

The impact of *Pentaclethra macroloba* on soil microbial nitrogen fixing communities and nutrients within developing secondary forests in the Northern Zone of Costa Rica

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Abstract: In the current study, we showed that *Pentaclethra macroloba* appears to influence the microbial communities within the soil of secondary forests in the Northern Zone of Costa Rica. The soil closest to the *Pentaclethra macroloba* trees ("tree soil") had a lower abundance of the N-fixing bacteria *Frankia*, but greater amounts of both nifH gene and *Rhizobium* than soils furthest away ("forest soils"). These soils also had greater amounts of microbial biomass and were more efficient at organic carbon use as indicated by lower metabolic quotient values (qCO₂). This suggests that the use of *Pentaclethra macroloba* in restoration of soils from harvested forests may provide a good strategy for recuperating nitrogen in the soils due to its association with N-fixing bacteria near the roots of the trees and in the nearby soils. As well, these trees were associated with enhanced microbial biomass, microbial activity, and microbial mediated enhanced efficiency of C use into the biomass, suggesting their value in improving soil richness. A tangential discovery during this study was that it was the first, to our knowledge to show the presence of *Frankia* in association with *Pentaclethra macroloba*. It is interesting to note that significant amounts of *Frankia* were found throughout the soils in this study, and it was present in all sites at levels far greater than *Rhizobium*. It is likely that *Frankia* is more critical in recuperating soil N within these developing secondary forests.

Resumen: En este estudio mostramos que *Pentaclethra macroloba* parece influir sobre las comunidades microbianas en los suelos de bosque secundario en la zona norte de Costa Rica. El suelo más cercano a los árboles de *Pentaclethra macroloba* ("suelo de árbol") tuvo una abundancia menor de la bacteria fijadora de N *Frankia*, pero cantidades mayores tanto del gen nifH como de *Rhizobium* que los más alejados ("suelos de bosque"). Estos suelos también tuvieron cantidades mayores de biomasa microbiana y fueron más eficientes en el uso del carbono orgánico, según lo indicaron los valores más bajos del cociente metabólico (qCO₂). Esto sugiere que el uso de *Pentaclethra macroloba* en la restauración de suelos de bosques cosechados puede brindar una buena estrategia para recuperar el nitrógeno del suelo debido a su asociación con bacterias fijadoras de N cerca de sus raíces y en los suelos aledaños. Así mismo, estos árboles estuvieron asociados con valores más altos de biomasa microbiana y de

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actividad microbiana, y una mayor eficiencia mediada por los microbios en el uso del C en la biomasa, lo que sugiere que ellos son valiosos para el mejoramiento de la riqueza del suelo. Un hallazgo tangencial durante este estudio fue que éste fue el primero, hasta donde sabemos, en mostrar la presencia de *Frankia* en asociación con *Pentaclethra macroloba*. Es interesante notar que se encontraron cantidades significativas de *Frankia* en todos los suelos estudiados, y que ésta estuvo presente en todos los sitios y en niveles muchos más altos que los de *Rhizobium*. Es probable que *Frankia* sea más crítica en la recuperación del N del suelo N en estos bosques secundarios.

Resumo: No presente estudo mostra-se que a *Pentaclethra macroloba* parece influenciar as comunidades microbianas no solo das florestas secundárias na região norte da Cota Rica. O solo mais próximo das árvores de *Pentaclethra macroloba* (“solo de folhada”) apresentava uma menor abundância de bactérias de *Frankia* fixadoras de N, mas maior quantidade do gene nifH e de *Rhizobium* do que os solos mais afastados (“solos florestais”). Estes solos também apresentavam maior quantidade de biomassa microbiana e eram mais eficientes no uso de carbono orgânico como indicado por menores valores do quociente metabólico (qCO₂). Isto sugere que o uso da *Pentaclethra macroloba* na restauração dos solos após abate das florestas pode proporcionar uma boa estratégia para a recuperação do azoto nos solos devido à sua associação com as bactérias fixadoras de N junto das raízes das árvores e nos solos próximos. Do mesmo modo, estas árvores estavam associadas com o acréscimo da biomassa microbiana sugerindo o seu valor na melhoria da riqueza do solo. Durante este estudo, uma descoberta adicional foi a de que este foi o primeiro trabalho, tanto quanto é do nosso conhecimento, a mostrar a presença da *Frankia* em associação com a *Pentaclethra macroloba*. Neste estudo, é interessante notar que uma quantidade significativa de *Frankia* foi encontrada através dos solos, e que estava presente em todos os locais em níveis muito maiores do que o *Rhizobium*. É provável que a *Frankia* seja mais crítica para a recuperação do N do solo nestas florestas secundárias em desenvolvimento.

