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Original Research Article

Effects of Selected Endophyte Actinomycete on Growth, Nodulation, Nitrogen Fixation and Yield for three leguminous

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ABSTRACT

Keywords

Actinomycete, *Bradyrhizobium japonicum, Rhizobium leguminosarum, Bradyrhizobium lianogensis,* Clover, Soybean, Faba bean

In Morocco, the effect of actinomycetes, endophytes and three strains of rhizobia on growth and nodulation of leguminous plants have been studied in the green house. Four treatments were applied as follows: the control plants with faba bean, clover and soybean non-inoculated (T0), inoculated plants based actinomycete endophyte (T1), inoculated plants based on three strains of rhizobia (T2) (Bradyrhizobium *japonicum* to Soybean, *Rhizobium leguminosarum* for faba bean, and *Bradyrhizobium lianogensis* for clover) and the third treatment consisting of inoculated basis of actinomycetes combination with the three strains of rhizobia (T3). Improving growth is almost universal in all species studied. Furthermore, the combination of strain A and rhizobia showed the same results as those observed in the single bacterium, the number of nodules per plant obtained and found that the diameter of bean plants have a significantly greater number of nodules higher than that recorded in clover. It was found that the strain A induces secretion of flavonoids.

Introduction

Nitrogen is an essential element for the development of living beings, it is present in large quantities in various forms in the different compartments of terrestrial ecosystems [12]. Indeed, about 80 % of the atmosphere consists of nitrogen gas, representing an almost inexhaustible source of nitrogen. Nitrogen is an element present in large quantities in living beings. [20] In some soils, the amounts of nitrogen available to plants are low.

This often is a major limiting factor in the growth of cultivated plants. This paradox is because the nitrogen molecule is very stable and higher organisms are unable to use.

The best known example is that of rhizobia, which are symbiotic associations with legumes such as bean, soybean, peanut, clover, alfalfa [6]. The roots of leguminous plants release chemicals recognition (type flavonoids / isoflavonoids) [10]. These exudates attract bacteria (rhizobia), which in turn synthesize and secrete nodulation factors (Nod factors) [5]. These Nod factors are different depending on the rhizobial species and a specific antigenic structure recognized by the plant [5].

In addition, legumes are an important source of fertilizer for the poor soil nitrogen. They are considered a natural source of nitrogen for soil enrichment. Agricultural engineers advise farmers to switch their crops by growing legumes [17].

Recently, several commercial products based on nitrogen-fixing bacteria namely species of Azospirillum, Rhizobium and Azotobacters appeared. The results obtained with microorganisms seem satisfactory. Moreover, essentially in the case of symbiotic bacteria Rhizobium, there are other microorganisms which may interact with the plant and Rhizobium. This interaction allows increased nodulation and thereby improving the nitrogen fixation.

Actinomycetes that produce secondary metabolites are known for their role in agriculture. Among other things, improving the soil structure, the production of plant growth factors (phytohormones, organic acids, siderophores). These bacteria are also capable of inducing the production of plant secondary metabolites such as polyphenols [2].

Research and demonstration of one or more strains, which can improve and increase nodulation and nitrogen supply to plants, has become one of the main objectives of agriculture.

In this work are interested in both study and understand the mechanisms used by the strain A to stimulate Rhizobium-legume association.

Materials and Methods

Materials

Microorganisms

Isolate A belongs to the collection of the Laboratory of Microbial Biotechnology center transfer and innovation.

The rhizobia used belong to the collection of the Laboratory of Microbial Biotechnology center transfer and innovation.

-*Bradyrhizobium japonicum*
- *Rhizobium leguminosarum*
- *Bradyrhizobium lianogensis*

Plant material

In this study there are three different légumineuses with today great economic interest to have:

- Seeds of Soybean (*Glycine max*)
- Seeds of Clover (*Trifolium pratense* L)
- Seeds of Faba Bean (*Vicia faba*)

Specificity to Strain A of the plant and Rhizobium

Seed sterilization is to immerse soybean, faba bean and clover in a mixture of distilled water and bleach for 10 min. Then, they were rinsed three times with distilled water and dried on sterile Whatman paper in the water extractor. The corresponding seeds are placed in each pot, three soybean at a depth of 1cm, three seeds faba bean at a depth of 2cm and 2g seed clover 3mm deep. They are then covered with a thin layer of potting soil to promote contacts seeds. The pots were irrigated by daily intakes of tap water. The terms of the processing performed in this test are presented in Table 1. Several combinations were performed in order to determine those promoting better symbiosis between plants and microorganisms tested. We repeated five times for each treatment and each plant.

