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Effects of Rhizobia and Mycorrhizae Inoculations on the Growth and Nodulation of *Chamaecytisus proliferus*

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Abstract
The present study aims to assess the effect of rhizobia and /or mycorrhizae inoculations on the growth and nodulation of Tagasaste "*Chamaecytisus proliferus* subsp. Palmensis". Three strains of mycorrhizae and twelve rhizobia strains were used. The mycorrhizal inoculation was done at the time of the seedling, mixing the spores of three arbuscular mycorrhizal fungi with a sterile peat. The rhizobial inoculation was performed twice by applying the inoculum that contained a mixture of 12 strains of Rhizobium multiplied before in the Yeast Mannitol Broth medium. The biomass and nodulation were evaluated under different treatments. The essay was realized at the greenhouse the Faculty of Sciences, Moulay Ismail University. The Inoculation with these root symbionts, separate or combined, increased the biomass accumulation of the Tagasaste treated compared to the absolute control. However, the best response result was that of the simple rhizobial inoculation which showed a very good growth as well as an important root infection assisted by the number and weight nodule compared to the single mycorrhizal inoculation and to the dual inoculation. Finally, the symbiosis with rhizobia and mycorrhizae is an important biological technology to improve the sustainable production of leguminous plant in different agro-ecological regions.

1. Introduction

The interaction rhizosphere-plant involves the plants root colonization (in and around the root) by microorganisms that may develop a symbiotic relationship, mutual, saprophytic or parasitic within the plant, depending on the type of microorganism, the plant defense system, its nutritional status and the soil environment [1]. These colonizers roots influence the growth and productivity of plants [1]. The application of symbiotic microorganisms in agriculture like the Arbuscular Mycorrhizal Fungi (AMF) [2, 3] and Rhizobium [4, 5], were able to provide significant benefits mainly on the rehabilitation of degraded soils [6]. Several studies concerning the dual inoculation with these two types of microorganisms have revealed their roles in the improvement of soil fertility, the growth, the mineral nutrition and the plant resistance against pathogens and drought [7, 8, 2, and 3]. The AMF play an important role in the mobilization of the non-bioavailable phosphorus in the soil, while the rhizobia fix atmospheric nitrogen [9]. Previous research studying the tripartite symbiosis, legume-rhizobia-mycorrhiza, had shown stimulatory effects [10] or inhibitors [9, 11].

The Tagasaste (*Chamaecytisus proliferus* subsp palmensis) is a perennial legume shrub, native to the Canary Islands [12], able to establish symbiotic relationships with the rhizobia and the AMF. Studies on Tagasaste (*Chamaecytisus proliferus*) began at the end of the nineteenth century, but its agronomic characteristics were determined until 1982 [14, 15, 16, and 13]. Indeed the Tagasaste was characterized by a high agronomic value [17]. This fast-growing shrub produces large amounts of biomass (usually more than 18 tons per hectare per year) [18], rich in protein (20-25%) [17], which gives it the importance to be used as forage for dairy goats in semi-arid regions [19]. Besides these nutrition qualities, Tagasaste can play a crucial role in preserving the environment. It offers significant advantages in terms of erosion control [20], salinity [21], soil fertility [22] and deforestation [13]. As so, it will contribute to the colonization of infertile ecosystems, especially as it grows easily on sandy soils and tolerates low annual rainfall (the lower limit is 300 mm). [13] Finally, in addition to all its benefits as a good fodder and an important element on preserving the environment, this plant has multiple industrial uses such as the paper fabrication [23] and the energy production [18].

As so our study assesses the effects of Double inoculation rhizobial and mycorrhizal on the growth and nodulation of the Tagasaste (*Chamaecytisus proliferus* subsp. Palmensis).

2. Material and Methods

2.1. Nodule Collection

Four legumes (red bean, white bean, groundnut and cowpea) were collected from the green house in the faculty of science at Moulay Ismail University in Meknes-Morocco. From each plant, 10 nodules were collected randomly. Isolation of bacteria was performed after nodule collection.