Key words: Carbon cycling, *Frankia*, microbial biomass carbon and nitrogen, microbial community, nitrogen cycling, *Pentaclethra macroloba*, *Rhizobium*, secondary forests.

Introduction

Pentaclethra macroloba is the dominant tree with nitrogen (N)-fixing root nodules in the lowland forests of Costa Rica (Hartshorn & Hammel 1994; Pons *et al.* 2007). As such, it is presumed to be an important early colonizing tree in secondary forests in this region. However, little is known about the N cycle dynamics and the nodular and free-living N-fixing bacteria associated with *P. macroloba*. In the temperate forests of the Pacific Northwest, N-fixing actinomycete bacteria in the genus *Frankia* are associated with the root nodules of Alder (*Alnus* spp.) trees and found as free-living microbes in the soil, and are critical to recuperation of soil N in these forests (Binkley *et al.* 1992; Hart *et al.* 1997; Wardle 2002). The genus *Rhizobium* includes N-fixing bacteria that are both free-living and nodular, and

are associated with a variety of legumes and non-leguminous plants, including in the tropics (e.g. Bala & Giller 2006; Singh *et al.* 2006). We assume that the nodular and free-living N-fixing bacterial communities associated with *Pentaclethra* trees are similar in function to alder, and would stimulate N recuperation in soil.

The lowland rainforests of Costa Rica's Northern Zone have experienced thirty years of extraction-based forest management, resulting in significant fragmentation of the remaining primary and secondary forests, and ecological degradation in floral and faunal forest species (Chassot *et al.* 2001, 2005; Monge *et al.* 2002, 2003; Sigel *et al.* 2006; Whitfield *et al.* 2007). Attempts have been made to remediate the impact of the extensive timber extraction with the development of secondary forests (Chassot *et al.* 2001, 2005; Monge *et al.* 2002, 2003; Oelbermann *et al.* 2004; Schelhas *et al.*

2006). The newly established Maquenque National Wildlife Refuge (MNWR) is such an attempt. The area contains old growth forests, selectively harvested primary forests with natural re-growth, cleared forest, pasture, agriculture, and silviculture, providing opportunities to study the impacts that different land management practices have on the structure and function of soil eco-systems. As *Pentaclethra* is considered an important early successional tree species like Alder, it is possible that it could aid forest restoration strategies if it enhances soil N recuperation through stimulation of the root nodular bacterial populations at the base of the trees, and/or the free-living N-fixing bacterial populations in soil away from the trees in managed lands. The current study compared the abundance of bacterial, *Rhizobium*, and *Frankia* rRNA and nifH gene (for N-fixation), and carbon (C) and N metrics as indicators of the effect of *Pentaclethra* on the surrounding forest soil, both adjacent to and at a distance from these trees.

Methods

Study sites and sample collection

Soil was collected from a *Pentaclethra maculoba*-dominated forest in the Maquenque National Wildlife Refuge (MNWR) in the Northern Zone, near Pital, Costa Rica (10.7151 - 84.1697) over 2 consecutive days in July 2009. The site had been a primary forest, and was harvested and converted into grassland in the early 1980s. In 1991, cattle were removed and the area was allowed to naturally regenerate into a secondary forest typical of the mixed species forests of the area, containing over 130 different tree species. For this study, work was conducted in large areas containing primarily *Pentaclethra* trees. Four 300 m² plots in this secondary forests were identified, with an average of about 100 *Pentaclethra* trees > 50 cm in height in each plot. In each plot, 20 samples of non-rhizosphere soil were collected from a distance of 1 m from 10 trees and pooled (called "tree soil"), and 20 non-rhizosphere soil samples were collected throughout the plot that were at least 4 m from any *Pentaclethra* tree ("forest soil") and pooled. All samples were examined at the time of collection to ensure there were no roots or root nodules present. Sample cores were 2 cm diameter x 15 cm deep, and were collected using sterile technique to avoid cross contamination. Samples were brought back to the lab and processed within 24 h. Soil samples were mixed and sieved at 5 mm to remove rocks,

insects and plant matter. Additional soil samples were collected at each plot for bulk density determination. All data were adjusted for soil dry weight and bulk density.

