EMF morphotype were sampled for DNA extraction and sequencing. Molecular identification of fungal symbionts was carried out by sequencing the amplified internal transcribed spacer (ITS) of rDNA. Polymerase chain reaction (PCR) product was obtained in nested PCR with outer primers NSA3/NLC2 or NSI1/NLB4 and internal primers ITS-1F and ITS-4B. Suitability of the PCR product for sequencing analysis was evaluated using agarose gel electrophoresis. Sequencing of the PCR product was performed by SeqMe (Prague, Czech Republic). The methods used for molecular identification of ectomycorrhizas in this study are described in detail in [4]. EMF colonization was determined for each seedling as the percentage of the number of ectomycorrhizas from the total number of short roots (ectomycorrhizas and nonmycorrhizal short roots).

### 2.5.3. Foliar Nutrient Concentration

The foliar nutrient concentration of each watering and additive treatment combination was determined on one combined sample of leaves sampled from the upper one-third of four to five randomly chosen seedlings in each block of the respective watering regime × additive combination in the first week of August. Foliar samples were analyzed in the laboratory of the National Forest Centre (Zvolen, Slovakia). The samples were dried at 70 °C for 60 h in an oven. Total C and N in leaves were determined using dry combustion (900–1250 °C) and subsequent oxidation–reduction reactions in a CN analyzer, FLASH EA 1112. Foliar concentrations of K, Ca, and Mg were measured using an inductively coupled plasma atomic absorption spectrometer (Thermo iCE 3000). The concentration of P was measured spectrophotometrically (PHARO 300) from the HNO<sub>3</sub> extract using the ammonium molybdate method after digestion of the ground material in concentrated HNO<sub>3</sub>.

### 2.5.4. Chlorophyll *a* Fluorescence

The fast kinetics of chlorophyll *a* fluorescence was measured for 10 seedlings (2 records per seedling) for each watering regime × additive × block combination at the time of leaf sampling for nutrient analysis. A Handy PEA fluorimeter (Hansatech Ltd., Kings Lynn, UK) was used for the OJIP transient measurements. After a 30 min dark adaptation period using leaf clips, the seedling leaves were irradiated using a one-second-long saturating pulse (2000 μmol m<sup>-2</sup> s<sup>-1</sup>) and basic fluorescence parameters were estimated. The dark-adapted leaves were illuminated with weak modulated measuring light (all PSII reaction centers were open) to measure the minimal fluorescence level in the dark (F<sub>0</sub>). Application of a saturating pulse to the dark-adapted leaves induced a maximum value of fluorescence (F<sub>m</sub>) by closing all PSII reaction centers. The variable fluorescence (F<sub>v</sub> = F<sub>m</sub> – F<sub>0</sub>) and maximal quantum yield of PSII chemistry (F<sub>v</sub>/F<sub>m</sub>) as a chlorophyll *a* fluorescence indicator of environmental stress were calculated.

### 2.6. Data Analysis

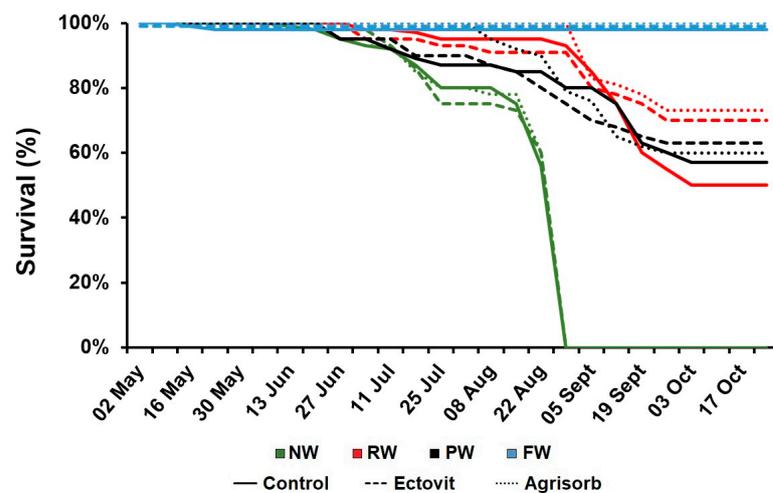
Prior to analysis, the normal distribution of the data was tested with the Shapiro–Wilk test. Percentage data (survival, EMF colonization) were log<sub>10</sub> transformed prior to analysis to correct non-normal distribution of the data. The data were analyzed using two-way analysis of variance (ANOVA) (with watering regime, additive, and their interaction as fixed effects and block as the random effect) followed by Tukey’s HSD test ( $p \leq 0.05$ ) to determine the significance of differences among treatments. The experimental unit was a replication (block) of the watering regime × additive combination (20 potted seedlings).

Statistical analysis was performed using the PC SAS statistical package (SAS Institute Inc., Cary, NC, USA).

