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MICROBIAL SYMBIONTS AND NUTRIENTS (N AND P) SHARING: EFFECT ON SOIL MICROBIAL ACTIVITY IN THE UPLAND RICE (*Oriza sativa*) AND BEAN (*Phaseolus vulgaris*) INTERCROPPING

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Abstract

The symbiotic association (plant-soil-microorganisms) has an important role in nitrogen (N) and phosphorus (P) uptake. The main objective of this study is to assess the potential of fungal and rhizobial symbionts as well as the importance of plant-soil-microorganism interactions on microbial dynamics. The upland rice and the beans were cultivated in mono or in co-culture using the rhizospheric soil of the upland rice and beans collected on plots treated with different levels of organic and mineral fertilizers. What about saying: Microbial (fungal and rhizobial) inoculate were constituted by root fragments (rice or bean) collected from previous crop and coded as I1 (root fragments from rice), I2 (root fragments from bean) and I3 (mixed root fragments from rice and bean). The results showed that soils inoculated with I1 and I3, were characterized by high phosphatase activity. These two treatments enhance also the amount of nitrogen and phosphorus in the aerial part of upland rice intercropped with bean. These results suggest that the bean with its symbiont can be considered as ecological engineers that stimulate the biological functioning of soils and is beneficial for upland rice cultivation.

Key word: bean, co-culture, inoculum, microbial symbionts, upland rice.

INTRODUCTION

About 45% of the hillsides, or "tanety", of the highland regions of Madagascar are potentially cultivable with an average slope of almost 9.23% (Ramifehiarivo et al., 2016). The increased use of tanety soils could alleviate the saturation of the lowlands, especially for flooded rice cultivation (Rasoamampionona et al., 2008) and provide local populations with new cultivable land. However, these tanety are characterized by ferralitic soils rich in iron and aluminium oxyhydroxides, which strongly adsorb phosphorus (P) (Hinsinger, 2001; Morel, 2002; Smith et al., 2003; Rabeharisoa, 2004; Richardson et al., 2009; Gérard, 2016)

limiting its uptake by plant species (Holford, 1997; Jarosch et al., 2015). These soil properties are also accompanied, by low nitrogen (N) content which, like phosphorus, is well known as a factor limiting plant production (Vance et al., 2003). The use of ferralitic soils for agriculture requires then prior improvements to reduce these deficiencies in available P and N. Direct inputs of P and N and crop rotation practice which contributes mainly to the intensification of major biological processes that influence nutrient acquisition, plant growth and plant productivity are the best and usual ways to correct such deficiency. It is also possible in some case to combine these options, i.e. by providing immediately available

nutrients and optimizing biological, processes. These different alternatives allow the improvement of nutrients availability, particularly P, and soil quality.

Several authors have demonstrated the importance of nitrogen-fixing rhizobia and mycorrhizal fungi, two major microbial groups involved in soil fertility improvement and plant productivity (Abdelkader et al., 2017). The nitrogen-fixing rhizobia are involved in atmospheric nitrogen (N₂) fixation, which can reach more than 200 to 300 kg N ha⁻¹ per year in some legumes (Mohammadi et al., 2012). Mycorrhizal fungi (mainly vesicular arbuscular mycorrhizal fungi or VAM), which are obligate symbionts (Duponnois et al., 2010), improve directly (mycorrhizae formation at the root level) or indirectly (extension of extramatrical hyphae in the soil), P acquisition by plant species (Smith and Read, 1997, 2008). Generally, legumes, except the lupines species (*Lupinus albus*), are able to contract mycorrhizal and nitrogen-fixing symbiosis at the same time (Jensen and Hansen, 1968; Amarger et al., 1977). These two forms of association are complementary.