Nutrient analysis of soil

Nitrate (as NO₃-N) and ammonium (as NH₄-N) levels were measured from 10 g of field-moist soil using 50 mL of 2M KCl for the extraction (Alef & Nannapiery 1995) and the ammonium salicylate and cadmium reduction spectrophotometric analysis using the HACH DR 2700 system (Hach Company, Loveland, Colorado, 80539-0389) and the HACH methods 8155 and 8192, respectively. Phosphate level was measured from 2 g of field moist soil using Bray 1 extracts and the molybdate reduction method (Method # 8048) and the HACH DR 2700 system. Microbial biomass C (C_{mic}) was measured by the fumigation-extraction method of Jenkinson (1988). The difference in the extractable dissolved organic C (DOC) from unfumigated soil samples (considered the measurement of the soil DOC) from 50 ml 0.5 M K₂SO₄ extracts of 10 g soil subsamples and the dissolved organic C from fumigated soil samples is used to calculate the C_{mic}. The DOC levels were determined from the extracts by dry combustion analysis at the CATIE labs in Turrialba, Costa Rica (Anderson & Ingram 1993). The rate of soil respiration was determined as CO₂ released using a Qubit SR1LP Respiration system (Kingston, ON, Canada). The metabolic quotient, qCO₂, was calculated by dividing respiration rate by C_{mic}.

DNA analysis

Microbial community DNA was extracted from three, 0.3 g replicate samples of pooled soil using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, Catalog #: 12888), and the DNA extracts from each replicate pooled. Endpoint PCR amplifications were performed on DNA samples to determine presence or absence of the target gene in the different samples using AmpliTaq Gold Complete Master mix (Applied Biosystems, Foster City CA) and the Universal Bacterial 16S rRNA primers 27f and 1492r and the conditions described by Martin-Laurent *et al.* (2001); the *Rhizobium* 16S rRNA primers 63f (AGGCCTAACACATGCAAGTC) and 1244r and the conditions described by Singh *et al.* (2006); and the *Frankia* 16S rRNA primers FGPS56-352 and FGPS1509'-153' and the conditions described by Normand & Chapelon (1997). A quantitative PCR

Table 1. Nutrient levels and microbial activity in soil collected within 1 m of *Pentaclethra macroloba* trees (called “tree soil”) and at least 4 m from any *Pentaclethra macroloba* tree (“forest soil”) in a *Pentaclethra macroloba*-dominant secondary forest within the Maquenque National Wildlife Refuge in the Northern Zone of Costa Rica. Mean values \pm standard deviation are presented.

	Forest Soil (n=4)	Tree Soil (n=4)	PD	<i>P</i> Value	Hedge's <i>d</i>
Total N ($\mu\text{g NO}_3\text{-N} + \text{NH}_4\text{-N cm}^{-3}$)	2.64 \pm 0.24	2.51 \pm 0.40	4.9	0.6	0.39
Nitrate ($\mu\text{g NO}_3\text{-N cm}^{-3}$ soil)	0.76 \pm 0.20	0.75 \pm 0.32	0.7	0.96	0.04
Ammonia ($\mu\text{g NH}_4\text{-N cm}^{-3}$ soil)	1.88 \pm 0.28	1.77 \pm 0.08	5.8	0.5	0.53
Phosphate ($\mu\text{g P cm}^{-3}$ soil)	5.64 \pm 0.41	2.15 \pm 0.89	62.1	0.0004	5.04
Organic C ($\mu\text{g C cm}^{-3}$ soil)	470.3 \pm 70.8	455.5 \pm 59.2	3.2	0.76	0.23
C_{mic} ($\mu\text{g C cm}^{-3}$ soil)	96.8 \pm 58.3	215.2 \pm 69.7	122.3	0.04	1.84
Respiration ($\mu\text{g CO}_2\text{-C g}^{-1}$ soil dwt h^{-1})	4.75 \pm 1.43	5.18 \pm 0.66	8.3	0.62	0.36
qCO ₂ (as respiration/ C_{mic})	0.073 \pm 0.057	0.026 \pm 0.007	64.4	0.153	1.16

PD = percent difference in mean values; *P* value = t-test *P* value; Hedge's *d* effect size value.

Table 2. A comparison of the abundance (fg of target gene/ng soil DNA \pm standard deviation) of nifH gene DNA, *Rhizobium* 16S rDNA, nifH gene, *Frankia* 16S rDNA, and bacterial 16S rDNA in soil collected within 1 m of *Pentaclethra macroloba* trees (called “tree soil”) and at least 4 m from any *Pentaclethra macroloba* tree (“forest soil”) in a *Pentaclethra macroloba*-dominant secondary forest within the Maquenque National Wildlife Refuge in the Northern Zone of Costa Rica. Mean values \pm standard deviation are presented.