### 3. Results

#### 3.1. Survival

Seedling mortality was low almost until the middle of July (two and a half months from the beginning of the experiment), regardless of treatments (Figure 2). From that time on, a higher mortality rate was observed in NW seedlings over a few days, and after dying had been discontinued for approximately two to three weeks, all seedlings quickly died until the end of August (after 17 weeks of desiccation). Almost all FW seedlings survived the entire growing season. The mortality of PW seedlings was moderate and balanced, regardless of additives, throughout the whole experimental period. Survival under RW was high (over 90%) until the end of August, after which approximately 20% of additive-treated and 35% of untreated seedlings died during September (Figure 2). At the end of the experiment, the watering regime had a significant effect on seedling survival (Table 1). The survival rates of both PW and RW seedlings (60 and 64%, respectively) were significantly lower and higher than those of FW and NW seedlings, respectively. The additives had no significant effect on survival, but a significant interaction between watering and additive was found (Table 1). The survival rates of Ectovit- and Agrisorb-treated seedlings under RW were 70% and 73%, respectively. These rates were significantly higher (approximately by 20%) compared to untreated seedlings, while differences within the other watering treatments were insignificant (Figure 2).



**Figure 2.** Time course of survival of 1 + 1 potted European beech untreated seedlings (control) and seedlings treated at the time of transplanting 1 + 0 bareroot seedlings to pots with ectomycorrhizal inoculum Ectovit and hydrogel Agrisorb, and exposed during second growing season to different watering regimes (NW—no watering, RW—reduced watering, PW—periodic watering, FW—full watering).

#### 3.2. Growth

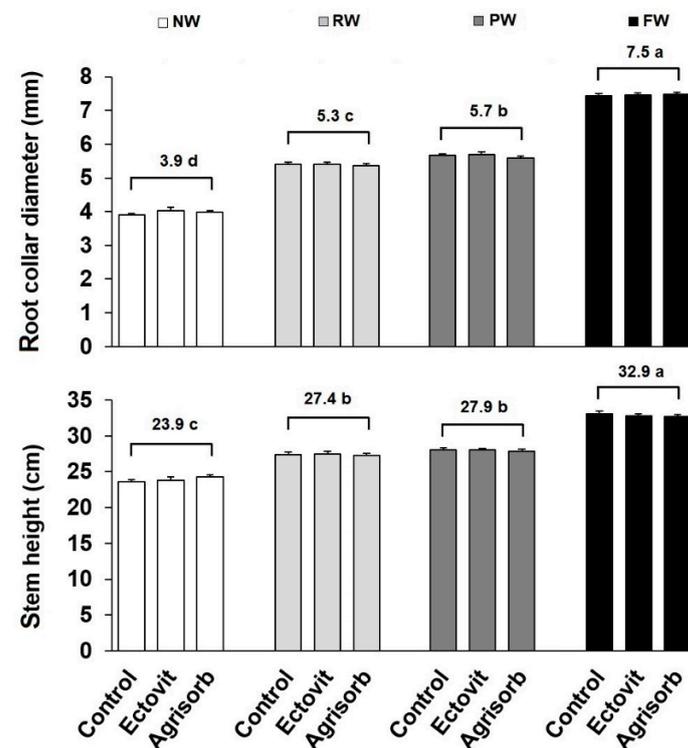
An analysis of variance showed a significant effect of watering on all growth parameters of seedlings (Table 1). The mean values of the growth parameters (except RDW/SDW) of FW seedlings were significantly higher, and those of NW seedlings were significantly lower than those under RW and PW treatments (Figures 3 and 4). The growth parameters of PW and RW seedlings reached similar values, except for the significantly larger root collar diameter of PW compared to RW seedlings. FW seedlings had significantly higher RDW/SDW than PW and NW seedlings; the ratio under RW was significantly higher than under NW but was not significantly different from FW and PW treatments. There was no revealed significant effect of the additives on any growth variable (Table 1), but slightly higher root and shoot dry mass and lower RDW/SDW values were detected in all watering

regimes with Ectovit- and/or Agrisorb-treated seedlings compared to control seedlings, except RDW/SDW under FW (Figure 4).

