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Brief Report

The Nurse Plant *Acacia spirorbis* Enriches Ectomycorrhizal Community Composition of a Target Species: *Tristaniopsis calobuxus*

Anne Houlès [1,2], Karine Gotty [1,*], François Joussemet [1,2], Bryan Vincent [1], Laure Hannibal [1], Magali Patrois [2], Philippe Jourand [1,3] and Marc Ducousso [1]

[1] Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), IRD, INRAE, CIRAD, Institut Agro, Univ. Montpellier, 34398 Montpellier, France; houles.anne@gmail.com (A.H.); francois.joussemet@uclouvain.be (F.J.); bryan.vincent@cirad.fr (B.V.); laure.hannibal@ird.fr (L.H.); philippe.jourand@ird.fr (P.J.); marc.ducousso@cirad.fr (M.D.)

[2] Koniambo Nickel SAS, Vavouto, BP 679, 98860 Koné, New Caledonia; patoum480@gmail.com

[3] Entropie, IRD, Université de la Réunion, Ifremer, CNRS, Université de Nouvelle-Calédonie, 97744 St Denis, France

* Correspondence: karine.gotty@inrae.fr; Tel.: +33-(0)4-67-59-37-63

Abstract: We investigated the suitability of *Acacia spirorbis* Labill., a tropical ectomycorrhizal (ECM) tree, as a nurse plant to improve the growth of *Tristaniopsis calobuxus* Brongn. & Gris seedlings for the restoration of nickel mines in New Caledonia. Rehabilitation of nickel mines in New Caledonia is a major concern. In such harsh soil conditions, ectomycorrhizal (ECM) symbiosis is important for tree growth, survival, and resistance. To improve ecological restoration in New Caledonia, new technical itineraries have undergone experimentation using ECM as a plant nurse, allowing ECM saplings to rapidly acquire a wide range of ECM fungi. We transplanted ECM seedlings of *Tristaniopsis calobuxus* from the nursery to bare ferralitic soils harbouring some scattered 12-year-old *Acacia spirorbis* to be used as ECM nurse plants. Using molecular characterisation of ITS rDNA, we characterised ECM fungal communities of *A. spirorbis* and of *T. calobuxus* saplings at transplanting time and 13 months later. We observed changes in the composition of fungal communities of *T. calobuxus* with an increase in diversity, notably the appearance of operational taxonomic units (OTUs) affiliated with /russula, /boletus and /pisolithus-scleroderma and a decrease in ubiquitous nursery order such as /sebacina. We also observed a higher number of shared OTUs between *T. calobuxus* and *A. spirorbis*. The vicinity of *A. spirorbis* enabled diversification and adaptation of the *T. calobuxus* ECM fungal community. These results led us to recommend *A. spirorbis* as a good nurse tree candidate in the framework of ecological restoration of mine sites.

Keywords: agroforestry; ectomycorrhizal share; forest nursery; fungal ITS; New Caledonia; tree legume; ultramafic soil

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1. Introduction

Tropical ecosystems have been the subject of far fewer studies than temperate ecosystems [1], thus our knowledge of the unique New Caledonian ecosystem is extremely new and fragmentary [2,3]. Recent work carried out in these ecosystems revealed the importance of ectomycorrhizal (ECM) symbiosis in New Caledonia (e.g., [4,5]). However, anthropogenic activities (bush fires, nickel mining and urbanization) are major threats to the preservation of these ecosystems in New Caledonia [6–9], and so this tropical archipelago is now considered as a world biodiversity hot spot [10,11]. In this context, studies on the use of trees to rehabilitate mine sites began in the 1970s [12]. Since then, only two major endemic tree species, *Acacia spirorbis* Labill. and *Casuarina collina* J. Poiss. ex Pancher & Sebert, have been shown to be able to grow rapidly in such extreme soil conditions [13].

However, these two tree species are not naturally present in most original mine ecosystems nor do they enable the spontaneous return of the original native endemic species. Thus, even if these two species grow well in these harsh conditions, they do not provide satisfactory ecosystem services, notably, because natural plant successions do not occur spontaneously [14,15].

To test the setting of a controlled plant succession, we took the opportunity to use the remains of a 12-year-old field plantation of *A. spirorbis*, of which the ECM community was recently described [16]. In this plantation, the mortality after planting had left some scattered isolated trees on bare soil surrounded by the native endemic ecosystem locally dominated by the ECM *Tristaniopsis guillainii* Vieill. ex Brongn. & Gris. Considering the vicinity of sources of ECM fungal propagules and the dispersal abilities of ECM propagules [17], we assumed that natural diversified ECM inoculums would not be a limiting factor for nursery saplings being colonised by the variety of established ECM fungi. However, the conditions in nurseries, i.e., confined space, highly fertile soil, the occasional use of fungicides and abundant watering, prevent ectomycorrhizal interactions [18,19], and mostly involve opportunistic species with high dispersal abilities [20–22]. As result, ECM fungal communities associated with nursery saplings are markedly different from those encountered in the field [23]. Transplanting from the nursery to the natural environment has been widely studied, especially in the framework of controlled inoculation trials using selected ECM fungal strains [24,25]. Nevertheless, after planting, opportunistic nursery species must make way for indigenous species, to ensure the survival and development of the saplings.

