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RESEARCH ARTICLE

Exploring rhizobial diversity in tara (*Caesalpinia spinosa*) by trapping with pea (*Pisum sativum*)

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Abstract

Tara (*Caesalpinia spinosa*) is an emblematic legume tree of Peruvian dry forests and is a multi-purpose tree for tannins and gum, in particular. Despite its importance, the microbiological aspects associated with tara are not currently considered in forest management, and its nodulation status remains contentious. This study sought to confirm or deny *C. spinosa's* nodulation status and, using *P. sativum* as a trap plant, to investigate the effects of *C. spinosa* on rhizospheric rhizobial communities. The study revealed a lack of tara nitrogenase activity and that *C. spinosa* is a non-nodulating species. Soil samples were collected from a tara plantation to investigate their effect on tara and pea growth, in a tara planting row (R), between 2 rows (IR), and outside the plantation (OP). For the total biomass growth parameter, soil R significantly promoted tara and pea growth. For root length and leaf chlorophyll content, there was a significant difference in favor of *C. spinosa* grown on R and IR soils compared with OP soil. Fifty-seven pea strains were characterized by analyzing the partial 16S-23S rDNA intergenic spacer. The phylogenetic tree showed high diversity with five clusters of *Rhizobium* spp. in the *R. leguminosarum–etli* clade and phylogenetic specificity according to soil origin. This study provides information of interest on the non-nodulating nature of *C. spinosa* and demonstrates the substantial influence of tara on rhizospheric bacterial communities. The results of this study highlight the need to integrate microbiological factors into forest management strategies to improve the ecological sustainability and agricultural yield of tara plantations.

Keywords: *Caesalpinia spinosa*; *Pisum sativum*; Rhizobium; 16S-23S rDNA intergenic spacer (IGS); agroforestry.

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1. Introduction

Caesalpinia spinosa (Molina) Kuntze belongs to the Caesalpinioideae sub-family of legumes and is a native species of Peru, commonly known as tara, which is widely distributed in Latin America from Venezuela to the north of Chile (de la Torre, 2018). *C. spinosa* is an evergreen thorny shrub or small tree that grows naturally in semi-arid regions with an annual rainfall of 230 mm-500 mm and average yearly temperatures of 14.7 ºC-27.5 ºC (Dostert et al., 2009). In Peru, it thrives in forests and in arid and semi-arid scrubland zones of coastal hills, inter-Andean coastal hills, and inter-Andean valleys (de la Torre, 2018). Tara is emblematic of an agroforestry system dating back to the Inca period, with this multi-purpose tree capable of intercepting fog and condensing mist, reflecting sophisticated Inca land management practices involving other native legume trees and husbandry (Balaguer, 2011). Tara was already used by pre-Inca and Inca populations for tanning, dyeing and medicinal purposes (Sangay-Tucto & Duponnois, 2018). Currently, Peru is the world's largest producer of tara pods and gum, with production mostly originating from natural forests: 75% of Peruvian pod production comes from natural forests and only 25% from plantations (Barriga Ruiz, C. A., personal communi-

cation, 2017). However, tara production in Peru varies significantly, ranging from 10 kg/plant to as much as 40 kg of pods per year in two harvests, depending on forest management (De la Cruz Lapa, 2004). Tara can be cultivated for soil protection (Mancero, 2009) and slope stabilization (De la Cruz Lapa, 2004), used in monocultures for industrial purposes, in relict forests converted to monospecific forests, and in agroforestry systems establishing tara plantations in association with pasture, or crops such as maize, peas, and shrubby plants or trees like *Vachellia macracantha* (formerly *Acacia macracantha*) (de la Torre, 2018). Cordero et al. (2016b) characterized the rhizobia associated with *V. macracantha* in Peruvian soils to select potential inoculants for ecological restoration based on potential synergistic effects between the two legumes. Certain rhizobial strains improved the photochemical efficiency of tara seedlings. Subsequently, Cordero et al. (2024) tested the effects of biofertilization with plant growth-promoting rhizobacteria (PGPR) on *C. spinosa*. Certain strains significantly improved tara's ability to withstand drought. Outside the cited studies, forest management techniques do not seem to consider microbiological interactions in the soil, or the microbial partners of tara, be they symbiotic or not. As regards the mycorrhizal status of *Caesalpinia spinosa*, Sangay-Tucto et al. (2017) showed tara to be primarily associated with arbuscular mycorrhizal (AM) fungi affiliated with the Glomeraceae family, while Zurita et al. (2021) showed that the rhizosphere of *C. spinosa* is associated with ectomycorrhizal fungi, mainly Basidiomycetes and *Rhizoctonia* species. In terms of *C. spinosa* nodulation status, several studies (Aleman, 2009; Mancero, 2009; Marien & Delaunay, 2010) reported that tara ought to host root rhizobial bacteria and fix atmospheric nitrogen. Indeed, Ogata (2006) confirmed the presence of nodules on tara roots in a plantation in the Peruvian department of Huanuco. However, within the formerly defined Caesalpinioideae large sub-family of legumes to which tara belongs, only between 10% and 20% of the subfamily species display root nodules. Currently, of the six defined legume subfamilies, nodulation is only present in two subfamilies, Papilionoideae and Caesalpinioideae, with the latter subfamily now including the mimosoid clade (LPWG, 2017). As for Cordero et al. (2016a), the authors suggested that tara is a non-nodulating species. Subsequently, Zurita et al. (2021) confirmed that although *C. spinosa* is a legume, it does not have nodules on its roots. The aim of this study was therefore, first, to corroborate or refute the existence of nodules on tara roots under different substrate and culture conditions. Regardless of the nodulation status of *C. spinosa*, the hypothesis that the presence of *C. spinosa* would have a significant impact on rhizospheric bacterial communities was evaluated. For such purpose, a phylogenetic analysis was conducted on rhizobia present inside and outside the tara plantation.

