RESEARCH ARTICLE

The nitrogen fixing specie *Sophora cassioides* (Fabaceae), is nutritionally favored and their rhizosphere bacteria modified when is co-cultivated with the cluster root forming *Embothrium coccineum* (Proteaceae)

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Abstract

The The hypothesis that *Embothrium coccineum*, a species able to solubilize P through cluster roots (CR) carboxylate exudation, makes P more available in volcanic depositions, thus facilitating the growth of other plant species such as the N-fixing *Sophora cassioides* was tested. In this work, seedlings of these two tree species were grown alone or co-cultivated for six months in greenhouse conditions with either pumice, a recent volcanic deposition with low P availability, or an organic commercial mix. Upon analyzing the aboveground growth of the two substrate treatments, we found no clear evidence of facilitation. Despite this, substrate type did influence the relative growth and some characteristics of CR and nodules. On the other side, the number of total soil bacteria, but not *Rhizobium* spp, was greater when *E. coccineum* and *S. cassioidea* were grown together than when only one species was grown alone. Nutritional parameters, such as the concentration of nitrogen (N) and phosphorous (P) in roots and total leaf protein content in leaves, indicated that the nutritional content of *S. cassioides* was greater when co-cultivated with *E. coccineum* in pumice. Specifically, co-cultivation tended to reduced P limitation and improved N acquisition by *S. cassioides*. Based on these results, our hypothesis is partially accepted.

Keywords: Facilitation, cluster roots, nodules, volcanic deposition plant colonization

1. Introduction

For centuries, volcanic activity has occurred throughout southern South American thus influencing the soil development and diversity of vegetation. In Chile, volcanic activity is responsible for many allophanic soils, which implies high capacities to fix P into the soil (Fleige *et al.,* 2016; Goebel *et al.*, 2017). Moreover, pyroclastic materials can induce crucial physical changes in soils that can negatively impact vegetation, preventing the successful establishment of new plants (Zúñiga-Feest *et al.*, 2014; Fleige *et al.*, 2016). Recent volcanic deposits have coarse textures and form unstable single grain substrates with low bulk densities, low nutrient contents, and low water retention (Goebel *et al.*, 2017).

Nutritional constraints, mainly due to low phosphate (P) concentrations, may be among the key factors why few plant species are able to colonize volcanic deposits commonly found in Chile (Delgado *et al.*, 2014; Zúñiga-Feest *et al.*, 2014). Plants able to colonize volcanic depositions have several mechanisms to acquire nutrients in impoverished soils. Among these is the ability to form cluster roots (CR), which are considered one of the main adaptive strategies for the acquisition of immobile nutrients, particularly mineral-P (Zúñiga-Feest *et al.*, 2014). Indeed, CR are able to exude carboxylic acids and acid phosphatase to the soil, thus mobilizing low available forms of P to be used by plants (Lambers *et al.*, 2012, Zúñiga-Feest *et al.*, 2014). Several plant species from Chile and Argentina, including the *Proteaceae Orites myrtoidea* and *Embothrium coccineum*, are able to colonize volcanic depositions by forming CR and releasing organic acids (Ávila, 2014; Delgado *et al.*, 2014). The rhizosphere of mature CR of these species have significantly more available forms of P than bulk soil, as has been described for adult E. coccineum trees growing in soils of volcanic origin (Delgado *et al.*, 2015a).

Competition and facilitation are often observed among plants growing together. As commonly defined, facilitation is a process in which a plant species has a positive effect on the fitness of another plant species or on the recruitment of other (beneficiary) plants (Callaway, 2007). In this way, facilitation can affect the plant community structure, dynamics, and maintenance of diversity in many ecosystems (Michalet *et al.*, 2006). Specifically, facilitating plants improve microclimatic conditions under the canopy, and this results in higher productivity and allows less adapted species to recruit despite adverse local conditions, thus increasing overall plant diversity (Callaway, 2007; Cavieres *et al.*, 2016). While facilitation has been studied in many habitats including those with adverse environmental conditions, such as arid and alpine climates (Cavieres *et al.*, 2016), as far we know, information about the possible belowground effects of facilitation have not been reported for colonizing species in southern South America.

Nitrogen fixing plants, such as legumes, have been used in intercalated crops due to their ability to increase the supply of nitrogen to the ecosystem (Valentine *et al.*, 2017). Several studies have evaluated facilitation between legumes and cereals including chickpeas/wheat, pea/barley, and fava beans/maize (Temperton *et al.*, 2007; Li *et al.*, 2016). Thus, facilitation is commonly applied in agronomic practices and could be used as a reforestation strategy (Brooker *et al.*, 2016). Moreover, in arid and semi-arid regions, about one-third of nurse plants are legumes capable of fixing N through bacterial symbiosis (Flores and Jurado, 2003). For example, the woody shrub *Retama sphaerocarpa* has been shown to recruit other woody plants in hostile Mediterranean ecosystems where this species has a relatively high tolerance to soil contamination (de la Fuente *et al.*, 2014).

Recently, it has been proposed that soil microorganisms might play a role in plant facilitation (Rodríguez-Echeverría *et al.*, 2016). Soil microorganisms provide essential ecosystem services such as nutrient cycling, carbon sequestration, and soil fertility (Bardgett and van der Putten, 2014). Additionally, soil microbes also strongly influence individual plant fitness as well as the structure and diversity of plant communities (Lozano *et al.*, 2014). Multiple studies have shown that plant associated microbial communities are significantly influenced by the plant host and the host's developmental stage (Bardgett and van der Putten, 2014; de Armas *et al.*, 2016). Moreover, it has been demonstrated that nurse plants can alter the composition of soil bacterial communities, selecting microbiota that promote plant growth and that mineralize nutrients (Rodríguez-Echeverría *et al.*, 2016). Despite this being known, the effects of microbiota on species with radicular adaptions growing in recent volcanic depositions in southern South America have yet to be tested.

