Agroforest Syst (2024) 98:369–381
https://doi.org/10.1007/s10457-023-00915-2

Effects of *Tuber borchii* inoculation on *Pinus pinea* 3 years after establishment along a latitudinal gradient in the Southern Hemisphere

Verónica Loewe-Muñoz · Claudia Delard · Rodrigo del Río · Gianluigi Gregori · Mónica Balzarini

Received: 24 November 2022 / Accepted: 26 September 2023 / Published online: 7 October 2023

Abstract Stone pine (*Pinus pinea*) produces a highly demanded dried fruit, the pine nuts; *Tuber borchii* produces a well-known commercialized truffle. Thus, the co-culture of *P. pinea* and *T. borchii* may represents an interesting productive option. This work evaluated the effect of *T. borchii* inoculation on stone pine tree growth, survival, health, entry into cone production, and mycorrhization level during the first 3 years after establishment along a 2000 km gradient in Chile. We tested two treatments (inoculation and non-inoculation) in a multi-environment trial (MET) involving seven sites along Chile. Plantations were repeatedly measured during 3 years after establishment. In all environments, inoculation enhanced tree height, root collar diameter (RCD) and crown diameter (6.9, 10 and 8.3% higher for inoculated than for non-inoculated plants). Vigor was also favored (14.1% more vigorous trees). Stone pine performance was enhanced in all sites, but mainly in those with extreme environmental conditions. *T. borchii* mycorrhizae were abundant (over 60% of mycorrhized root apexes) 3 years after establishment. The truffle-host plant combination was not previously evaluated in Chile. The results showed high root colonization levels and the persistence of mycorrhization 3 years after establishment, and an enhanced effect of mycorrhization on tree growth and vigor under different soil and climate conditions, confirming the high plasticity of both species.

Keywords Mycorrhization · Stone pine · Truffle · Plantation establishment · Co-cultivation

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10457-023-00915-2.

V. Loewe-Muñoz (✉) · C. Delard
Chilean Forest Institute (INFOR), Sucre 2397, Ñuñoa, Santiago, Chile
e-mail: vloewe@infor.cl

V. Loewe-Muñoz · R. del Río
Centro Nacional de Excelencia para la Industria de la Madera (CENAMAD), Pontificia Universidad Católica de Chile, Vicuña Mackenna 4860, Santiago, Chile

G. Gregori
Centro Sperimentale di Tartuficoltura, Via Macina 1, 61048 Sant'Angelo in Vado, Italy

M. Balzarini
CONICET UFYMA Biometry Unit, Universidad Nacional de Córdoba, Av. Haya de La Torre S/N, Córdoba, Argentina

Introduction

Pinus pinea L. is a Mediterranean tree mainly cultivated in Spain, Portugal, Italy, Turkey and Tunisia. It is one of the most important nut species in the world (INC 2020) due to the high nutritional (Evaristo et al. 2013; Nergiz and Dönmez 2004) and culinary value of its pine nuts, which were consumed as far back as 150,000 years ago (Cortés-Sanchez et al.

2011). Despite the increasing pine nut demand (Sen et al. 2016) and high prices (INC 2019), production in Europe is costly and limited (Vanhanen and Savage 2013). Consequently, efforts are being made to cultivate stone pine for pine nut production in non-native habitats with Mediterranean climate, such as Argentina (Lutz et al. 2017), Australia (Holman 2020), New Zealand (Vanhanen and Savage 2013) and Chile (Loewe et al. 2016).

In many areas of Chile, the favorable conditions for the species cropping (Loewe et al. 2016) as well as technological advances (Loewe et al. 2017; Loewe-Muñoz et al. 2020a, b, 2021, 2022) have boosted an increase of the planted area from 100 to over 4000 hectares in the last decade. Indeed, stone pine is considered an emerging crop in Chile (Dube et al. 2015).

In ancient times Mesopotamians, Egyptians, Greeks and Romans appreciated truffles (Kaounas 2020), i.e., edible hypogeous fungi of some *Tuber* species. *Tuber borchii* Vittad. is the most noteworthy species of the *Puberulum* group (known as "Whitish truffle" or "bianchetto truffle" due to the peridium color), with a very complex and peculiar garlic-like flavor. It is ubiquitous in Europe, where it grows in cold, temperate and Mediterranean regions, usually in calcareous sub-alkaline soils, from coasts to hilly inland areas. *Tuber borchii* was first cultivated in Italy (Zambonelli et al. 2000) and is currently cropped in non-native countries such as New Zealand, Australia (Hall et al. 2017) and the US (Lancellotti et al. 2016). Despite the high prices of truffles, the life cycle of *T. borchii* is still poorly understood (Belfiori et al. 2016).

The association of *P. pinea* and *T. borchii* has been used to promote trufficulture in Europe (Bagnacavalli et al. 2012; Baglioni et al. 2016) as an attempt to develop cultivation strategies for truffle species other than *T. melanosporum* Vittad. (Incredible 2021). In non-native habitats, the association has been less explored, with some successful plantations having been reported in New Zealand (Hall et al. 2017).

In Chile, many farmers are interested in profitable and sustainable crops that can be established on marginal lands with low fertility and steep slopes, which do not compete with traditional food systems. *T. melanosporum* culture is an interesting option due to the high prices paid for its truffles in the national and international markets, and to the country climatic and soil conditions (FIA 2009; Ramírez et al. 2011; Cordero et al. 2011). *T. borchii* represents other appealing agroforestry alternative given its broad ecological requirements, allowing its cultivation in different environments, even in areas unsuitable for other truffle species, being its association with stone pine a novel strategy to boost rural disadvantaged economies that lack alternative profitable crops. Technical advantages that favor the implementation of this associated system include timing of biological cycles (stone pine cones are harvested in fall-winter, and truffles are collected in spring), and the lack of toxic residual chemical applications to stone pine, which could negatively affect truffle development.