Study of the mechanisms employed by the strain A to stimulate nodulation

Study of the effect the Strain A on the rhizobia

Rhizobia strains contained in a liquid YMA medium were co-cultured with the juice and the cells of strain A separately. The reading of the results was carried out every day by measuring the optical density at a wavelength of 600 nm.

Study of the effect of the Strain A in stimulating the production of flavonoids

After the surface sterilized seeds of soybean, they are placed in vials containing the different treatments:

- Sterile water
- 100% of filtrate of strain A
- 100% of filtrate of *R. japonicum*
- 50% of the filtrate of strain A
- 50% of *R. japonicum* filtrate
- Stem Cell
- Cell *R. japonicum*

Then, these seeds are germinated in the dark under agitation in the growth chamber for 4 days at 28 °C. These seeds are then removed; the supernatant was extracted with ethyl acetate at a 10:10 ratio (v/v). The organic fractions of ethyl acetate are obtained evaporated in rotavapor. The dry residue is recovered in 95% methanol, put in vials and injected in HPLC (gradient isocratic mobile phase 40: 60 Methanol (100%) and (0.5%) orthophosphoric acid at a flow rate of 1ml min by a temperature of 25°C and a detection wavelength of 370 nm.

The peaks obtained are identified by their retention time and absorbance spectrum by comparing them to standard commercially available from Sigma Aldrich, such as quercetin, luteolin, Genistein, Daidzein and β naphtho flavone. Standards are prepared at concentrations of 5 mM in methanol.

The nod factor was analyzed by an HPLC system using a column of type XBridge C18, 5μm 4.6 μ X 150mm. The nod factor was separated by isocratic gradient Water {Water (C) / methanol (B) (80: 20)} and detected at 280 nm with a visible wavelength of 220 nm UV detector [8].

Result and Discussion

Specificity to Strain A of the plant and Rhizobium

To confirm the above study on the growth of legumes, we wondered if the isolate A is only a species of Rhizobium or all existing species. Similarly, if we isolate A. is subservient to all legumes or only a few species (Figure 1). Thus, a test was conducted in a greenhouse with several legumes and rhizobia strains specific.

Figures 1 and 2 represent the different results recorded after the test. These figures show that the treatment of the seed with strain A improves significantly the growth of soybean plants, faba bean and clover. Indeed, increases were recorded in treated plants with strain A, the concerned increases growth factors (length of the aerial part (Figure1a) length of the root part (Figure1b), fresh weight from the aerial part (Figure 1c) and fresh weight from the root part (Figure1d).

These improvements in growth are widespread in almost all species studied. Furthermore, the combination of A and rhizobium strain showed the same results as those observed in the single bacterium.

Table.1 Different treatments undertaken when testing the specificity of the strain A

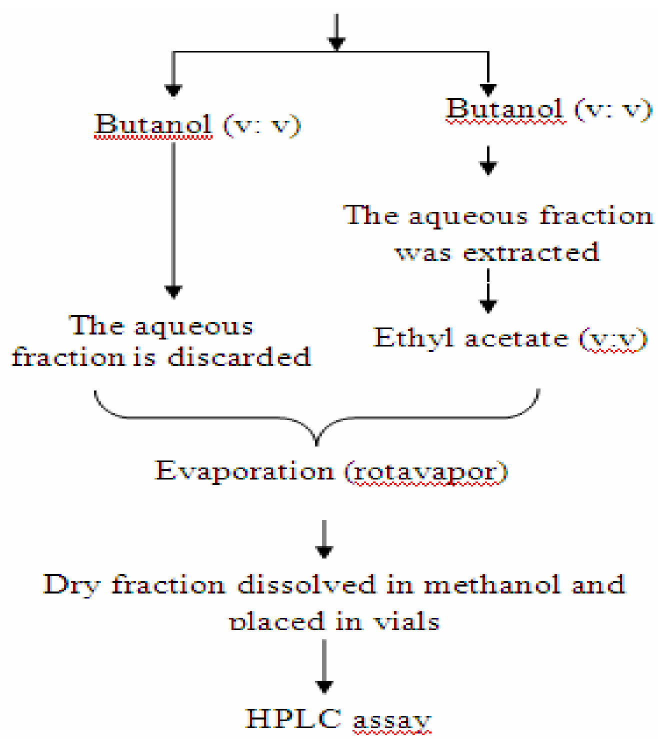
Plant Tests	Treatment 0	Treatment 1	Treatment 2	Treatment 3
Faba bean, Soybean and Clover	1ml water	1ml S A	1 ml Rhz	1ml SA+1ml Rhz

