Effect of Quercetin on Mycorrhizal Synthesis between *Tuber borchii* and *Arbutus unedo* L. In Vitro Plants

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Abstract: *Arbutus unedo* L. is a Mediterranean species used for fruit production; it is tolerant to drought and shows regeneration ability following forest fires. Mycorrhizal plants with *Tuber borchii* add resilience and value. This study aims to test the effect of quercetin on mycorrhizal synthesis between *T. borchii* and *A. unedo*. Two genotypes selected for fruit production and hydric stress tolerance, were micropropagated for mycorrhizal synthesis, accomplished during ex vitro rooting in perlite, using lyophilized spores of *T. borchii* suspended in culture media with different quercetin levels (0–10 μM). Six months after inoculation, plants were transferred to pots and maintained in nursery. Ten and 12 months after inoculation, roots were morphological examined and molecularly characterized using ITS1-5.8SITS2 rDNA region and specific primers. Results showed that mycorrhizae establishment was dependent on studied factors (genotype, quercetin level, and culture medium) and their interaction (genotype X culture medium). Quercetin levels up to 2.0 μM favored mycorrhizae establishment and plant growth, although levels superior to 4 μM showed a toxic effect. Quercetin showed to be an efficient factor on inducing mycorrhiza thriving independent of the genotype. Morphological observations and molecular analysis confirmed the permanence of the fungus association 10 and 12 months after inoculation.

Keywords: arbutoid mycorrhizae; ex vitro rooting; micropropagation; spores' inoculation; strawberry tree

1. Introduction

Arbutus unedo L. (known as Strawberry tree) is a Mediterranean species that can be found across Iberian Peninsula, southern Europe (Mediterranean basin countries), northern Africa, and Ireland [1–3]. It is widely spread across Portugal and can grow from 20 to 1000 m in altitude, withstanding drought and degraded soils [4]. It is commonly found on the understory of oaks, particularly *Quercus suber* on the south and *Quercus robur* on the north, as well as on pine stands. It is typically a shrub type plant of 1 to 3 m height, although in optimal conditions of water and soil, it can grow up to 15 m [4].

Recently, *A. unedo* has been recognized as an interesting species for economic and ecological purposes, such as its great ability to survive wildfires, due to sprouting, even if let without a full crown. The lignotubers linked to the stem are a source of new meristems,

allowing its resprouting, which is an important survival strategy [5,6]. *A. unedo* is also considered a valuable species in regards to beekeeping and high-quality honey production [7]. Flowering occurs simultaneously with the ripening and falling of the fruits of the previous year due to its long phenological cycle (1 year). During the fall period, flowers and fruits are simultaneously in the same plant, a trait that is linked to its use for gardening and landscape purposes. The species hermaphrodite blossoms are pollinated by insects, particularly bees, due to its melliferous traits, once it is their main source of food during the winter months [3,7].

For all of these reasons, the *A. unedo* species has been submitted to a breeding program in order to make pure crops available, aiming for fruit production for fresh fruit consumption, of jams and honey. There is also the traditional income associated with the production of alcoholic beverages [8]. More recently, our group established a protocol for mycorrhizal synthesis between *Tuber borchii* and *Arbutus unedo* L. micropropagated plants [9].

The use of artificially inoculated plants in forestry can improve plant potential to withstand environmental pressure, as well as stimulate plant growth, leading to improved crop productivity [10,11]. *A. unedo* forms mycorrhizae with a wide-ranging group of fungi; ca. 44 *taxa* were identified in plants growing in an adult Mediterranean forest [12]. The type of mycorrhizae that occurs naturally in species from genus, such as *Arbutus* and *Arctostaphylos*, is classified as arbutoid mycorrhizae [13]. They are characterized by the mantle, the well-developed Hartig network, and the development of hyphae at the intercellular level—but only in the first layer of the root cells [11,13–17].

The majority of fungi that form arbutoid mycorrhizae are basidiomycetes; however, some ascomycete fungi (e.g., *Tuber* sp., which include important edible mushrooms) have been shown to be able to form arbutoid mycorrhiza with *A. unedo* plants, as first identified by Lancellotti, et al. [18].

Tuber borchii Vittad, commonly called whitish truffle, is a mycorrhizae fungus from the order *Pezizales,* and produces edible truffles that represent an interesting gastronomic product with high value. Although its economic value is not as great as other truffles, such as *Tuber melanosporum* [18], *T. borchii* has a wide distribution range in Europe, from north to south of the continent—from Finland (as one of the coldest region) to Sicily (as one of the southeast regions of the Mediterranean), and from Ireland (in the western edge) to Hungary and Poland (on the eastern side of Europe) [18]. Some considered it as the *Tuber* species with the broadest ecological extent, allowing to establish truffle production in environments unfit for other truffles, greatly expanding cultivated areas to very varied environmental conditions [17].