Diverse effects of mycorrhization along environmental conditions and management have been reported (Bledsoe 1992; Rincón et al. 2001; Baciarelli Falini et al. 2012). In this work, we evaluated the effect of in-nursery inoculation with *T. borchii* on stone pine tree growth, vigor, survival and health, as well as mycorrhization levels, during the first 3 years after establishment in a multi-environment trial (MET) involving seven environments along a wide latitudinal range in Chile. We hypothesized that *T. borchii* mycorrhizal development and its effects on *P. pinea* tree performance would be positive along different latitudes.

Material and methods

Multi-environment trial

A MET involving seven sites was established in Chile in 2018–2019, and annually monitored until 2021. Environmental characteristics of plantation sites are presented in Table 1 and their distribution in Fig. 1.

The experimental trials were planted in winter with 1-year old *P. pinea* seedlings produced at INFOR's nursery. Inoculated and non-inoculated seedlings were grown in sterile substrate in 500 cm^3 containers. *T. borchii* used to inoculate seedlings was certified by the EU, and the species verification in each ascomata was performed by the Istituto Sperimentale per la Tartuficoltura, Italy, following guidelines on the truffle morphological features (peridium, gleba, asci, spores, smell) (ASSAM 2004). *T. borchii* was confirmed through molecular authentication (100%). Soil preparation in the plantation sites was performed considering soil texture, including complete ploughing and ripping

Table 1 Characteristics of test sites in the multi-environment trial

Site description	Unit	Environment						
		Pirque	Estrella	Cabrero	Yumbel	Chacaico	Villarrica	Exploradores
Location								
Latitude		33° 42′ 38.50″ S	34°12′29.17"S	36°56′42.51″ S	36° 57′ 27.67″ S	37° 31′ 58.91″ S	39° 12′ 14.37″ S	46° 35′ 34.01″ S
Longitude		70° 34′ 33.62″ W	71° 38′ 1.34″ W	72° 28′ 54.10″ W	72° 33′50.16″ W	72° 32′ 39.98″ W	72° 8′ 33.93″ W	72° 53′ 15.71″ W
Altitude	m a.s.l	686	213	113	127	100	295	300
Climate								
Rainfall	mm $year^{-1}$	457	515	1097	1163	1213	2450	1484
Average temperature	°C	20.1	18.8	18.4	18.2	17.7	15	9.6
Soil								
pH		5.8	6.6	6.7	6.2	6.3	6.7	8.1
Sand	%	85	68	95	74	74	55	77
Lime	%	11	16	3	4	22	40	13
Clay	%	3	16	2	22	4	5	10
Salinity, EC	mmhos cm^{-1}	0.24	0.02	0.10	0.82	–	0.09	0.48
Organic matter	%	1.49	0.4	0.7	5.4	1.0	9.6	1.6
N	mg kg^{-1}	103	26	3	48	2	11	40
P	mg kg^{-1}	23	< 4	2	8	6.7	2	6
K	mg kg^{-1}	148	< 50	73	165	1787	25	25
B	mg kg^{-1}	0.06	0.12	0.10	1.3	0.40	0.10	na
Zn	mg kg^{-1}	0.43	0.34	0.10	1.3	0.20	0.20	na
Mg	cmol + kg^{-1}	1.08	1.24	0.32	2.53	0.55	0.49	0.20
Plantation year		2018	2019	2018	2018	2018	2018	2018

on the plantation line at 30–40 cm depth. Seedlings were manually planted with a 6 × 6 m spacing. After planting, seedlings were irrigated (20 L $plant^{-1}$), and the following techniques were used: manual weed control three times a year; fertilization (zinc sulphate 20 g $plant^{-1}$, boronatrocalcite 25 g $plant^{-1}$, manganese sulphate 35 g $plant^{-1}$ at planting); formation pruning 3 years after planting; and complementary irrigation. Irrigation was provided from spring to the beginning of autumn through a drip irrigation system. Water supply was monthly calculated considering soil conditions and the hydric deficit of the previous year, and 25% of the hydric deficit was provided, following Olivera et al. (2014), who reported that mycorrhizal proliferation of black truffle is promoted with a certain degree of water deficit.

In each environment, a complete randomized block design was used to assess the effects of inoculation. Four replicates per treatment (inoculation and non-inoculation) were used in each trial. Experimental units (648 m^2 plots) had 18 plants, with a two-row isolating border. Growth, vigor, and health were annually measured in each plant, and strobili presence was monitored in spring.