S: Strains A

Rhz: Rhizobia equivalent specific of each plant. *Bradyrhizobium japonicum* to Soybean, *Rhizobium leguminosarum* for Faba bean and *Bradyrhizobium lianogensis* for clover.

Study of the production the factor Nod by Strain A

The extraction was performed according to the following scheme:
500 ml of juice of the Strain A



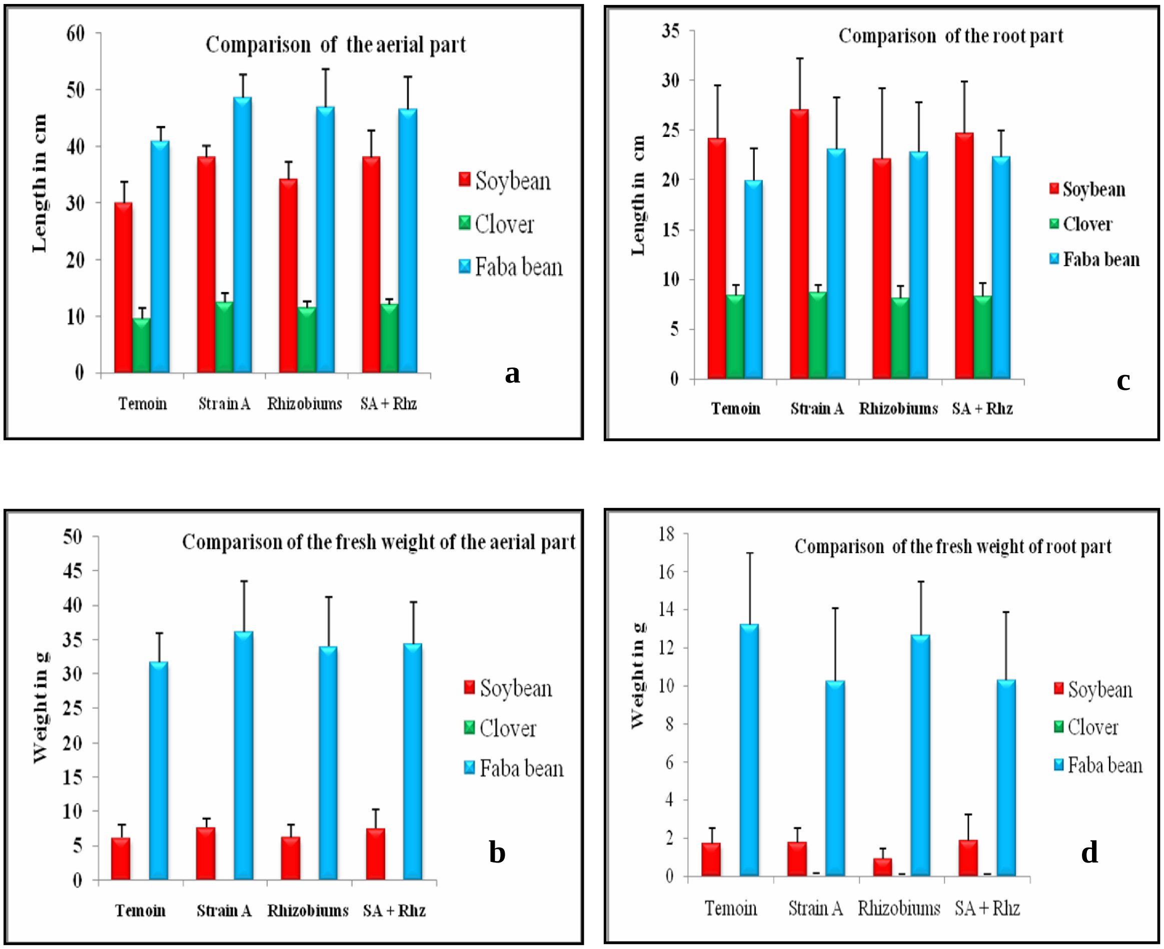


Figure.1 Study the selective effect of plant-rhizobia (Soybean, Clover, Faba bean) by Strain A: **a.** Size of the air part, **b.** Size of the root part, **c.** Fresh weight of the aerial part and **d.** Fresh weight of the root part.