It is widely known that mycorrhizal fungi are considered very important symbionts to the majority of plants, whose existence is stimulated under phosphorus deficiency in the soil [19]. The fungi grow from spore germination or hyphae extension (response to root chemical signals). Hyphae then penetrate their host root tissue to form different structures according to the specific type of mycorrhiza (ecto/endomycorrhiza) [19–23]. When this occurs, some of the host plants involved can exudate a substance that stimulates spore germination, as well as hyphae branch growth, leading to root colonization. The exudates have been identified as flavonoids [19–24]. Flavonoids are members of one of the most diverse phenolic groups, and their occurrence is widely distributed throughout the *Plantae* kingdom. These compounds participate in a vast collection of biochemical reactions, as quenchers of free radicals, (reactive oxygen species) and as binders to metallic ions, enhancing their bioavailability [19]. The concentrations of flavonoids as daidzein, coumestrol, and quercetin found in the root were greater than in the seed and showed to be related with the number of entry points and facilitating root colonization in a variety of species, such as soybean, clover, and tomato [20,23].

The aim of this study was to test the effect of different concentrations of the flavonoid quercetin on the mycorrhiza synthesis between in vitro plants of *A. unedo* and lyophilized spores of *T. borchii*. For this purpose, micropropagated clones, previously selected from adult plants, according to fruit production and stress hydric tolerance, were used.

2. Materials and Methods

2.1. Fungal and Inoculum Characterization

Lyophilized spores of *Tuber borchii* were purchased from Micologia Florestal and Aplicada (Barcelona, Spain). Two suspended solutions of lyophilized spores of *T. borchii* (3 g L^{-1}, each) were prepared using either Knop medium without P$_2$O$_5$ [25] or tap water. Both suspensions were kept in agitation overnight at room temperature to complete hydration. The number of spores was evaluated by using the Neubauer-ruled bright line-counting chamber, which showed an average of 3200 spores per ml of the inoculum suspension. In both inoculum suspension media, Knop vs. water, different concentrations of quercetin were added: 0.5 μM, 2.0 μM, 4.0 μM, 7.0 μM, and 10.0 μM, to be compared to controls with no quercetin.

2.2. Plant Material

Adult plants of *A. unedo* from different provenances have been selected, according to fruit production and quality (°Brix, total acidity, reducing sugar, and pH). The selected plants were established in vitro and micropropagated [26]. According to the multiplication and survival rate after subcultures with higher concentrations of sucrose [27], several clones were tested in field to confirm their tolerance to hydric stress and identify their interaction between genotype and environment [28]. From this survey, clone AL1 (101) was chosen together with clone PF1 (401), from the southernmost region of Portugal, as flagged in Figure 1. These clones were from soils of sedimentary rocks of schist and limestone, respectively.
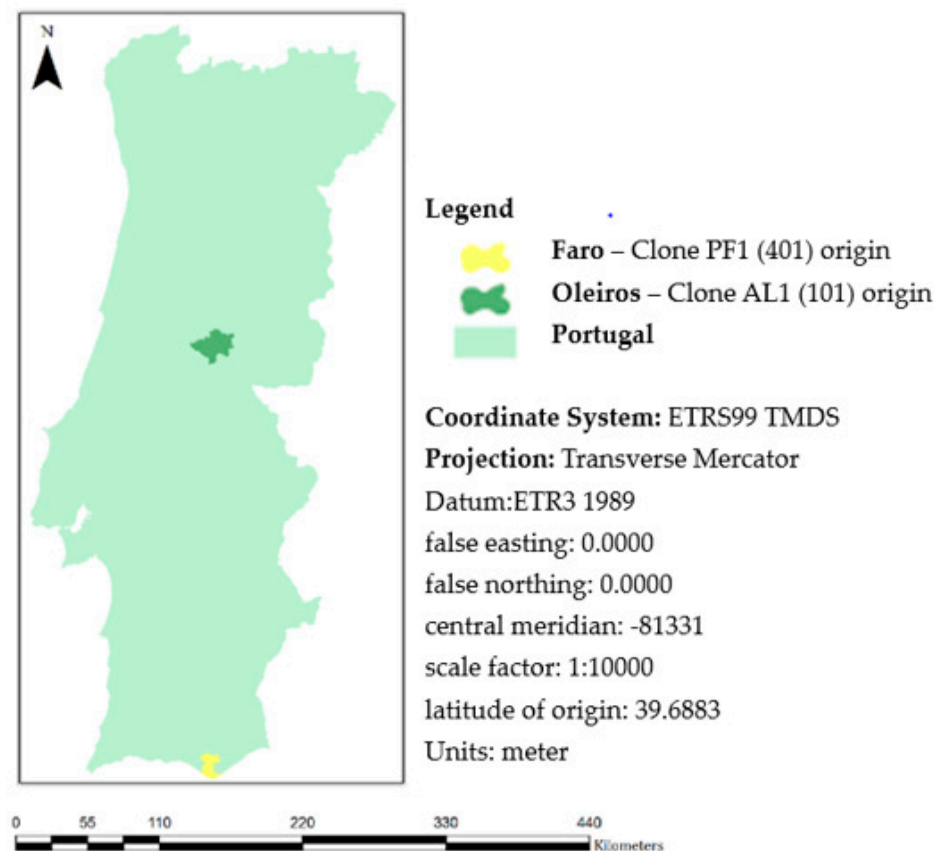

Figure 1. Provenances of the two genotypes tested (the clone AL1(101) from Oleiros, Alvéolos Muradal Mountain and clone PF1 (401) from Faro).