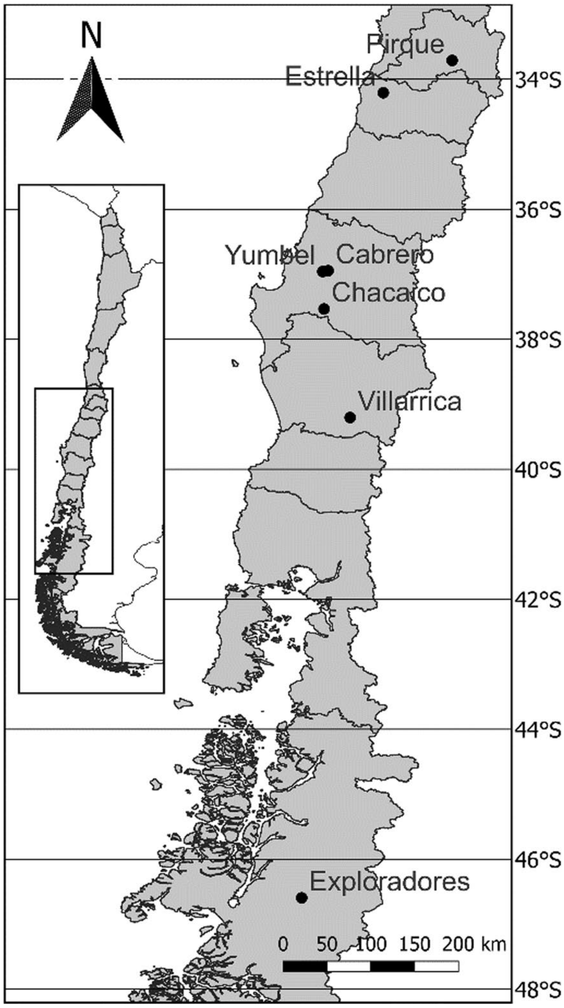

Fig. 1 Location of the multi environmental trial sites in Chile, South America

Measurements

Tree growth

The recorded growth variables were root collar diameter (RCD), measured with a digital caliper; tree height with a height pole; and crown diameter (distance between the crown projections of the longest living branches) with a tape. Vigor was evaluated in all trees by using a using a binary scale: (1) vigorous trees (abundant foliage, dark green needles) and (2) non-vigorous trees (abundant to regular foliage, light green to yellow needles, scarce foliage, strong discoloration), based on Lakatos and Mirtchev (2014). Plant health was evaluated as a binary trait (healthy and damaged plants, the latter category including any biotic or abiotic damage). To determine strobili entry into production, all branches of each tree were annually checked for the presence of male or female strobili. At age 3, the total number of vegetative shoots was recorded in each tree.

Root mycorrhization

The evaluation of root micorrhization in nursery and in the field one and 3 years after planting in each inoculated tree was performed with the same method. *T. borchii* presence was assessed by checking for mycorrhizal system, mantle and cystidia features as in Agerer (1996). For this procedure, each sample was placed on moist filter paper in a plastic bag, labeled, and kept at −18 °C until evaluation. Roots were observed under OMAX 2×-90×stereoscope and OMAX 40×-2500×light microscope, following the ordinal scale used in France (Giraud 1988) and Italy (Gregori et al. 2008). Scale scores were 0: absence; 1: (0–20%]; 2: (20–40%]; 3: (40–60%]; 4: (60–80%]; 5: (80–100%] of root apexes mycorrhized with *T. borchii*. Any other unidentified fungi were recorded as morphotypes and quantified using the same scale; when different fungi were simultaneously present in the same root area, their occupancy was recorded independently. Meanwhile in nursery we assessed the whole root system, in field established plants the evaluation was performed in a root sample randomly selected from one cardinal quadrant (North–East, South–East, South–West, North–West) around each tree (Giraud 1988). To describe each morphotype, anatomo-morphological characteristics described by Agerer (1996) were used.

Additionally, molecular identification was performed in frozen truffles used for inoculation and in randomly selected mycorrhized root tips collected 3 years after establishment on the field. To perform the molecular authentication, DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The complete nrDNA internal transcribed spacer (ITS) sequence was amplified with the primer pair ITS1/ITS4 (White et al. 1990). DNA was sequenced at CD Genomics (New York, US). The sequences obtained from the isolates were processed using pipeline DADA2 (Callahan et al. 2016). Then, sequences were identified to the highest similarity using an online BLASTN algorithm and comparing them with those available in the GenBank database from NCBI.

Statistical analyses

Tree height, RCD and crown diameter measurements were fitted using a generalized linear mixed model (Stroup 2012). A MET model was used including terms for inoculation, measurement year, and their interactions as fixed terms, and environment and block within environment effects as random effects normally distributed with zero mean and homogeneous variances. When the interaction among environments was significant, treatments were compared within each environment using the standard errors estimated from the longitudinal MET data.

As non-normal variables, we analyzed survival, vigor and health using a generalized linear mixed model for proportions with a logit link function (Stroup 2012). Test sites involved in the study were ordinated with Principal Component Analysis (PCA) using climatic variables as source of variability. Statistical analyses were performed using the software Infostat (Di Rienzo et al. 2022) and its interface with R (https://www.r-project.org/).

Results

The PCA analysis showed the main climatic differences along the studied latitudinal gradient. Villarrica and Exploradores are characterized by the highest rainfall and hydric index throughout the year, whereas Pirque has the highest maximum temperature; Yumbel, Cabrero and Chacaico share similar climatic conditions, and Estrella has a low rainfall (Fig. S1).

Tree growth, vigor and survival

3 years after establishment, inoculation effect was statistically significant for stone pine tree height ($p<0.0001$), RCD ($p<0.0001$), crown diameter ($p<0.0001$) and vigor ($p=0.0001$), being higher in inoculated than in non-inoculated trees (Table 2, Fig. 2). No differences in survival ($p=0.94$) or healthy trees ($p=0.99$) were observed between inoculated and non-inoculated trees.