In these conditions, following Walker and del Moral [26], who defined facilitation as any positive influence of one species on another, we hypothesised that a species such as *A. spirorbis* could act as an ECM facilitator [27] for another species such as *Tristaniopsis calobuxus* Brongn. & Gris. ECM facilitation is effective only if most ECM fungi are not specific [28–32]. We thus needed to check the ability of ECM fungal species associated with *A. spirorbis* to associate with *T. calobuxus* before and after planting. The remains of a 12-year-old plantation of *A. spirorbis* established on bare soil on the top of the Koniambo Massif (altitude 851 m above sea level) was used to test the ability of *A. spirorbis* to act as a nurse plant.

2. Materials and Methods

2.1. Study Site and Experimental Design

The Koniambo Massif in New Caledonia is currently a nickel mine exploited by the company Koniambo Nickel SAS. The general features of the massif have been described in Perrier et al. [33]. The plantation site is a 60 × 30 m rectangle located on the Koniambo Massif, between the latitudes 21°00′20–21″ S and longitudes 164°50′17–19″ E at an altitude of 851 m. The main characteristics of the soil in the study site are listed in Table S1.

The experimental plantation was established in May 2015 and contained the remains of a former plantation of *A. spirorbis* dating from 2003 in the form of scattered 12-year-old trees. The site is surrounded by diversified *maquis* vegetation with the recurring presence of the ECM endemic shrub species *T. guillainii.* Such surrounding vegetation is a potential source of ECM inoculums for planted *T. calobuxus* saplings. We thus delimited three zones in the maquis in which *T. guillainii* was sampled to assess ECM fungal diversity (Figure 1).

A preliminary prospective study was designed to select the better species to test the ability of *A. spirorbis* to act as a nurse plant through the integration of the target species in a common ectomycorrhizal network, in respect to the project timeline.

Tristaniopsis calobuxus was growing quickly in nursery conditions and needed a shorter co-planting period compared with *T. guillainii* to obtain relevant results (data not shown). These two *Tristaniopsis* species share some similarities and have trouble growing in a post-mining ultramafic soil on their own. Therefore, we chose *T. calobuxus* rather than *T. guillainii*.

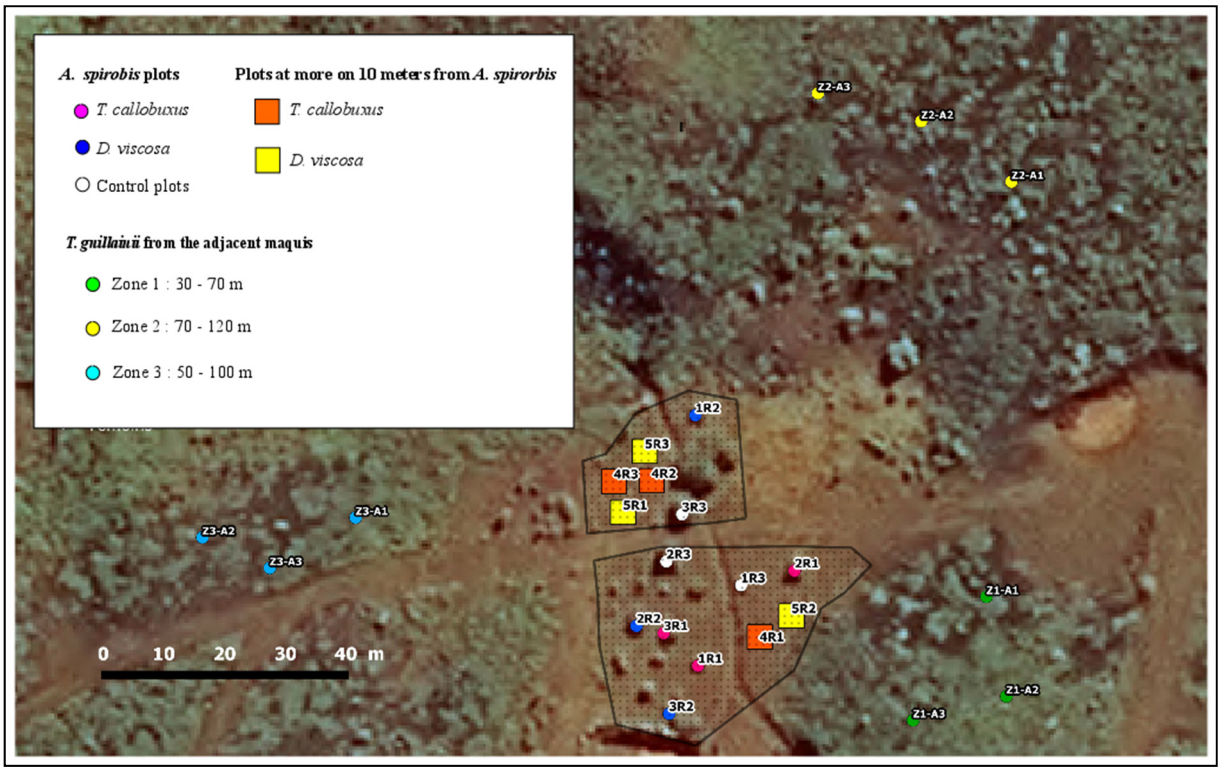

Figure 1. View of plantation site and surrounding area on the Koniambo Massif, New Caledonia. Plantation plots are located inside areas surrounded by black lines. The circles inside planting areas represent plots planted in a radius around *A. spirorbis* (see Figure 2a). The squares represent plots at least 10 m away from *A. spirorbis* (see Figure 2b). The circles outside planting areas are the exact positioning of harvested *T. guillainii* trees in the natural maquis surrounding the planting areas.