2. Methodology

2.1. Soil and plant materials

Study site

The study site was the Canchacalla tara plantation, located in the Huanuco region (S 10°10.654'; W 076° 10.29'), Ambo province, at an altitude of 2459 m, in Peru. Soil was collected at a depth of 20 cm from the planting row (first treatment called "R"), the interrow at one meter from the base of the *C. spinosa* trunk (second treatment called "IR"), and in a field outside the plantation where tara had never grown (third treatment called "OP"). The Canchacalla tara plantation of 175 ha had been established seven years earlier and was temporarily irrigated and fertilized by drip 6 months per year from May to October along the tara planting row (R). The results of the soil chemical analysis are presented in Table 1.

C. spinosa root observation

To determine the nodulation ability of *C. spinosa*, 30 root samples (5 trees x 3 replicates x 2 locations) were harvested at a depth of 20 cm at the foot of trees in the planting rows (R). Root samples were thoroughly rinsed and observed under a stereomicroscope (Olympus SZH10).

Table 1
Chemical characteristics of the soil samples collected under adult *Caesalpinia spinosa* trees (R), between the *C. spinosa* planting rows (IR) and outside the *C. spinosa* plantation (OP) in the Canchacalla plantation, Peru

	Soil origin		
	OP	IR	R
pH (H_2O)	6.11 (0.14) [1] b [2]	6.06 (0.29) ab	5.34 (0.29) a
Total nitrogen (%)	0.04 (0.009) a	0.07 (0.02) b	0.05 (0.01) ab
Total carbon (%)	0.48 (0.11) a	0.86 (0.23) b	0.64 (0.13) ab
C/N	13.8 (0.82) a	13.4 (0.41) a	13.9 (0.71) a
Total phosphorus ($mg.kg^{-1}$)	408.1 (68.8) a	560.9 (64.1) b	894.1 (156.7) c
Soluble phosphorus ($mg.kg^{-1}$)	2.95 (0.81) a	8.16 (1.76) b	82.3 (31.9) c

[1] Standard error. [2] Data in the same line followed by the same letter are not significantly different according to the Newman-Keul's test ($p < 0.05$).

2.2. *Caesalpinia spinosa* nodulation tests

Three *in vitro* nodulation assays

In vitro nodulation test with the inoculation of a Huanuco planting row (R) soil suspension: test tubes (Gibson, 1980) were filled with 40 ml of Jensen medium (Vincent, 1970) and tara seedlings were inoculated with 2 ml of soil suspension (1 g of soil and 20 ml of distilled water).

In vitro nodulation test of tara in a sand and soil mixture: tara seedlings were transferred to test tubes filled with sterile sand and 14 g of (R) soil.

In vitro nodulation test of tara in an attapulgite and soil mixture: tara seedlings were transferred to test tubes filled with sterile calcined attapulgite (Oil Dri US Special, Damolin, Denmark) and 14 g of (R) soil. All plants (each assay in ten replicates) were kept in a growth chamber at 22 °C / 18 °C during the day/night, with a 16: 8 L:D photoperiod and a relative humidity of 60% - 70% and were watered once a week with sterile distilled water. *C. spinosa* was harvested eight, five, and five months after inoculation, respectively.

C. spinosa nodulation test under greenhouse conditions

The nodulation test took place in plastic pots (5 replicates) with one tara seedling per pot filled with 300 ml of a mix comprising 20% soil (R, IR, or Huanuco OP) and 80% sterile sand. They were kept under greenhouse conditions at 22 °C / 15 °C during the day/night, with a 14:10 L:D photoperiod. Tara plants were watered three times per week with deionized water. The plants were harvested six months after inoculation.