2.2. Isolation of Strains from Nodules

Each nodule was crushed in 1 ml of sterile distilled water. The suspension was flooded on Yeast Mannitol Agar (YMA) plates and incubated at 28°C. Single typical colonies of rhizobia were subjected to the Gram staining. Purity of the cultures was ensured by repeatedly streaking the bacteria on YMA and verifying a single type of colony morphology.

2.3. Inoculum Preparation

After their purification, we have choose 12 isolates that were grown separately for 72 h in Yeast Mannitol Broth (YMB) at 28°C on a rotary shaker at 200 rpm. Growth was monitored by optical density measurement of the cell suspension at 600nm. Cell densities were related to viable cell numbers measured as $cfu.ml^{-1}$ by standard plate count and the number of bacterial cells was adjusted to$10^9 cfu.ml^{-1}$[24].

2.4. Plant Inoculation Test

2.4.1. Plant Inoculation Test

The plant inoculation test of Tagasaste was performed in the alveoli placed in a greenhouse at the Faculty of Science, of Moulay Ismail University. Seeds were hand-sorted for size uniformity and freedom from damage, surface-disinfected by soaking in 1‰ mercuric chloride for 3 min followed by rinsing four times with sterile distilled water for 3, 5, 10 and 15 min as previously described [25, 26]. Surface-disinfected seeds were placed on water agar plates and incubated at 28°C for 3 days to ensure their pre-germination. The experiment was conducted in the alveoli filled with 500g of sterilized peat (pH 7, 23% Organic Carbon and Organic Nitrogen 0.4%). The mycorrhizal inoculation was applied simultaneously during the seedling with a mixture of soils containing 50 spores/g of soil of the three AMF used (*Glomus clarum*, *Glomus* sp1 and *Glomus* sp2). The Rhizobial inoculation was performed by applying 20 ml ($10^9 cfu.ml^{-1}$) of the rhizobial inoculum per plant. Two reminders of the rhizobial inoculation were done after 15 and 30 days of the first application with the aim to enhance the infection chance.

Treatments were arranged in a randomized complete block with three replicate for each strain inoculated. The plants were watered twice a week. The treatments were maintained in a greenhouse under natural lighting and day/night temperatures.

The plant harvesting was made after 190 days of the seedling and the roots were removed and rinsed carefully. Thereafter, the number and the fresh weight of root nodules, the dry shoot and root parts weight (oven-drying for 72 hours at 65°C) were measured to assess the effect of the double inoculation rhizobial and/or mycorrhizal on the growth of the Tagasaste.

2.4.2. Mycorrhizal Root Infection

After thorough rinsing with tap water, the roots were stained with trypan blue to assess the mycorrhizal infection.

2.4.3. Isolation of Strains from Tagasaste Nodules

Nodules were chosen randomly and each nodule was crushed in 1 ml of sterile distilled water. Isolation and purification of all isolates were conducted as described previously in the section (2.2).

2.4.4. Phenotypic Characterization

(i) Colony Morphology

The isolates morphology was evaluated on Yeast Mannitol Agar (YMA) agar plates which were incubated from 3 to 7 days at 28°C. On the basis of the size, color, shape, transparency, borders and elevation the colonies were characterized [27].

(ii) Phosphorus Solubilization Test

The selection of bacteria able to solubilize phosphorus

manifested is based on the appearance of a clear halo around the colony deposited on solid medium containing tri calcium phosphate that is the only source of phosphorus in the medium. Each dish was divided into four parts, and on each quartile, 10 µl of the bacterial suspension were deposited. All plates were incubated in the dark at 28 ° C for 10 days.

2.4.5. Genotypic Characterization

The molecular study involved 12 selected bacteria and was carried out at functional genomic platform, of the Technical Support Unit for Scientific Research, CNRST in Rabat - Morocco.