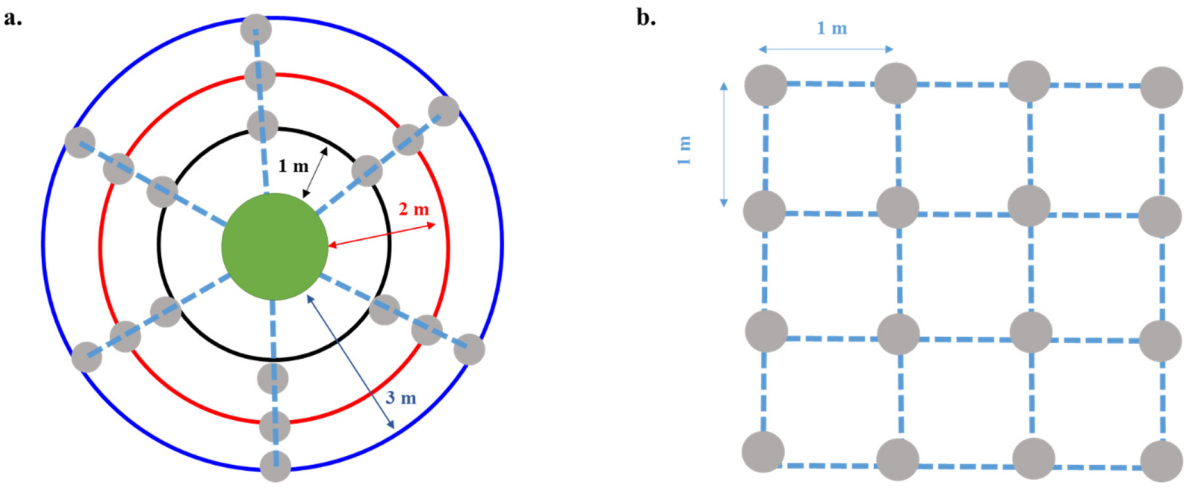

Figure 2. Schemes of the planting plot designs. (**a**) Using present *A. spirorbis* with the green circle representing the canopy, the target species, represented with grey circles, are planted in a radius around *A. spirorbis* trees at 3 different distances: the black line represents an area with 6 target species, planted at 1 m from the *A. spirorbis* canopy limit, the red line is at 2 m, and the blue line is at 3 m. (**b**) On bare soil at least 10 m away from the closest *A. spirorbis*, the target species, represented with grey circles, are planted in quadra of 4 plants by 4, spaced 1 m from each other.

In each zone, three *T. guillainii* were sampled, and the coordinates of each tree are given in Table S2. In these conditions, two types of plots were planted: one in the close

vicinity of a 12-year-old *A. spirorbis* and the second outside the vicinity of an *A. spirorbis*. The first type of plantation was a circle with an *A. spirorbis* at the centre; *T. calobuxus* with three repetitions were planted on each radius, the first at 1 m, the second at 2 m and the third at 3 m from the limit of the *A. spirorbis* canopy; three *A. spirorbis* were not associated to *T. calobuxus* and were used as controls. The second type of plot was a standard 4 row/4 line plantation with 1 m space between the lines and the rows; 3 plots were planted with *T. calobuxus*, at least 10 m away from any 12-year-old *A. spirorbis* (Figure 2). The plant survival and growth (collar diameter and height) were measured quarterly after transplantation from May 2015 until July 2016 (Table S3).

2.2. Nursery Growth

All saplings came from the same nursery. Seeds used in this nursery are always sterilized as already described in Jourand et al. [34]. Briefly, seeds were pre-treated for 2 min in 70% ethanol supplemented with 0.1% *(v/v)* Tween 20, then surface sterilized for 5 min in H_2O_2 (30%) and washed 3 times in sterile water.

2.3. Sampling Ectomycorrhizal Root Tips and Molecular Analyses

In order to describe the original ECM fungal communities at planting time, in May 2015, 15 ECM root tips were sampled per plant for DNA analysis on three *T. calobuxus* saplings randomly selected in each plot (for a total of $3 \times 6 \times 15 = 270$ root tips). Using the root tracking method described in Perrier et al. [33], 15 root tips from the nine *A. spirorbis* were used as nurse plant candidates and nine *T. guillainii* from the surrounding maquis (Figure 1 and Table S2) were harvested (total of $15 \times (9 + 9) = 270$ root tips).

To describe ECM fungal communities after a growth period of 13 months (July 2016), three randomly chosen *T. calobuxus* per plot were uprooted, and 15 root tips were harvested per plant (total of $3 \times 6 \times 15 = 270$ root tips). A root tip was considered ECM when a fungal mantle surrounded it. The root tips were preserved in 2% cetyltrimethylammoniumbromide (CTAB), 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA) at −20 °C until molecular analysis.