2.3. *C. spinosa* growth, nitrogenase activity and leaf chlorophyll content under three soil conditions

After the greenhouse experiment, tara plants were collected and N_2 effectiveness was estimated by measuring leaf chlorophyll content with a SPAD52 chlorophyll meter (Minolta Soil-Plant Analyses Development). Nitrogenase enzyme activity by the acetylene reduction assay (ARA) was determined by gas chromatography as described by Renier et al. (2011). For growth estimation, roots were scanned at 300 dpi (Epson Perfection 2450 photo; Seiko Epson) and root length was measured using Optimas 6.1 image analysis software (Media Cybernetics). Lastly, tara plants were dried for 7 days at 50 °C to measure the aerial and root dry biomass.

2.4. Trapping of rhizobia from tara plantation soils (R, IR) and from soil outside the plantation (OP) with *P. sativum*, strain isolation, and effect of the soils on *P. sativum* growth

Native rhizobial populations on a tara plantation in Ambo province were studied using *Pisum sativum*, a crop traditionally associated with tara, as a trap plant. This was done to assess the share of symbiotic bacterial biodiversity on this tara plantation, along with a control in an area outside the plantation where tara had never grown before. Thus, rhizobia from the three soils (R, IR, and OP) were trapped using *P. sativum*. The pea seedlings in five replicates were grown under greenhouse conditions at 22 °C / 15 °C during the day/night, with a 14:10 L:D photoperiod in 250 g pots filled with a soil/sand mixture (1/4; v/v) used for each soil. One month later, pea nodules were thoroughly rinsed and surface-sterilized with 70% ethanol for 1 minute and with 30% hydrogen peroxide (H_2O_2) for 3 minutes and 30 seconds. Crushed nodule suspensions were streaked on Yeast Extract Mannitol Agar (YMA) plates and incubated at 28°C. Single colonies were obtained by the streak-plate method. Pea growth was obtained by scanning the total leaf area and the total root length of the trapping plants using Optimas 6.1 image analysis software (Media Cybernetics). Plants were dried for 7 days at 50 °C to measure the aerial and root dry biomass.

2.5. Molecular characterization of the internal transcribed spacer region (ITS) of isolated *P. sativum* strains

Molecular characterization of their partial 16S–23S ribosomal DNA internal transcribed spacer region (ITS) was carried out on all the strains obtained after pea trapping (as described in 4) as per Haro et al. (2018). For bacterial characterization, multiple alignments and phylogenetic tree construction were carried out using SeaView version 5 multiplatform software (Gouy et al., 2021). The fifty-seven ITS sequences of *Rhizobium* spp. strains isolated from *P. sativum* were deposited in the GenBank database under accession numbers from KY073436 to KY073492.

2.6. Statistical analysis

All the data were processed with a one-way analysis of variance (ANOVA). Means were compared using the Newman-Keul's test ($p < 0.05$). The distributions of different groups of rhizobial sequences (within which the sequences were identical) were compared between each soil origin with 2 by 2 contingency tables, a chi-square test (2 test), and Yates correction for small numbers.

3. Results and discussion

3.1. Tara is a non-nodulating legume tree

Contrary to the referent study by Ogata & Zuniga (2008a), no nodules were found in our study. Thirty *C. spinosa* root samples were examined directly to determine whether nodules were present. No nodules

were found on these tara roots, nor after each of the three *in vitro* tests (3x 10 plants), nor after the nodulation test (fifteen plants) in the greenhouse.

A further quantitative test of nitrogen fixation by acetylene reduction assay was carried out and it also proved to be negative. The ARA test showed a lack of nitrogenase activity with an average of 0.10 µmol C_2H_4 $plant^{-1}min^{-1}$ (Table 2) and no significant difference could be found between the three soil treatments.

In the MCC clade, Mimosoideae-Caesalpinieae-Cassieae, Sprent et al. (2017) reported that nodulated Caesalpinioideae are more common in the New World tropics than in the Old World, but that there are nevertheless many non-nodulated caesalpinioid legumes in the New World tropics, such as in the *Caesalpinia* genus. This would appear to be due to the basal position of Caesalpinioideae *sensu lato* in the evolution of legumes, as suggested by Rathi et al. (2018). More specifically, Cordero et al. (2016a) and Zurita et al. (2021) stated that *Caesalpinia spinosa* is a non-nodulating tree legume species, which the present study confirmed.