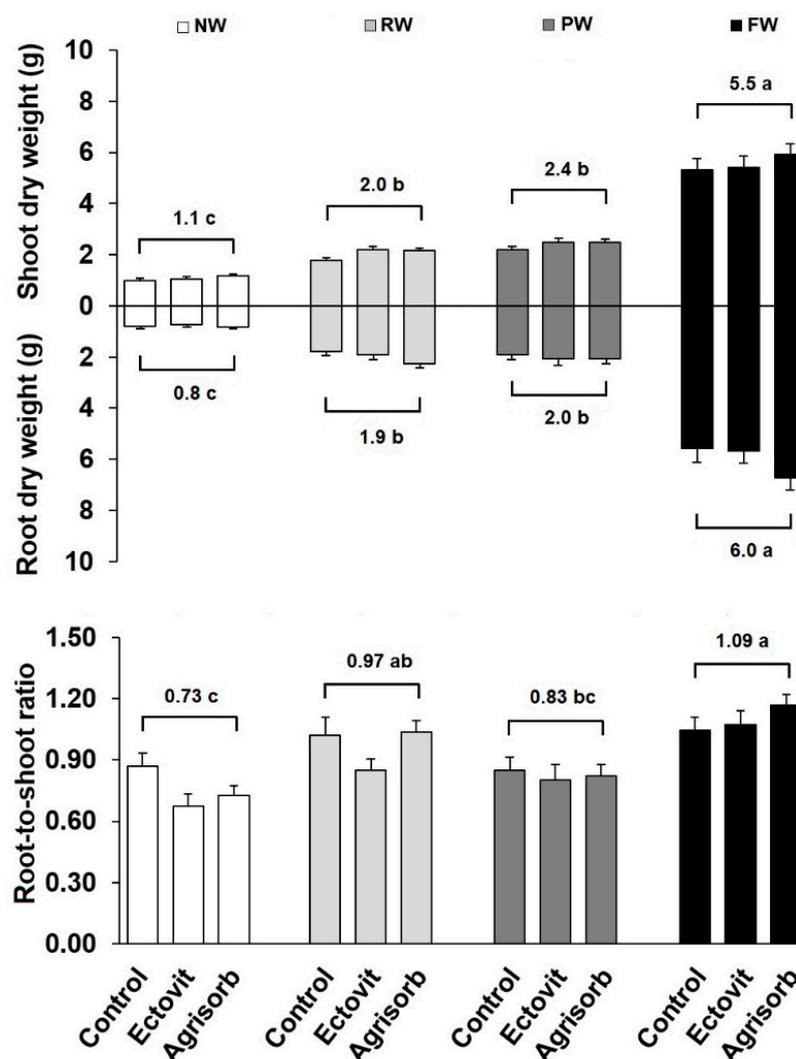
**Table 1.** Analysis of variance (F and *p*-values) of effects of watering regime and additive (Ectovit, Agrisorb) on survival, growth, short roots (frequency and ectomycorrhizal fungi colonization), and chlorophyll *a* fluorescence parameters of 1 + 1 potted European beech seedlings.

Variable	Watering		Additive		Watering × Additive	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
Survival	311.4	0.001	6.4	0.056	4.1	0.017
Root collar diameter	2276.1	0.001	1.2	0.368	0.4	0.853
Stem height	207.4	0.001	0.1	0.999	0.5	0.776
Shoot dry weight	164.3	0.001	0.9	0.455	0.2	0.973
Root dry weight	656.1	0.001	3.4	0.135	0.7	0.634
Root-to-shoot ratio	14.5	0.004	1.2	0.386	1.1	0.428
Short root frequency	12.9	0.005	0.1	0.861	0.1	0.991
EMF colonization	516.0	0.001	0.3	0.789	0.2	0.971
F0	2.8	0.128	0.7	0.561	0.5	0.799
Fm	14.8	0.003	5.3	0.075	0.7	0.636
Fv	17.9	0.002	5.9	0.063	0.6	0.748
Fv/Fm	14.5	0.004	3.3	0.143	0.4	0.882

Degrees of freedom (dfs): watering 3, additive 2, block 2, watering × additive 6, watering × block 6 (error for watering), additive × block 4 (error for additive), watering × additive × block 12 (error for watering × additive), model 35; dfs for growth and short roots parameters: residual 266, total 301; dfs for chlorophyll *a* fluorescence parameters: residual 647, total 682; dfs for survival: watering 3, additive 2, watering × additive 6, Model 11, residual 24 (error for watering and additive), total 35. Residual and total dfs for the growth and chlorophyll *a* fluorescence parameters do not match with the number of samples intended for assessment and stated in the Section 2 due to fewer seedlings surviving in the water-deficit regimes.



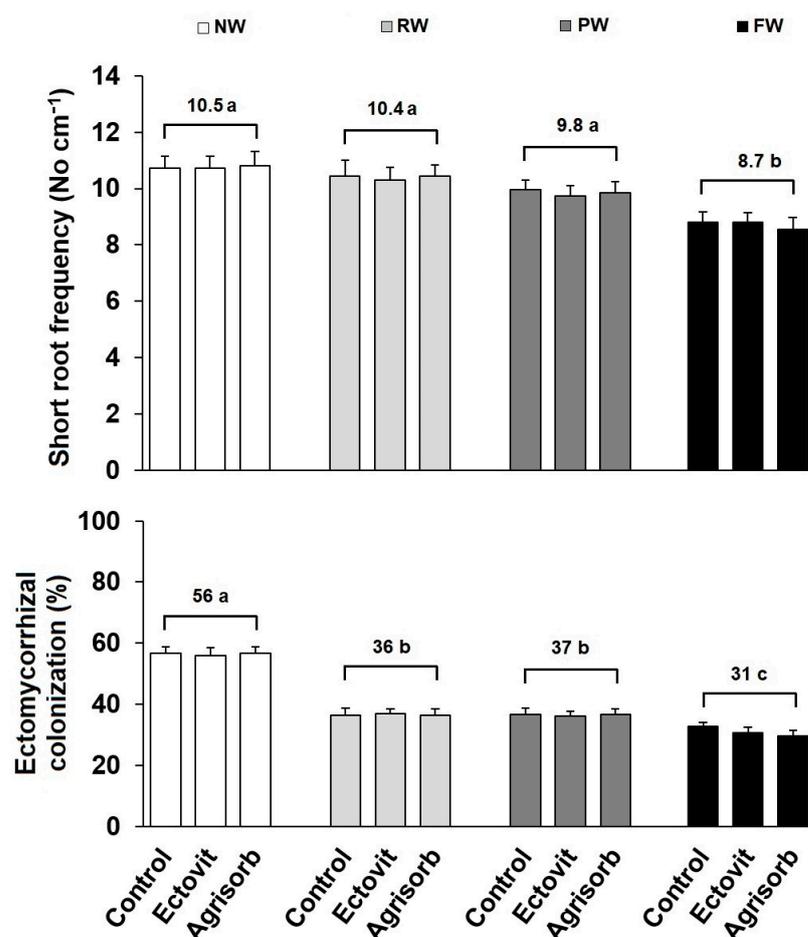
**Figure 3.** Root collar diameter and stem height of 1 + 1 potted European beech untreated seedlings (control) and seedlings treated at the time of transplanting 1 + 0 bareroot seedlings to pots with ectomycorrhizal inoculum Ectovit and hydrogel Agrisorb, and exposed during second growing season to different watering regimes (NW—no watering, RW—reduced watering, PW—periodic watering, FW—full watering). Means of watering regimes followed by different letters are significantly different ( $p < 0.05$ ). Differences among means of additive treatment within each watering regime are insignificant ( $p < 0.05$ ). Error bars indicate standard errors of the means.