(i) Genomic DNA Extraction of Isolates

DNA extraction from bacterial strains on liquid culture using the kit "GenElute Bacterial Genomic DNA kit" from SIGMA, Aldrich according to the protocol provided.

(ii) 16S Ribosomal DNA Gene Amplification

To amplify the 16S rDNA gene, two primers were used FD1 (5′ AGAGTTTGATCCTGGCTCAG 3′) and rp2 (5’ ACGGCTACCTTGTTACGACTT 3’) [28]. PCR amplification was carried out in a 25 µl reaction volume-containing template DNA (30 ng), Taq buffer (10x), MgCl2 (50 mM), dNTP mixture (10mM), fd1 primer (100 µM), rp2 primer (100µM), and (5 U/µl) of Taq DNA polymerase. PCR amplification was performed with a « Verity » thermal cycler model from ABI (Applied Biosystems, Foster City, USA). The PCR temperature profile used was 96°C for 4 min followed by 35 cycles consisting of 96°C for 10 s, 52°C for 40 s, 72°C for 2 min, with a final extension step at 72°C for 4 min. Reaction efficiency was estimated by horizontal agarose gel electrophoresis (1% w/v) using a molecular weight marker of 100 bp and photographed. The photos were displayed by the "G Box" photo documentation system.

(iii) 16S rRNA Gene Sequencing

Sequencing was performed on the 515 bp to 907 bp region of the 16S rRNA gene using the 3130XL Dye Terminator Cycle Sequencing (DTCS) Quick Start kit (Applied Biosystems) according to manufacturer instructions with 25–100fmol template DNA and 0.2 µM 515F and 907R primers (515F: GTGCCAGCMGCCGCGGTAA, 907R: CCGTCAATTCCTTTRAGTTT) [29 and 30]. For the purposes of this study, both strands of the 16S rDNA gene were sequenced for 12 samples, only the forwards strand was sequenced for the remainder. The optimal thermocycling conditions for the cycle sequencing reaction were as follows: 25 cycles of 96 °C for 1 min, 96 °C for 10s, 50 °C for 5s, and 60 °C for 4 min, followed by a 4 °C infinite hold. The Sephadex G50 superfine (Sigma Aldrich) was used to remove unincorporated dye terminators from the cycle sequencing reaction, according to manufacturer's instructions with an additional 300 µl wash of the column with distilled H_2O and centrifugation at 1500×g for 3 min prior to applying the sample to the column.

DNA sequencing was performed on the ABI PRISM 3130XL Genetic Analyzer (Applied Bio systems) using the POP-7 polymer and ABI PRISM Genetic Analyzer Data Collection and ABI PRISM Genetic Analyzer Sequencing Analysis software. Preliminary identification was performed by FASTA search of the Ezbiocloud database and identification that is more precise was performed by phylogenetic analysis with type strains of the nearest neighbors. Isolates were regarded as belonging to a species when sequence similarity with the species type strain was at least 99% and to a genus when sequence similarity with a type strain was at least 97%.

3. Results

3.1. Plant Nodulation

The figure 1 (a) shows the absence of nodulation for absolute control and for the simple mycorrhizal inoculation. While for plants inoculated with the rhizobia, we note that there was nodulation on the roots.