3.2. Soil from the tara planting row was the most suitable for *C. spinosa* and *P. sativum* grown in the greenhouse

When considering biomass parameters after growing tara in the greenhouse for six months, no differences could be found between the three soil treatments for dry shoot biomass and dry root biomass (Table 2). Nevertheless, it can be seen from Table 2 that total biomass was significantly greater for tara grown on row soil (R) than on soil from Outside the Plantation (OP) and that the R and IR treatments were favorable for tara growth according to the root length and leaf chlorophyll

content parameters, the latter parameter being an indicator of plant health (Nagy et al., 2017). The presence of tara may have had a stimulating effect on the nodulating bacteria of the rhizosphere, and an effect in structuring bacterial communities by stimulating the growth of species that can catabolize its root exudates, as suggested by Ormeño-Orrillo et al. (2012). Indeed, host legumes can enrich their immediate soil environment with rhizobia through rhizosphere effects, as noted by Thies et al. (1995), as attaching to roots, or living in the rhizosphere, offers a competitive advantage for rhizobial survival (Hirsch, 2010).

The pea trapping results are presented in Table 3. After growing *Pisum sativum* in the greenhouse for one month, an average of ten root nodules were collected from five replicates of *Pisum sativum* in the OP treatment, as opposed to more than forty nodules in each of the other two treatments R and IR. *P. sativum* nodulation was less profuse in the OP treatment reflecting a weak rhizobial density. Given the significant results, the root and total biomass results in treatment R showed better pea growth than in the IR and OP treatments, with 32% and 44% higher biomass for treatment R compared to treatment OP. Furthermore, the IR and OP treatments were not significantly different for the root biomass and total biomass parameters. Neither did root length show any significant differences between the three treatments. In the R and IR treatments, the leaf area appeared to be significantly higher and more than twice that of the OP treatment.

Table 2
Effect of soil origin (R, IR and OP) from the Peruvian Canchacalla tara plantation on dry biomass, root length and nitrogen fixation of greenhouse-grown tara (*Caesalpinia spinosa*) plants

Treat-ment	Greenhouse					
	Dry shoot biomass (mg)	Dry root biomass (mg)	Dry total biomass (mg)	Root length (cm)	Nitrogenase activity (µmolC_2H_4 $plant^{-1}min^{-1}$)	Chlorophyll (SPAD units)
IR	233.5 (74.2)a	290.5 (60.8)a	524(129.8)a.b	108.8 (5.3)a	0.10 (0)a	49.5 (1.8)a
R	274.0 (23.6)a	218.8 (28.3)a	492.8 (27.3)a	105.2 (21.7)a	0.11 (0.010)a	47.8 (1.3)a
OP	95.6 (25.6)b	144.9 (22.7)a	240.4 (32.8)b	49.8 (4.1)b	0.09 (0.007)a	23.3 (9.1)b

(1) Standard error. (2) Data in the same line followed by the same letter are not significantly different according to the Newman-Keuls test ($p < 0.05$).

Table 3
Trapping of rhizobial strains from the Peruvian Canchacalla tara plantation and effect of soil origin (R, IR and OP) on the growth of *Pisum sativum* after one month of growth

Treat-ment	Greenhouse					
	Total nodule numbers	Dry shoot biomass (mg)	Dry root biomass (mg)	Dry total biomass (mg)	Leaf area (cm^2)	Root length (cm)
IR	> 40a	348.3(51.4)[1]a[2]	522.4(49.9)b	870.8(71.0)b	28.3 (4.2)a	161.7 (189.2)a
R	> 40a	484.6 (68.6)a	637.2(19.8)a	1121.8(52.9)a	30.5 (6.3)a	176.4 (125.9)a
OP	10b	297.3 (77.0)a	482.1(22.3)b	779.4 (60.4)b	13.3 (1.3)b	114.8 (222.2)a

[1] Standard error. [2] Data in the same line followed by the same letter are not significantly different according to the Newman-Keuls test ($p < 0.05$).

However, the positive effects of soil treatment R, and to a lesser extent of soil treatment IR, on *C. spinosa* and *P. sativum* growth could not be attributed solely to the presence/absence of the legume *C. spinosa*. Indeed, it was established that in the cultivation procedures of the tara plantations, fertilizer and irrigation were provided six months per year from May to October using a drip irrigation system, with both factors influencing the development of the two tested plants apart from biotic factors. However, it should be noted that at the time the three soils R, IR and OP were sampled, there had been no fertilization or irrigation for 4 months. Moreover, Table 1 shows that the total nitrogen values were very low (under 0.08%) and that organic matter (total C) values were also very low (under 0.9%) for each of the 3 soils. Consequently, in our study, as the level of N fertilization was very low pea nodulation in the trapping experiment was neither suppressed nor limited. On the contrary, nodulation was more profuse on *Pisum sativum* in the R and IR treatments compared to OP, where fertilizer was never applied. Moreover, pea was able to nodulate in the three soil origins showing, as Muniz et al. (2017) found in Brazil, the presence of native nodulating bacterial populations even without a known history of pea cultivation in the study area.