**Figure 4.** Shoot and root dry weight and root-to-shoot ratio of 1 + 1 potted European beech untreated seedlings (control) and seedlings treated at the time of transplanting 1 + 0 bare-root seedlings to pots with ectomycorrhizal inoculum Ectovit and hydrogel Agrisorb, and exposed during second growing season to different watering regimes (NW—no watering, RW—reduced watering, PW—periodic watering, FW—full watering). Means of watering regimes followed by different letters are significantly different ( $p < 0.05$ ). Differences among means of additive treatment within each watering regime are insignificant ( $p < 0.05$ ). Error bars indicate standard errors of the means.

### 3.3. Short Roots and Ectomycorrhizas

Significant differences were found among the mean values of short root frequency and ECM colonization affected by watering regime, but not with respect to additives (Table 1). FW seedlings had significantly lower short root frequencies than those in the other watering treatments. The highest EMF colonization of short roots was in NW seedlings (56%), which was significantly higher than in RW, PW (36%–37% both), and FW seedlings (31%) (Figure 5). Five EMF morphotypes were distinguished and described regardless of treatments on the basis of macromorphological features of ectomycorrhizas (Table 2). All five morphotypes were found in all treatments. Any treatment-specific morphotype was neither formed by the EMF applied in the inoculum nor found in the other treatments. No short-root colonization by EMF contained in Ectovit was detected using molecular methods. DNA analysis revealed the presence of indigenous EMF *Hebeloma sacchariolens* Quél. and *Hymenogaster vulgaris* Tul. and C. Tul.



**Figure 5.** Short root frequency and ectomycorrhizal colonization of 1 + 1 potted European beech untreated seedlings (control) and seedlings treated at the time of transplanting 1 + 0 bareroot seedlings to pots with ectomycorrhizal inoculum Ectovit and hydrogel Agrisorb, and exposed during second growing season to different watering regimes (NW—no watering, RW—reduced watering, PW—periodic watering, FW—full watering). Means of watering regimes followed by different letters are significantly different ( $p < 0.05$ ). Differences among means of additive treatment within each watering regime are insignificant ( $p < 0.05$ ). Error bars indicate standard errors of the means.

**Table 2.** Macromorphological description of ectomycorrhizal morphological types (EMF morphotypes) and identification of ectomycorrhizal fungi using molecular methods on roots of 1 + 1 potted European beech seedlings treated with ectomycorrhizal and hydrogel additives and exposed to different watering regimes.

EMF Morphotypes (25× Magnification)	Description of EMF Morphotypes and Identification of Ectomycorrhizal Fungi via DNA Sequencing
	White color, ramification absent, short and straight shape with inflated distal end, shiny mantle, white tips, and whitish hyphae. Ectomycorrhizal fungus identified: <i>Hebeloma sacchariolenis</i> Quél. NCBI database record, Accession No: MT929352, similarity 99%.
	Ochre to yellowish-brown color, monopodial pinnate ramification, straight or bent shape, loosely cottony mantle covered with soil particles, brown tips, yellowish hyphae. Ectomycorrhizal fungus identified: <i>Hymenogaster vulgaris</i> Tul. and C. Tul. NCBI database record, Accession No: MT929774, similarity 99%.

Table 2. Cont.

EMF Morphotypes (25× Magnification)	Description of EMF Morphotypes and Identification of Ectomycorrhizal Fungi via DNA Sequencing
	Yellowish-brown color, ramification absent, sinuous shape, smooth densely woolly mantle, yellowish-brown tips, and whitish hyphae. Unsuccessful DNA sequencing.
	Yellowish-brown color, monopodial-pinnate ramification, straight or bent shape with inflated distal end, shiny loosely cottony mantle, ochre tips, no emanating hyphae. Unsuccessful DNA sequencing.
	Brown color, ramification absent, straight or bent shape, smooth, loosely cottony mantle, brownish tips, no emanating hyphae. Unsuccessful DNA sequencing.