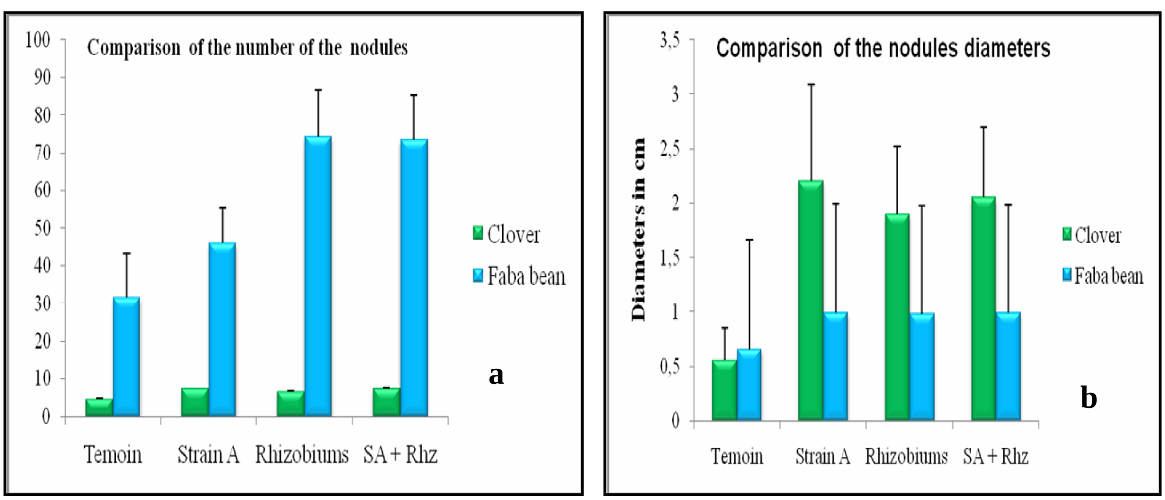


Figure.2 Study the selective effect of plant-rhizobia (Soybean, Clover, Faba bean) by Strain A : **a.** Number of nodules and **b**. Diameters of the nodules