The R and IR soils were conducive to tara and pea growth for certain parameters. Tara leaf chlorophyll content was twice as high in the R and IR treatments compared to OP. For pea, nodulation was at least 4 times more abundant under R and IR soil conditions when compared to OP. Furthermore, when comparing the chemical analysis in the Canchacalla tara plantation in the Huanuco region (Table 1) with those in the study by Cordero (2016b) with nine other sites in Peru, where *C. spinosa* and *Vachellia macracantha* naturally grow together, we found an average N content of 0.05% and 0.145%, respectively, which further confirms the low nitrogen levels in the tara plantation of our study. In any event, fertilization that was as moderate as in our study could promote the growth of rhizobia, as indicated by Simonsen et al. (2015), and especially isolates with a greater ability to utilize fertilizer for free-living growth.

Table 1 also shows that phosphorus contents were clearly higher in the IR treatment and even much higher in the R treatment than in OP. It should be noted that in the above-mentioned study (Cordero et al., 2016a) at the different Peruvian study sites, the P_2O_5 content displayed a large amplitude between the different sites ranging from 70 to 3200 mg/kg, with an average of 794 mg/kg. In the Canchacalla tara plantation, total phosphorus was 621 mg/kg on average (Table 1), which is also a high content. These high levels were explained by Dick (1994) who reported that P fertilization had taken place at least since the 16th century in Peru when the pre-Colombian farmers cultivated agricultural terraces with high inputs of P-rich guano. The highest P contents of 1200 mg/kg were identified on terraces in the Colca Valley, where the farmers had to abandon their cultivated terraces because of the Spanish conquest. Sandor & Eash (1995) obtained significantly the same results and stated that P generally has low mobility in soil. Dick (1994) concluded that the high P levels in abandoned agricultural soils were probably from P fertilization more than four centuries earlier.

The last factor to consider between the three soils was the temporary irrigation for the R and IR treatments and, by comparison, drought or at least water stress and possibly a more elevated temperature for the OP treatment in the absence of tree cover. Ampofo et al. (2016) found that irrigation had a positive effect on soybean nodulation, growth and yield. Sharaf et al. (2019) reported that water status influenced genetic bacterial composition and functioning. Indeed, soil moisture is an important edaphic factor that affects soil microorganisms and an increase in temperature also has an effect in reducing soil moisture (Furtak & Galazka, 2019). Outside the tara plantation, in the control OP soil, it can be imagined that the persistence and survival of rhizobia might be impaired, especially in the absence of a host legume with, as a consequence, a reduced rhizobial population in the soil, as mentioned by Simon et al. (2014) and probably an inadequate number of effective legume-nodulating bacteria (Ngo Nkot et al., 2015), in accordance with rhizobial ecological tolerance, given that beyond the tolerance limits microorganisms will lose their viability or die (Furtak & Galazka, 2019).

In any event, under our experimental conditions we were unable to separate the roles of the various edaphic factors (including fertilization and irrigation) from the biotic factors (including the presence of tara and its rhizosphere). Indeed, the effect of soil on rhizobial diversity can be the result of a combination of many factors (Palmer & Young, 2000) and as interactions exist between rhizobia, host legumes, and biotic and abiotic factors (Yan et al., 2014), no conclusion can be drawn from this part of the study. Indeed, these factors are too interwoven to identify a potential effect of the presence of *C. spinosa* during the experiment on the growth of *C. spinosa* and *P. sativum* under the three soil conditions. However, *C. spinosa* may have particular rhizospheric microbial diversity, which we

attempted to reveal through a nodulating legume, *Pisum sativum* as a trap plant, since pea is a relevant crop in Peru (Santillana et al., 2008).

3. 3. *Pisum sativum* revealed five-cluster rhizobial diversity associated with tara and a phylogenetic gradient according to the soils

Partial rhizobial sequence phylogeny of the 16S-23S rDNA intergenic spacer was used to analyze the diversity of pea-associated rhizobia inside and outside the tara plantation. All the isolated bacterial nodulating strains were characterized exclusively as *Rhizobium* spp. and belonged to the *R. leguminosarum–etli* clade, as defined by Young et al. (2021).

The phylogenetic tree (Figure 1) shows that the partial sequences were distributed in five distinct groups, of which group 5 had a separate single OP11 isolate. The ITS sequences of all the isolated strains were affiliated with different genera, of which *R. hidalgonense*, *R. leguminosarum bv. vicieae*, *R. etli*, *R. mesocinicum* and *R. acidisoli*.

