Physiological characteristics of *Medicago truncatula* shoot growth from vegetative stage to fructification

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ABSTRACT: To develop more our knowledge on the model plant *Medicago truncatula,* we followed the shoot growth of inoculated plant from the vegetative stage to the fructification. The development of *M. truncatula* is characterized by a slow rhythm of growth. Once the plant satisfies their needs in nutriments, and especially in nitrogen via the symbiotic association, the matter production becomes more important. We register a decrease in the total soluble sugar beyond 4 weeks post inoculation (wpi). The fluctuation of the protein amount and the proteolytic activity reveal a change of the growth stage and indicate a remobilization of the plant reserve to sink organs. In addition, *M. truncatula* is characterized by a stability of the chlorophyll and potassium amount under control condition of growth. In fact, we register during the cyclical growth of *M. truncatula* a variation on the shoot calcium. The nitrogen elements change during the different stages of growth. This characterization of the physiological parameters of the development of this model plant presents a basis to study the molecular and the genes of different growth stages.

*Keywords***:** *Medicago truncatula,* Growth stages, Chlorophyll, Protein, Proteolytic activity, Nitrogen elements.

INTRODUCTION

Legumes represent the third largest group of angiosperms and are the second largest group of food and feed crops grown globally. It regroups varied food and feed crop species, such as clover, chickpea, soybean, pea, alfalfa and mung bean. Grain legumes provide about one-third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Graham and Vance, 2003). Indeed, same variety such as soybean and *Pongamia pinnata* takes more attention because of their high seed oil content how can be used like a biofuel (Scott et *al*., 2008). Also, for human, legumes present a source of: essential minerals (Grusak, 2002a), of secondary compounds that can protect against human cancers (Grusak, 2002b; Madar and Stark, 2002) and insulin (Jenkins et *al.,* 2003). One-third of the genus *Medicago* is composed by annual medic species, majority of which are diploid, self-pollinated and present a faster growth, good feed quality, and crude protein value (Zhu et *al*., 1996).

However, *Medicago* is able to evolve a symbiotic relationship with specific soil bacteria, called *Rhizobia* (de Faria et *al.,* 1989). The legume-rhizobia symbioses play a bigger role in the biological nitrogen fixation. The fixation is estimated roughly to 200 million tons of nitrogen annually (Graham and Vance 2003; Peoples et *al*., 2009).

To develop more knowledge on legumes, *Medicago truncatula* get more attention because of its small genome (500-550 Mpb) (Young et *al.,* 2005) and its short growth cycle. This model plant is the subject of numerous researches that studies the symbiotic and the mycorrhizal symbiosis (Ané et *al.,* 2004), the abiotic stress tolerance (Narasimhamoorthy et *al.,* 2007), the disease resistance (Ameline-Torregrosa et *al.,* 2008). But what are the physiological characteristics of this model plant under controlled conditions?

In this work, we will try to present some physiological, biochemical, and nitrogen composition of *M. truncatula* shoots during different growth stages. The kinetics of development of inoculated *M. truncatula* with *Sinorhizobium meliloti* is followed during ten weeks post-inoculation.

MATERIALS AND METHODS

Biological Material and Growth Conditions

Seeds of *Medicago truncatula* A17 were sterilized with Sodium hypochlorite (0,6 %), rinsed with distilled water and imbibed during 2h. Seeds are placed on moist filter paper in a Petri dish and incubated at 25°C. After 5 d, the seedlings were planted in pots filled with a melange sable/vermiculite (2/3-1/3).

The watering solution contained macronutrients: $CaCl_2H_2O$ (1mM); $MgSO_47H_2O$ (25mM); K_2SO_4 (0,51mM); KH_2PO_4/K_2HPO_4 (5,5mM); Fe-EDTA (0,05mM), and micronutrients (for 1L): H_3BO_3 (2g); $MnSO_44H_2O$ (2,23g); $ZnSO_47H_2O$ (0,287g); $CuSO_45H_2O$ (0,125g); $CoCl_26H_2O$ (0,065g), $NaMoO_42H_2O$ (0,12g) (Frendo et *al.,* 1999). The NH_4NO_3 (5mM) is added to the watering solution just after plantation. One week old, *M. truncatula* was inoculated with *Sinorhizobium meliloti* RCR2011 strain previously grown as described in Frendo et *al.* (2005). Seedlings were grown in a growth room at 25°C/20°C (day/night) with 18h/6h (day/night) photoperiod and 75% relative humidity.