The presence of two or more plant species with root adaptations that enhance N-fixation and P-solubilization could positively affect growth in volcanic soils. Li *et al.*, (2014) have suggested that complementary root adaptations result in increased agricultural yields due to increased nutrient availability in the rhizosphere of neighboring plants. Species with CR inhabiting volcanic soils may also provide nutritional benefits to neighboring plants given that the roots of these plants increase available forms of P (Delgado *et al.*, 2015a) and other nutrients in the soil. As such, Muler *et al.*, (2014) have shown that *Banksia attenuata*, a cluster-root forming species, facilitates the acquisition of manganese by neighboring *Scholtzia involucrata* species that lack such specialized roots. These authors suggest that CR-forming species could facilitate nutrient acquisition by neighboring plants.

It has been well described that the presence of N-fixing legumes increases N availability in soil and therefore can favor neighboring non-leguminous species (Temperton *et al.*, 2007; Li *et al.*, 2016). However, legumes have high P demands to synthesize ATP, which is needed for symbiotic N-fixation (Aerts and Chapin, 2000). Because plant species able to solubilize soil P could make this nutrient more available to other plants, we proposed that the CR bearing species *Embothrium coccineum* (Proteaceae) and the N-fixing species *Sophora cassioides* (Leguminosae) could positively interact in recent volcanic depositions, thus facilitating the growth of one another. To test this hypothesis, we grew seedlings in two different substrates: a) pumice, a nutrient-poor substrate derived from recent volcanic depositions; and b) a commercial organic mix rich in nutrients. We evaluated the survival, growth, and foliar protein content of the *E. coccineum* and *S. cassioides* seedlings in the two treatments and grown either separately or together. Belowground effects were also assessed by determining CR, nodules, and soil bacteria colony forming units. If a positive association between the two plant species occurs, we expected to find higher: survival, relative growth rates, leaf protein content, P root concentration and number of bacteria and *Rhizobium* spp. associated with the rhizosphere of the plants when grown together. Additionally, we expected that the positive effects of this association could be higher when both species are grown in pumice compared to both are grown in the organic mix.

2. Materials and Methods

2.1. Plant material and cultivation

Four month-old *E. coccineum* and *S. cassioides* seedlings were produced from seeds, collected in Valdivia (2013) and maintained in greenhouse conditions at the

Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile. The climate where the seeds were collected is temperate and rainy, with a mean annual temperature and precipitation of 12 °C and 2,300 mm, respectively. The mean temperature of the warmest month (January) is 17 °C (Luebert and Pliscoff, 2006). The organic mix consisted of a homogenized commercial preparation obtained from a local supplier (ANASAC®) and the pumice was collected in Choshuenco located in the Region of Los Ríos, Chile (39°47' S - 71° 57' O). The pH of the substrates was measured using a digital pH meter (Orion 3 star pH Benchtop, Thermo Fisher Scientific Inc, Waltham, MA, USA) with the substrates suspended in water (ratio 1:2.5; w/v H_2O). The chemical characteristics of the soil were determined at the beginning of the experiment according to Sadzawka *et al.*, (2004) (Table 1). The plants were cultivated in 1 L pots. Six treatments were evaluated, involving *E. coccineum* and *S. cassioides* cultivated both individually or together (co-cultivated) in organic mix or pumice. The experimental design was four blocks with five replicates for each treatment randomly distributed. Thus, each treatment corresponds to 20 plants in total. The experiment was carried out during September-2013 to March-2014. Seedlings were irrigated twice per week to maintain field capacity with tap water.

Table 1. Chemical properties of the recent volcanic and organic substrates at the beginning of the experiment.

Properties	Substrate type	
	Pumice	Organic mix
Organic matter (%)	0.53	22.38
pH	6.57	5.25
P total (mg kg $^{-1}$)	278.98	492.78
P Olsen (mg kg $^{-1}$)	2.19	15.13
N total (%)	0.03	0.33
N mineral (mg kg^{-1})	8.40	43.40
Al extractable (mg kg^{-1})	19.68	4.99

2.2. Morphological evaluations

Several growth parameters, including height and biomass, were evaluated. When harvested, the leaves, stems, roots, and CR or nodules were separated. These tissues were then dried in an oven at 60 °C for 48 hrs and later weighed using an analytical balance.

To calculate the relative growth rate in terms of height (RGRh) and weight (RGRw), all living plants were used, and the results were expressed as the percentage related to the initial value. To calculate the average weight of the nodules per plant, we only used the plants that presented nodules at the end of the experiment.

2.3. Nutrient concentration in roots

After harvesting the plants from all of the treatments, the roots were washed with distilled water. Following this, they were dried for 48 h at 60° C. Dried samples were grounded to analyze P and N concentrations. To evaluate the concentration of P in the roots, the samples were calcinated and digested in HCl, and P was determined colorimetrically using the vanado-phosphomolybdate method. To determine the concentration of N in the roots, the digestion and distillation method from Kjeldahl was used (Sadzawka *et al.,* 2004). As an indicator of N or P limitation, the N/P ratio was then calculated for the roots (Koerselman and Meuleman, 1996).

2.4. Extraction and determination of total leaf protein

Fresh leaf tissue (0.5 g) was homogenized with 3 mL of a buffer containing 50 mM Tricine-HCl (pH 7.8), 0.4 M Sorbitol, 10 mM NaCl, and 5 mM MgCl$_2$ using a chilled pestle and mortar at 4 °C. The homogenate was centrifuged at 13,400 rpm for 20 min at 4 °C. The supernatant was stored (fraction A) and the pellet was resuspended in a wash buffer containing 50 mM Tricine-HCl (pH 7.8), 10 mM NaCl, and 5 mM MgCl$_2$. This washing step was repeated twice and the obtained supernatant pooled (Fraction B). After both washes, the final supernatant was discarded and the pellet was suspended in a solubilizing buffer containing 60 mM Tris-HCl (pH 8.8), 0.003 mM SDS, 1 mM EDTA di-Na, 12% Sucrose, and 50 mM DTT, thus obtaining the fraction C. The protein concentrations in all the obtained fractions samples were quantified according to the method of Bradford. Total protein content was then calculated and expressed as mg of protein per gram of fresh leaf weight.