Sample	nifH gene	Rhizobium	Frankia	Bacteria
Forest soil	12.4 \pm 9.9	12.7 \pm 8.3	1.5x10 ⁶ \pm 7.3x10 ⁵	4850 \pm 2081.7
Tree soil	17.1 \pm 20.6	33.8 \pm 32.6	1.4x10 ⁵ \pm 1.7x10 ⁵	6615.4 \pm 8293.8
PD	37.9	166.1	91.1	36.4
<i>P</i> value	0.744	0.256	0.01	0.694
Cohen's <i>d</i>	0.242	0.887	2.621	0.292

PD = percent difference in mean values; *P* value = t-test *P* value; Hedges's *d* effect size value.

was used to estimate the abundance of each target gene. The endpoint PCR conditions and methods were used except that aliquots were collected at the end of a cycle and the fluorescence values determined for sample DNA and for known concentrations of control DNA (6.5 to 28.4 ng μL^{-1} of cloned target gene DNA with sequences confirmed in GenBank). These values were used to compare the threshold cycle (C_t) for sample DNA to the C_t of the positive control DNA and then to calculate the abundance of the target gene DNA concentration for each sample. From these abundance calculations, a RC value was determined that represented a comparison of the percent of an individual target gene present in the tree and the forest soil samples. For example, RC nifH gene forest soil = 100 x [(nifH gene forest soil) / (nifH gene forest soil + nifH gene tree soil)], and RC nifH gene tree soil = 100 x [(nifH gene tree soil) / (nifH gene forest soil + nifH gene tree soil)]. It should be recognized that comparisons of this

nature-[A/(A+B)] to [B/(A+B)] will result mathematically in identical standard deviation values.

Statistical analysis

Statistical analyses were performed using RT4Win software (Huo *et al.* 2006) to determine if there were meaningful differences in the mean values of the parameters measured from the different locations. The standard deviation (S.D.) and percent difference in the mean values (PD) were calculated, and the t-test *P* values and Hedge's *d* effect size statistic (> 0.7 is considered a large effect size difference) were used as indicators of biologically meaningful differences between mean values of parameters measured, as recommended for analysis of small sample sizes by Di Stefano *et al.* (2005).

Results

There were no notable differences in levels of inorganic N, organic C or respiration in the soils from

Table 3. A comparison of the percent relative contribution (% RC) of *nifH* gene DNA, *Rhizobium* 16S rDNA, *nifH* gene, *Frankia* 16S rDNA, and bacterial 16S rDNA in soil collected within 1 m of *Pentaclethra macroloba* trees (called “tree soil”) and at least 4 m from any *Pentaclethra macroloba* tree (“forest soil”) in a *Pentaclethra macroloba*-dominant secondary forest within the Maquenque National Wildlife Refuge in the Northern Zone of Costa Rica. Mean values \pm standard deviation are presented.

Sample	% RC <i>nifH</i> gene	% RC <i>Rhizobium</i>	% RC <i>Frankia</i>	% RC Bacteria
Forest soil	44.9 \pm 12.1	25.6 \pm 8.6	88.9 \pm 14.9	43.64 \pm 33.2
Tree soil	55.1 \pm 12.1	74.4 \pm 8.6	11.1 \pm 14.9	56.46 \pm 33.2
PD	18.5	190.6	87.5	22.7
<i>P</i> value	0.278	0.0002	0.0003	0.606
Cohen's <i>d</i>	0.843	5.674	5.221	0.386

PD = percent difference in mean values; *P* value = t-test *P* value; Hedge's *d* effect size value.

the two forest types; however, soil P levels were greater in the forest soil. The metabolic quotients (qCO₂) were used as indicators of efficiency of use of organic C, with smaller values suggesting more efficiency than larger values (Anderson 2003). The microbial biomass was greater and the metabolic quotient was lower in the tree soil (Table 1). Differences in the abundance of the *nifH* gene and the bacterial 16S rRNA gene DNA between the two locations were not meaningful (Table 2). The abundance of *Rhizobium* 16S rRNA was greater in the tree soils and the abundance of the *Frankia* 16S rRNA was greater in the forest soils (Table 2). The RC values were used to determine the level of contribution of each of the different target genes in the different soil types. The RC of the bacterial 16S rRNA was about the same in both locations. The RC of the *Frankia* 16S rRNA gene was greater in the forest soils (Table 3), while the RC of *nifH* gene and *Rhizobium* 16S rRNA was greater in the tree soils (Table 3).