### 3.4. Foliar Nutrient Concentration

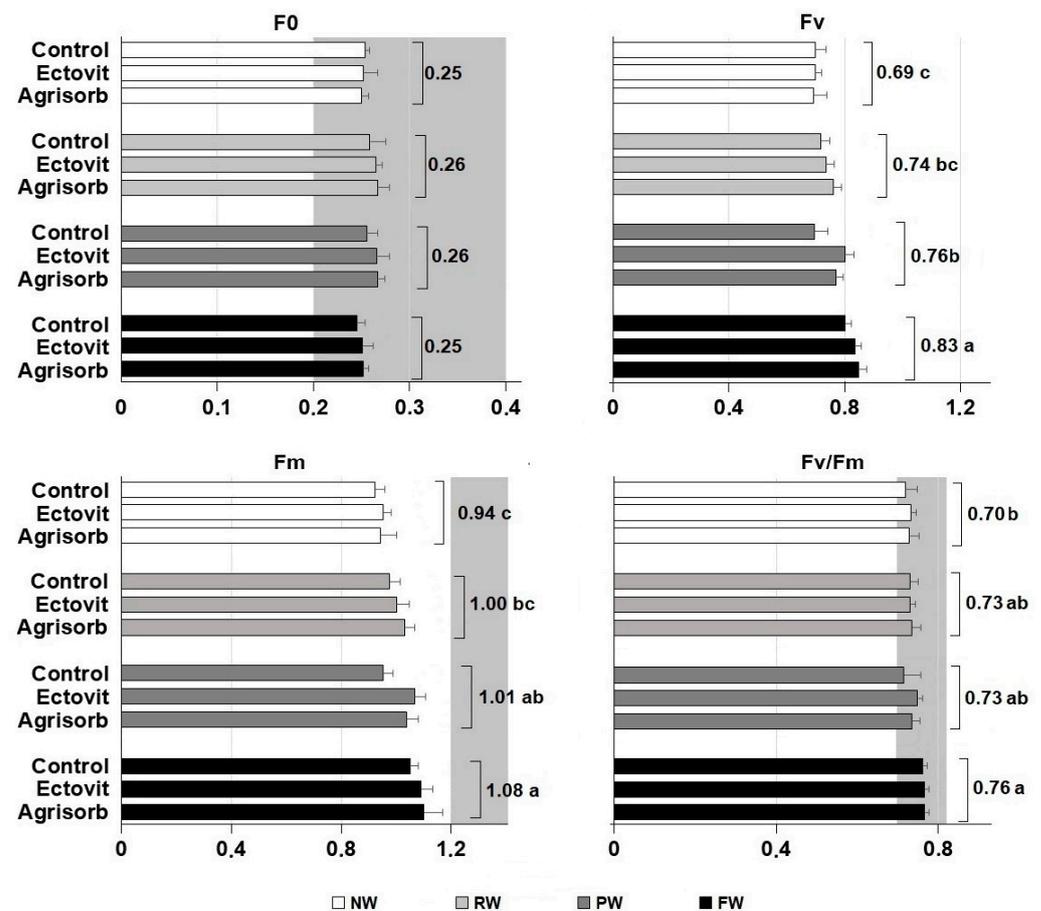
Differences in foliar nutrient concentrations among both watering and additive treatments were mostly indistinctive (Table 3). However, FW seedlings exhibited lower Ca and Mg concentrations and those treated with Ectovit also had lower P concentrations than those in the other watering treatments. Similar low P concentrations were found in NW control seedlings. Ectovit and Agrisorb increased the concentrations of all macronutrients under PW and the P concentration under NW; the hydrogel also increased the Ca concentration in RW seedlings (Table 3).

**Table 3.** Nutrient concentrations in the leaves of 1 + 1 potted European beech untreated seedlings (control) and seedlings treated at the time of transplanting 1 + 0 bareroot seedlings to pots with ectomycorrhizal inoculum Ectovit and hydrogel Agrisorb, and exposed during the second growing season to different watering regimes.

Treatment	N (%)	P (mg kg <sup>-1</sup> )	K (mg kg <sup>-1</sup> )	Ca (mg kg <sup>-1</sup> )	Mg (mg kg <sup>-1</sup> )
No watering					
Control	2.9	4650	10,200	11,500	2640
Ectovit	2.9	5630	10,100	12,100	2420
Agrisorb	3.3	5820	10,500	11,700	2820
Reduced watering					
Control	3.2	6730	9050	12,300	2120
Ectovit	3.2	6540	8900	13,300	2170
Agrisorb	3.2	7180	8910	14,800	2560
Periodic watering					
Control	2.9	5910	8380	9160	1990
Ectovit	3.2	7060	9510	12,200	2590
Agrisorb	3.2	7080	9460	10,700	2630
Full watering					
Control	2.9	6010	9990	8830	1910
Ectovit	2.9	4500	8380	8170	1640
Agrisorb	2.8	5970	9070	8740	1800

### 3.5. Chlorophyll *a* Fluorescence

Analysis of variance showed a significant effect of the watering regime on Fv, Fm, and Fv/Fm parameters (Table 1). Fv and Fm values of FW seedlings were significantly higher than those of the other watering treatments (the lowest values under NW), except for the difference between FW and PW for the Fm parameter. The Fv/Fm value for FW was significantly higher than that of the NW treatment only (Figure 6). The mean values of F0 ranged in very close intervals, regardless of the watering regime and additive applied. The additives had no significant effect on chlorophyll *a* fluorescence parameters (Table 1); however, slightly higher values for Ectovit- and Agrisorb-treated seedlings than those of control were recorded for the PW regime.