The provenances are, according to the Köppen–Geiger climate classification, under "Mediterranean, hot dry-summer" (classified as Csa) and "Mediterranean, warm

dry-summer" (classified as Csb), respectively PF1 (401) and AL1 (101). In the natural environment, the heat stress showed to be related to morphological and functional changes in mycelium and mycorrhizae of different strains of *Tuber borchii* [29].

Concerning natural conditions, it may be said that clone PF1 (401) is in worse climatic conditions, with a long, hot, and dry summer period, although in a more favorable limestone soil (which promotes water retention), since the presence of calcium is relevant in the establishment of mycorrhiza between truffles and host plant [30].

2.3. Mycorrhizal Synthesis Procedures

The mycorrhizal synthesis procedure was accomplished, promoted once contact occurred of the spore inocula during the ex-vitro rooting of the micropropagated shoots, as previously reported [9].

Inoculum suspensions (of both media tested Knop vs. water), with different levels of quercetin, were added to the substrate. As substrate, 500 mL of perlite was used inside transparent containers of 750 mL volume. The inoculum suspension was added to perlite, in a proportion of 10 % (*v/v*).

The ex vitro rooting was performed simultaneously with acclimatization, regarding a previously described procedure [31]. In brief, after the multiplication period (1 month), micropropagated shoots (±1.5 cm of length) were dipped in auxin (9.8 mM indole-3-butyric acid/IBA, during 5 s) and straight inserted into the previously inoculated perlite. The containers were maintained in a culture chamber (under temperatures of 25/20 °C and photoperiods of 16/8 h, and keeping the substrate and inoculum in the dark by wrapping the containers with aluminum foil).

For each treatment (2 culture media tested for spore suspensions × 6 quercetin levels), 50 shoots × 2 genotypes from in vitro clones (101 and 401) were tested, a total of 1200 shoots (initial time of the experiment, Figure 2).

Figure 2. Timeline from inoculation to end of the experiment and final collection of root branches to confirm mycorrhiza establishment.

The containers were kept for 5 months, closed to preserve high levels of humidity, and during the 6th month, humidity gradually decreased to hardening by gradually opening the containers. Next, plants were transferred to nursery pots (80 cm^3) with peat and perlite mix (75:25%; *v/v*) and placed under controlled conditions with moderate light intensity (50% shadow net) and watering. According to the Timetable of Figure 2, during plant transplant, first morphological observations of the mycorrhizae were registered (6 months). Then, four months later, i.e., 10 months after inoculation, plants were transferred to larger containers (220 cm^3) using the same substrate mix (peat and perlite, 75:25%; *v/v*) for plant development (timetable in Figure 2), where these plants remained until 12 months when the last morphological observations and molecular analyses were performed.

2.4. Morphological and Molecular Identification of Mycorrhizae

Six months after inoculation, during the plant transfer from perlite substrate to the mix of peat and perlite (75:25%; *v/v*), the following parameters were evaluated: mycorrhizae establishment, shoot length (SL), number of primary roots (NPR), number of leaf pairs, and root length (the length of the longest root/LLR and the length of the smallest root/**LsR**), as well as root morphological observation.

The mycorrhizae establishment was evaluated using the following notation: **0**—absence of fungi, **1**—mycelium, **2**—baby branch, and **3**—mycorrhiza, when a typical cruciform branching was observed.

Morphological observations were performed every time the plants were transferred to a new container, e.g., 6 and 10 months after inoculation and finally 12-month old plants (Figure 2). Arbutoid mycorrhizal roots were carefully examined with the aid of a stereomicroscope. A few samples were prepared using a fresh freezing microtome (Jung AG Heidelberg) and observed under a microscope (Olympus BX41).

For molecular analysis, mycorrhizal branches were collected 10 and 12 months after the inoculation (Figure 2).

Genomic DNA from root branches was extracted using DNeasy®Plant Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Molecular characterization was based on sequence analysis of the ITS1-5.8SITS2 rDNA region with ITS1F-ITS4 primers [32,33].

PCR reactions contained 10 mM Tris–HCl (pH 9.0), 10 mM NaCl, 7.5 mM MgCl$_2$, 0.4 mM for each dNTP, 0.4 μM for each primer, 0.6 μg·μL^{-1} of bovine serum albumin, and 1.25 U of Taq DNA polymerase (GoTaq® DNA Polymerase, Promega) and 1 μL of template DNA with approximately 15 ng·μL^{-1} in a 25 μL volume reaction. Thermal cycling was performed on a T gradient thermal cycler (Biometra GmbH, Göttingen, Germany) with the following conditions: 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s, with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 7 min. Amplicons were resolved by electrophoresis at 5V·cm^{-1} in 1% of agarose gel with 1× TBE running buffer and visualized with ultraviolet (UV) light after staining with 0.5 μg·mL−1 of ethidium bromide and visualized by VersaDoc Gel Imaging System (Bio-Rad, Hercules, CA, USA). PCR product purification was carried out with Ron's PCR Pure Mini Kit (BIORON GmbH, Germany) following the manufacturer's instructions.