2.5. Total bacteria and Rhizobium determination in soil samples

To recover rhizospheric soil from the organic substrate, each plant was carefully removed from the soil in order to retain the soil attached to the roots. Briefly, 0.5 g of the recovered soil was then transferred to a sterile tube and suspended in 10 mL of NT solution (0.9 % NaCl, 0.02% Tween 20). The tubes were agitated at 150 rpm for 10 min, serially diluted, and the aliquots were plated on various types of media. A yeast extract plus Congo Red medium (Vincent, 1970) was used to characterize *Rhizobium* spp in the soil samples, and a yeast and malt extract plus glucose and agar medium (0.1; 1.0; 2.0 and 1.5 % respectively) was used to estimate the total bacteria. To obtain non-rhizospheric soil, the remaining soil content of each pot was transferred to a plastic bag and homogenized. Then, 1 g was placed in a sterile tube, suspended in 15 mL NT solution, and treated as before. Non-rhizospheric soil from pumice was collected in the same way except that 1 g was suspended in 5 mL of NT solution. Plates were incubated at 25 °C for up to four days after which the colonies were counted. The results were expressed as colony forming units (CFU) per gram of soil.

2.6. Statistical analysis

A two-way ANOVA was applied to determine if there were significant differences between treatments (*E. coccineum* and *S. cassioides* cultivated individually in organic mix, *E. coccineum* and *S. cassioides* cultivated individually in pumice, *E. coccineum* and *S. cassioides* co-cultivated in organic mix, and *E. coccineum* and *S. cassioides* co-cultivated in pumice) in terms of the variables analyzed (relative growth rate, cluster root and nodule formation, ratio structures/total dry weight,

ratio structures/total root dry weight, and colony forming units of the total bacteria and *Rhizobium* spp in the rhizospheric and non-rhizospheric soils). A Tukey test was used to identify values that differed significantly. Both analyses were performed with Sigma Plot 11.0 (SPSS, Chicago, IL). Differences between the values were considered significant at $P \leq 0.05$. A Chi-squared test (X^2) was applied to determine significant differences in the percentage of seedlings with cluster roots (*E. coccineum*) and nodules (*S. cassioides*).

3. Results

3.1. Substrate properties

The main chemical properties of both substrates were characterized at the beginning of the experiment (Table 1). The organic mix had 42 times the organic matter content and 10 times the total nitrogen compared to the pumice. It is relevant to note that although the pumice also had low available P (P-Olsen) content (about seven times lower than that of the organic mix), the total P content of the pumice was only half that of the organic mix.

3.2. Survival rates and growth parameters

Seedling survival for both species and both substrates was almost 100%. However, seedling growth was higher for plants cultivated in the organic substrate. In fact, the RGRh was about two times higher for *E.*

coccineum grown in organic mix compared to when grown in pumice, and the RGRh of *S. cassioides* grown in organic mix was eight times higher than when grown in pumice. Despite this, plant height was not affected when both species were co-cultivated (Figure 1A).

In terms of biomass weight, the growth of *S. cassioides* in organic substrate was higher than when grown alone or together with *E. coccineum* in pumice (about eight and six times higher, respectively, Figure 1B). In contrast, the growth of *E. coccineum* in pumice and in the organic mix did not differ (Figure 1B). Co-cultivation did not affect the growth (in terms of weight) of either species or of either substrate.