Vegetative growth analysis

Growth parameters of shoots were evaluated after 4, 6, 8 and 10 weeks post-inoculation (wpi). Fresh weights (FW) were immediately determined for shoots. Dry weights (DW) were obtained by weighing the plant material after drying at 80 °C until a constant mass was reached.

Chlorophyll

Chlorophyll measurement was performed according to Wintermans and De Mots (1965), and total chlorophyll concentration was calculated as in Horchani et *al.* (2008).

Ions and soluble sugar

Ions were extracted from dried plant material in a nitric acid (HNO_3). K^+ was assayed by flame emission photometry (Corning, UK). Ca^{2+} was determined by atomic absorption spectrophotometer (Perkin Elmer, Courtaboeuf, France). Total soluble sugars were determined in shoots as in Horchani et *al.* (2009).

Soluble protein and proteolytic activity

Total soluble proteins were assayed according to Bradford (1976) using γ-globulin as a standard. Proteolytic activity in shoot crude extracts was determined spectrophotometrically by following the digestion of azocasein at 440 nm (Brouquisse et *al.,* 1998).

Nitrogen compounds

Fresh shoot samples were ground thoroughly with mortar and pestle in Tris-HCl (pH 7,5), and centrifuged at 20000 g for 10 min. The supernatant was analyzed for nitrate, nitrite, and ammonium, was extracted. NO_3^- and NO_2^- were assayed in plant tissues using the salicylic acid-sulfuric acid method (Cataldo et *al.,* 1975) and the Griess reagent method (Miranda et *al.,* 2001), respectively. Ammonium was determined by the phenol-hypochlorite method (Brouquisse et *al.*, 1991).

Statistical treatments

Three biological replications were used in this study. Statistical data analysis was made using Student's t-test. The results are given as means with standard errors of at least 10 replicates per treatment. The significance of differences in comparison to values obtained at 4 wpi was determined at the significance level of p 0,05.

RESULTS AND DISCUSSION

Results
Dry weight production and hydration status

The following of the growth of *M. truncatula* from a vegetative stage to fructification is characterized by the accumulation of the dry matter. Indeed, the speed growth of the aerial part is slow during the vegetative phase (between 4 and 6 wpi). This slow growth during this stage can be explained by the time required for the inoculation process and for the development of nitrogen-fixing nodules. During flowering (8 wpi) and fructification (10 wpi), the dry matter became 5 and 10 times higher (Figure1A). However, the hydration status of aerial organs remains stable over time. Indeed, alfalfa keeps stable rate of hydration and close to 90% during different growth phases (Figure1B).
Chlorophyll:
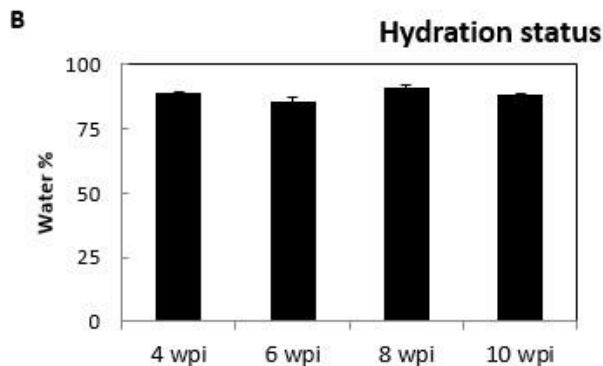
B