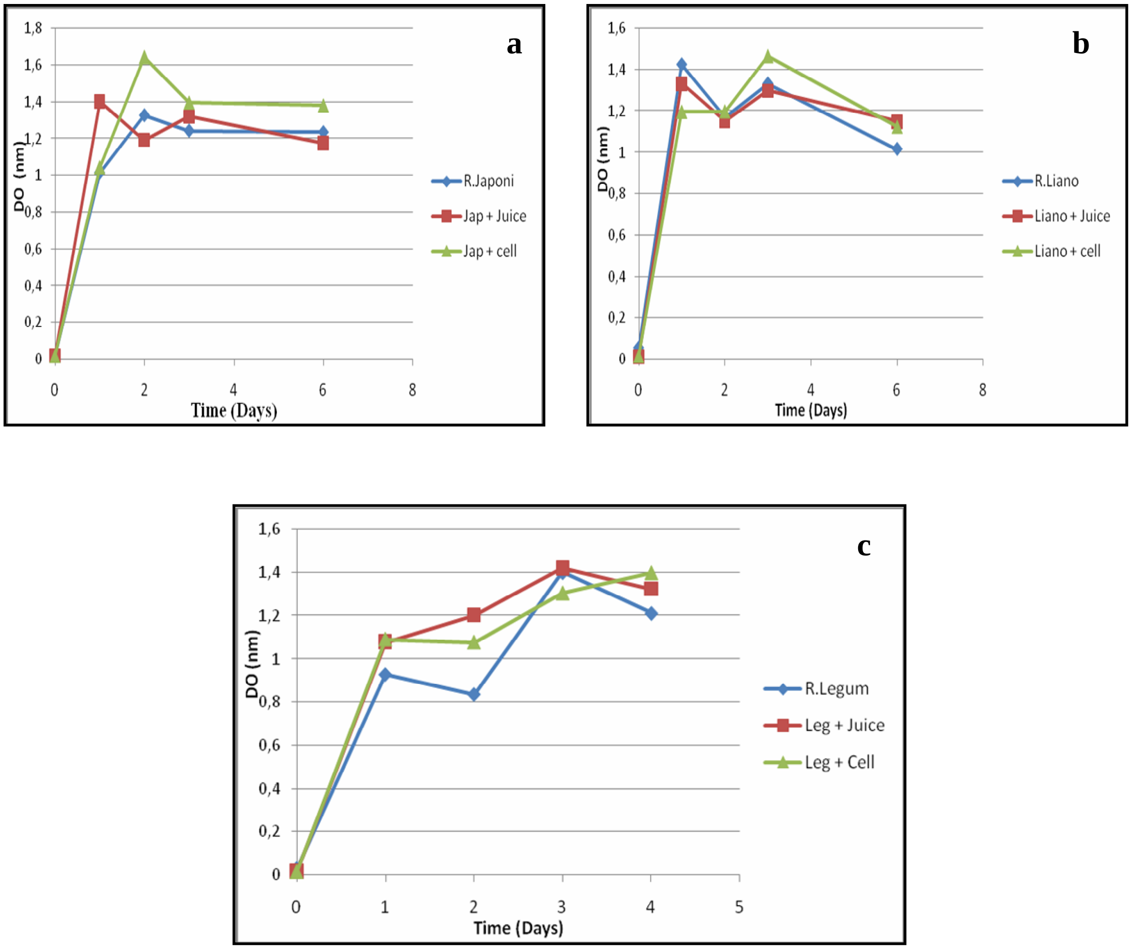


Figure.3 Effect of strain A on the growth of rhizobia **a.** has Growth kinetics of *Bradyrhizobium japonicum* **b.** Growth kinetics of Bradyrhizobium lianogensis and **c.** Growth kinetics of *Rhizobium leguminosarum*

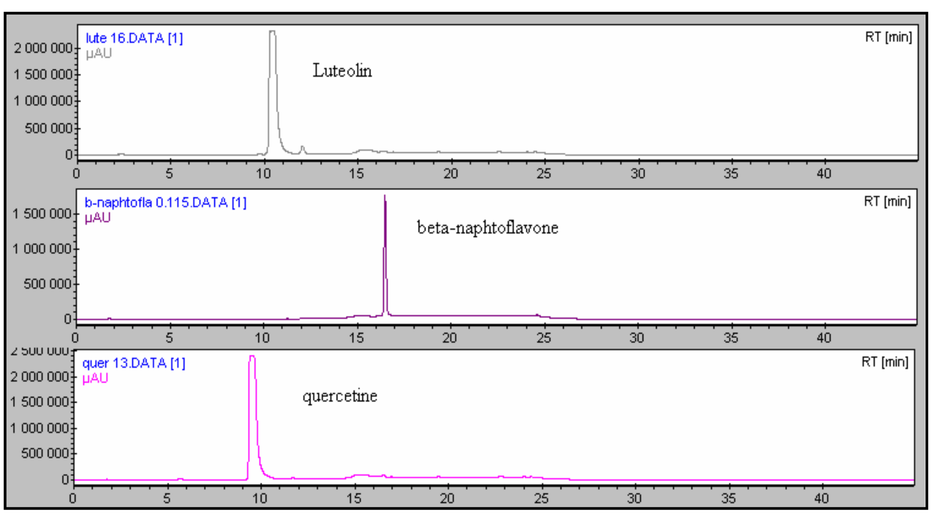


Figure.4 Chromatograms of standards. Luteolin (tr: 10mn) Beta-naphthoflavone (tr: 16mn) Quercetin (tr: 9mn).