3.3. Formation of CR in E. coccineum and nodules in S. cassioides

Cluster root formation occurred in all *E. coccineum* seedlings, both those grown in pumice and in the organic mix, and CR formation was not affected by co-cultivation (Figure 2). Thus, the mean number of CR per plant was around 30 (Figure 2A), which corresponded to 14% of the total plant dry weight (Figure 2B). Differences were found in the proportions of CR weight to total root weight for *E. coccineum* grown alone. Specifically, the CR weight of *E. coccineum* was about 60% lower when grown in the organic mix than when grown in pumice (Figure 2C). When co-cultivated with *S. cassioides*, no significant differences in CR weight were found among treatments (Figure 2C).
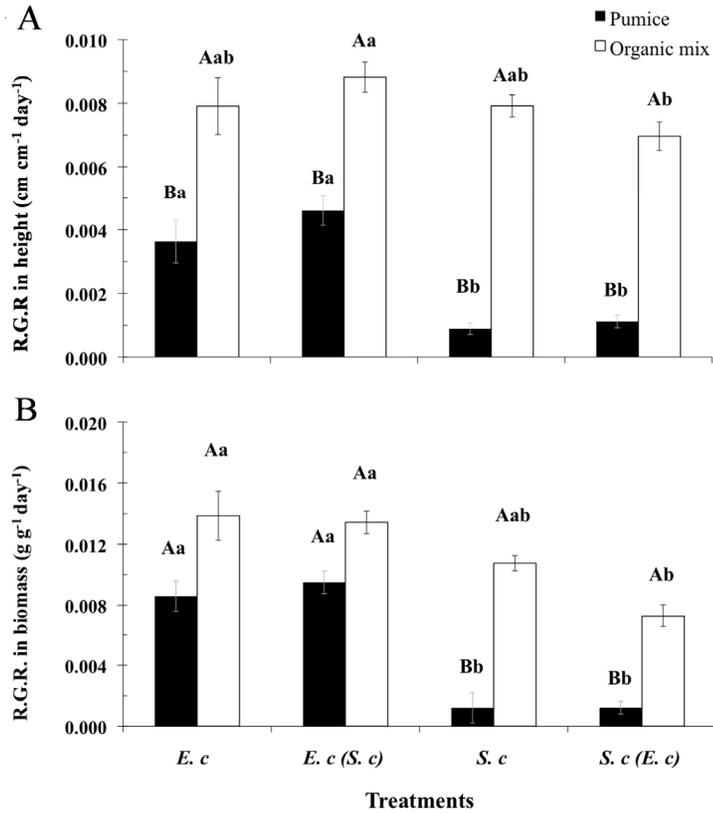
Figure 1. Relative growth rate in height (A) and biomass weight (B) of *E. coccineum* and *S cassioides* seedlings growing individually and in combination in pumice or organic mix. Values are presented as means of the seedlings (n=15-20) in each treatment ± standard error. E.c: *E. coccineum*, S.c: *S. cassioides*, E.c (S.c): *E. coccineum* co-cultivated with *S. cassioides*, S.c (E.c): *S. cassioides* co-cultivated with *E. coccineum*. Capital letters show significant differences between substrates ($P \leq 0.05$). Lower case letters indicate significant differences between treatments ($P \leq 0.05$).
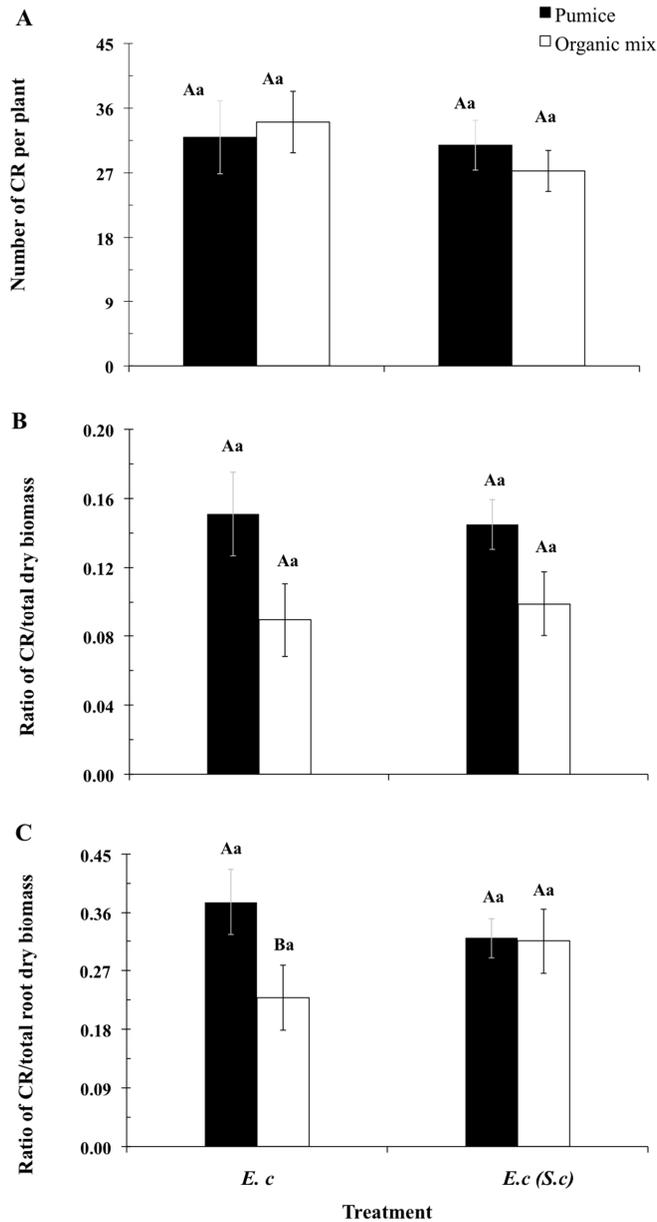
Figure 2. Effect of co-cultivation on the CR formation of *E. coccineum* seedlings. Number of CR per plant (A), Ratio of CR/total dry weight (B), and Ratio CR/total root dry weight (C) in *E. coccineum* seedlings growing individually and in combination with *S. cassioides* in pumice or organic mix. Values are presented as means of seedlings (n=15-20) in each treatment ± standard error. E.c: *E. coccineum*, E.c (S.c): *E. coccineum* co-cultivated with *S. cassioides*. Capital letters show significant differences between substrates (P ≤ 0.05). Lower case letters indicate significant differences between treatments (P ≤ 0.05).

Nodule formation in *S. cassioides* occurred in almost all the seedlings when grown alone, however, when grown with *E. coccineum* only 82% and 88.8% of *S. cassioides* seedlings produced nodules in pumice and organic mix, respectively (data not shown). Overall, but these differences between treatments were not significant. Nodule weight accounted for up to 2.5% of the total weight of the plant (Figure 3A). Substrate typeand co-cultivation did not affect the ratios of nodule weight to total plant or root dry weight (Figure 3A and 3C). Despite this, substrate type did have a significant effect on individual nodule weight. As shown in Figure 3B, when *S. cassoides* was grown alone in organic mix, the weight of individual nodules were about four times higher than the weight of the nodules of plants grown in pumice. Co-cultivation did not affect nodule weight.

3.4. Total leaf protein content

Regardless of treatment, the total leaf protein content of E. coccineum was lower than that of *S. cassioides* (Figure 4). There were, however, significant differences in the total leaf protein content among S. cassioides treatments (Figure 4). The highest leaf protein content for *S. cassoides* was found when plants were grown alone in the organic mix, and these values were about 2.5 times higher than those of plants grown in pumice. The opposite pattern was found for the co-cultivation treatment; S. cassoides leaves had about double the protein content when grow in pumice together to *E. coccineum*.

3.5. Nitrogen and phosphorous content in roots

The lowest root P concentrations were when the plants, regardless of species, were grown in pumice. Conversely, the highest root P concentrations were found when the plants were grown in the organic mix, and the values were particularly high in *S. cassioides* (Figure 5A). Slight increases in root P concentrations were found for

the co-cultivation treatments, yet these increases were not significant. Regarding N concentration, the highest values were found for *S. cassioides* grown in both substrates. The lowest N content was found for *E. coccineum* grown in pumice. Overall, N concentration was not affected by co-cultivation (Figure 5B).

When analyzing the above data to obtain the N/P ratios, the lowest ratios were found for both plant species grown in the organic mix (Figure 5C). For *E. coccineum*, the N/P ratio was higher for plants grown in pumice compared to those grown in organic mix. Additionally, there were slight effects of co-cultivation. Conversely, the N/P ratios of *S. cassioides* were highest when plants were grown in pumice, and these values decreased significantly (by about 40%) under co-cultivation (Figure 5C). 3.6. Presence of total bacteria and *Rhizobium* in rhizospheric and non-rhizospheric soil.

Among the treatments, significant differences were found for the bacteria content of the non-rhizospheric soils (NRS) (Figure 6). *E. coccineum* cultivated alone and in pumice had the highest total bacteria counts. When co-cultivated, the amount of bacteria found was similar to that found for the NRS of *E. coccineum* growing alone (Figure 6A). The amount of *Rhizobium* spp found in the NRS of *E. coccineum* growing alone in organic mix was high compared to the NRS of *S. cassioides*. Additionally, the amount of *Rhizobium* spp was not affected by co-cultivation (Figure 6B). Due to the properties of the pumice substrate, it was not possible to obtain rhizospheric soil. Therefore, the bacterial content of this substrate was not determined.