Table 2 Stone pine (*Pinus pinea*) inoculated (I) and non-inoculated (NI) with *T. borchii* 3 years after establishment

Site	Height (cm)		Root collar diameter (mm)		Crown diameter (cm)		Vigorous trees (%)		Survival (%)		Healthy trees (%)	
	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI
Pirque	84.4 ± 2.0 a	74.8 ± 2.8 b	32.0 ± 2.0 a	24.9 ± 2.2 b	64.8 ± 3.7 a	50.9 ± 4.2 b	59 ± 8 a	38 ± 10 a	86 ± 4 a	86 ± 6 a	100 ± 0 a	100 ± 0 a
Estrella	104.9 ± 3.2 a	110.8 ± 3.7 a	51.0 ± 1.4 a	52.3 ± 2.1 a	55.7 ± 1.9 a	58.3 ± 2.1 a	90 ± 3 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Cabrero	122.7 ± 4.2 a	100.1 ± 4.8 b	58.9 ± 2.0 a	47.3 ± 2.2 b	81.7 ± 2.2 a	72.5 ± 2.5 b	55 ± 10 a	37 ± 11 a	89 ± 5 a	95 ± 4 a	100 ± 0 a	100 ± 0 a
Yumbel	109.2 ± 8.3 a	109.7 ± 8.8 a	59.5 ± 3.7 a	59.1 ± 4.1 a	88.9 ± 6.1 a	88.5 ± 6.7 a	100 ± 0 a	100 ± 0 a	97 ± 2 a	86 ± 6 a	100 ± 0 a	100 ± 0 a
Chacaico	119.9 ± 5.4 a	132.8 ± 6.5 a	63.7 ± 2.1 b	69.5 ± 2.7 a	98.8 ± 4.1 a	100.9 ± 5.9 a	82 ± 5 a	88 ± 6 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Villarrica	117.6 ± 1.8 a	99.9 ± 2.5 b	41.5 ± 0.7 a	35.0 ± 1.0 b	41.9 ± 2.5 a	37.8 ± 2.6 b	99 ± 1 a	97 ± 3 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Exploradores	46.9 ± 1.1 a	35.1 ± 2.4 b	23.7 ± 1.9 a	13.5 ± 2.4 b	26.8 ± 1.8 a	16.4 ± 2.3 b	29 ± 11 a	0 ± 0 b	95 ± 5 a	89 ± 12 a	91 ± 5 a	60 ± 17 b
Mean†	100.9 ± 1.8 A	94.4 ± 2.1 B	47.3 ± 0.9 A	43.0 ± 1.1 B	65.6 ± 1.8 A	60.6 ± 2.1 B	73 ± 5 A	64 ± 7 B	95 ± 2 A	93 ± 2 A	90 ± 5 A	86 ± 7 A

Mean values ± standard error. For each environment, different letters indicate statistical differences among treatments ($p<0.05$)

†Mean across sites except for Estrella, which was planted 1 year later

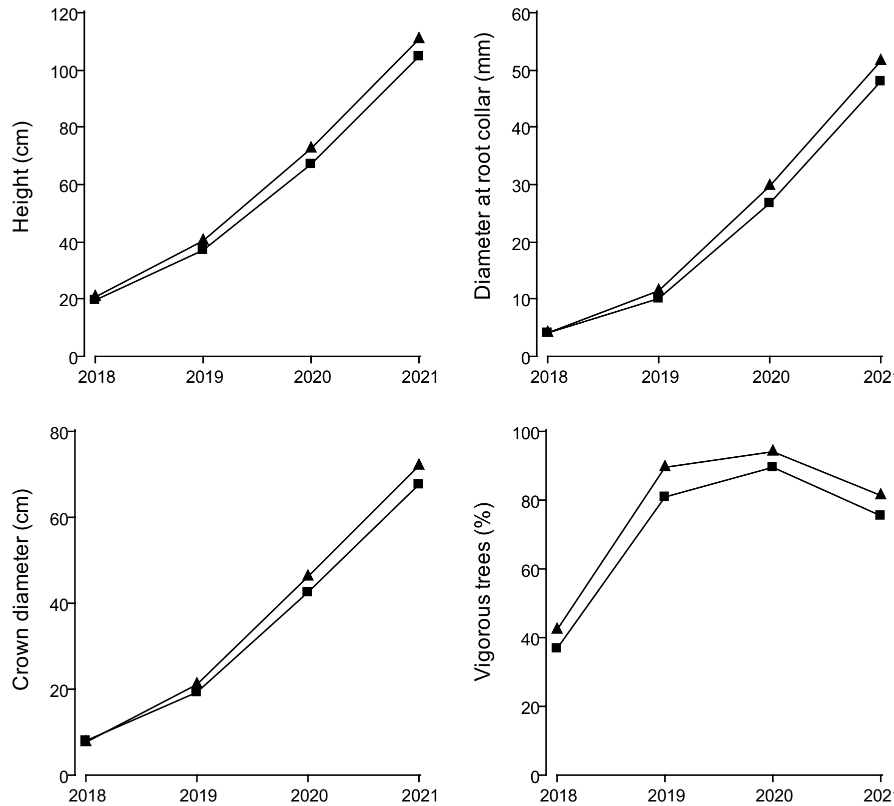

Fig. 2 Stone pine tree growth profiles in seven environments by treatment. Inoculated with *T. borchii*: triangles; Non-inoculated: squares

Inoculated trees across sites had a 6.9% higher height than non-inoculated trees, 10% higher RCD and 8.3% higher crown diameter (Table 2). Inoculated trees showed important increases in height in Pirque (> 12.8%), Cabrero (> 22.6%), Villarrica (> 17.7%) and Exploradores (> 33.6%); in RCD in Pirque (> 28.5%), Cabrero (> 24.5%), Villarrica (> 18.6%) and Exploradores (> 75.6%); and in crown diameter in Pirque (> 27.3%), Cabrero (> 12.7%), Villarrica (> 10.8%) and Exploradores (> 63.4%). Inoculation also significantly increased vegetative shoots in four out of seven sites (Pirque (> 62.4%, $p < 0.0001$), Cabrero (> 20.1%, $p = 0.0012$), Villarrica (> 46.8%, $p < 0.0001$) and Exploradores (> 2.75 times, $p < 0.0001$) (Fig. 3).