Using Sanger sequencing, each root tip was subjected to molecular analyses to characterise the ECM fungus putatively involved in the ECM symbiosis. For this purpose, the total DNA was extracted from each ECM root tip using the Wizard Genomic DNA Purification (Promega, Charbonnière-les-Bains, France) following the protocol described by Carriconde et al. [35]. We used the nuclear ribosomal internal transcribed spacer (ITS) as a universal fungal barcode [36]. A fragment of approximately 650 bp was amplified using primers ITS1F (5′-TTTCCGTAGGTGAACCT-3′) [37] and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [38]. The PCR reaction used for this amplification was prepared in a total volume of 25 µL containing a 1 µL aliquot of genomic DNA diluted 1/20, 1 µM of each primer, 1.5 units of Taq DNA polymerase (Promega, Charbonnières, France), 5 µL of 5X Promega Taq polymerase buffer, 2 mM of $MgCl_2$ and 200 µM of dNTP Promega. Amplification was performed with a Eppendorf® Mastercycler® thermocycler (Eppendorf, Hamburg, Germany) programmed as follows: one cycle of 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C and a final extension of 10 min at 72 °C. PCR products were checked by electrophoresis on a 1% agarose gel (Sigma) in 0.5% TBE buffer with 2 µL ethidium bromide (BET, 10 µg/mL for 100 mL gel). A size marker 1000 pb (Promega) was used to estimate the weight of the different fragments. The DNA bands were then visualised by fluorescence under UV light and photographed. Only for samples with simple well-defined bands, bidirectional sequencing with primers ITS4 and ITS1F was carried out by an external service (Genoscreen, Lille, France) on an automated sequencer using genetic analysis based on capillary electrophoresis ABI3730XL.

The resulting sequences were analysed and assembled into contigs with Geneious® 7.1.9 software (http://www.geneious.com, accessed on 6 July 2021) (Biomatters Ltd., Auckland, New Zealand). DNA sequences were assigned to operational taxonomic units (OTU) using a sequence identity cut-off of 97% of the ITS sequences, as reported in Smith et al. [31]

using software CD-Hit Est® [39]. The taxonomic identification and lineage attribution of the generated OTUs were carried out using the UNITE database, in relation to the fungal nomenclature reported by Tedersoo et al. [40].

2.4. Data Analysis

Statistical analyses were performed in the R environment [41], with the following packages: vegan [42] and RVAidememoire [43]. To test the sampling method, the observed and estimated sample completeness curves for each plot were constructed using iNEXT online software (http://chao.stat.nthu.edu.tw (accessed on 6 July 2021; [44]). The species richness (^{0}D), Shannon (^{1}D) and Simpson (^{2}D) indexes were calculated using the iNEXT *R* package [45] in order to evaluate ECM fungal diversity among the treatments [46]. The estimated and asymptotic richness index ^{0}D counts species equally without considering their relative abundance, the Shannon diversity index ^{1}D counts species in proportion to their abundance, and the Simpson diversity index ^{2}D discounts all but the dominant species in the assemblage. Venn diagrams were created online, with the help of the Venny2 website [47]. The total species richness of each community was estimated using Specpool in the vegan package [42] with the first-order jackknife estimator [48]. The global representation of the lineages identified in the sample is represented with an NMDS, based on a dissimilarity matrix of Bray–Curtis. Supplementary analyses such as Chi^2 tests, were carried out to test if several parameters, such as host specificity, the year of sampling, the distance of sampling from *A. spirorbis*, or the combination of all treatments could affect the structuration of ECM fungal communities. All OTUs belonging to the /non-ectomycorrhizal lineage were excluded from the analyses.

3. Results

Diversity and Comparison of ECM Fungal Communities Associated with the Different ECM Tree Species

From a total of 810 root tips, 312 ECM ITS DNA sequences were obtained. These sequences were merged into 97 ECM OTUs which were grouped into 11 fungal lineages. One representative sequence of each OTU was deposited in the DNA Data Bank of Japan (DDJB) with the accession numbers LC271284 to LC271380. The taxonomic identification of all OTUs is available in Table S4. Summarized data of the ITS sequences are presented in Table S5. The sampling effort is represented with completeness curves (Figure 3).

The distribution of lineages according to the OTUs is presented in Figure 4. At transplantation, we observed 26 OTUs grouped in seven lineages on *A. spirorbis*, 14 OTUs grouped in four lineages on *T. guillainii*, and five OTUs grouped in four lineages on *T. calobuxus* saplings. Thirteen months after transplanting, the number of OTUs associated with *T. calobuxus* was 21 (multiplied four times), and the number of shared OTUs between *A. spirorbis* and *T. guillainii* increased from one to five (Table 1; Figure 5). Concerning lineages, we observed four new lineages on the *T. calobuxus* 13 months after transplantation: */boletus*, */pisolithus-scleroderma*, */russulales* and */coltricia*. Except for */boletus*, which was present only on *T. guillainii*, all other lineages were observed on *A. spirorbis* ECM (Figure 4).