Sequencing reactions were performed at STABVIDA (Portugal) on a DNA analyzer ABI PRISM 3730xl (Applied Biosystems). The chromatograms were edited, with FinchTV Version 1.4.0 (Geospiza Inc., Seattle, WA, USA). Consensus sequences for all isolates were compiled and aligned using Clustal X v. 2.0 [34]. When necessary, subsequent manual adjustments were made with BioEdit version 7.0.5.3 (Hall 1999). Consensus sequences were searched using the Basic Local Alignment Search Tool (BLAST) available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov (accessed on 1 January 2021)). All sequences generated in this study were deposited in GenBank.

A nested PCR was performed using previous amplification PCR product with *T. borchii* specific primers, TBA/TBB [35]. PCR reactions were performed as previously described except the addition of 4% DMSO and 1 μL of template DNA (the previously PCR product).

Thermal cycler conditions were the following: 40 cycles of 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 45 s, with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 8 min. Amplicons were analyzed as the previous reaction except with 1.5% of agarose gel.

2.5. Statistical Analysis

Statistical analysis was conducted using Statistica 7.0 software; complementary approaches were performed: analysis of variance (ANOVA) and the means were compared using the Duncan test ($p < 0.05$) and a principal component analysis (PCA). When variables were abnormally distributed, as the assessment of the mycorrhiza establishment variable (as notation 0–3, i.e., non-parametric data), means were compared using the Kruskal–Wallis test [36].

3. Results

3.1. Morphological Characterization

Six months after inoculation, when in vitro plants were transferred to nursery pots for the first time, root systems were examined for mycorrhizal formation. It was possible to observe that mycorrhizae had already developed, forming a mantle with visible external hyphae and the typical cruciform appearance, as shown in Figure 3.
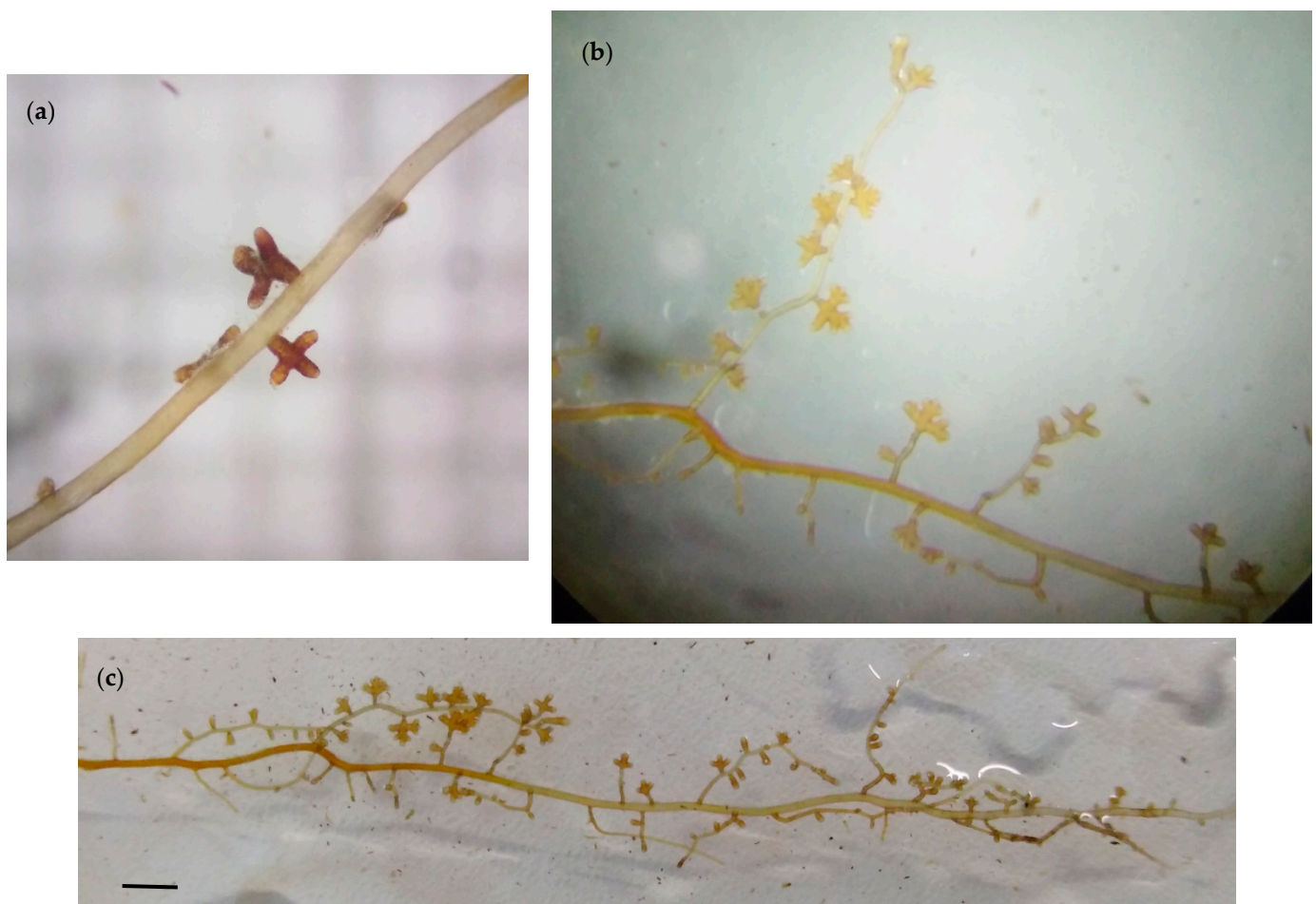