Differences in the bacteria content of the rhizospheric soil (RS) was found for plants grown in the organic substrate (Figure 7). Specifically, *E. coccineum* grown alone had the highest total bacteria and *Rhizobium* spp counts. These values were two and three times higher than that found for the NRS (Figure 6 and Figure 7) of the same treatment. Overall, the bacterial content of RS was not affected by co-cultivation. *Sophora*

cassioides grown alone had the lowest total bacteria and *Rhizobium* spp content. These values were similar to those detected in the NRS. Under co-cultivation, a higher number of total bacteria (about 4.6 times higher) was found for the RS of *S. cassioides* compared to that found when this species was cultivated alone. Under co-cultivation, the amount of *Rhizobium* spp found was similar to that found for *S. cassioides* growing alone. Overall, higher total bacterial content, but not *Rhizobium* spp content, was found for the co-cultivation treatments (Figure 7).

4. Discussion

Aboveground parameters, i.e. relative growth based on height and biomass weight, did not provide evidence of a positive interaction between *E. coccineum* and *S. cassioides* when co-cultivated in pumice or organic mix. The data of the belowground parameters, i.e. CR and nodule characteristics as well as bacteria content in the soil, indicated some differences linked to the kind of substrate in which the plants were grown. Despite these results, *S. cassioides* was positively influenced by co-cultivation in pumice as indicated by the following findings: (i) the high number of total soil bacteria (but not *Rhizobium* spp.); (ii) the low N/P ratio of the roots, and; (iii) the high protein content of the leaves

The aboveground analyses clearly showed that the type of substrate affected plant growth, particularly the growth of *S. cassioides*. While *E. coccineum* was able to grow in both kinds of substrates, *S. cassioides* grew much better in the organic mix. This finding is to be expected as *E. coccineum* is known to be an early volcanic soil colonizer (Donoso, 2006; Zúñiga-Feest *et al.*, 2014; Delgado *et al.*, 2014). The low performance of *S. cassioides* in pumice can be attributed to several environmental and physiological factors. First, this leguminous tree is widely distributed

in southern Chile, from Maule to Aisén (Rodríguez *et al.*, 1983), yet S. cassioides inhabits humid valleys along streams and lakes where the soil has high organic matter content and high humidity compared to recent volcanic substrates (Donoso, 2006). Thus, the low relative growth rate observed for the *S. cassioides* seedlings grown in pumice could be a reflection of the low nutrient content and water retention capacity of pumice. However, some *Sophora* species are able to resist adverse environmental conditions such as those present in volcanic soils. For instance, it has been shown that *S. viciifolia* is capable of growing in lead and zinc contaminated soil in China due to the presence of arbuscular mycorrhizal fungi (AMF) and a unique soil bacterial community (Xu *et al.*, 2012). Unfortunately, here we did not evaluate the presence of AMF, despite that these fungi have been reported to associate with *S. cassiodes* in Chile (Zúñiga *et al.*, 1998).

The belowground analysis indicated differences among treatments, specifically the ratio of CR/root dry weight was higher for *E. coccineum* seedlings grown alone in pumice compared to the organic mix. Despite this trend, the differences in mean number of CR per plant were not significant. As has been widely reported before, the *E. coccineum* seedlings formed CR in all of the soil conditions tested here (Zúñiga-Feest *et al.*, 2014). As has been observed previously (Ávila, 2014), the lower CR/ root dry weight ratios of the plants grown in the organic mix compared to those grown in pumice could be due to the higher P content of the organic mix (Table 1). In addition, *E. coccineum* seedlings grown in pumice exuded phosphatases and carboxylates at faster rates than plants grown in the organic mix (Ávila, 2014); we expect that something similar occurred in our experiment. *Sophora cassioides* formed nodules in all of the experimental conditions, yet individual nodule weight was highest when these plants were grown alone in the organic mix.

The nodules of plants grown in the organic mix were four times heavier than those grown in pumice (Figure 3C). As far we know, this is the first evidence of nodule formation by this species growing in a recent volcanic substrate; however, nodulation in this species has been recently observed by our research group for plants cultivated in sandy volcanic material (data not published). These results suggest the presence of nodulating microorganisms in volcanic depositions or the involvement of some endophitic microbes. Despite the formation of nodules when growing in pumice, these root adaptations were not enough to support higher growth. Moreover, the reduced growth of *S. cassioides* in the organic mix and subjected to co-cultivation (Figure 1) could be due to the exudation activity of *E. coccineum*. Specifically, *E. coccineum* exudes mainly carboxylates that decrease the pH of the rhizosphere. Thus, *E. coccineum* exudates could create a soil environment that is directly or indirectly less favorable for *S. cassioides*. For example, these exudates could negatively affect microorganisms associated with the plant (see below). The soil associated bacteria of both species were significantly affected by co-cultivation and the type of substrate in which the plant was grown. When grown alone and in pumice, the total bacteria and *Rhizobium* spp. NRS counts of *E. coccineum* were higher than those of *S. cassioides* (Figure 6). Overall, however, the total bacteria counts were high for all co-cultivation treatments. This suggests that *E. coccineum* is able to stimulate bacterial growth even in the rhizosphere, potentially via CR carboxylate exudation (Delgado *et al*., 2015b) and perhaps the exudation of other compounds. It is thought, however, that the effect of exudation on soil microbes would be more notable in the RS, which could only be evaluated for plants grown in the organic mix. Here, *E. coccineum* again had the highest total bacteria and *Rhizobium* spp. counts, and *S. cassioides* had the lowest. Under co-cultivation, total bacteria counts remained high, and the