Regarding strobili presence, no female or male conelet was observed in any tree. As per vigor, a positive and significant effect of inoculation was observed across sites (> 14.1%); in Exploradores no non-inoculated trees were vigorous, whereas 29% of the inoculated trees were vigorous. Survival was high in all sites and in both treatments, without statistical significance, ranging between 86 and 100%. Finally, in all sites no differences in percentage of healthy trees were found between inoculated and non-inoculated trees.

Mycorrhization

Seedlings in nursery had an average mycorrhization score of 3.7, with at least 40% of micorrhized root apexes. In field, all inoculated trees presented a high percentage of *T. borchii* mycorrhization 3 years after establishment (Table 3). At age 3, inoculated trees presented an average mycorrhization score of 4.9, i.e. over 60% of root apexes mycorrhized with *T. borchii* (Table 3, Fig. S2). Although the climatic characteristics differ along sites located along a wide latitudinal range, mycorrhization with *T. borchii* was high in all sites. Figure 4 shows the biplot of the two principal components by sites, *T. borchii* mycorrhization and climatic variables, which explained 90.6% of the variability; it shows that mycorrhization with *T. borchii* was favored by higher minimum temperatures (higher than 5 °C). Exploradores and Villarrica present high rainfall and low hydric deficit, and Pirque

Fig. 3 Number of vegetative shoots in 3-year-old stone pine trees inoculated (black) and non-inoculated (white) with *T. borchii* in seven environments. Estrella trial was evaluated 2 years after establishment

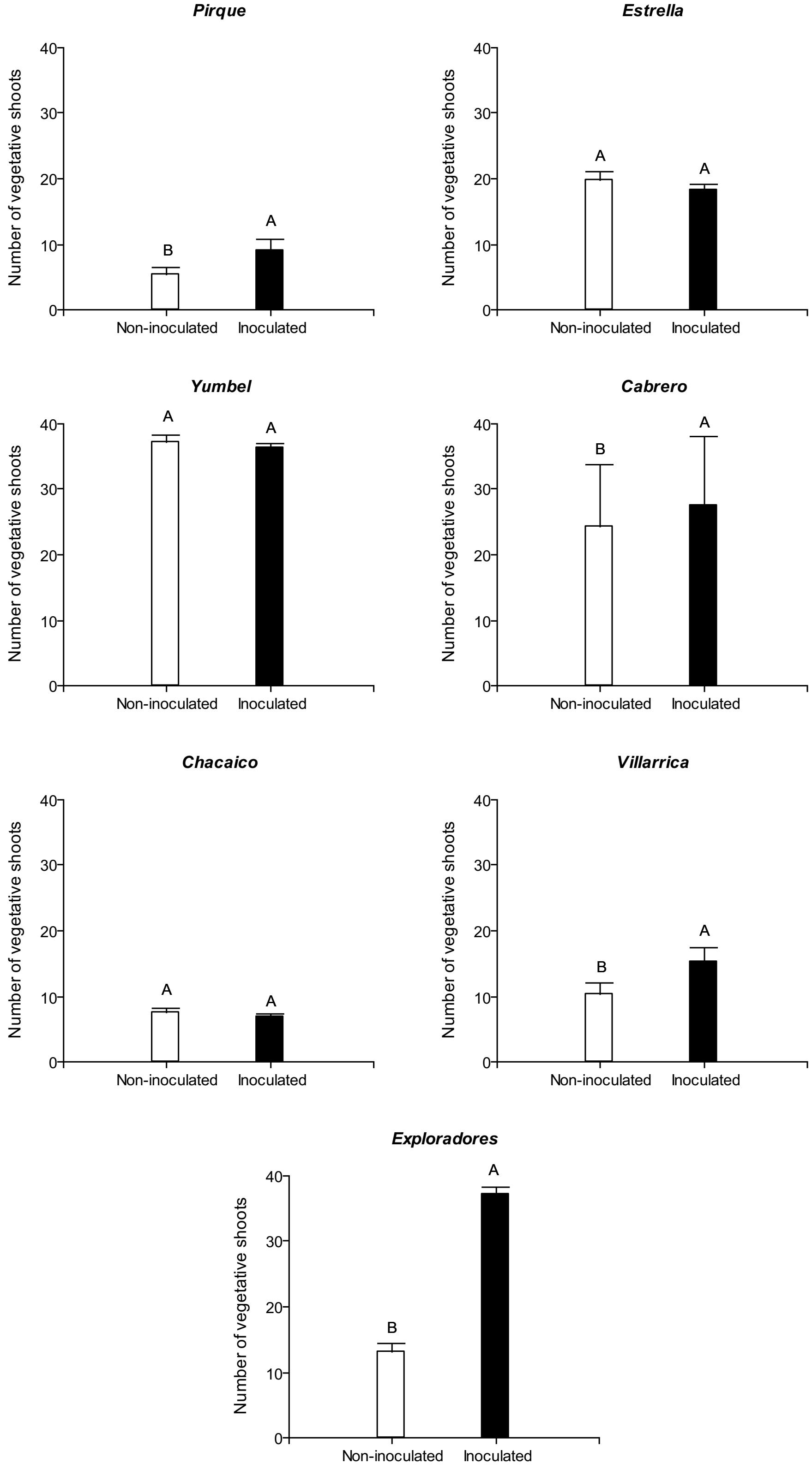

Table 3 Characteristics of root apexes of stone pine plants inoculated with *T. borchii* 1 and 3 years after establishment in seven sites along Chile