Figure 3. Mycorrhizae of *Tuber borchii* observed at the transfer of in vitro plants to nursery pots, six months after inoculation, using perlite as substrate. Mycorrhizae showed a typical cruciform appearance (**a**—21×). Cruciform ramifications on secondary roots (**b**—8×; **c**—scale bar =1 cm).

The presence of mycorrhizae formed by *T. borchii* was also confirmed by morphological observation 12 months after inoculation in nursey conditions. Figure 4 shows a completely formed thick mantle and the typical cruciform appearance, as well as highlights the unequivocal presence of *Tuber borchii* cystidia.
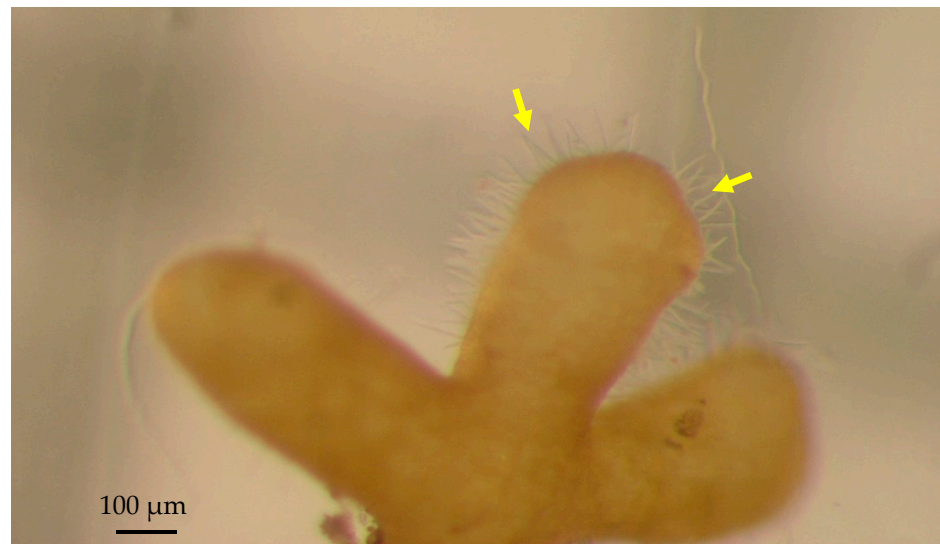

Figure 4. Mycorrhizae of *Tuber borchii* observed twelve months after inoculation, in nursery conditions. Mycorrhizae showed a thick mantle and the typical cruciform appearance. Notice the presence of cystidia (yellow arrows).

3.2. Plant and Mycorrhizal Evaluation

The plant survival rate observed six months after inoculation treatments, performed during ex vitro rooting, simultaneously with acclimatization, was 95% (1140 plants). However, mainly due to the long period of acclimatization (six months) for mycorrhizae establishment, associated with an inorganic substrate (perlite), only 65.9% showed a good root development; thus, 751 plants were transferred to nursery pots.

During the plant transfers, different parameters were evaluated to conclude about the effects of the main factors (quercetin addition, genotype, media culture for spore suspensions, and ulterior substrate inoculation) or their interactions on the mycorrhiza establishment and plant development. The addition of quercetin showed significant differences on mycorrhizal establishment and plant development (Table 1).

The addition of quercetin allowed a superior mycorrhizal establishment ($p < 0.05$), when it was added at 2.0 μM concentration, without significant differences, from 4.0 μM. Similar results were observed with the variables shoot length and the number of leaves. These results show that when quercetin was added at 2.0 μM concentration, plants showed a higher level of mycorrhiza establishment and higher growth. No significant differences were observed, due to quercetin treatment, for the number of primary roots and root length (LLR).

When quercetin was added in a concentration equal to or greater than 7.0 μM, a deleterious effect was observed on micropropagated plants: shorter shoot length and lower number of leaves ($p < 0.05$); a higher number of primary roots, quite thin, without the formation of secondary roots, and consequent mycorrhizae establishment; and a lower survival rate.

Table 1. Effect of quercetin treatment on mycorrhiza establishment, shoot length (**SL**), number of primary roots (**NRP**), leaf numbers, and the length of the longest and shortest root (**LLR, LsR**), observed 6 months after inoculation.