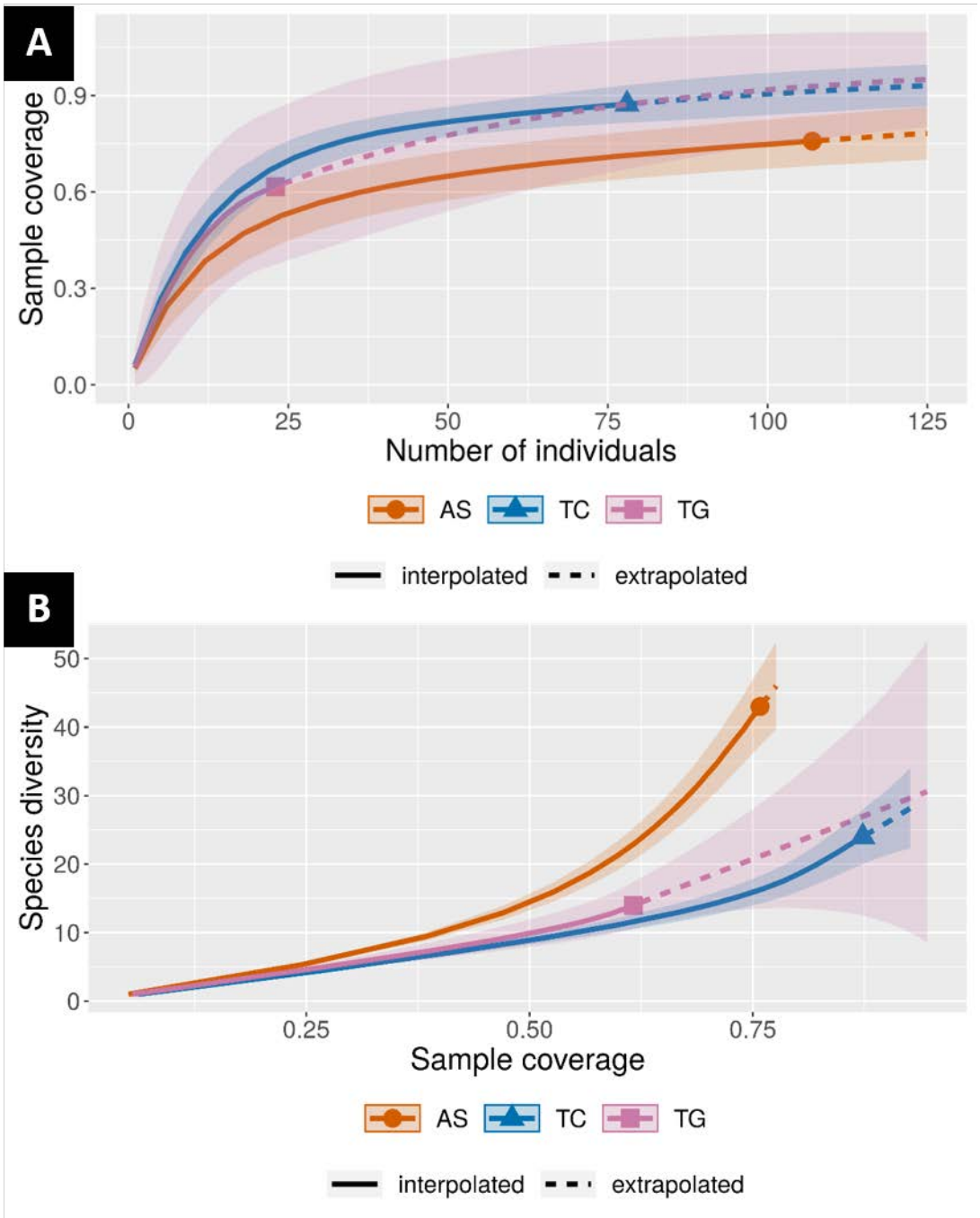

Figure 3. Non-asymptotic approach based on interpolation and extrapolation of species diversity and sample coverage based on iNEXT online software (http://chao.stat.nthu.edu.tw, accessed on 6 July 2021) as reported by Chao et al. [44]. Solid lines represent rarefaction of the sampling curves, the observed sampling effort; dotted lines represent extrapolation of the sampling curves, based on Hill numbers for q = 0.1 and 2. (**A**) Sample completeness curve according to host tree species. (**B**) Coverage-based rarefaction and extrapolation sampling curve according to the host tree species. Legend: AS = *A. spirorbis*; TC = *T. calobuxus*; TG = *T. guillainii*.