Site	Number of root apexes micorrhized with *T. borchii*		Number of root apexes with presence of at least one morphotype		Dead root apexes		Alive non-mycorrhized root apexes	
	Year 1	Year 3	Year 1	Year 3	Year 1	Year 3	Year 1	Year 3
Pirque	4.8 ± 0.2 ab	4.7 ± 0.3 b	0.6 ± 0.3 bc	0.0 ± 0.0 d	0.0 ± 0.0 a	0.3 ± 0.3 a	0.6 ± 0.3 a	0.2 ± 0.1 ab
Estrella†	–	5.0 ± 0.0 a	–	1.1 ± 0.3 c	–	0.0 ± 0.0 a	–	0.1 ± 0.1 ab
Cabrero	4.8 ± 0.1 b	5.0 ± 0.0 a	0.6 ± 0.2 bc	2.6 ± 0.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.5 ± 0.1 a	0.2 ± 0.1 ab
Yumbel	4.8 ± 0.1 ab	5.0 ± 0.0 a	2.2 ± 0.3 a	2.6 ± 0.1 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.3 ± 0.2 ab	0.0 ± 0.0 b
Chacaico	4.9 ± 0.1 ab	5.0 ± 0.0 a	1.6 ± 0.6 ab	2.1 ± 0.1 b	0.0 ± 0.0 a	0.0 ± 0.0 a	0.1 ± 0.1 c	0.3 ± 0.1 a
Villarrica	5.0 ± 0.0 a	4.9 ± 0.1 b	1.1 ± 0.3 ab	0.8 ± 0.2 c	0.0 ± 0.0 a	0.0 ± 0.0 a	0.1 ± 0.1 bc	0.3 ± 0.1 a
Exploradores	5.0 ± 0.0 a	–	0.2 ± 0.1 c	–	0.1 ± 0.1 a	–	0.1 ± 0.1 bc	–
Mean	4.9	4.9	1.0	1.5	0.1	0.1	0.3	0.2

Mean values ± standard error. Different letters indicate statistical differences among environments ($p < 0.05$)

Ordinal scale [0 to 5]: 0: absence; 1: up to 20%; 2: (20–40%]; 3: (40–60%]; 4: (60–80%]; 5: (80–100%]

†Evaluation 2 years after establishment

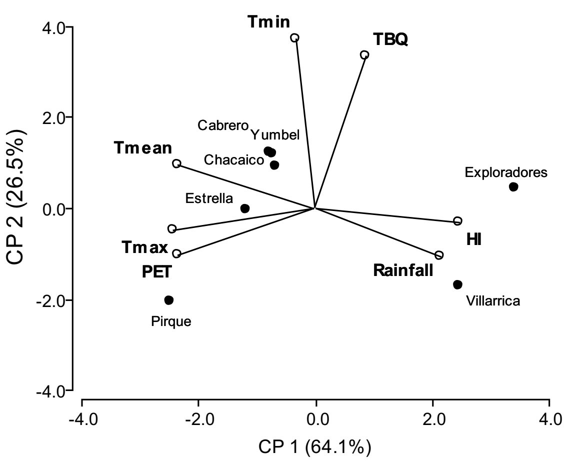

Fig. 4 Biplot of the principal component analysis by sites, *T. borchii* mycorrhization and climatic variables. Tmax: maximum average temperature, Tmean: average temperature, Tmin: minimum average temperature, PET: Potential Evapotranspiration, HI: Hydric index (HI = Rainfall minus Potential Evapotranspiration), TBQ: *T. borchii* mycorrhization abundance

is characterized by high PET and maximum temperature, and along with Villarrica, high minimum temperature.

The mean score for alive non-mycorrhized root apexes was 0.2, indicating a high colonization of the root system by *T. borchii*. No dead root apexes were recorded in six environments; in the remaining environment, the score was 0.1 (Table 3).

Results showed that mean mycorrhizal colonization with *T. borchii* can be overlapped with other fungi colonization, as for example in Yumbel and Chacaico. 1 year after establishment, four morphotypes were identified, with a higher abundance in Yumbel and Chacaico (Table 3). Morphotypes were descript as follows: (1) semi-bright cottony white mycelium covering adjacent roots and dead mycorrhizae; (2) beige irregular cottony mycelium covering root tips *T. borchii* mycorrhiza; it adheres to the substrate; (3) semi-bright white padded mycelium that forms mycelial cords and covers *T. borchii*; and (4) almost transparent filamentous mycelium, covering mycorrhizae and roots; it adheres to the substrate.

At age three, all trees had presence of the same most prevalent morphotype 1 (Fig. 5), with an inferior abundance in the driest and warmest environment, Pirque; Cabrero, Yumbel and Chacaico presented higher abundance of other mycorrhizal fungi (Table 3). The average score of number of root apexes with presence of at least one morphotype across sites increased from 1.0 to 1.5 from year 1 to 3.