Plants throughout their development cycle benefit from the positive contributions of these microorganisms but also constitute an indispensable support for their propagation in different forms resulting from sexual or asexual multiplication (Bonfante and Anca, 2009), such as cells, spores, hyphae (Hartnett and Wilson, 1999) and root pieces with nodules or mycorrhizae (Scheublin et al., 2004). Indeed, these beneficial microorganisms (rhizobia and mycorrhizal fungi) in addition to their symbiotic potential, also excrete in the soil different substances (enzymes, natural antibiotics, growth hormones...) (Bashan, 1998; García-Garrido and Ocampo, 2002) that by their activities strongly contribute to the maintenance of soil fertility. Thus, on the one hand, a gap in terms of microbial density in the soil should be corrected by introducing active populations of beneficial microorganisms (technique called inoculation), and on the other hand, a significant wealth could be used to enrich other soils according to compatibilities (Koide and Mosse, 2004). In this case, the inoculum consists essentially of propagules (germs, spores, hyphae, mycorrhizal root

pieces) whose effectiveness has already been proven (Adholeya, 2003; Smith and Read, 2010).

In the highlands of Madagascar, the most adopted technique is the leguminous-cereal crop rotation (most often voandzou- upland rice) (Andriamananjara, 2011) and the crop association involving upland rice and maize, rarely upland rice-bean. The latter one deserves a particular attention in order to better understand the mechanism of the "soil-microorganism-plant" interaction. Thus, the main objective of this study was to assess the potential of the "rice-bean" intercropping system to promote the propagation of microbial symbionts and the sharing of nitrogen and phosphorus while stimulating the activity of soil microorganisms under different controlled fertilization.

MATERIALS AND METHODS

Two different plant species were used in this study: the upland rice (*Oryza sativa* L.) *Poaceae* family and the common bean (*Phaseolus vulgaris* L.), *Fabaceae* family.

Soil preparation

The soils used during this study were taken from "rice-bean" plots located in Lazaina, an agronomic experimental site owned by the University of Antananarivo. These plots were divided according to the type of fertilizer used, i.e. Triple SuperPhosphate with 45% P₂O₅ (S2), manure (S3) and Triple Super Phosphate + manure (S4) and Control (without any fertilizer input) (S1).

The soils (S1, S2, S3 and S4) were respectively mixed with sterilized sandy soil (120°C for 40 min) and distributed into 2 liter buckets. Microbial (fungal and rhizobial) inoculate were constituted by root fragments (rice or bean) collected from previous crop and coded as I1 (root fragments from rice), I2 (root fragments from bean) and I3 (mixed root fragments from rice and bean).

Greenhouse experiment

Each soil (S1 to S4), were respectively inoculated with I1, I2 and I3. Three techniques were adopted for the experiment: monoculture

of rice (MoR), monoculture of bean (MoH) and coculture rice and bean (CoRH).

Nitrogen and phosphorus content of above-ground biomasses

The determination of soil phosphorus content was carried out according to the ammonium molybdate blue-ascorbic acid method described by Murphy and Riley (1962) and the total plant nitrogen was determined by the Kjeldahl method.

Soil microbial enzymatic activities

Acid phosphatase activity and total microbial activity through Fluorescein diacetate or FDA hydrolysis activities were assessed *in vitro*. For the first one, the para- Nitrophenyl Phosphate (pNPP) substrate in contact with the soil was hydrolyzed to p-Nitrophenol by phosphatase enzymes and the FDA in contact with the soil was hydrolyzed by different enzymes producing the fluorescein (Alef, 1998; Schnurer and Rosswall, 1982).

Mycorrhization rate evaluation

Fresh roots were stained with Trypan Blue (Phillips and Hayman, 1970) and the percentage of root length colonized by the mycorrhizal fungus was quantified by observing 30 root segments under microscope and calculating the mycorrhization rate according to the method of Giovannetti and Mosse (1980).

RESULTS AND DISCUSSION

Effect of soil type/fertilizer on the acquisition of mineral elements (nitrogen and phosphorus) in the aerial parts of rice and bean.

In this study, high levels of fertilization [S3 (manure), S4 (TSP+manure)] increase the N and P content in the aerial part of each plants, rather than their growth. With regard to N acquisition, a 20 and 10% increase was observed in rice and beans in S3 and S4 soil, respectively. For P content, this increase was 16 and 24% in rice and beans, respectively.

These effects can be explained by a direct supply of P from fertilizers applied in the mineral form of TSP. Manure is both an additional intake of P, an intake of N (14 g N

kg⁻¹) (Henintsoa, 2018) and other nutrients such as macronutrients (K, Ca...) and micronutrients, necessary for plant growth. S3 soil (manure) is the source of N, mainly in mineral form, which is immediately available to the plants.