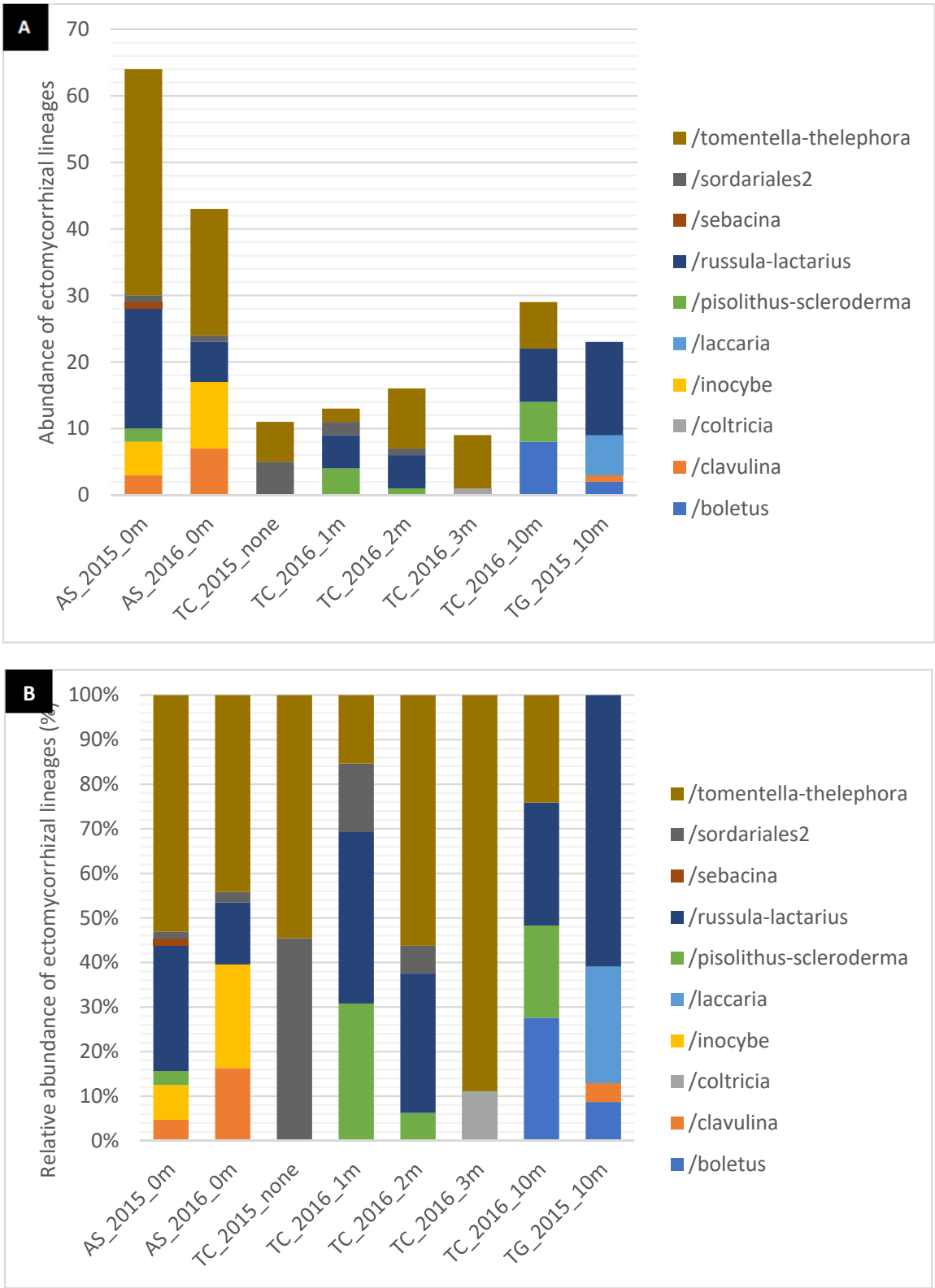

Figure 4. Ectomycorrhizal lineages found in nurse trees (*A. spirorbis* = AS) and target plants (*T. calobuxus* = TC) at successive distances (1, 2, 3 and 10 m away from *A. spirorbis*) after one year of plantation. Bar name corresponds to the plant (AS or TC), the year of sampling (2016) and the minimum distance of sampling from AS (0 m for AS; 1, 2, 3 and 10 m for TC; more than 10 m for TG). Data are presented according to the true abundance of lineages (**A**), and relative abundance expressed in % (**B**).