Treatment	N	Mycorrhiza [1] Mean ± SE		SL (mm) Mean ± SE		Leaves No. Mean ± SE		NRP Mean ± SE		LLR (mm) Mean ± SE		LsR (mm) Mean ± SE	
0 µM	153	1.95 ± 0.10	b	21.3 ± 0.63	a	8.03 ± 0.26	a	5.90 ± 0.43	a	58.40 ± 2.80	a	13.85 ± 0.84	b
0.5 µM	130	1.92 ± 0.11	b,c	20.91 ± 0.60	a	8.38 ± 0.27	a	5.14 ± 0.37	a	61.18 ± 2.99	a	15.88 ± 1.22	b
2 µM	111	2.50 ± 0.09	a	22.22 ± 0.60	a	8.52 ± 0.29	a	4.91 ± 0.39	a	51.81 ± 3.05	a	15.70 ± 0.94	b
4 µM	121	2.07 ± 0.11	ab	22.47 ± 0.89	a	7.75 ± 0.26	b,c	4.79 ± 0.35	a	55.44 ± 3.08	a	15.95 ± 1.03	b
7 µM	118	1.40 ± 0.12	c	19.04 ± 0.51	b	7.30 ± 0.27	c	5.39 ± 0.39	a	55.72 ± 3.01	a	19.55 ± 1.47	a
10 µM	118	1.72 ± 0.13	b,c	18.58 ± 0.69	b	6.47 ± 0.29	d	5.78 ± 0.47	a	59.63 ± 2.60	a	21.47 ± 1.39	a
Total	751	1.92 ± 0.05		20.78 ± 0.28		7.76 ± 0.11		5.34 ± 0.17		57.20 ± 1.20		16.93 ± 0.48	

[1] Notation 0–3 (0—absence of fungi; 1—mycelium; 2—baby branch; and 3—mycorrhiza cruciform branching), non-parametric data. Values are mean ± SE (n = 751). Values followed by different letters in columns are significantly different (*p* < 0.05) according to Duncan's multiple range test or Kruskal–Wallis test to non-parametric data.

Considering the effect of the medium tested for spore suspension on the mycorrhizal establishment, significant differences were also observed. The best results were observed when water was tested compared to the Knop medium (Table 2). However, globally, the plant development (shoot length, number of leaves, root length/LLR) was greater (*p* < 0.05) with Knop medium.

Table 2. Effect of medium used for spore suspension on mycorrhiza establishment, shoot length (**SL**), number of primary roots (**NRP**), leaf numbers, and the length of the longest and shortest roots (**LLR, LsR**), observed 6 months after inoculation.

Medium	N	Mycorrhiza [1] Mean ± SE		SL (mm) Mean ± SE		Leaves No. Mean ± SE		NRP Mean ± SE		LLR (mm) Mean ± SE		LsR (mm) Mean ± SE	
H₂O	402	2.15 ± 0.06	a	19.87 ± 0.38	b	7.53 ± 0.16	b	5.17 ± 0.22	a	53.84 ± 1.55	b	15.61 ± 0.58	b
KNOP	349	1.66 ± 0.08	b	21.81 ± 0.40	a	8.02 ± 0.15	a	5.54 ± 0.25	a	61.07 ± 1.84	a	18.60 ± 0.78	a
Total	751	1.92 ± 0.05		20.78 ± 0.28		7.76 ± 0.11		5.34 ± 0.17		57.20 ± 1.20		16.93 ± 0.48	

[1] Notation 0–3 (0—absence of fungi; 1—mycelium; 2—baby branch; and 3—mycorrhiza cruciform branching), non-parametric data. Values are mean ± SE (n = 751). Values followed by different letters in columns are significantly different (*p* < 0.05) according to Duncan's multiple range test or Kruskal–Wallis test to non-parametric data.

Considering the effect of the clone/genotype tested on mycorrhizal establishment, significant differences were observed on mycorrhiza establishment, the number of leaves, and number of primary roots, meaning that clones showed a different response to the conditions tested (Table 3).

Table 3. Effect of clone/genotype on mycorrhiza establishment, shoot length (**SL**), number of primary roots (**NRP**), leaf numbers, and the length of the longest and shortest roots (**LLR, LsR**), observed 6 months after inoculation.

Clone	N	Mycorrhiza [1] Mean ± SE		SL (mm) Mean ± SE		Leaves No. Mean ± SE		NRP Mean ± SE		LLR (mm) Mean ± SE		LsR (mm) Mean ± SE	
AL1(101)	435	2.27 ± 0.05	a	20.84 ± 0.38	a	8.59 ± 0.15	a	6.00 ± 0.24	a	51.70 ± 1.23	b	15.28 ± 0.53	b
PF1(401)	316	1.45 ± 0.08	b	20.68 ± 0.39	a	6.62 ± 0.16	b	4.44 ± 0.20	b	64.78 ± 2.22	a	19.32 ± 0.87	a
Total	751	1.92 ± 0.05		20.78 ± 0.28		7.76 ± 0.11		5.34 ± 0.17		57.20 ± 1.20		16.93 ± 0.48	

[1] Notation 0–3 (0—absence of fungi; 1—mycelium; 2—baby branch; and 3—mycorrhiza cruciform branching), non-parametric data. Values are mean ± SE (n = 751). Values followed by different letters in columns are significantly different (*p* < 0.05) according to Duncan's multiple range test or Kruskal–Wallis test to non-parametric data.