Effect of soil type/fertilizer on the mycorrhization rate of plants

The results obtained (Tables 1 and 2) showed that P input to S3 and S4 soils slightly increase mycorrhization rate, which increase by about 10% (65-75% on rice and 70-80% on beans). These high mycorrhization rate values could explain better phosphate nutrition of the plants and thus a higher P content. Moreover, it has been confirmed by Boukcim and Mousain (2001) that the level of mineral or organic fertility in the soil or growing medium and/or the nutritional status of the host plant has a major influence on plant mycorrhization. Compared to the roots of the two plants grown with the control soil without fertilizer, mycorrhizal infection of plants grown on the fertilized soil is significantly high. However, generally, the intensity of root colonization by symbiotic fungi decreases when the level of phosphorus in the soil increases (Dickson et al., 1999; Wang et al., 2014; Guo et al., 2016). But, the establishment of mycorrhizal symbiosis on plants within an ecosystem generally promotes the availability of major soil nutrients, especially phosphorus (Boullard, 1968; Mosse, 1981; Strullu, 1985; Vassilev et al., 2001; Khasa et al., 1992) and improves plant health. This subsequently maintains growth and productivity of the host plant (Van Der Heijden et al., 1998; Scheublin et al., 2007; Shah et al., 2009).

Effect of the inoculum on the acquisition of mineral elements (nitrogen and phosphorus) from the aerial parts of rice and bean.

Tables 1 and 2 give the results on the phosphorus and nitrogen contents in the aerial part of plants inoculated with I1, I2 and I3. Results showed that I1 (1112.48 mg·kg⁻¹) and I3 (1131.77 mg·kg⁻¹) increase significantly the phosphorus content of the aerial part of bean. A similar result was obtained with I3 (1040.93 mg·kg⁻¹) for rice aerial part. Maximum nitrogen gain of the aerial part of beans and rice was observed with I3.

Table 1. ANOVA results and comparison of means for bean productivity parameters as a function of soil/fertilizer type, inoculum and cropping system

Parameters Factors	P (mg·kg ⁻¹)		N (mg·kg ⁻¹)		Mycorrhization rate (%)		
	F	P(F)	F	P(F)	F	P(F)	
Soil type/fertilizer (S)	21,91	< 0,0001	12,51	< 0,0001	18,31	< 0,0001	
Inoculum (I)	4,34	0,02	17,42	< 0,0001	15,31	< 0,0001	
Cultivation system (MoH/CoRH)	57,37	< 0,0001	0,73	0,39	16,85	0,00	
S*I	2,61	0,02	2,91	0,01	4,75	0,00	
S*MoH/CoRH	4,17	0,01	2,61	0,06	5,12	0,00	
I*MoH/CoRH	18,93	< 0,0001	1,69	0,19	6,53	0,00	
S*I*MoH/CoRH	1,29	0,27	3,03	0,01	2,01	0,08	
Comparison of means		Mean	group	Mean	group	Mean	group
Soil type (S)	S1	916,30	c	18025,14	b	70,71	b
	S2	1089,64	b	18386,48	b	68,62	b
	S3	1217,12	a	19665,77	a	83,33	a
	S4	1152,50	ab	20099,57	a	81,86	a
Inoculum (I)	I1	1112,48	a	19141,05	b	75,98	b
	I2	1037,43	b	17982,36	c	82,17	a
	I3	1131,77	a	20009,31	a	70,24	c
Cultivation system	MoH	1198,53	a	19164,79	a	72,52	b
	CoRH	989,25	b	18923,69	a	79,75	a

Table 2. ANOVA results and comparison of averages for rice productivity parameters as a function of soil/fertilizer type, inoculum and cropping system