The highest rhizobial diversity was obtained from the R treatment, where isolates belonged to three phylogenetic groups (1, 2 and 3). Sequences of the IR trapped strains were distributed in two groups (3 and 4) and those of the OP trapped strains were found in group 4 and group 5.

When considering the soil origin of the phylogenetic groups of strains, we found that strain groups 1 and 2 were 100% soil R origin, group 3 was 40% soil R and 60% soil IR origin, and group 4 was 48% IR and 52% OP origin.

The strains belonging to group 1 were related to *Rhizobium* sp. sv. *phaseoli* strain IE4803 (CP007641), formerly *R. etli*, isolated from *Phaseolus vulgaris* in Mexico with 93.82% sequence identity, to *Rhizobium* sp. PEPSM14 which was isolated from *Pisum sativum* in Peru with 90.47% sequence identity and to the *R. etli* CFN42 type strain originating in Mexico (Depret et al., 2004) with 88.69% sequence identity. The single strain OP11 was closely related to the *R. mesosinicum* strain CCBAU 25217 isolated from *Kummerowia stipulacea* (tribe Desmodieae) in China (Lin, 2009), and to *Rhizobium* sp. CI-39Bx isolated from *Cajanus cajan* (pigeonpea) in Ivory Coast, with 98.21% and 98.09% sequence identity, respectively.

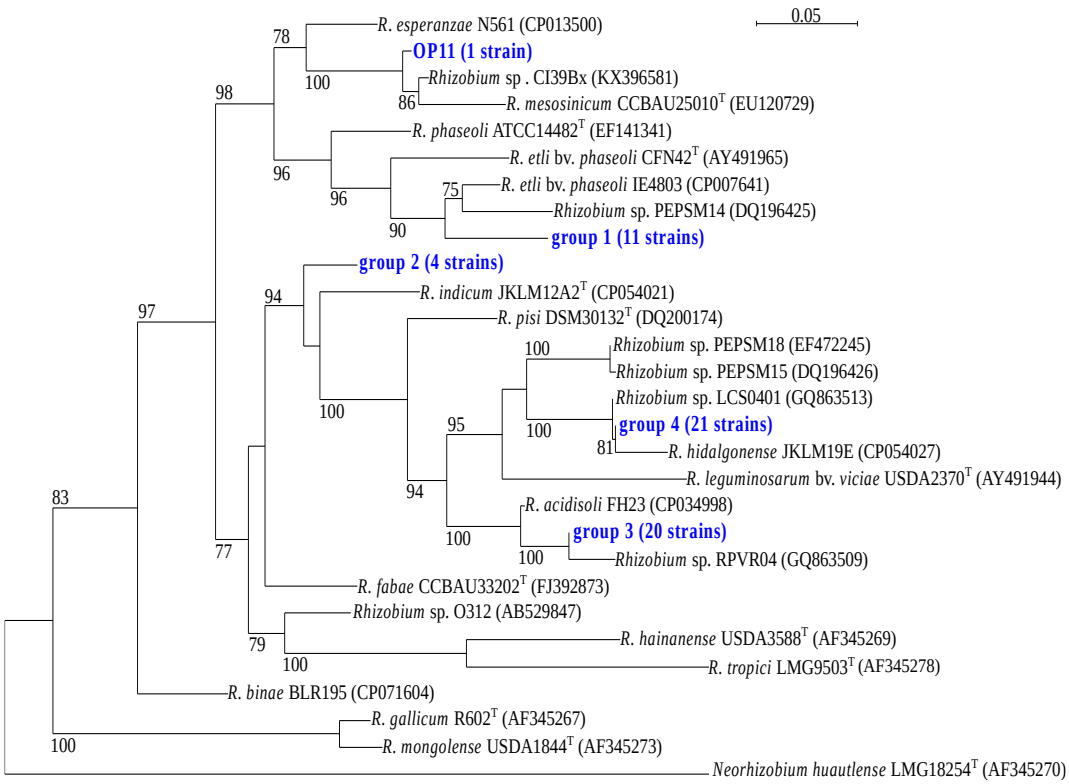

Figure 1. PhyML phylogenetic tree based on 16S-23S rDNA intergenic sequences of 57 *Pisum sativum Rhizobium* spp. strains, reference and related strains. Only bootstrap probability values higher than 70% (100 replications) are given at the branching points. Gaps were not considered. Scale indicated 5% sequence divergence. The *Neorhizobium huautlense* type strain was chosen as an outgroup. Pea (*Pisum sativum*) was nodulated on collected soils from the tara planting row (R), from the tara interrow (IR) and outside the tara plantation (OP). In each group (1 to 4) the *Rhizobium* spp. sequences were identical. Strains represented in each group: group 1: R5, R6, R10, R13, R21, R28, R29, R40, R55, R56, R58, group 2: R2, R3, R4, R14, group 3: R7, R12, R17, R20, R30, R31, R32, R34, IR2, IR3, IR8, IR10, IR12, IR15, IR20, IR22, IR23, IR25, IR27, IR60, group 4: IR1, IR4, IR6, IR7, IR11, IR13, IR24, IR29, IR31, IR58, IR61, OP1, OP2, OP3, OP4, OP5, OP6, OP9, OP10, OP12, O13 and OP11.