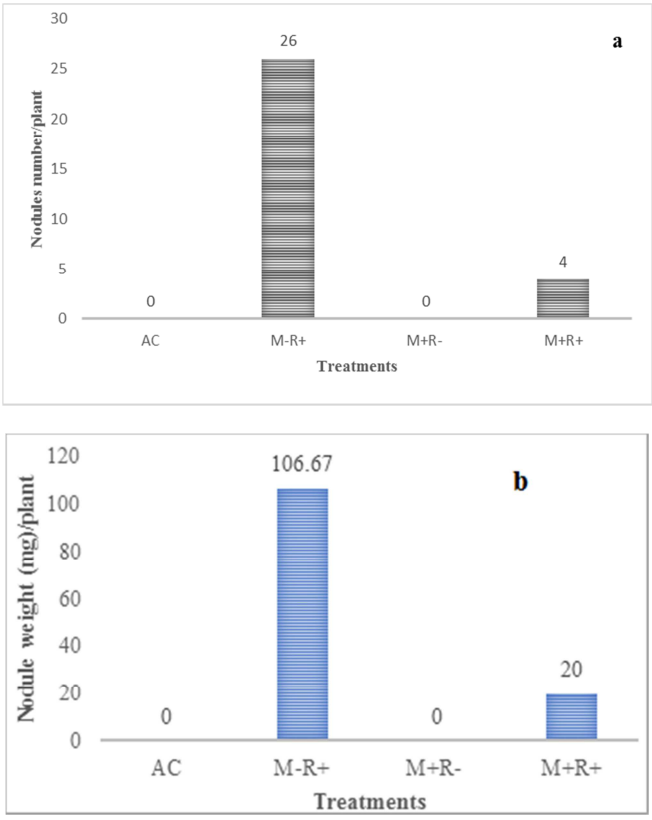

Figure 1. *Response of Tagasast ((a) the number of nodules and (b) fresh weight of nodules)) to the effects of mycorrhizal and / or rhizobial inoculation. AC: Absolute control; M^-R^+: Plants inoculated by rhizobia alone; M^+R^-: Plants inoculated with mycorrhiza alone; M^+R^+: Plants co-inoculated with rhizobia and mycorrhizae.*

Thus, for plants inoculated only by rhizobia showed a high rate of infection (number of nodules), that explains the heavy weight of nodules per plant treated. However, the co-inoculated plants, by both the rhizobia and the AMF showed a low infection rate (number and weight of nodules) compared with the single rhizobial inoculation.

3.2. Strains Characterization

Isolation of rhizobial strains from Tagasast nodules:

Four strains were isolated from nodules removed from Tagasast roots with the aim to characterize them.

3.2.1. Phenotypic Characterization

The Gram staining and a microscopic observation of the isolates showed that all isolates were Gram negative. Generally, the greater part of the isolates had the same colony morphology over and above a fast and high growth rate on YMA medium. 90% of the studied isolates, formed transparent to creamy mucoid colonies with 3 to 5 mm in diameter within 3 days of incubation on YMA medium.

3.2.2. Phosphorus Solubilization Test

The results of the phosphorus solubilization test of non-bioavailable phosphates, while the tricalcium phosphate was the sole source of phosphorus in the medium, showed that the four bacteria isolated from nodules and tested on GES medium showed a positive result by the development of a clear halo around the colonies.

3.2.3. Genotypic Characterization

The four bacterial isolates were subjected to 16S rDNA gene sequence analysis. For our isolated strains, the 16S rDNA sequences determined in this study comprised 600 to 1500. The 16S rDNA gene sequence analysis showed that there was a high similarity (≥ 99%) between the test strains and their closest phylogenetic relative, which may indicate that 16S rDNA gene sequence data are helpful for identification of isolates at the species level. The sequences of the isolates were identified using the Ezbiocloud database. The isolates $T_4M^-R^+$ and $T_1M^+R^+$ had respectively 99.75% and 99,77% of similarity with the *Citrobacter youngae* with AJ389904 as its accession number. The isolate $T_1M^-R^+$ showed 99,26% of similarity with *Klebsiella oxytoca* (AB004754). The isolate $TagM^-R^+$ had 99,58% sequence identity with *Rhizobium lusitanum* (AY738130).