Parameters Factors	P (mg·kg ⁻¹)		N (mg·kg ⁻¹)		Mycorrhization rate (%)		
	F	P(F)	F	P(F)	F	P(F)	
Soil type/fertilizer (S)	16,45	< 0,0001	37,76	< 0,0001	8,76	< 0,0001	
Inoculum (I)	54,02	< 0,0001	88,79	< 0,0001	13,38	< 0,0001	
Cultivation system (MoR/CoRH)	252,95	< 0,0001	14,87	0,00	0,05	0,82	
S*I	5,95	< 0,0001	5,94	< 0,0001	1,72	0,13	
S*MoR/CoRH	3,05	0,03	3,23	0,03	1,92	0,13	
I*MoR/CoRH	20,93	< 0,0001	0,64	0,53	0,51	0,60	
S*I*MoR/CoRH	3,28	0,01	7,54	< 0,0001	1,79	0,11	
Comparison of means		Mean	group	Mean	group	Mean	group
Soil type (S)	S1	772,85	b	14337,64	b	64,84	b
	S2	952,21	a	14846,51	b	65,71	b
	S3	929,85	a	16404,38	a	72,92	ab
	S4	972,93	a	17169,35	a	78,71	a
Inoculum (I)	I1	923,59	b	15286,36	b	74,99	a
	I2	756,35	c	14171,29	c	74,17	a
	I3	1040,93	a	17610,76	a	62,48	b
Cultivation system	MoR	728,28	b	15274,83	b	70,30	a
	CoRH	1085,63	a	16104,11	a	70,79	a

This increase in P and N content may be closely linked to the abundance of beneficial microorganisms which have the ability to release or transform complex elements into some mineral forms that are immediately available to plant species. In this case,

arbuscular and vesicular mycorrhizae have a high affinity P absorption mechanism that improves the P nutrition of the plants. Arbuscular and vesicular mycorrhizae are able to improve the availability of P through their hyphae, which explore large areas of soil and

act as a bridge between the soil and plant roots for P transfer (Liu et al., 2000; Bianciotto and Bonfante, 2002). It was reported also that soil inoculation with rhizobia improved the acidification of the rhizosphere which subsequently stimulated P mobilization from a poorly soluble P source (Ögüt et al., 2011) and improved N nutrition of plants (Peoples et al., 1995). In addition to the effects of rhizobia and mycorrhizae, other groups of beneficial microorganisms can play an important role in improving plant nutrition. These include PGPR bacteria, whose association with plant roots plays a key role in P nutrition in many agroecosystems, particularly in P-deficient soils (Goldstein, 2007; Jorquera et al., 2008). Considering that some PGPRs have a capacity to solubilize phosphate, they could be useful in improving bean production by increasing soil P content and improving nodulation and nitrogen fixation. The inoculation of plants with PGPRs could increase the native population through various mechanisms that convert insoluble inorganic and organic P into the available form and thus improve plant nutrition (Guiñazu et al., 2010; Qureshi et al., 2012; Sharma et al., 2013; Singh, 2013). Thus, these PGPRs have enormous potential in biofertilizer formulations to be exploited to increase crop yields through the solubilization of soil-fixed P (Hayat et al., 2012).

Effect of inoculum on plant mycorrhization rate

Our results showed that the presence of a high diversity of microorganisms at the root level explains the response to inoculation. Thus, the high presence of arbuscular and vesicular mycorrhizae colonizing the roots of legumes and cereals, which cause highly stimulated plant growth, has already been reported (Youpensuk et al., 2005; Scheublin and Van Der Heijden, 2006; Malina Singha and Sharma, 2013).

Effect of cropping system on the acquisition of mineral elements (nitrogen and phosphorus) in the aerial parts of rice and bean.

The nitrogen rate of the bean was slightly reduced by competitiveness in crop association, that of the rice gave an excellent yield (I don't understand what you are saying here? Could

you please reformulate this phrase?). These results show the role of legumes and symbiotic nitrogen-fixing rhizobacteria in the leguminous-grass system, as well as the possibility of nitrogen transfer from one plant to another in the intercropping system (He et al., 2007; Shen et al., 2004). In addition, according to Zheng and Song (2000) vesicular-arbuscular mycorrhizae cooperate with legume N-fixing bacteria by affecting nodulation. Therefore, there would be an effect on nitrogen acquisition. In an intercropping system, mycorrhizal symbiosis transfers nutrients such as nitrogen between plants via hyphae networks (Johansen and Jensen, 1996). Thus, mycorrhizal symbiosis allows better phosphate nutrition and may play an important role in the sustainability of our agricultural systems (Plenchette et al., 2005; Hijri et al., 2006).