Hydration status

Figure 1: Dry weight (A) and hydration status (B) of *Medicago truncatula* shoots after 4; 6; 8 and 10 weeks post-inoculation (wpi). Results are the mean ±SE of ten measurements. * The significance of differences in comparison to values obtained at 4 wpi was determined by the Student's t-test at the significance level of $p < 0,05$
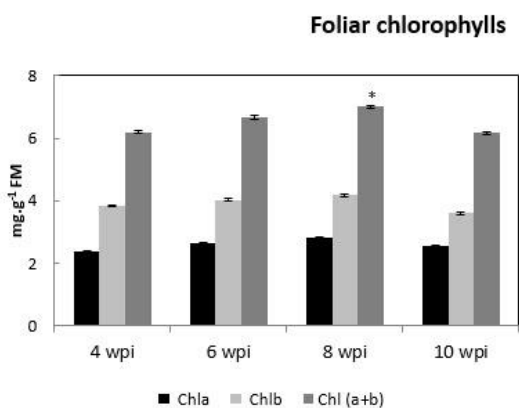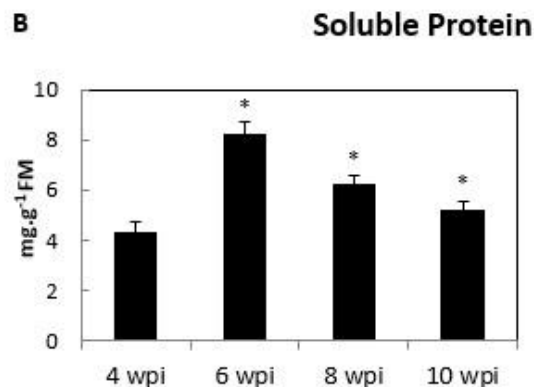
Foliar chlorophylls

■ Chla   ■ Chlb   ■ Chl (a+b)

Figure 2: Chlorophylls of *Medicago truncatula* shoots after 4; 6; 8 and 10 weeks post-inoculation (wpi). Results are the mean ±SE of ten measurements. * The significance of differences in comparison to values obtained at 4 wpi was determined by the Student's t-test at the significance level of $p < 0,05$

The examination of Figure 2 shows that the shoot chlorophyll shows a slight increase in chlorophyll a and b. The maximum is unregistered at 8 wpi. However, during flowering, we have a slight decrease in the rate of chlorophyll a and b. The total chlorophyll concentrations follow the same allure as the previous ones. It decreases by 12% at 10 wpi.

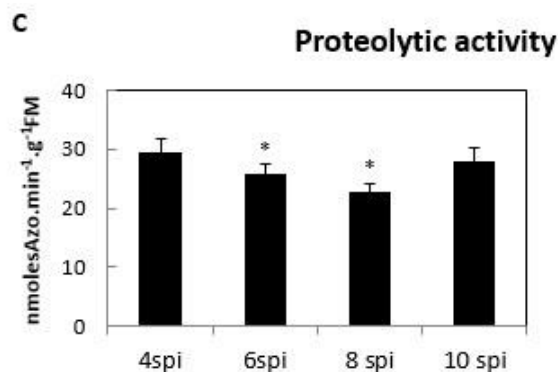**A**   **Total soluble Sugar**   **B**   **Soluble Protein**
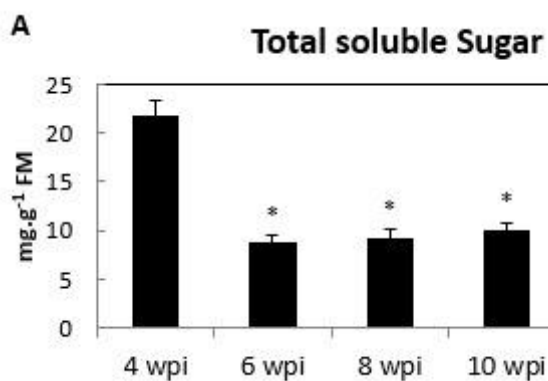
Figure 3: Total soluble sugar (A), soluble protein (B) and proteolytic activity (C) of *Medicago truncatula* shoots after 4; 6; 8 and 10 weeks post-inoculation (wpi). Results are the mean ±SE of ten measurements. * The significance of differences in comparison to values obtained at 4 wpi was determined by the Student's t-test at the significance level of p < 0,05

Sugar, proteins and proteolytic activity
Sugar: In shoot of *M. truncatula* inoculated with *S. meliloti*, we find that the highest content of total soluble sugars is registered at 4 wpi. The quantity is estimated at 12 mg.g^{-1} FM (Figure 3A). However, this level decreased significantly by 60% beyond this stage of growth.