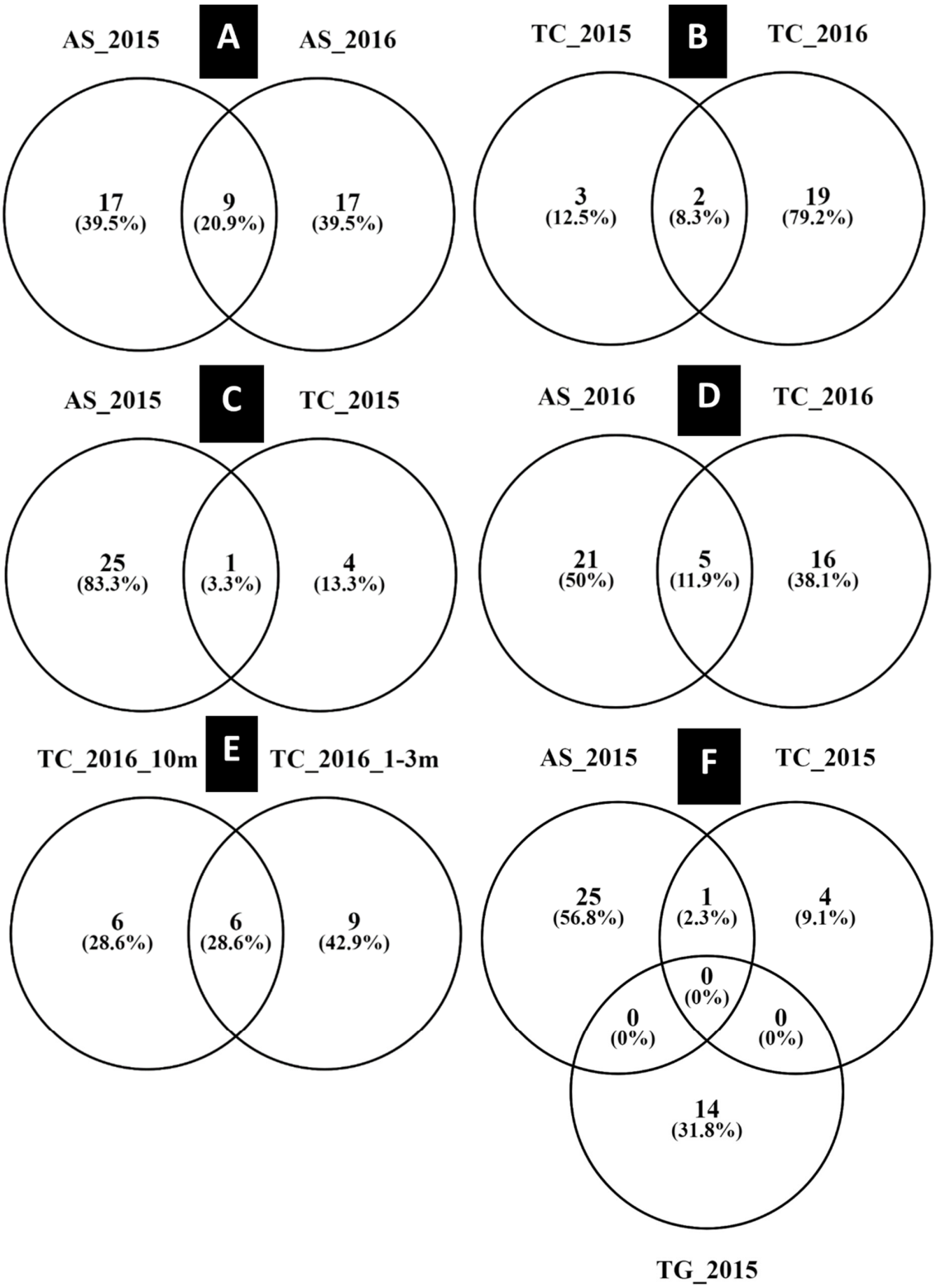

Figure 5. Venn diagrams of the ectomycorrhizal communities (based on OTUs) of *A. spirorbis* and *Tristaniopsis* species, in relation to host and year. Data are presented in multiple ways, as follows: (i) evolution of OTUs from 2015 to 2016 in *A. spirorbis* (**A**) and *T. calobuxus* (**B**), (ii) shared and unique OTUs between *A. spirorbis* and *T. calobuxus* in 2015 (**C**) and 2016 (**D**), (iii) shared and unique OTUs found between *T. calobuxus* planted inside a 3 m radius around *A. spirorbis*, versus *T. calobuxus* planted in square plots at 10 m away from *A. spirorbis* (**E**), and (iv) the shared and unique OTUs at the beginning of the experiment in 2015, between *A. spirorbis*, *T. calobuxus*, and *T. guillainii* (**F**).

Table 1. Ecological diversity indexes of the ECM fungal OTUs according to all treatments. The names of the treatments include the host species (AS: *A. spirorbis*; TG: *T. guillainii*; TC: *T. calobuxus*), the year of sampling (2015 or 2016) and the distance of sampling from *A. spirorbis*, expressed in meters (where none correspond to TC grown in nursery). The estimated and asymptotic richness index ^{0}D counts species equally without considering their relative abundance, the Shannon diversity index ^{1}D counts species in proportion to their abundance, and the Simpson diversity index ^{2}D discounts all but the dominant species in the assemblage.

	Diversity Indexes				
Treatment	**Richness ^{0}D**	**Chao1**	**ACE**	**Shannon ^{1}D**	**Simpson ^{2}D**
AS_2015_0m	26	50 ± 16	54 ± 3.5	2.8	0.90
AS_2016_0m	26	57 ± 20	67 ± 4.3	3.0	0.94
TG_2015_10m	14	26 ± 11	27 ± 2.4	2.5	0.90
TC_2015_none	5	5.5 ± 1.3	6.4 ± 1.1	1.5	0.74
TC_2016_1m	9	30 ± 17	27 ± 1.8	2.1	0.85
TC_2016_2m	6	6.5 ± 1.2	7.2 ± 1.0	1.6	0.78
TC_2016_3m	6	9.0 ± 4.1	12 ± 1.49	1.7	0.79
TC_2016_10m	13	13 ± 1.6	15 ± 1.7	2.3	0.88

As shown in Figure 5, the ECM OTUs of *A. spirorbis* (Figure 5A) and *T. calobuxus* (Figure 5B) shifted over time, with the emergence of 17 and 19 unique OTUs in 2016. Moreover, the number of ECM OTUs shared between *A. spirorbis* and *T. calobuxus* increased from one to five after one year of plantation (Figure 5C,D). Regarding the ECM OTUs of *T. calobuxus* in relation to the sampling distance from the nurse plant, 28.6% were shared between a 3 m radius and a sampling 10 m away (Figure 5E). A strong host effect was detected in 2015, where 43 of 44 OTUs were unique to their host species (Figure 5F).

A non-metric multidimensional scaling (NMDS) based on a dissimilarity matrix of Bray–Curtis revealed that ECM fungal communities differed in relation to the host species (*A. spirorbis*, *T. calobuxus*, or *T. guillainii*), and changed after a year of plantation (Figure 6). These results were further supported by Chi square tests (Table S6; must be read in parallel with the Venn diagrams (Figure 5)).

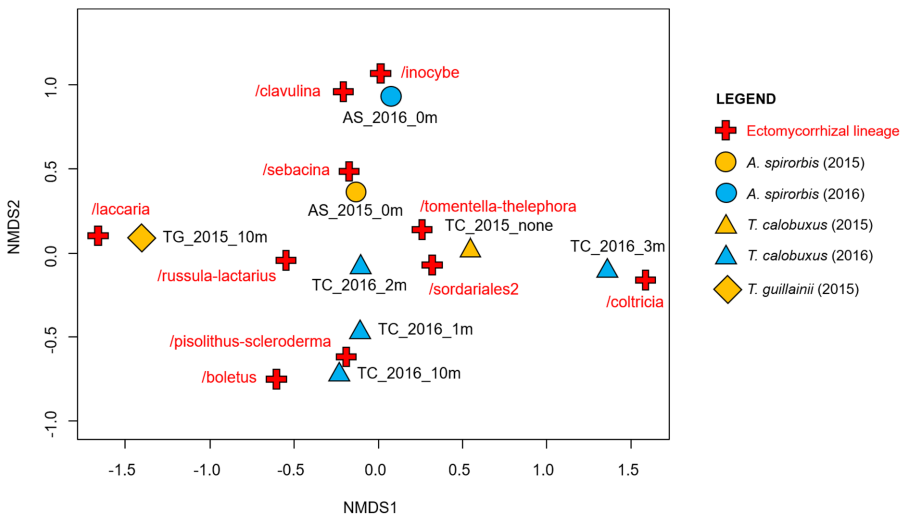

Figure 6. Non-metric multidimensional scaling representation of the ectomycorrhizal lineages (red cross and text) found in all samples. Hosts are symbolized as follows: *A. spirorbis* with circles, *T. calobuxus* with triangles, and *T. guillainii* with a diamond. Two different years of sampling (2015 and 2016) are indicated in blue and orange, respectively.