Rhizobium spp. counts were not affected (Figure 7). These results suggest that *E. coccineum* might aid *S. cassioides* in recruiting more soil associated bacteria. The effects on soil microbiota by other CR-forming plants have been reported for species such as Banksia in Australia (Marschner *et al.*, 2005) and Leucospermun in South Africa (Stafford *et al*., 2005). These studies have shown that these CR-forming species strongly influence the rhizosphere microbiota probably due to the exudation of carboxylates. Additionally, it has been shown that *Lupinus albus*, another CR-forming species, selects *Burkholderia* species, which dominate the CR rhizospheric soil (Kost *et al*., 2014). The species thus identified were able to use oxalate as a carbon source, which is released by *L. albus* CR. Because several *Burkholderia* species have been described to promote plant growth *L. albus* CR could be selecting beneficial bacteria (Kost *et al*., 2014). The influence of CR on the rhizospheric microorganisms of neighboring plants has not yet been described. For S. cassioides, we found more total RS bacteria in the co-cultivation treatments, but the amount of *Rhizobium* spp. was not affected by co-cultivation. It could be speculated that some recruited bacteria under cocultivation could be involved in to promote *S. cassioides* growth by P or N uptake improvement or other way as described for *L. albus* (Kost *et al*., 2014). However, it is clear that further studies are needed to determine how differences in the soil bacterial community affect belowground plant interaction and nutrition.

Our results of the N and P concentrations in roots and the protein content in leaves strongly suggest that *S. cassioides* benefits from co-cultivated with *E. coccineum* when both are grown in pumice. Under this condition, both plant species were under a P limitation - particularly *S. cassioides*- as the high N/P ratios indicates (Figure 5C) (Koerselman and Meuleman, 1996). Moreover, co-cultivation reduced this ratio significantly for *S. cassioides*, indicating a

lower P limitation. This fact can be explained due to the slightly increase in P root concentration observed under pumice (Figure 5A, C). Moreover, the protein content in the leaves of *S. cassioides* was greater when plants were co-cultivated in pumice compared to the plants growing alone. These results indicate that co-cultivation likely positively effects N fixation, uptake, and accumulation in leaves. This is important for legumes, because N-fixing capacity has been shown to be highly dependent on soil P availability (Valentine *et al.*, 2017). This result is also in agreement with Li *et al.* (2016), who studied co-cultivated legumes/non-legume pairs (faba bean/maize), where maize exudates promoted N-fixation by faba bean, resulting in increased total protein in leaves. Thus, it is likely that active *E. coccineum* CR increase P availability in P limited soils and this, in turn, is taken up by *S. cassioides.*

Co-cultivation likely causes both plants species to compete, and especially for P. In pumice, *E. coccineum* was more successful than *S. cassioides* at obtaining P, while the opposite seemed to occur in the organic mix. This is supported by the higher P concentrations obtained by *S. cassioides* when grew on the organic mix, thus the P concentration showed an order of magnitude greater in the organic mix than in pumice (Figure 5A). This result was also similar for leaves and stems of *S. cassioides* (data not showed). When grown in organic substrate, *S. cassioides* was more efficient at releasing and assimilating available P and, perhaps, reduced the soil P available for *E. coccineum*. Some legumes are able to release carboxylates in order to acquire P (Suriyagoda *et al.*, 2010), though this trait has not yet been described for *Sophora* species. Another mechanism of P acquisition is association with AMF, which has been described for several *Sophora* species (Xu *et al.*, 2012). Additionally, *S. tomentosa* in Brazil has been shown to be associated with both AMF and nodulating N-fixing bacteria (Toma *et al.*, 2017). Further to this, AMF associations and nodulation have been described in native Chilean *Sophora* species growing in organic soil in a botanical garden (Zuñiga *et al.*, 1998) but not in volcanic depositions. Despite this, here we did not measure the presence of AMF in *S. cassioides* roots, yet it is possible to speculate that mycorrhizal associations could occur in the organic mix, thus improving P uptake.