An interaction between the genotype and suspension culture medium was observed (Figure 5). For clone AL1 (101), the best results were observed when Knop medium was tested, for both variables, the mycorrhiza establishment and leaf number (*p* < 0.05; 205 plants) compared to water (230 plants). For clone PF1 (401), significantly higher results

for mycorrhiza were observed when water was tested (172 plants), although it showed similar growth compared to Knop medium (144 plants). These results show an interaction between the genotype and suspension culture medium for mycorrhizal establishment.
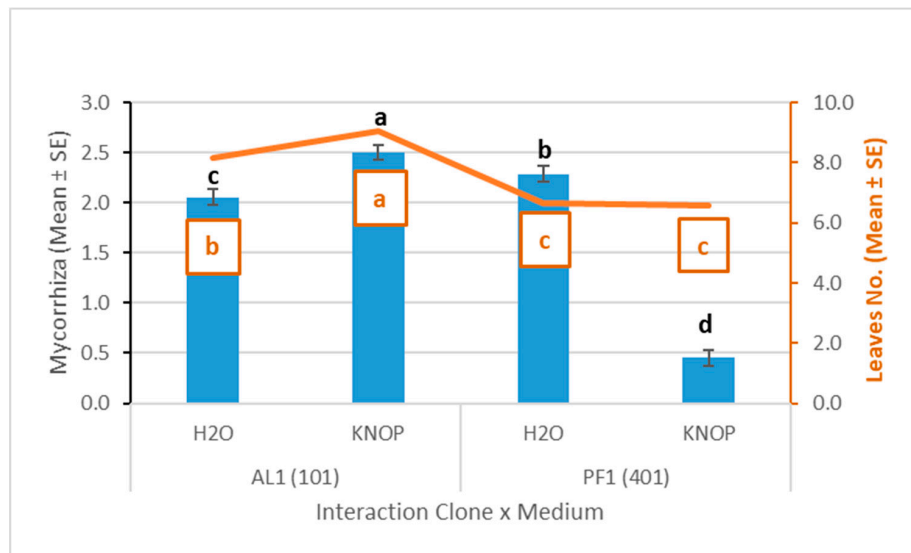

Figure 5. Interaction (clone x medium) on mycorrhiza establishment (notation 0–3, represented in columns) and plant growth (leaves No., represented by brown line). Different letters represent significantly different values ($p < 0.05$; n = 751).

The plant growth, evaluated by the number of primary roots (**NPR**) followed the same pattern distribution (**Leaves No.**), but without significant differences, suggesting higher presence of mycorrhiza on secondary roots.

The PCA (Figure 6) shows the interaction among different variables as the mycorrhizal establishment, suspension medium, genotype, quercetin concentration, leaf number, and root length.

PCA	Factor Loadings (F) Red label > 0.700		
Variables	**F 1**	**F 2**	**F 3**
Medium[1]	-0.006	-0.525	0.746
Clone	**0.643**	0.081	0.071
Quercetin Treatment	0.325	-0.341	-0.225
LsR (mm)[2]	0.262	-0.807	-0.287
Leaf No.[3]	-0.741	-0.335	-0.007
NRP[4]	-0.514	0.076	0.440
Mycorrhiza[5]	**-0.681**	-0.085	-0.483
Eigenvalue	1.864	1.176	1.121
Total Variance (%)	*0.266*	*0.168*	*0.160*

[1] Medium spores suspension
[2] Length of the shortest Root
[3] Leaves number
[4] NRP number of primary roots
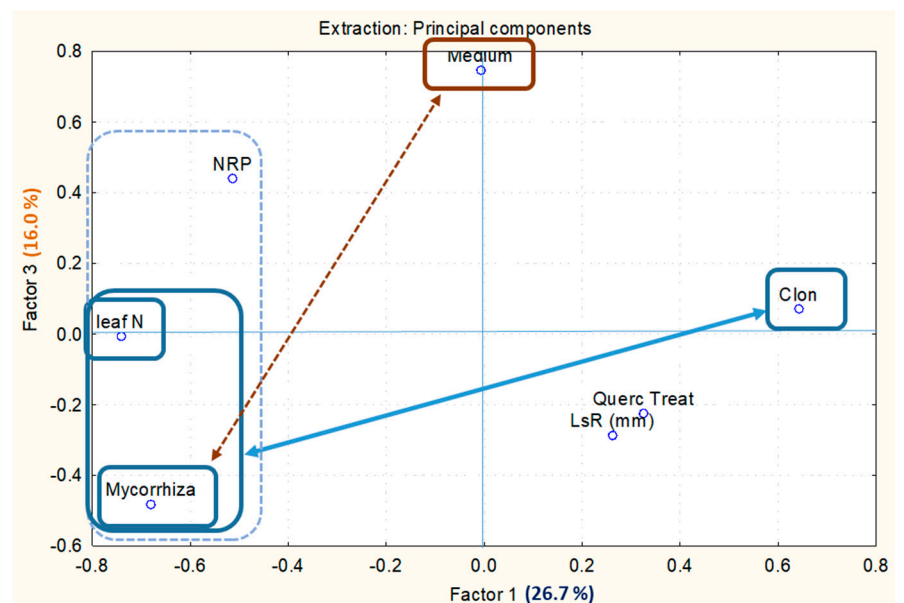[5] Mycorrhizal establishment

Figure 6. Principal component analysis (PCA) of the variables, observed 6 months after inoculation. The variables association with factors 1–3 are according to the coefficient factor loadings, on the left table. The highest factor loadings (>0.70) in the red label are relevantly correlated, explaining the total variance expressed by the three factors. Each factor explains a percentage of total variance with a cumulative value of 59.44%. The figure presents the relation between factors 1 and 3, which explains 26.7% and 16.0% of the total variance, respectively.