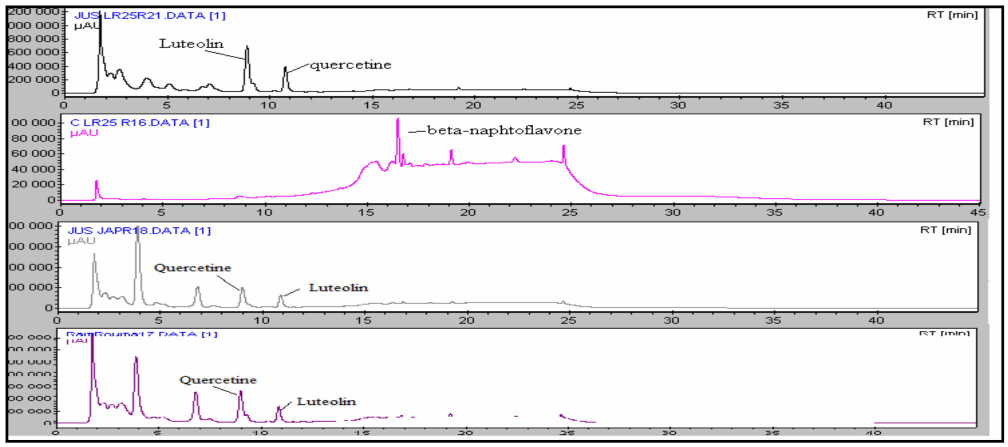


Figure.5 Chromatogram of different treatments filtrate of strain A, Strain A, filtrate of *R. japonicum*, association filtrate and filtrate *R.japonicum* and strain A.

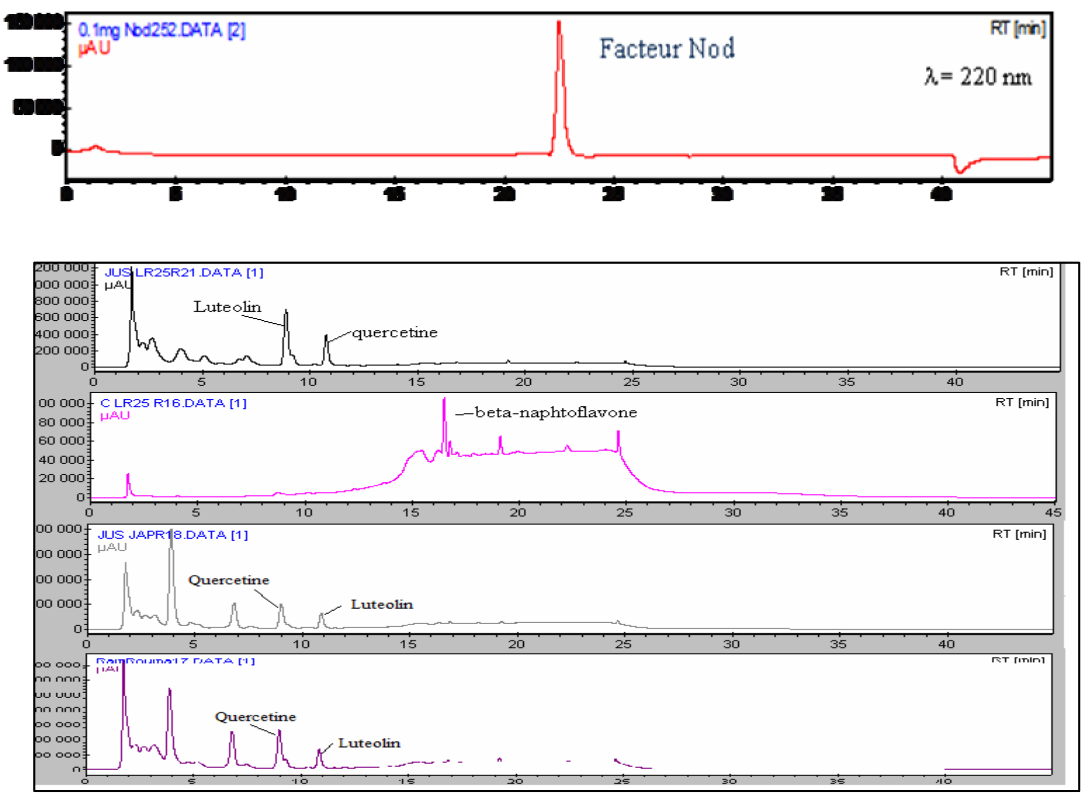


Figure.6 Chromatogram of the butanol extracts, filtrate of strain A

Counting the number of nodules per plant obtained and found that the diameter of bean plants have a significantly higher number than that recorded in nodules clovers. Soybeans showed no nodule formation. This could be explained by the fact that the plucking period plants were relatively early compared to that required for the formation of nodules in this species.

Counting the number of nodules per plant obtained and found that the diameter of bean plants have a significantly higher number than that recorded in nodules Clover (Figure 2a). Soybeans showed no nodule formation. This could be explained by the fact that the period of uprooting plants was quite early compared to that required for the formation of nodules in this species. On the diameter of nodules, those observed in the bean are also much larger than the nodules noted in clover (Figure 2b).