Finally, it must be noted that the experiment conducted here was temporally short and did not involve natural conditions. Moreover, the lower growth of *S. cassioides* compared with *E coccineum* could be masking facilitation. Therefore, it is possible that some of the positive interactions between these two plant species could require time to stabilize. Overall, although it is possible to propose that plant species with different and complementary nutrition systems might positively interact, such interactions must be proven as these kinds of relationships seems to be species-specific (Callaway, 2007; Li *et al.*, 2014; Rodríguez-Echeverría *et al.*, 2016).
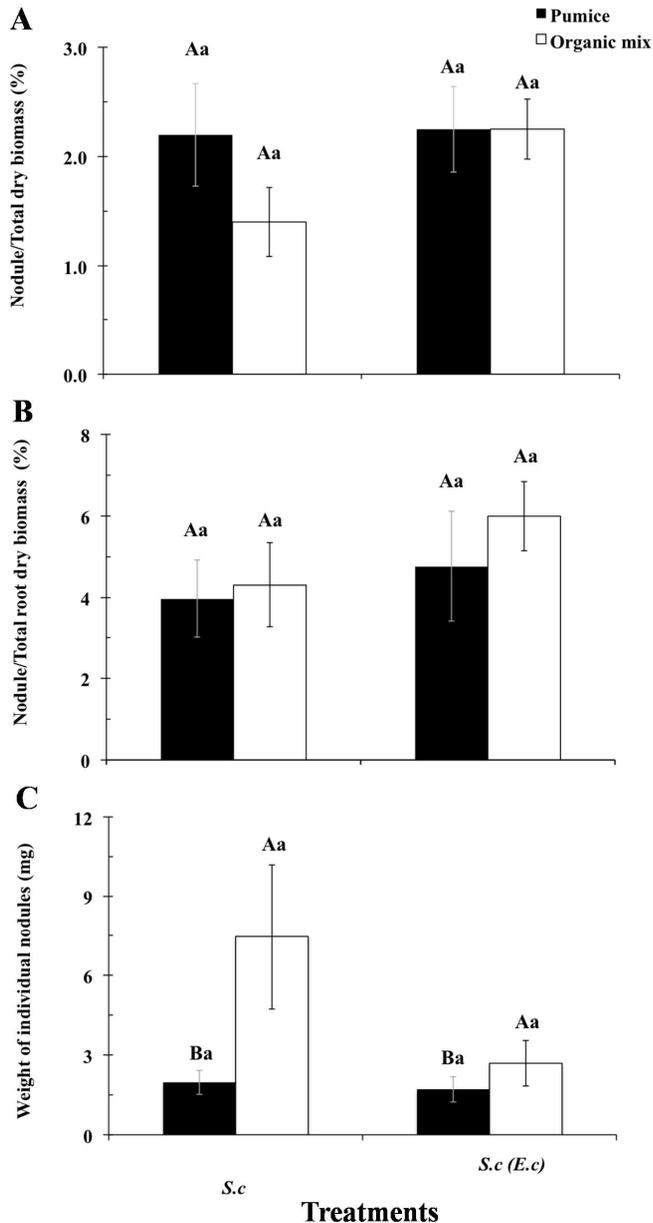
Figure 3. Effect of co-cultivation on the nodule formation of *S cassioides* seedlings. Nodule/total weight ratio calculated using total dry weight (A), nodule/total root weight ratio calculated using dry weight (B), and weight of individual nodules (C) in seedlings of *S. cassioides* growing individually and in combination with *E. coccineum* in pumice or organic mix. Values are presented as means (n=15-20) of seedlings in each treatment ± standard error. S.c: *S. cassioides*, S.c (E.c): *S. cassioides* co-cultivated with *E. coccineum*. Capital letters show significant differences between substrates (P ≤ 0.05). Lower case letters indicate significant differences between treatments (P ≤ 0.05).
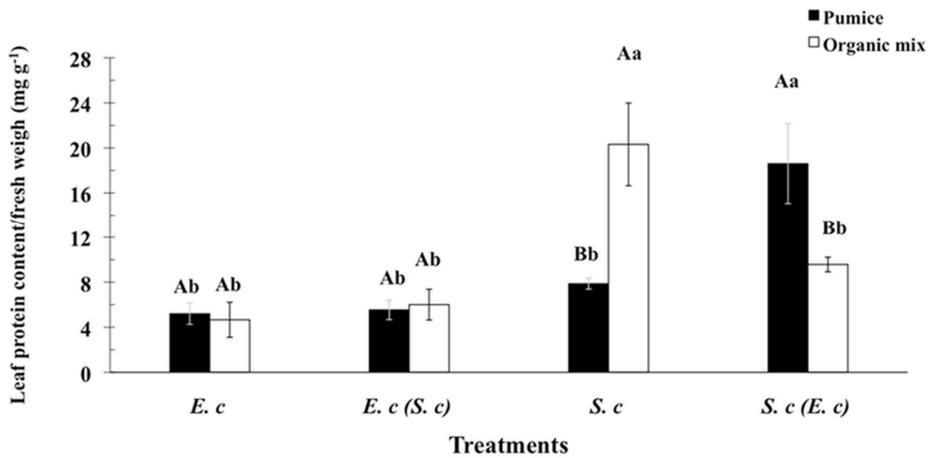
Figure 4. Leaf total protein content per fresh weight of *E. coccineum* and *S. cassoides* seedlings growing in pumice and organic mix. Values represented are means of leaf samples (n = 3-4) in each treatment ± standard error. Capital letters show significant differences between substrates (P ≤ 0.05). Lowercase letters indicate significant differences between treatments (P ≤ 0.05). E.c: *E. coccineum*, S.c: *S. cassioides*, E.c (S.c): *E. coccineum* co-cultivated with *S. cassioides*, S.c (E.c): *S. cassioides* co-cultivated with *E. coccineum*.
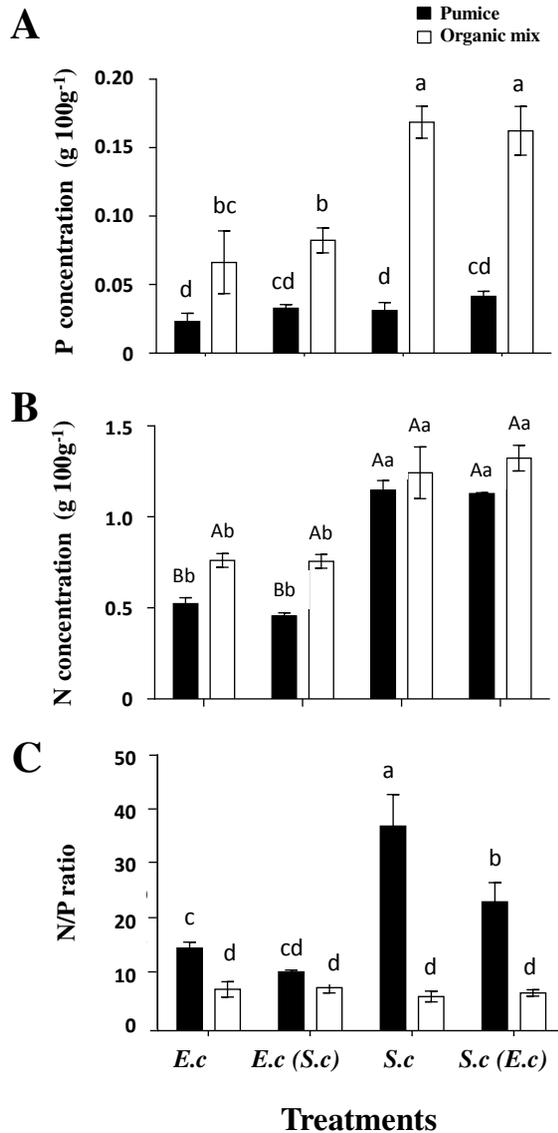
Figure 5. Phosphorous (A) and nitrogen (B) concentration and N/P ratio (C) in roots of *E. coccineum* (E.c) and *S. cassioides* (S.c) seedlings growing individually or in combination in pumice and organic mix. Values are presented as means ± standard error. E.c: *E. coccineum*; S.c: *S. cassioides*; E.c (S.c): *E. coccineum* co-cultivated with *S. cassioides*; S.c (E.c): *S. cassioides* co-cultivated with *E. coccineum*. In panel A and C, lower case letters indicate significant interactions (p ≤ 0.05) between species combinations and substrates. In panel B, there was not a significant interaction between these factors; thus, capital letters show significant differences between substrates (P ≤ 0.05), and lower case letters show significant differences between species combinations (P ≤ 0.05).