The sequences of the four strains composing group 2 had a separate position. The best score for sequence identity was obtained by blast with the strain *Rhizobium* sp O312 isolated from *Lathyrus japonicus* in Japan, with 93.07% sequence similarity. Pea strains belonging to groups 3 and 4 were grouped around the *R. leguminosarum* type strain LMG14904^T which is the same strain as *R. leguminosarum* bv. *viciae* USDA2370^T isolated from *Pisum sativum* (Ramírez-Bahena et al. 2008). *R. pisi* DSM30132^T, situated in the same cluster, was also isolated from *P. sativum*. The twenty strains from group 3 were affiliated to *R. acidisoli* FH23 isolated from root nodules of *P. vulgaris* in acid soils in Mexico, to *Rhizobium* sp. RPVR04 isolated in Spain from root nodules of *P. vulgaris* (García-Fraile *et al.*, 2010) and to a lesser extent to *R. pisi* strain DSM 30132^T, with 98.11%, 97.70% and 91.01% sequence identity, respectively.

The twenty-one strains from group 4 were related to *Rhizobium* sp strain PEPSM15 isolated from *P. sativum* var. *macrocarpum* in Peru by Santillana et al. (2008), but more closely to *Rhizobium* spp. strains isolated from *P. vulgaris*: strain LCS0401 (García-Fraile *et al.*, 2010) with 99.87% sequence identity and *R. hidalgonense* strain JKLM 19E with 99.25% sequence similarity.

The results presented here are consistent with other studies showing diversified groups of *Pisum sativum* nodule symbionts, whether in Morocco (El-Idrissi et al., 2020), in Tunisia (Ilahi et al., 2021), in Algeria (Belhadi et al., 2018), in the Indian Himalayan region (Rahi et al., 2020), or in Poland (Wielbo et al., 2015). Indeed, as *Pisum* belongs to the *Vicia* cross inoculation group, Flores-Felix et al. (2020) confirmed the existence of substantial genetic variability within the symbiovar viciae worldwide. Yang et al. (2008), using 16S–23S IGS analysis, found four groups with pea-nodulating strains in subtropical regions of China, one clustered with *R. leguminosarum* USDA2370^T, another with *R. etli* CFN42^T and the other two groups were separate. Likewise, in our study, potentially new genospecies were found in groups 2 and 1 as, with a similarity < 97%, they were not close to previously identified strains.

Five phylogenetic branches were identified in Figure 1 of this study. Our strains of phylogenetic groups 3 and 4, although in the same cluster with *R. leguminosarum bv. Viciae*, were nevertheless closer to *R. acidisoli* and *R. hidalgonense*, respectively. Curiously, Ilahi et al. (2021) had already shown that in a 16S rRNA gene sequence analysis, strains trapped by *P. sativum* in Tunisia mainly belonged to the *R. leguminosarum* complex group and, moreover, that *R. acidisoli* and *R. hidalgonense*, while not strictly speaking part of the *R. leguminosarum* complex (Rlc), were included in this branch with exactly the same 16S rRNA gene sequences as other strains of the complex. To our knowledge, Rahi et al. (2020) were the first to observe a *P. sativum* strain (strain JKLM 19E) close to the *R. hidalgonense* FH14 type train in the Indian trans-Himalayas.

Surprisingly, group 3 of trapped strains was related to *R. acidisoli* and R. sp. RPVR04, both isolated from *P. vulgaris*. To our knowledge, this *P. sativum* rhizobial affiliation has never been reported before, as there exists host nodulation specificity in strains of *R. leguminosarum* according to the biovars. However, it has been established that beans (*Phaseolus spp*. L.) are one of the most ancient crops of the New World and specifically from the Andean zone, including Ecuador and Peru (Broughton et al., 2003), while the origin and domestication of *P. sativum* lie in the Middle East (Smykal et al., 2011). Our hypothesis would be that in the absence of *R. leguminosarum* bv. viciae, the pea, which is more promiscuous than faba beans in the selection of nodulation genotypes (Efstathiadou et al., 2020), could be nodulated by *R. leguminosarum* bv. Phaseoli, which has existed for thousands of years in Peruvian soils in association with beans. Indeed, Efstathiadou et al. (2020) reported that *R. hidalgonense* surprisingly belonged to symbiovar viciae, while this strain was isolated from nodules of *P. vulgaris*, and then belonged to symbiovar phaseoli, according to Yan et al. (2017). In the same way, Figure 1 shows that all the isolated strains in this study were close to strains isolated from *P. vulgaris*, whether it be *R. leguminosarum bv. phaseoli*, *R. etli* (Aguilar et al., 2004), *R. hidalgonense* (Yan et al., 2017), *R. esperanzae* (Cordeiro et al., 2017), or *R. acidisoli* (Roman-Ponce et al., 2016).