Effect of cropping system on plant mycorrhization rate

Mycorrhizal infection rates in beans and rice in monoculture or co-culture reach values almost 80% and 70%, respectively (Tables 1 and 2). It is well known that arbuscular and vesicular mycorrhizae can easily colonize legume roots (Xiao et al., 2010). Many previous studies have also shown that most legume crops, including beans, are hosts for MVA fungi (Haugen and Smith, 1992; Khasa et al., 1992; Lin et al., 2001; Kasiamdari et al., 2002; Sprent and James, 2007). In co-culture, the rate of mycorrhization was more marked on bean roots under soil treated with manure alone.

Effect of soil type/fertilizer on the microbial activities of the crop soil.

Fertilizer inputs to the soil can be expressed indirectly through their effects on soil microbial communities. In this study, soil type/fertilizer did not have a significant effect on phosphatases activity and does not contribute to the P uptake. Indeed, the inorganic phosphate available to the plant is often released by phosphate-solubilizing bacteria that secrete phosphatases (Smith and Read, 1997). Results showed a significant increase in global microbial activity (FDA) for high fertility levels (S3-S4 > S1-S2) in soils under beans (Table 3) and rice (Table 4). Indeed, some authors explain that microbial

biomass activities increase after the addition of energy sources such as organic amendments

(Bolton et al., 1985; Goyal et al., 1993; Höflich et al., 2000).

Table 3. ANOVA results and comparison of averages for parameters related to soil microbial activities under beans as a function of soil/fertilizer type, inoculum and cropping system

Parameters Factors	P.ase Ac ($\mu\text{g-pNPh}^{-1}\text{g}^{-1}$)		P.ase Al ($\mu\text{g-pNPh}^{-1}\text{g}^{-1}$)		FDA ($\mu\text{g-h}^{-1}\text{g}^{-1}$)		
	F	P(F)	F	P(F)	F	P(F)	
Soil type/fertilizer (S)	4,751	0,006	1,412	0,251	2,958	0,042	
Inoculum (I)	154,750	< 0,0001	25,993	< 0,0001	2,855	0,067	
Cultivation system (MoH/CoRH)	0,679	0,414	22,165	< 0,0001	1,374	0,247	
S*I	2,696	0,025	4,318	0,001	0,250	0,957	
S*MoH/CoRH	5,667	0,002	2,769	0,052	1,821	0,156	
I*MoH/CoRH	5,497	0,007	4,764	0,013	0,386	0,682	
S*I*MoH/CoRH	2,694	0,025	3,150	0,011	0,153	0,988	
Comparison of means	Mean	group	Mean	group	Mean	group	
Soil type (S)	S1	300,966	ab	122,407	a	88,705	b
	S2	252,447	b	117,077	a	112,509	ab
	S3	289,656	ab	142,778	a	113,446	ab
	S4	329,524	a	119,008	a	121,811	a
Inoculum (I)	I1	245,308	b	117,738	b	109,449	a
	I2	164,940	c	85,585	c	96,842	a
	I3	469,196	a	172,629	a	121,061	a
Cultivation system	MoH	287,116	a	101,852	b	113,969	a
	CoRH	299,180	a	148,783	a	104,266	a

*P.ase Ac: Acid phosphatase activities; P.ase Al: alkaline phosphatase activities; FDA: Fluorescein diacetate hydrolysing activities

Table 4. ANOVA results and comparison of averages for soil microbial activity parameters under rice according to soil/fertilizer type, inoculum and cropping system