Proteins: During the vegetative stage, we register duplication of the soluble protein quantity in shoots of inoculated *Medicago* between 4 and 6 wpi. Beyond that, the quantity of soluble protein in aerial organ of *M. truncatula* present a significant diminution estimated by 24 and 36 % respectively during flowering (8 wpi) and fructification (10 wpi) of this legume. However, they remain significantly higher than that registered at 4 wpi in these organs (Figure 3B).

Proteolytic activity: During growth, the proteolytic activity of *M. truncatula* shoots present a significant diminution in comparison to 4 wpi. The decrease is estimated by 12 and 23 % respectively at 6 and 8 wpi. During the flowering stage (10 wpi), the proteolytic activity augments, and we register the same activity calculated at 4 wpi (Figure 3C).

Calcium and Potassium
The content of shoot calcium decrease by 34; 25 and 35% respectively in 6; 8 and 10 wpi in comparison to the quantity registered at 4 wpi (Figure 4A). For potassium, the growth of *M. truncatula* is characterized by a stability of shoot potassium during vegetative stage (Figure 4B). Belong this phase, we detect a slight reduction of shoot potassium (7 and 9 % respectively during flowering and fructification stage).
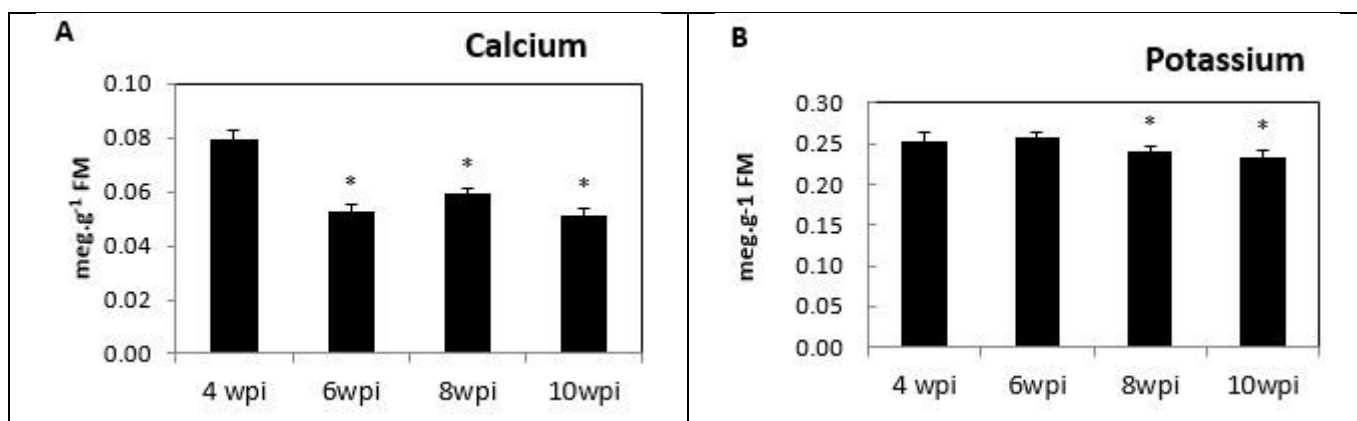

Figure 4: Calcium (A) and potassium (C) content of *Medicago truncatula* shoots after 4; 6; 8 and 10 weeks post-inoculation (wpi). Results are the mean ±SE of ten measurements. * The significance of differences in comparison to values obtained at 4 wpi was determined by the Student's t-test at the significance level of p < 0,05

Nitrogen compounds
Nitrates: In shoot of *M. truncatula*, the pool of nitrates is stable during the vegetative stage. During flowering and fructification we register an augmentation of shoot nitrates respectively by 24 and 69 % (Figure 5A).