4. Discussion

First, in terms of ECM diversity, our results were consistent with those reported by Wasseem et al. [49] who recorded seven ECM lineages associated with *T. guillainii*, and with those of Houlès et al. [16] who observed 12 ECM lineages associated with *A. spirorbis*, data both collected partly from the Koniambo Massif where our field trial was located. In contrast, none of the ECM fungi were shared with *Tristaniopsis* in Bangka Island (Indonesia) [50].

The spontaneous ECM communities of nursery saplings usually differ from those associated with plants growing in the natural environment [20–23,51]. Indeed, nursery conditions, i.e., confined space, high soil fertility and abundant watering, significantly influence mycorrhizal interactions resulting in the selection of opportunistic ECM species and contribute to low ECM fungal diversity [51,52]. In the absence of any inoculation practices, we can assume that, at the end of the nursery stage, the *T. calobuxus* ECM fungal community was composed of ruderal species, which are easily dispersed by spores [17,40] and mostly adapted to survive in nursery conditions. Our observations of ECM diversity associated with *T. calobuxus* before planting were consistent with the observations reported by these authors.

Field conditions may eliminate or at least reduce populations of opportunistic nursery fungi to select typical forest ECM species [53]. At thirteen months after transplantation, the number of OTUs associated with *T. calobuxus* doubled and the number of OTUs shared with *A. spirorbis* increased from four to 10, also consistent with marked changes in the lineages' composition. Notably, we observed the disappearance of one ruderal lineage: /sebacina. At the same time, in *T. calobuxus* roots, we observed the appearance of new lineages shared with *A. spirorbis* such as /boletus, /scleroderma-pisolithus and /russula. These changes in the ECM fungal community associated with *T. calobuxus* suggest that 13 months after transplantation, *T. calobuxus* was at least partially integrated with the common ECM network previously established by *A. spirorbis*. The decrease in the number of nursery OTUs was probably filtered by the in situ soil properties.

Saplings planted at least 10 m away from *A. spirorbis* seemed to have better growth. Although the decrease in height was not significant in other treatments, two hypotheses could explain this observation: (i) higher competition to access resources under *A. spirorbis* coverage, and (ii) a shading effect of *A. spirorbis* on other *T. calobuxus*.

Considering our results from this field experiment, and our experience in ecological restoration in extreme New Caledonian soils whose aim is to recreate the previous plant diversity (or a similar one) and maintain it through time, we recommend as the first step, planting *A. spirorbis* with 1 m by 2 m spacing. At this density (5000 trees per hectare), the canopy will rapidly close, litter will begin to accumulate, and a new mycorrhizal network will establish itself.

Between three and five years after planting, thinning should be carried out to leave *A. spirorbis* with 10 m by 10 m spacing. A range of endemic species should be planted within one to five metres of the remaining *A. spirorbis*. Beyond the direct influence of the remaining *A. spirorbis*, it would be better to plant endemic ECM species such as *Tristaniopsis* spp.

Although there are no OTUs shared between *T. guillainii* and *T. calobuxus* at first, this situation may evolve according to time. One example is the dynamic of mycorrhization of *T. calobuxus*, where more and novel OTUs were found after one year of plantation (Figure 6). Reforestation is a long process, where each species brings their own benefits to the ecosystem. After some time, plants can be interconnected through a mycorrhizal network, increasing their resilience. Even if some species (e.g., *T. guillainii* and *T. calobuxus*) do not share OTUs at first, they could still be interconnected later through a mycorrhizal network including *A. spirorbis*. Moreover, other parameters as important as ECM should also be considered, such as arbuscular mycorrhizal fungi, plant growth promoting bacteria, etc. To conclude, we would recommend the use of both *Tristaniopsis* species in the reforestation process, even if they do not share common ECM OTUs at first.

Sannantha spp. or *Melaleuca* spp. could even benefit from the established ECM network.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d14020107/s1, Table S1: Chemical characteristics of soil from the plantation site located on the top of the Koniambo Massif, New Caledonia; Table S2: Coordinates of the 9 *T. guillainii* sampled for the characterization of their associated ECM fungal partners; Table S3: Average plant growth and survival of *T. calobuxus* according to plantation plots and distance from the *A. spirorbis* canopy; Table S4: Taxonomic and lineage identification of all OTUs according to UNITE database. Non-ectomycorrhizal lineages are not included in ECM community analyses; Table S5: Summary data of ITS sequences of ectomycorrhizal root tips deposited on DDBJ (http://www.ddbj.nig.ac.jp/, accessed on 5 September 2017); Table S6: Results of Chi square tests multiple comparisons of ectomycorrhizal lineages. Several data pools were created, to determine if the lineage community differs in relation to multiple factors: (A) host specificity (AS = Acacia spirorbis; TG = Tristaniopsis guillainii; TC = T. calobuxus), (B) the year of sampling (2015 versus 2016), (C) the distance of sampling from AS, expressed in meters (the "none" level corresponds to TG grown in nursery), and (D) the combination of all factors mentioned above (host + year + distance). For each table, the results report that both the Chi square values (bottom left part of the table) and their respective significance (top right part of the table), where NS is $p > 0.05$; * is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$.

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