3.3. Mycorrhizal Infection of the Tagasast Root

As showed in the Figure 2, no mycorrhizal infection was observed either in the absolute control roots of Tagasaste and in the single inoculation with rhizobia

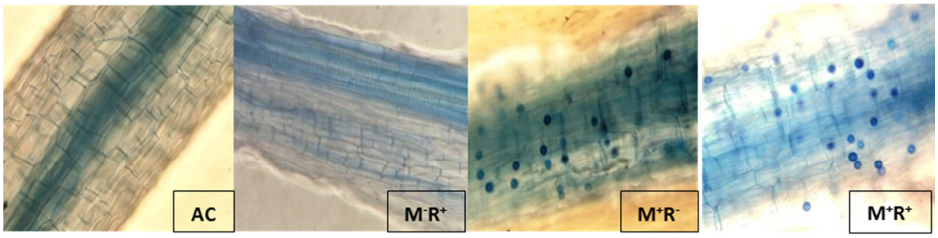

Figure 2. *Microscopic Observation of the Tagasaste roots with (x400). AC: absolute control; M^-R^+: Plants inoculated by rhizobia alone; M^+R^-: Plants inoculated with mycorrhiza alone; M^+R^+: Plants co-inoculated with rhizobia and mycorrhizae.*

However, in the case of the single mycorrhizal inoculation and for the dual inoculation, we noticed the presence of spores in the roots.

3.4. Effects of the AMF on the Relationship between the Growth and Biomass of Nodules

The efficiency of the rhizobial symbiosis was evaluated on assessing the relationship between the plant growth and the nodules biomass (Figure 3). The slope of the linear regression in figure 3 varies according to the presence or the absence of the AMF. Thus, rhizobial symbiosis was significantly higher than in the interaction treatment with the AMF.

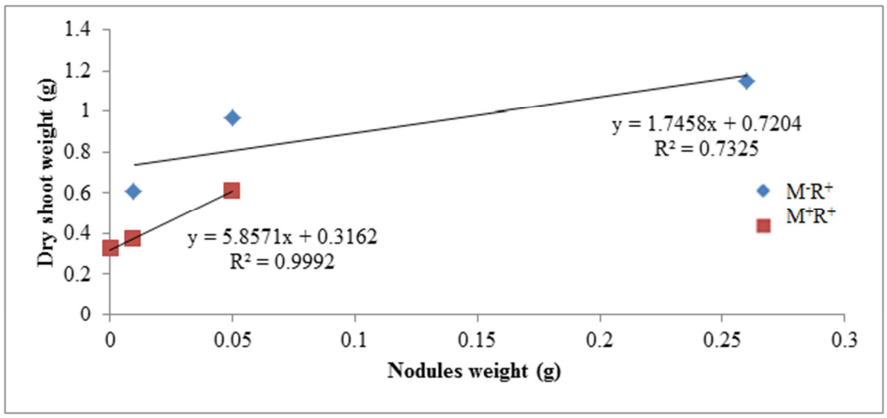

Figure 3. *Effect of the AMF on the relationship between growth (g / pot) and nodules biomass (g / pot). With M^-R^+: Plants inoculated by rhizobia alone; M^+R^+: Plants co-inoculated with rhizobia and mycorrhizae.*