Sequencing of randomly selected ectomycorrhiza from inoculated *P. pinea* trees confirmed the identity with *T. borchii* (96.4% to 100%). Other ectomycorrhizal fungi identified were S*uillus luteus* (L. Fries) Gray*, Suillus pseudobrevipes* A.H. Sm. & Thiers*, Wilcoxina mikolae* (Chin S.Yang &

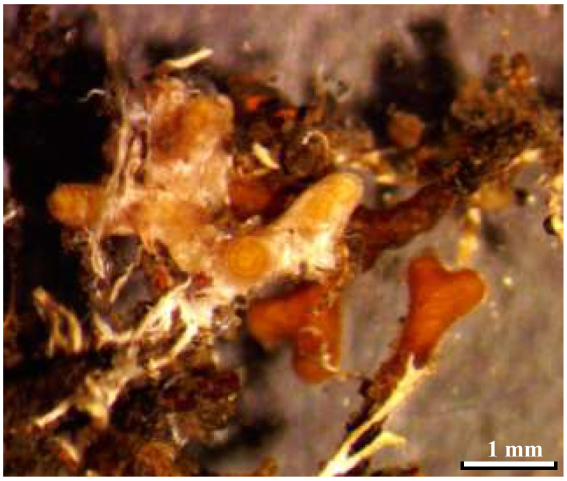

Fig. 5 Morphotype 1 found on stone pine trees mycorrhized with *T. borchii* 1 and 3 years after establishment in seven environments

H.E.Wilcox) Chin S.Yang & Korf, *Tomentella coerulea* Höhn. & Litsch. and *Tomentella viridula* Bourdot & Galzin.

Discussion

Tuber borchii cultivation associated with *Pinus pinea* it is not a scientific novelty. Indeed, the first productive plantation using the same combination host plant-truffle species was published in 2000 in Italy (Zambonelli et al. 2000). In the last decade, *T. borchii* cultivation with *P. pinea* was successfully introduced also in Australia and New Zealand (Vanhanen and Savage 2013; Hall et al. 2017; Holman 2020). However, the published results come from reduced areas. In Chile, *P. pinea* has been introduced long ago (Loewe-Muñoz and Noel 2021) and its cropping has expanded in a wider latitudinal range, generating the need of studying the association with *T. borchii* along plantations from different latitudes. The effects of *T. borchii* inoculation on stone pine growth, survival and mycorrhization are here explored for the first time along a wide latitudinal gradient of the Southern Hemisphere, demonstrating the possibility to introduce also in Chile the co-cropping of pine nuts and truffles.

Tree growth and survival

In *Pinus* species, mycorrhization with *Tuber* was reported as beneficial for growth (Harley and Smith 1983), i.e., a positive effect of *T. borchii* inoculation on seedling growth was reported by Angelini et al. (2004). This effect has been attributed to the ability of mycorrhizae to increase water and nutrient uptake and CO_2 assimilation by plants (Domínguez-Nuñez et al. 2004; Morcillo et al. 2015). Similarly, we also found a beneficial effect of inoculation. In average across sites, inoculated 3-year-old stone pine trees had greater stem and crown diameters, heigher height and superior vigor than non-inoculated trees. However, in Estrella, Yumbel and Chacaico, the impact of *T. borchii* on growth was not observed, which could be attributed to extra water supply from other sources. Although control plants were produced in a sterile substrate and not inoculated, they could have been mycorrhized after establishment on the field with native mycorrhizal fungi which could also promote plant growth.

The beneficial impacts of *Tuber borchii* on stone pine growth observed in most of the sites agree with findings published for other species. Bratek (2008) reported a greater height in *Tuber*-mycorrhized *Pinus nigra*, and Martínez et al. (2004) a three-fold height increase in mycorrhized seedlings of forest species. Mycorrhization also had a positive effect on RCD (10%), as reported for *Pinus halepensis* plants inoculated with *Tuber melanosporum* (Domínguez-Nuñez et al. 2004).

Growth of inoculated trees was significantly higher in four environments, with the highest gains (up to 33.6% in height, up to 75.6% in RCD, up to 63.4% in crown diameter, and up to 2.7 times in number of vegetative shoots) being found in Exploradores, the southernmost and coldest environment. Similarly, Gómez-Romero et al. (2019) found that the inoculation of *Pinus pseudostrobus* with *Pisolithus tinctorious,* another ectomycorrhizal fungus, growing together with *Eysenhardtia polystachya*, significantly increased growth (number of branches). Vigor was also enhanced by inoculation in Exploradores.

Even though the beneficial effect of *Tuber* mycorrhization on tree survival was reported (Yin et al. 2018), as well as of other ectomycorrhizal fungi on *Pinus thunbergii* (Nakashima et al. 2016), in our study no differences were observed in tree survival, probably because it was very high (over 86%) in all sites regardless of the inoculation status.

No differences were found in the tree sanitary status, showing a robust performance of stone pine along different latitudes. Regarding strobili appearance, no male or female conelets were observed at age three, which is expected considering the species biological cycle (Loewe et al. 2016).

The high stone pine plasticity along more than 2000 km of latitude observed in Chile, with a good adaptation to a broad range of environments, can be understood as an advantage of the species to buffer climate variability, as reported by Pardos and Calama (2018).