On the other hand, like Wielbo et al. (2015), we found some 16S-23S ITS phylogenetic groups to be soil-specific. Indeed, the rhizobia from the tara plantation trapped by the associated plant, *P. sativum*, were very specific and different from the rhizobia present in the soil outside the plantation. A phylogenetic gradient was observed from the tara planting row to the field outside the plantation. Indeed, the planting row was associated with phylogenetic groups of *R. spp.* strains 1, 2 and 3, the plantation interrow with the phylogenetic groups of *R. spp*. strains 3 and 4, and outside the plantation were found group 4 of *R. spp*. strains and the single strain OP11.

Pea trapping was used to survey the genetic diversity in the rhizosphere of the non-nodulating species *C. spinosa*. *Pisum sativum* revealed part of

the rhizobial diversity associated with the tara plantation, a diversity with five phylogenetic branches of *R. spp*. Moreover, phylogenetic specificity was shown along a geographical gradient from the core plantation to outside the plantation, and this was in the absence of an agroforestry system linking pea to the tara plantation. The tara rhizosphere hosted diverse rhizobia, which could also be more widely investigated using more than one species of trap plant when assessing the diversity of legume-nodulating bacteria in native populations, as recommended by Jaramillo et al. (2013). Although a non-fixing species, tara and its growing conditions have a significant impact on the symbiotic and rhizospheric functional diversity of soil microflora.

4. Conclusions

It has been established that *C. spinosa*, like most species of Caesalpinioideae, is a non-nodulating leguminous tree, with no nitrogenase activity. The present study had highlighted an interesting part of the rhizobial biodiversity associated with a tara plantation, but this diversity was still underestimated with a single trapping species. Tara cultivation in plantations and the associated growing conditions significantly influence the functional diversity and composition of soil microflora. This implies that growing tara may have a beneficial effect on soil biodiversity by encouraging a varied microbial environment that may improve soil productivity and health. The unique circumstances found on tara plantations, such as temporary irrigation and light fertilization, seem conducive to plant development. However, given the vulnerability of Peruvian dry forest ecosystems and the negative ecological impacts of fertilization, this study may be an opportune time to raise questions regarding the adequacy of tara forest management, which, thus far, has not taken microbiological factors into account.

This could be an opportunity to try and steer the hitherto conventional practice of tara plantation management towards agroforestry systems "in line with the paradigms of agroecological transition in the context of global change". Agroforestry is an agricultural diversification practice that purposely grows trees together with crops and/or animals and can provide essential goods and services by improving soil conservation and fertility, microclimatic conditions, water conservation, and biodiversity protections, among other things. Moreover, agroforestry has the potential to enhance farming systems' climate resilience and sustainability. Since the tara legume is not a nitrogen-fixing tree, it will be necessary to find an association with nitrogen-fixing legumes, be they crops such as *P. sativum*, or shrubs or trees. Every stage of tara forest management must be closely examined: tree cover; crop species and system type (alley cropping, silvo-arable, silvo-pastoral); and management practices including variety, pruning, fertilization, irrigation, and harvest time in a meta-analysis of different but equally drought-affected ecosystems in Mediterranean countries, to maximize synergies between different associated species.

While Peru promotes the adoption of agroforestry systems, soil conservation practices are often deficient. The same authors recommended organic soil amendments in Peru, when possible, to help sustain the establishment of agroforestry systems.

Further long-term research will therefore be required, including tara plantation management practices, soil amendments, and, in the shorter term, the study of tara rhizobacteria and the potential effectiveness of the strains isolated in the present study, both inside and outside the tara plantation, as well as the search for efficient native bacterial inoculants.

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Conflict of interest declaration

The authors declare no conflicts of interest.

Authors' contributions

S. Sangay-Tucto: Formal analysis, research, validation, visualization, writing – first draft, writing - review & editing. C. Le Roux: Formal analysis, research, visualization, writing – first draft, writing - review & editing, supervision. D. Zúñiga-Dávila: Resources, supervision, project management. R. Duponnois: Conceptualization, project management, supervision, resources.

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