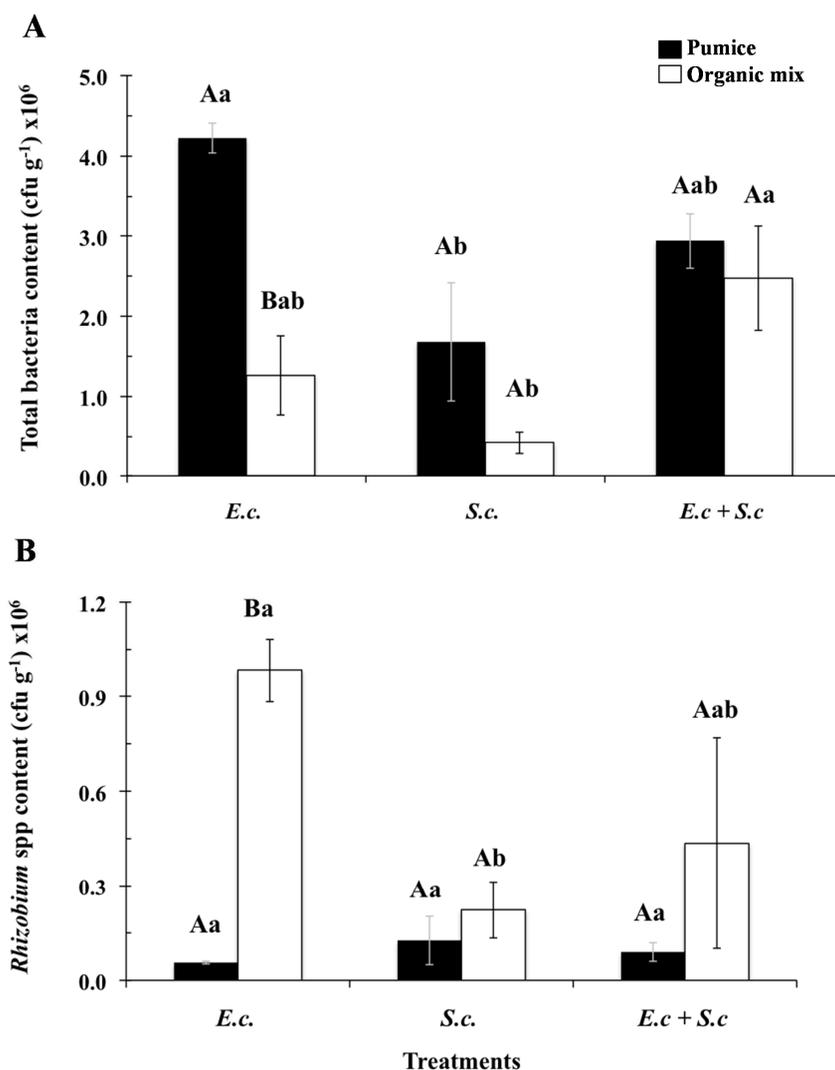
Figure 6. Colony forming units (CFU) of total bacteria (A) and *Rhizobium* (B) obtained from the non-rhizospheric soil of *E. coccineum* and *S. cassioides* seedlings grown in pumice and in organic mix. The values correspond to the average of three determinations per treatment ± standard error. E.c: *E. coccineum*, S.c: *S. cassioides*, E.c (S.c): evaluation of *E. coccineum* growing along with *S. cassioides*, S.c (E.c): evaluation of *S. cassioides* growing along with *E. coccineum*. Capital letters show significant differences between substrates (P ≤ 0.05). Lower case letters indicate significant differences between treatments (P ≤ 0.05).
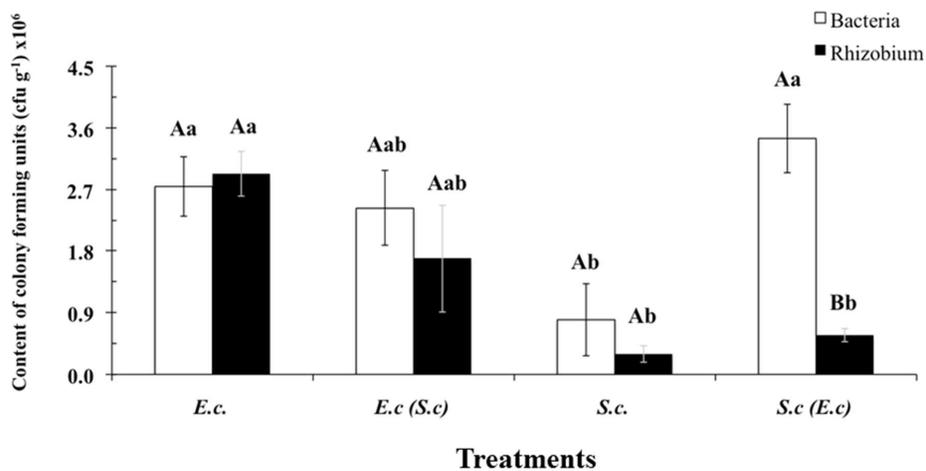
Figure 7. Colony forming units (CFU) obtained from the rhizosphere of *E. coccineum* and *S. cassioides* seedlings grown in organic mix. The values correspond to the average of three determinations per treatment ± standard error. E.c: *E. coccineum*, S.c: *S. cassioides*, E.c (S.c): *E. coccineum* co-cultivated with *S. cassioides*, S.c (E.c): *S. cassioides* co-cultivated with *E. coccineum*. Capital letters indicate statistically significant differences between microorganism counts (bacteria and *Rhizobium*) (P ≤ 0.05). Lowercase letters indicate significant differences between treatments (P ≤ 0.05).

5. Conclusions

The CR-forming species *E. coccineum* and the N-fixing species *S. cassioides* grow together in young volcanic depositions, yet relative growth analyses did not provide any evidence of facilitation in terms of aboveground growth. Belowground, the kind of substrate in which these plants are grown effects characteristics of the CR and nodules. However, co-cultivation increases the number of total soil bacteria but not *Rhizobium*. Analyzing nutritional parameters, such as the concentration of N and P in roots and the total protein content in leaves, indicated that co-cultivation positively affects *S. cassioides*, at least when this species is grown in pumice with *E. coccineum*.

Acknowledgements

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