Discussion

Our results suggest that *Pentaclethra macroloba* may have an important influence on the non-rhizosphere soils in these secondary forests. The microbial biomass C and the efficiency of C use was greater in the soils closer to the *Pentaclethra* trees (i.e. tree soils). As well, there was a greater abundance of *Frankia* DNA than any of the other target genes in both locations, with the abundance and RC being greater in the forest soils, and the abundance and RC of *Rhizobium* being greater in the tree soil than the forest soil.

This is the first study to show the presence of *Frankia* in the soils of these secondary forests dominated by *Pentaclethra macroloba*. It appears

that the free-living *Frankia* community becomes more important (or at least more abundant) in these non-rhizosphere soils moving out from the trees into the forest. If *Frankia* is the root nodule bacteria for *Pentaclethra*, and serves as a source of these microbes to move out into the forest soils to help recuperate N, then it would be following a model similar to that for *Frankia* in the root nodules Alder trees (*Alnus* sp.) and the forest soils in the Pacific Northwest of the USA-however, this needs to be tested and confirmed.

The levels of inorganic N were about the same in both soils, but the RC of *Rhizobium* and to a lesser extent *nifH* gene were greater in the tree soils, closer to the *Pentaclethra macroloba* trees. This suggests that there may be other important free-living N-fixing bacteria in the non-rhizosphere forest soil besides *Rhizobium* playing critical roles in N-fixation. This could be accounted for, in part, by the greater amount of *Frankia* in these soils, and could also be due to the presence of other *nifH*-positive bacteria. Lowe *et al.* (2012) recently showed that Archaea and methanotrophic bacteria were present in these soils, and both groups have been shown to contain the *nifH* gene (Aumen *et al.* 2001; Miyazaki *et al.* 2009; Pernthaler *et al.* 2008; Quaiser *et al.* 2002).

The N-fixing microbial community structure in the forest soils of the *Pentaclethra*-dominated secondary forests is different from the tree soils, where the abundance and RC of *Rhizobium* rRNA was greater, but that of *Frankia* rRNA was lower. The microbial communities of rhizosphere soils are more dependent on (and affected by) the roots of the plant species they are associated with, as opposed to the microbial communities of non-rhizosphere soils that are not closely linked to living roots (Garland 1996; Grayston *et al.* 1998).

It may be that the microbial community of the *Pentaclethra* tree soils are more influenced by the rhizosphere microbial community than those of the forest soils, resulting in some of the differences in microbial communities observed in this study.

The greater amounts of microbial biomass C and lower qCO_2 found in the tree soils are indicators of a larger microbial community that uses organic C more efficiently (Anderson 2003), and could be occurring in the tree soils due to proximity to - and benefiting from - the rhizosphere soil. It is well-accepted that the soil microbial community and nutrient dynamics are more complex and active in rhizosphere soil and the area immediately adjacent to it (for a review, see Cardon & Whitbeck 2007), and that the amount of nutrients going into the microbial biomass in this part of the soil is greater than in other parts due to rhizodeposition, as mediated by the microbial community (Nguyen 2003; Patterson 2003; Patterson & Sim 1999). This would account for the greater amounts of organic C, microbial biomass C, and microbial activity often found in the rhizosphere of soils, and observed in the tree soils from the current study.

In the current study, lower levels of inorganic P were associated with greater microbial biomass and more efficient use of C. However, the role of inorganic P in soil microbial activity in the tropical soils is a complicated story, with differences in observations reported from soils in similar forests of the Northern Zone of Costa Rica since 2002. Some have found increases in P were associated with increases in soil microbial biomass and activity (Cleveland *et al.* 2002), while others have found either the opposite relationship (Eaton *et al.* 2011; Schwendenmann & Veldkamp 2006) or relationship (McGroddy *et al.* 2004). More work is needed to understand the role of P in microbial biomass development in these soils.

After four decades of extraction-based land management strategies in the Northern Zone of Costa Rica, the development of secondary forests is becoming a land remediation strategy for this region. This provides an opportunity to study the structural components of soil ecosystems, and the rates at which the soil C and N nutrients and biomass development occur under different restoration strategies. There has been some discussion in Costa Rica about the potential use of *Pentaclethra macroloba* in restoration activities (Pers. Comm. Nelson Zamora, National Biodiversity Institute of Costa Rica), the idea of which is supported by the results of this current study. To help land managers make decisions on possibly

using this tree in restoration activities, future studies should focus on determining how *Pentaclethra* affects the rates of soil biomass development, nitrification, N-fixation, and inorganic P development in these soils.

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