Study of the mechanisms employed by the strain A to stimulate nodulation

Study of the effect the Strain A on the rhizobia

The effect of isolate A on the growth of rhizobia in liquid medium was studied. The results in Figure 3 show that the growth kinetics of rhizobia (*Bradyrhizobium japonicum, Bradyrhizobium lianogensis, Rhizobium meliloti and Rhizobium leguminosarum*) is not affected by the presence or absence of strain in the strain A.These results allow us to conclude both that strain A has no negative effect on rhizobia. It can be used in co-treatment of the seeds or roots of plants. Moreover the hypothesis stimulating increased nodulation by rhizobia bacteria growth is not valid.

Study of the effect of the Strain A in stimulating the production of flavonoids

This study was done only on soybeans; thereof were germinated in vials tubes isolates tested in the presence and flavonoids were extracted from root exudates and assayed by HPLC. These results showed that seed treatment of soybean with the filtrate of strain A induces the production of the release of Quercetin and Luteolin in the plant root exudates, while seed treatment by cells of the strain induced the production of β-naphtofavone. Thus, a difference in behavior was noted between the soybean in the presence of the bacterium and the soybean in the presence of only bacterial filtrate. However, these results suggest that the strain filtrate and allow the production of flavonoids in the plant (Fig. 4 and 5).

Analysis of the chromatograms showed that also the co-treatment of seeds by the filtrate of strain A and *Bradyrhizobium japonicum* induced increases the amount of flavonoids by *Bradyrhizobium japonicum* These results rise that isolate A induces the secretion of flavonoids. Also, the combination of both microorganisms Rhizobium and strain A amplifies this secretion. This will test our hypothesis that the strain A stimulates the production of flavonoids in the roots and root exudates, which could explain the increase in the number of nodules and their diameters mentioned above.

Study of the production the factor Nod by Strain A

After the extracts by HPLC analysis, the resulting chromatograms (Fig.6) were compared with those of the standard used (Penta-N-acétylchitopentaose; Sigma Aldrich).

The chromatogram of the butanol extract showed the presence of certain molecules, but without the presence of the standard molecule. This result shows that the A strain does not produce similar Nod factor molecules used in this study, it may be that it produces other molecules of different structures or products does not.

The positive effect of strain A stimulate nodulation and plant growth is consistent with the results of studies [16], [1] and [14] in the study [18], the roots of peas colonized endophytes by actinomycetes (Streptomyces lydicus WYEC 108) resulted more nodules in the roots. In our study, the double inoculation of strain A improves considerably the number of nodules in plants and clover bean, while in soybean was not forming nodules, this could be explained by the fact that period plucking plants was quite early compared to that required for the formation of nodules in this species. These results are consistent with the findings of [19].

In this study, the existence of strain A in tissues of the plant has not been studied. But the ability of the strain to colonize the P4 host soybean plant was previously tested by [17], [18].

Studies have shown that the phytohormones are involved in the interaction between the plant and the rhizobacteria promote plant growth, by IAA that is one of the most important improvements of phytohormones in the growth of plants. Cytokinins and auxins may act synergistically to initiate cell division and the formation of the primary nodules, which are intermediate between the symbiotic assotion plants and rhizobia [11]. The analysis of the kinetics of the formation of nodules in the case co-inoculation (A + strain Rhizobium) (Figure 2a and b) and the dosage of flavonoids suggested that the effect of running actinomycete early infection between plant roots and the genus rhizobium bacteria. These results are consistent with the work of [16] and [15].

Based on analyzes mentioned in Figure 3 and the results in the HPLC technique figures 4, 5 and 6. We found that strain A does not affect the growth of rhizobia (*B. japonicum, B. lianogensis, R. meliloti and R. leguminosarum*). From Figure 6, we can say that this strain is unable to produce Nod factor, which is responsible for the physical and physiological changes in the host plant, such as root deformation, cell division and organization of the primordium nodular [7] [8], [11] and [4]. The figures 4 and 5 it can be concluded that the strain A increases the amount of flavonoids, which have attractors effects on some soil microorganisms.

With the legume symbiotic association between plants and rhizobial bacteria type, an important nitrogen fertilizer saving can be achieved. For example, in Brazil the inoculation of soybean (*Glycine max* L.) with field provides up to 300 kg N/ha, resulting in the estimated 3 billion [13] nitrogenous fertilizer savings.

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