Parameters Factors	P.ase Ac ($\mu\text{g-pNPh}^{-1}\text{g}^{-1}$)		P.ase Al ($\mu\text{g-pNPh}^{-1}\text{g}^{-1}$)		FDA ($\mu\text{g-h}^{-1}\text{g}^{-1}$)		
	F	P(F)	F	P(F)	F	P(F)	
Soil type/fertilizer (S)	2,637	0,060	1,322	0,278	5,988	0,001	
Inoculum (I)	218,216	< 0,0001	23,459	< 0,0001	4,123	0,022	
Cultivation system (MoR/CoRH)	0,085	0,772	17,712	0,000	3,505	0,067	
S*I	2,621	0,028	2,897	0,017	0,055	0,999	
S*MoR/CoRH	1,909	0,141	1,541	0,216	2,816	0,049	
I*MoR/CoRH	1,804	0,176	8,220	0,001	0,082	0,922	
S*I*MoR/CoRH	1,382	0,241	2,482	0,036	0,753	0,610	
Comparison of means	Mean	group	Mean	group	Mean	group	
Soil type (S)	S1	325,450	a	129,643	a	79,311	b
	S2	276,548	a	114,352	a	93,957	ab
	S3	278,214	a	140,873	a	101,282	ab
	S4	307,950	a	113,016	a	117,936	a
Inoculum (I)	I1	200,893	b	81,657	b	101,707	a
	I2	177,034	b	114,673	b	85,214	b
	I3	513,194	a	177,083	a	107,445	a
Cultivation system	MoR	294,901	a	100,159	b	91,978	a
	CoRH	299,180	a	148,783	a	104,266	a

*P.ase Ac: Acid phosphatase activities; P.ase Al: alkaline phosphatase activities; FDA: Fluorescein diacetate hydrolysing activities

Effect of inoculum on the microbial activities of the crop soil.

It has also been shown by our results (Tables 3 and 4) that the effect of the inoculum of rice

roots (I2) and bean roots (I1) is less important than the inoculum of rice-bean mixed roots (I3). The presence of a high diversity of

microorganisms at the root level justifies this inoculation response.

Many studies have led to the identification of rhizobacterial microorganisms such as the Proteobacteria and Endobacteria group (Sun et al., 2008; Joshi and Bhatt, 2011; Pereira et al., 2011), vesicular-arbuscular mycorrhizae (Youpensuk and Yimyam, 2005; Scheublin and Van Der Heijden, 2006; Malina Singha and Sharma, 2013) that colonize the roots of legumes and cereals. Thus, the composition of the microbial community in the root zone depends on root type, plant species, plant age and soil/fertilizer type (Campbell, 1985).

Effect of cropping system on the microbial activities of the crop soil.

According to the results (Tables 3 and 4), alkaline phosphorus activities and global microbial activities were significantly affected by the cropping system. The highest values were found on CoRH (combined rice-bean crop) for alkaline phosphorus activity and on MoH (bean monoculture) for global microbial activity. Studies have already shown significant increases in microbial community activity in the rhizosphere of legume alone or associated with other plant families (Song et al., 2007; Wang et al., 2007; Li et al., 2010).

In addition, Wang et al. (2007) pointed out that in an associative system, legume root mixtures lead to a larger microbial community through the mixing of the respective communities of each of the two species. This root mixing zone is richer in organic compounds that are derived from root exudates in equally important quantities. Hartmann et al. (2009) and Dennis et al. (2010) thus confirm that the quantities of root exudates and rhizodepots have a significant influence on the diversity of the microbial community and their activity. These facts demonstrate the importance of community abundance and microbial activity in the soils studied under associational cultivation. According to Barea et al. (2005), soil biological quality refers to the abundance, diversity, and activity of the living organisms in the soil. Indeed, microorganisms associated with bean roots, such as arbuscular endomycorrhizae and rhizobacteria or nitrogen-fixing bacteria, are thus strongly involved in the coexistence

mechanism of plants as well as in maintaining soil fertility.

CONCLUSIONS

In conclusion, the choice of an associative crop between rice and bean, the inoculation technique using roots pieces, and the rational use of chemical and organic fertilizers at optimal doses could improve the yield of upland rice. In this study, we recorded that the P and N contents of the aerial parts of upland rice were positively affected by the combined cultivation of rice and beans, and by the inoculum from bean roots. In addition, the involvement of soil microorganisms (rhizobacteria and endomycorrhizae) as well as the tripartite interaction, arbuscular endomycorrhizae - nitrogen-fixing bacteria - legumes, in the mechanism of plant coexistence showed particularly better N and P acquisition and improved nutrient sharing in rice plants. It has also been shown that soils under the associative cropping system maintain soil fertility that corresponds to the abundance of community and soil microbial activities.

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