**Figure 6.** Chlorophyll *a* fluorescence of 1 + 1 potted European beech untreated seedlings (control) and seedlings treated at the time of transplanting 1 + 0 bareroot seedlings to pots with ectomycorrhizal inoculum Ectovit and hydrogel Agrisorb, and exposed during second growing season to different watering regimes (NW—no watering, RW—reduced watering, PW—periodic watering, FW—full watering). Means of watering regimes followed by different letters are significantly different ( $p < 0.05$ ). Differences among means of additive treatment within each watering regime are insignificant ( $p < 0.05$ ). Error bars indicate standard errors of the means. The ranges of values of F0, Fm, and Fv/Fm expressed by gray rectangular areas show normal values indicating optimal state of chlorophyll structures (Ritchie [41]). Range of appropriate Fv values is not available.

## 4. Discussion

### 4.1. Effect of Water Availability on Seedling Survival and Growth

For plant survival under extreme drought, the ability to prolong the desiccation process and keep hydraulic integrity as long as possible seems to be a key adaptive issue [42]. All potted beech seedlings cultivated in different types of soil died 30 days after the termination of watering [30]. Reduction of the conventional irrigation dose by 75% has decreased the

total dry mass of beech seedlings by five times, while lower biomass reductions of sessile oak (*Quercus petraea* (Matt.) Liebl.) and Scots pine (*Pinus sylvestris* L.), and even no reduction in silver fir (*Abies alba* Mill.), have been detected in [14]. These results suggest the high sensitivity of beech seedlings to water deficits. However, the survival of 1-year-old beech in a sand-peat potting mixture ranged 29%–67%, depending on seedling provenance, 8 weeks after watering was stopped [12]. Moreover, contradicting the general belief that beech is particularly sensitive to environmental stressors, Pflug et al. [43] suggest a highly resilient physiological response of beech to drought. According to [44], beech is less drought-sensitive than *Acer pseudoplatanus* L. and *Picea abies*, but more susceptible than most other native broadleaf trees of Central Europe. Although a significant adverse effect of water deficiency on seedling survival and growth was found in our study, the total mortality of seedlings after as much as 17 weeks of watering exclusion supports the suggestion in [43] and confirms the various responses of beech to drought stress depend on seedling traits and experimental conditions [13,14,38].

Beech seedlings [45], as well as seedlings of other tree species [46,47], react to reduced water availability by intensifying root growth to colonize soil layers with a more balanced water supply. In a study by Robakowski et al. [14], biomass allocation in response to reduced irrigation was species-specific, whereby the root dry mass and RDW/SDW of beech were not affected. No effect of mild drought stress on the RDW/SDW of beech seedlings was also reported in [31]. In our experiment, in proportion to increasing the substrate water deficit, the root biomass and RDW/SDW significantly decreased; the lowest values were recorded under NW. In addition, the severe water deficit increasing the physical resistance of the dry substrate against penetration by roots could limit root growth [48]. Poorter et al. [46] explained inconsistent seedling biomass allocation by the intrinsic pattern of young plants tending to have a low fraction of shoots and a large fraction of leaves; this effect possibly confounds the impact of experimental treatments or environmental factors.

#### 4.2. Effect of Hydrogel on Seedling Water Supply

The ameliorative effect of hydrogel application on the photosynthetic performance of beech seedlings during 50 days of dehydration has previously been reported [18]. Orikiriza et al. [30] observed that under simulated watering exclusion, soil amendment with hydrogel prolonged the survival time and increased the root and shoot biomass of beech seedlings compared to the control. Whereas neither Ectovit nor Agrisorb affected seedling growth regardless of the watering regime, a significant effect of the additives on survival was recorded under RW. An insignificant effect of the additives on survival under the most severe regime (NW), along with a promoting effect in a more favorable RW treatment, was not expected. However, the inefficiency of the hydrogel under NW corresponds with [49], suggesting that the amount of water held by hydrophilic amendments is sufficient to keep the seedlings alive for a short time, but it is not great enough to increase survival or growth under long-term, very dry conditions. Similarly, in [50], dipping the roots of bareroot red oak (*Quercus rubra* L.) seedlings into hydrogel provided protection to aid survival under short-term desiccation, but did not improve seedling performance following a 45-day drought stress exposure. Conversely, in [17], it was shown that mixing hydrogel with a peat-based growth medium increased the water status and survival of bareroot cork oak (*Quercus suber* L.) seedlings during a severe drought period. Dipping root plugs of container-grown Norway spruce and eucalypt (*Eucalyptus* sp.) seedlings in hydrogel slurry prior to planting also enhanced seedling survival, but dipping roots of bareroot spruce seedlings was not effective [4,16]. The abovementioned observations and our results indicate that mixing hydrogel with growth substrate and dipping container root plugs into hydrogel should be a more efficient application technique than dipping the roots of bareroot seedlings.