The PCA (Figure 6) shows that mycorrhiza is directly related to plant growth, evaluated by leaf number. Moreover, plants showing mycorrhiza also have a superior number of primary roots. On the other hand, the mycorrhiza is also dependent on the genotype tested, as well as of the spore suspension medium.

3.3. Molecular Characterization

Confirming the presence of mycorrhizae formed by *T. borchii* was possible by using a molecular identification. This study generated sequences in the ITS1-5.8SITS2 rDNA region submitted to the GenBank database (accession numbers: MW115315, MW115316, and MW115317). BLAST results for accession no. MW115315 are query cover: 95%; percent identity: 99.63%; and total scores: 987. The values for accession no. MW115316 are query cover: 96%; percent identity: 99.63%; and total scores: 976; and for accession no. MW115317, query cover: 95%; percent identity: 98.69%; and total scores: 948. All sequence have an e-value of 0.0 when compared with accession no. FJ554490.1, a *T. borchii* species. In addition, nested PCR showed an expected DNA band of 432 bp characteristic of *T. borchii* species.

4. Discussion

Six months after inoculation, when in vitro plants were transferred to nursery pots, morphological root observations showed consistent arbutoid mycorrhizae formation with *T. borchii* analogous to that referred in previous studies with *A. unedo* [37].

Molecular analysis performed ten and twelve months after inoculation confirmed the presence of a *T. borchii* species, assuring the persistence of this symbiotic association. Our results are in line with those referred to in previous studies with *A. unedo* plants [11,18,38,39]. Moreover, the nested PCR showed the predictable DNA band of 432 bp characteristic of *T. borchii* species as previously referred [35].

The spore suspension culture medium effect on the mycorrhizal establishment showed significant differences. Although globally the best results were observed for water when compared with Knop medium, a strong interaction was observed between suspension culture medium and genotype, showing that the choice of the suspension culture medium should be linked to the genotype to achieve the best mycorrhizal establishment. The effect of the genotype of the donor plants of *A. unedo* showed to be a relevant factor interfering with the multiplication and rooting. This stresses, once more, generally importance of the genotype [23] and particularly in the case of the *A. unedo* species [40].

Several authors observed that flavonoids are produced and released into the rhizosphere, in response, modulated to by the colonization process [20–22,41], and are linked to the establishment of arbuscular mycorrhizal fungi, particularly in the early stages of the interaction [20–23]. In our study, quercetin addition of up to 2.0 µM showed a superior mycorrhizal establishment ($p < 0.05$). Scervino et al. [23] refer that quercetin is the most widely distributed flavonoid in plants where mycorrhizal symbiosis is present and has a positive effect on germination or hyphal length of arbuscular mycorrhiza (AM) spores. The authors showed that quercetin in arbuscular mycorrhizal (AM) clover roots, stimulated the penetration and root colonization of tomato by *Gigaspora*. Our results show similar effects of the quercetin on the formation of Arbutoid mycorrhiza on *A. unedo* plants by *T. borchii* [23].

Quercetin concentrations above 7.0 µM showed a deleterious effect, reflected on shorter shoot length, less leaf numbers, and a reduced number of secondary roots, seriously compromising the mycorrhiza establishment and the plant survival rate. Flavonoids were shown to be involved in the AM colonization in plant root, but its effect was highly complex, dependent not only on flavonoid concentration, but also on the AM fungal genus (or even species) [23]. However, in our study, within the species of *A. unedo*, the quercetin effect did not seem to be related to the genotype, as no statistic interaction was observed. Thus, quercetin was shown to be an efficient factor, when it added up to 2.0 µM on the induction of mycorrhiza, thriving independently of the genotype.

Morphological observations and molecular analysis confirmed the permanence of mycorrhizal association 12 months after inoculation in nursery conditions. These plants were established in field trials; further morphological and molecular studies will be performed to confirm the persistence of *T. borchii* mycorrhizae.

5. Conclusions

This study showed that mycorrhizal development between *T. borchii* and *A. unedo* micropropagated clones, maintained in nursery conditions, was dependent on the three main factors studied: genotype, quercetin concentration, and spore suspension culture media, and on their interactions.

Quercetin can be added to the spore suspension culture media in an expedited form during inoculation procedures, with a significant positive effect on the establishment and ulterior development of mycorrhiza, independently of the genotype.

Further studies should be performed to confirm the long-term persistence of mycorrhizae in field trials and evaluate the fungal colonization level required to guarantee mycorrhiza persistence in a natural environment.

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