Mycorrhization

In the field, inoculated trees presented a high percentage of *T. borchii* mycorrhized root apexes (over 60%) 3 years after establishment, indicating the successful persistence of mycorrhization with this cultivated truffle in different environments. Furthermore, *T. borchii* colonization was found in all trees in all of the tested environments. This is relevant, since Zambonelli et al. (2000) reported a reduction in colonization (from 95.8% to 42.8%) of stone pine trees inoculated with this truffle in nursery 4 years after planting. The average colonization with *T. borchii* found by Benucci et al. (2012) in 10 month-old pecan seedlings was 62%. The very low percentage of live non-mycorrhized root apexes found in this work indicates a high mycorrhization of the root system. This result could be explained by the average temperature (below 34 °C) in all tested sites, a condition that favors root colonization (Leonardi et al. 2017). In Chile, average temperature and thermal oscillation are lower than in stone pine (Loewe et al. 2016) and *T. borchii* (Salerni et al. 2014) native ranges.

We found that *T. borchii* was also able to compete with other native ectomycorrhizal fungi present in the soil. In fact, the low presence of other fungi observed in all sites, and especially in the most extreme environments is positive, and agrees with results from New Zealand (Hall et al. 2017). One of the major reported problems affecting other truffle orchards has been the competition with other fungi after the establishment in the field (Bratek 2008). We found a slight increase in the abundance of other fungi from the first to the third years after establishment, with no fungal competition in this combined system so far, confirming the close association between *P. pinea* and *T. borchii*. In fact, Zambonelli et al. (2000) reported an increase of colonisation (from 0% to 34.7%) by other fungi in a stone pine plantation inoculated with *T.borchii* 4 years after planting. It is interesting that the composition of other fungi present along *T. borchii* decreased along time to just one morphotype at age three. A molecular characterization of the most prevalent morphotype 1 could be useful to know if this contaminant is the same one found in European *Tuber borchii* truffle grounds. Ori et al. (2023) performed a molecular characterization of ectomycorrhizae from a plantation of *Pinus pinea, Quercus pubescens, Quercus robur* and *Corylus avellana* inoculated with *T. borchii*, established in a farming area where this truffle is not present, and identified 21 ecotomycorrhizal species, including *Tomentella coerulea* among the most abundant. This species was also found in our study.

T. borchii is the truffle with the broadest ecological amplitude, a characteristic that favours its cultivation in different environments, including those unsuitable for other *Tuber* species (Zambonelli et al. 2002). On the other hand, the high plasticity of stone pine and its adaptability to different conditions has been reported (Mutke et al. 2010). Therefore, the co-cultivation of this precious truffle on stone pine is feasible under several different environmental conditions. 3 years after planting in New Zealand, the first ascomata were found (Iotti et al. 2016); we checked for ascoma production at age three in four plantations and although dogs heavily marked couldn't extract any, probably because of their small size and their location deeper than 25 cm that was the depth in which dogs were allowed to explore in order to avoid damaging the roots of these young trees.

Even though the assessment of the degree of mycorrhizal colonization using an ordinal scale is not only used to certify commercial mycorrhized plants (Giraud 1988; Gregori et al. 2008) but also in scientific evaluations (Donnini et al. 2014; Murat 2015), future evaluations should be performed counting all apexes from each sample.

In some areas with high hydric deficit, irrigation from spring to early autumn is a recommended practice in truffle orchards (Garcia-Barreda et al. 2020), inhibiting the formation and growth of other mycorrhizal fungi (Le Tacon 2021). A positive effect of a limited irrigation (100 mm during summer to prevent reaching the wilting point) in an adult *T. melanosporum* orchard production was reported by Le Tacon (2021), indicating that a favorable water regime during summer is essential to ensure a proper initiation of carpophores.

Inter-annual variations in *T. melanosporum* production are associated with climate (Garcia-Barreda and Camarero 2020); hence, climate variability may become a major threat to truffle production. In our study, a low irrigation was provided in all sites with a hydric deficit above 750 mm. Thus, future studies testing different doses of water supply are necessary to deliver information of practical importance for boosting the association of stone pine and *T. borchii*.

The cultivation of *T. borchii* is already structured in European countries and New Zealand, but for South America it can be an important challenge. In this context, this study developed at different latitudes can be useful for agroforestry applications throughout the American continent.

Conclusions

This study reports the successful establishment of *P. pinea* trees mycorrhized with *T. borchii* in Chile. 3 years after establishment, stone pine tree growth and vigor was enhanced by the inoculation with this truffle, which maintained high root colonization levels along a 2000 km latitudinal gradient in Chile. This indicates the adaptation potential and stability of the stone pine and *T. borchii* association, which opens the possibility of this co-cropping to add commercial value to pine nut production in South America.

Acknowledgements The authors thank Dr. Davide Sisti from Urbino University, Italy, for assisting in seedling mycorrhization certification in nursery, and Jorge González from INFOR, for assisting with molecular authentication procedures.

Author contributions VL wrote most of the paper. CD directed the project that funded the study, and designed the study along with MB, GG and VL. CD was in charge of measurements and field activities and contributed to the paper elaboration. RDR and MB performed the statistical analyses, elaborated figures and collaborated in information gathering and analysis. GG directed the inoculation and evaluation of mycorrhization and participated in the preparation of the paper. All authors discussed the results and implications, and commented on the manuscript at all stages.

Funding Trial establishment and measurements were funded by the Foundation for the Agriculture Innovation (FIA) [Development of a combined truffle and stone pine nut productive model, profitable productive alternative in a scenario of increasing hydric restrictions, PYT-2016-0038]. Analyses were funded by the Chilean Ministry of Agriculture [Development and contributions for the use of high value forest and fruit-forest species for Chile, INFOR] and by ANID BASAL FB210015.

Data availability The datasets generated and analyzed during the current study are not publicly available due to institutional guidelines. They are available from the author upon request.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare that they have no conflict of interest.

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