More efficient absorption and release of water by the regular, compared to periodically, hydrated polymer network structure of the hydrogel [51,52] could at least partly explain the difference between the positive and insignificant hydrogel effects on beech

survival under RW and PW, respectively. This implication is supported by [19,20,49], who reported the substantial release of water from the structure of hydrogel during a longer period of drought, which may result in contraction and shrinkage of the applied hydrogel slurry with possible negative effects on the vitality of fine roots. Moreover, especially in porous substrates (e.g., peat), constituted discontinuities and air pockets around the shrunk slurry can decrease the effectiveness of water absorption by the hydrogel during rewetting events [34].

#### 4.3. Effect of EMF Inoculation on Seedling Development

Positive effects of EMF inoculation on the formation of ectomycorrhizas, seedling survival, and/or growth have been observed in some nursery and field studies [28,53–55]. However, the effectiveness of introduced EMF on seedling development is not warranted [4,24,25,56]. The absence of inoculation treatment-specific EMF morphotypes, the lack of short root colonization by EMF applied in Ectovit traced via molecular methods, and no difference in EMF colonization between Ectovit-treated and control seedlings in our experiment suggest that the applied EMF did not successfully compete with native EMF that formed the same EMF morphotypes in all treatments. Beniwal et al. [5] reported a stimulative effect of soil amendment with a mixture of hydrogel and mycelial inoculum of *Paxillus involutus* on the EMF colonization of beech seedlings exposed to air before planting; however, the observation of EMF morphotypes on roots indicated that the effect did not need to be specific to the *P. involutus* applied. Inoculum and inoculation patterns, inter- and intraspecific tree-fungus variation and consequential (in)compatibility, environmental conditions (growth medium, microclimate, microorganisms, etc.), seedling production practices, and other circumstances affect seedling responses to inoculation [21,53,56,57].

The rating of EMF colonization of seedlings after transplanting and one year of growth in pots was considerably reduced (from 80% for 1 + 0 to 31%–57% for 1 + 1 depending on watering treatment). As well as ineffective inoculation, the most likely reasons are differences in fertility and composition (physical, biological, and chemical properties) between the growth media of 1- and 2-year-old seedlings. The rapid utilization of photosynthate for seedling growth in highly fertile substrates may reduce the root concentration of carbohydrates and consequently decrease the susceptibility of short roots to infection by EMF [58]. The decline of the transformation of short roots to ectomycorrhizas due to a high level of nutrients or other unfavorable circumstances (e.g., unavailability of EMF propagules, low peat aeration/high water retention, fast root growth in containers) was demonstrated, e.g., in [21,59–61]. The growing conditions of 1 + 1 seedlings in this study were advantageous to accelerate seedling growth (approximately a 6-fold increase in biomass of 1 + 1 against 1 + 0 seedlings) and decreased the possibility and need for ectomycorrhizal development.

In contrast to the better growth of 1 + 1 seedlings under FW compared to water-deficit regimes, an opposite effect of watering on short root frequency and EMF colonization rate was detected; non-watered seedlings exhibited the highest EMF colonization. The lower EMF colonization recorded on watered seedlings was probably caused by intensive root growth due to the high water supply and by circumstances retarding ectomycorrhiza formation as discussed above. By contrast, slower root growth and the ability of obligatory ectomycorrhizal plants to release root exudates, allowing fungi to colonize roots in harsh environments [22,62,63] likely enabled increased ectomycorrhizal development under NW. In addition, Manzanedo et al. [13] found increased EMF colonization of the roots of beech seedlings with increasing drought, and Ortega et al. [27] detected a low degree of EMF colonization of *Pinus radiata* D. Don. Seedlings, presumably because of the high water content of the substrate and competition by native nursery EMF. Conversely, decreased soil water availability had negative [32] and insignificant effects [63] on the EMF colonization of beech seedlings. As EMF symbionts can in certain circumstances retard seedling growth due to increased carbohydrate demands [22,64], the withdrawing of seedling carbohydrates

by EMF might, along with the negative influence of the increasing water deficit, has contributed to the decreasing seedling biomass production also in our experiment.

The structure and functional diversity of EMF assemblages are important circumstances influencing plant responses to drought stress [27,65,66]. Shi et al. [63] reported that drought did not change the number of EMF types, but different EMF types responded to drought differently in terms of their abundance. As the inoculation resulted in survival-promoting effects explicitly under RW and did not improve survival in the other watering regimes, the EMF-forming ectomycorrhizas in our study could likewise respond differently to the different levels of substrate water availability. However, as quantification of the abundance of EMF morphotypes was not performed, the EMF community structure depending on treatments is unfortunately unknown. Moreover, considering the relatively low amount of samples of ectomycorrhizas analyzed and the unsuccessful identification of EMF via molecular methods from some visually distinct EMF morphotypes, participation of the introduced fungi in ectomycorrhiza formation and their nonspecific effects [67–69] are not entirely impossible. Thus, we have assumed that the higher survival of inoculated compared to uninoculated seedlings under RW (both exhibiting an equal EMF colonization rate) was affected by the components contained in the inoculum Ectovit and/or different EMF community structure (proportion of morphotypes) and functionality. A better assessment of the fungal community composition will be necessary in the future to clarify its role in seedling drought response.