3.5. Dry Shoot Weight

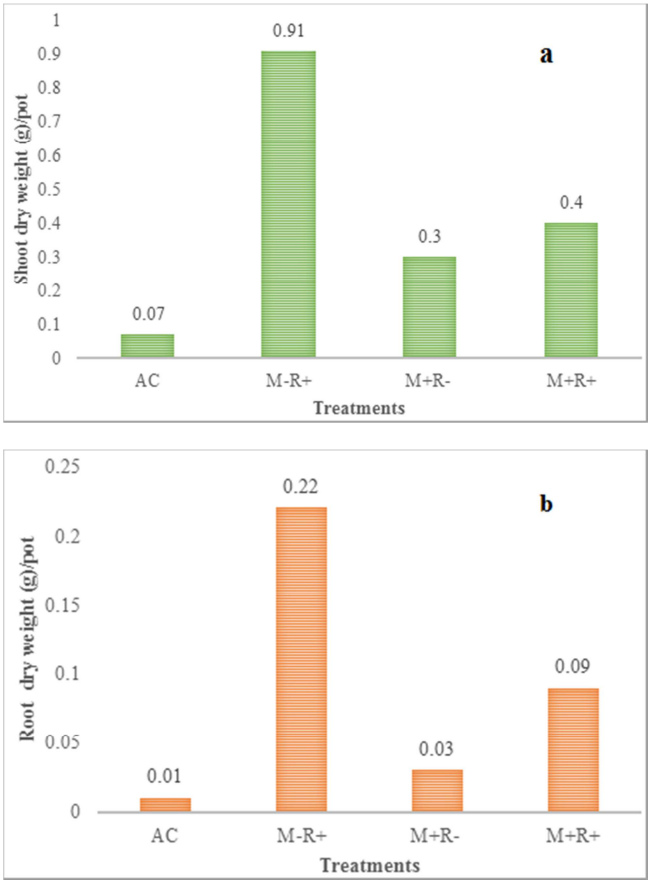

Figure 4. *Effects of mycorrhizal and / or rhizobial inoculation on (a) shoot dry matter accumulation and (b) root dry weight. AC: absolute control; M^-R^+: Plants inoculated by rhizobia alone; M^+R^-: Plants inoculated with mycorrhiza alone; M^+R^+: Plants co-inoculated with rhizobia and mycorrhizae.*

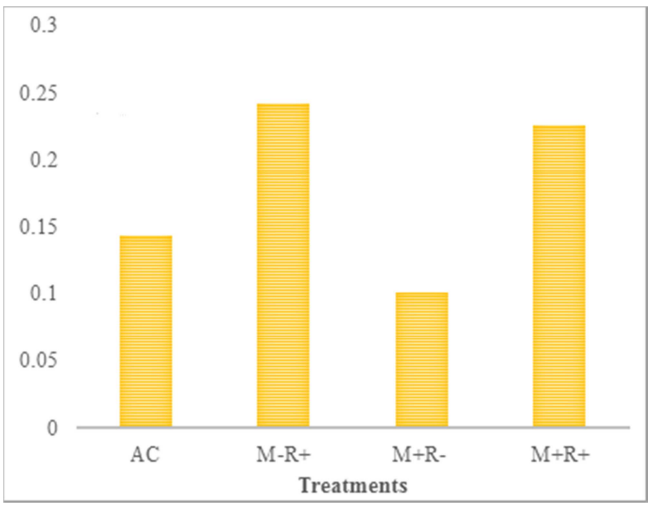

Figure 5. *Effects of mycorrhizal and / or the rhizobial inoculation root / shoot report. AC: absolute control; M^-R^+: Plants inoculated by rhizobia alone; M^+R^-: Plants inoculated with mycorrhiza alone; M^+R^+: Plants co-inoculated with rhizobia and mycorrhizae*

The results in the Figure 4 showed that the shoot and root dry matter of the absolute control was very low; compared to the absolute control, the effect of single inoculation with rhizobia was very beneficial, it increase 13 times the rate of the shoot dry biomass and 22 times for the root biomass. The same result was found in the case of single inoculation with mycorrhizae with a rate of 4.3 and 3 times for shoot and root biomass respectively, which remained low compared to that of the single rhizobial inoculation.

The dual inoculation improved shoot and root dry weight in comparison to the absolute control and also to the single mycorrhizal inoculation However, compared to the single rhizobial inoculation, a decrease of accumulation was observed in shoot and root dry matter of 56% and 59% respectively.

3.6. The Distribution of the Shoot and Root Biomass

The distribution of the shoot and root biomass was significantly affected by the simple rhizobial inoculation as well as the double inoculation (Rhizobium + AMF) in comparison to the absolute control; However the simple mycorrhizal inoculation decreased significantly this ratio (root/shoot).