#### *4.4. Effect of Water Availability and Additives on Foliar Nutrient Concentration*

Although low soil water availability may decrease the nutrient mobility in a soil solution and consequently decrease nutrient uptake by plants [70], the foliar nutrient concentration of seedlings in the water-deficit treatments in our study was slightly higher than that of those under FW treatment. FW probably accelerated the leaching of the nutrients from the growth substrate [71], consequently affecting the decline in the nutrient content in the leaves. However, regardless of treatments, none of the nutrient concentrations detected in the leaves declined below the recommended range [72]. Nutrient analyses carried out in our experiment and in [45] showed Ca and Mg foliar macroelements as being mostly affected by substrate water availability. Concentrations of N and K were, regardless of treatments, balanced in this study, contrary to foliar beech N content being distinctly affected by drought as reported in [45]. The increased inorganic N uptake of drought-stressed beech seedlings accomplished via stress-induced activation of beneficial EMF taxa was also detected in [68], suggesting functional diversity within EMF assemblages.

Furthermore, Peuke and Rennenberg [73] found P to be the element markedly responding to drought due to reduced mobility. This finding is in line with the low P concentration in additive-untreated NW seedlings in this experiment, which was increased by both Agrisorb and Ectovit. Both additives were efficient, particularly in the PW treatment, increasing the foliar concentration of all analyzed macroelements. The nutrient-promoting effect of Ectovit could act through the nutrient-containing compounds present in the inoculum and/or the impact of the EMF community structure discussed above in relation to the survival of the inoculated/uninoculated seedlings. The low P concentration in Ectovit-treated FW seedlings indicates at least some effect of this inoculum on P, depending on the watering regime. In hydrogel-treated seedlings, the increased concentration of nutrients may be provided by the accumulation of salts dissolved in absorbed water that are gradually embedded in the hydrogel slurry encasing seedling roots [74,75]. However, [74,75] also reported that the mechanism of the continuous accumulation of cations in salts (especially  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) increases the amount of available nutrients and associates with the formation of ionic bridges between carboxyl groups, resulting in the gradual collapse of the hydrogel structure.

#### 4.5. Effect of Water Availability and Additives on Chlorophyll *a* Fluorescence

Although significant differences among watering treatments in chlorophyll *a* fluorescence (chl fluorescence) parameters (except F0) were recorded and Fm values were lower in this experiment than those recommended by Ritchie [41], values of F0 and Fv/Fm fell within the recommended range [41] regardless of treatments (Figure 6). The reduction in soil water availability did not substantially affect parameters F0 and Fv/Fm in [38,43]. These results illustrate the resilience of the photochemical apparatus in beech with decreasing soil water availability. Decreasing water availability may favor ecophysiological traits that optimize carbon assimilation to counterbalance the reduced growing period [38]. However, these findings may also indicate that the setup water deficit levels were not severe enough to induce under-limited values of the fast kinetics of chl fluorescence. Nevertheless, chl fluorescence was measured early in August in our study, more than 90 days after the beginning of seedling drying under NW and just before the wilting point was reached under PW, i.e., in the time that very likely sufficiently warranted a severe drought. However, the wilting point was derived for agricultural plants, and young trees, such as European beech, may deplete water resources near the fine roots at lower soil water potentials than the wilting point [38]. In contrast to our results, Fv/Fm was clearly reduced below the bottom bound of the recommended range [41] on day 50 after termination of watering at a soil water potential of  $-900$  kPa [18], equivalent to a higher water content than under NW and PW at a chl fluorescence measurement in this experiment.

Chl fluorescence parameters indicated chronic photoinhibition in beech seedlings under drought treatment, while no significant changes in Fv/Fm were recorded under drought treatment with hydrogel Stockosorb applied compared to watering treatment in [18]. By contrast, although the additives slightly enhanced chl fluorescence values under PW, no significant effect of either the hydrogel or EMF inoculum on photosynthetic processes was detected in our study. Similarly, in [50], hydrogel-treated red oak seedlings did not show greater differences in net photosynthesis compared with the control following a 45-day drought stress exposure.

## 5. Conclusions

This study can be considered a contribution to the findings on the effects of growth medium water deficit and rhizosphere amendment with water-holding and EMF additives, potentially mitigating impact of drought stress on the development of European beech seedlings. The results of this study confirm the importance of water supply for seedling development. The EMF inoculum was not effective in treatment-specific ectomycorrhiza formation or enhancement of the EMF colonization level, and as well as the hydrogel, it did not substantially support the development of seedlings. However, both additives significantly increased seedling survival at continuous reduced irrigation and slightly increased the values of some parameters of chl fluorescence and foliar nutrient concentrations during drought periods. These inconsistent effects of the additives resemble the results of previous studies and suggest uncertainty in relation to the additives' positive effects. Despite this, we have assumed that the application of such amendments should be a prospective tool by which to alleviate the adverse effects of drought and facilitate the adaptation process and survival of out-planted seedlings. Consequently, further research focusing on the influence of soil properties and emphasizing soil moisture, and in respect of the additives' application time, techniques and doses on seedling performance would be useful to achieve findings for the use of additives in reforestation programs considering planting beech seedlings on water-deficient planting sites.

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