4. Discussion

The current environmental problems (deforestation, pollution, erosion, ...) have been aggravated hence the use of the remediation and rehabilitation tools is needed to maintain the productivity of agricultural systems [31and 32] through the use of biological systems such the introduction of fast-growing plants such as Tagasaste [14] in order to improve the physico-chemical properties of the soil [33], to increase its flora and fauna diversity as well as the yield and the quality of subsequent crops [34, 35 and 36].

The AMF are microorganisms having a great agronomic interest. These bio-fertilizers are often more suited to soil conditions hence their competitiveness varies depending on soil conditions and soil-plant interactions. Consequently, the AMF have different strategies to colonize the roots that help to improve the shoot and root biomass [37] as well as reducing the need into plants fertilizer [38]. This result is in perfect agreement with ours which showed that the shoot and root biomass were enhanced 4.3 and 3 times respectively compared to the absolute control.

Therefore, the characteristics of the AMF make them an interesting tool to select elite species and promote the domestication and the improvement of recalcitrant species mainly in soils characterized by their low fertility [39].

As another tool recognized by its importance in the rehabilitation of poor soils especially on nitrogen, rhizobia are characterized by their ability to tolerate the main environmental factors [40 and 41]. These microorganisms improve the growth and biomass of legumes and therefore increased yields. These results confirm ours and can be explained by the fact that the rhizobial inoculation ensures

better absorption of nutrients, protection against pests and pathogens and the induction of the systemic resistance of legumes [42, 43, 44, 45 and 46]. In addition to its role in the mobilization of nutrients, these rhizobia secrete auxins that promote root growth which in turn stimulates root infection [47 and 48]. This could explain our results that showed a good infection assisted by the number and the weight of nodules.

However, it is recommended to identify combinations with other microorganisms promoting the growth of plants such as phosphate solubilizing bacteria or the AMF, which act synergistically to improve the productivity of different legume crops [41]. A previous study showed that the symbiotic effectiveness is dependent on the particular combination of the strain of Rhizobium and *Glomus* species, indicating the selective and specific compatibilities between bacterial and fungal strain [22]. This result is verified by our study through which we noticed a significant improvement in shoot and root biomass of Tagasaste inoculated with rhizobia and/or AMF. This is explained by the fact that the growth responses of the inoculated legumes are influenced by the symbionts and the compatibility of the interactions between them and those with the host plant [18, 20 and 22]. However, there was a significant decrease in the number (85%) and the fresh weight (81%) of the nodule; this could be explained by the inhibitory effect exerted by the AMF on the rhizobia that is in perfect agreement with the results proved by Franzini et al [11]. Moreover, this inhibitory effect could be due either to interaction between the two symbionts or depending to the stage of their development inside the roots of the host [21].

Beside the importance of the rhizobia and the AMF for the maintain of the environment, the third interesting tool known by its ability to make the phosphorus in its bioavailable form in the soil, the phosphate solubilizing bacteria (PSB) play a fundamental role in the biogeochemical cycling of phosphorus in agricultural systems [41]. These BSP transform the insoluble phosphorus into a soluble form bioavailable to plants thus by the medium acidification, chelation, exchange reactions, and the formation of polymeric substances [12, 23 and 41]. According to our results, we have found that all the rhizobial strains isolated from Tagasaste nodules were PSB strain. This result is in perfect agreement with those reported by Saghir Khan et al [49]. This confirms the dual ability of Rhizobium once to nodule Tagasaste and the second to dissolve the non-bioavailable phosphates, and therefore their use as biofertilizer may have a direct application to improve soil fertility, to reduce chemicals and consequently participate to the protection of the environment.

5. Conclusion

In conclusion, our results have verified the important effects of the co-inoculation with rhizobia and AM fungi on the growth of Tagasaste. These results show an interesting rehabilitation of marginal lands that are often deficient in nutrients. Symbiosis with rhizobia and mycorrhizae is a biological technology that can improve the sustainable production of leguminous plant in different agro-ecological regions.

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