Nitrites: The amount of nitrites shoots increases between 4 and 6 wpi by 56%. The elevation is 9 folders more important at flowering. During fructification, the content of the shoot on nitrites decreases by 64% in comparison to 8 wpi (Figure 5B).

Ammonium: The content of ammonium in shoot of *M. truncatula* presents a fluctuation during the different stage of development. However, the shoot ammonium decreases by 29% at 6 wpi. After an augmentation at 8 wpi, we register a re-diminution by 48% at fructification stage (10 wpi) (Figure 5C).
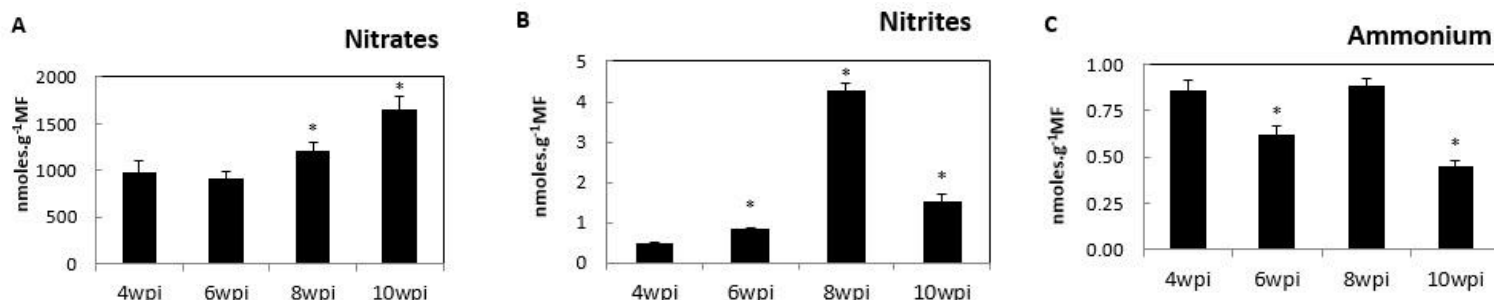

Figure 5: Nitrates (A), Nitrites (B) and Ammonium (C) of *Medicago truncatula* shoots after 4; 6; 8 and 10 weeks post-inoculation (wpi). Results are the mean ±SE of ten measurements. * The significance of differences in comparison to values obtained at 4 wpi was determined by the Student's t-test at the significance level of $p < 0,05$

Discussion

In this study, we characterized some physiological and metabolic changes that occur during the shoot growth of *Medicago truncatula* inoculated with *Sinorhizobium meliloti*. The study describes some change between the vegetative stage (4wpi) and fruiting stage (10wpi). During this period, the production of dry biomass of shoot increases with time post-inoculation. The stimulation of vegetative growth is the result of the positive interactions that develops the plant with microorganisms of the rhizosphere, such as bacteria (Höflich et *al.,* 1994; Lodwing et al., 2003), or mycorhize (Berthelin and Leyval, 1982). In this interaction, the plant supplied its partner with organic compound in exchange for mineral elements essential to its growth.

The vegetative growth of *M. truncatula* consumes relatively high amounts of carbohydrates. The decreases of the total sugar between 4 and 6 wpi can be the result of the symbiotic process. Indeed, our results corroborate those obtained in soybean, where the establishment of mycorhize symbiosis is concomitant with a high demand for photo-assimilates (Kucey and Paul, 1981; Bethlenfalvay et *al.,* 1982).

It was shown that the inoculation process uses up to 60% of the photo-assimilates produced during the photoperiod. This high demand for carbohydrates by inoculated roots is compensated by a high nitrogen fixation (Minchin and Witty, 2005; Antolin et *al.,* 2010). This could explain the lower levels of total soluble sugars in the initiation of flower development (6wpi), flowering (8 wpi) and fruiting (10 wpi). In addition, the biological cycle development of *M. truncatula* shows that the flowering is at 8 wpi and the start fruiting is about 10 wpi. These two phases are characterized by a decrease in foliar soluble protein and an increase of the proteolytic activity. This could be due to remobilization of protein reserves to pod filling. This stage is characterized in soybean by the increasing of the intensity of the nitrogen fixation (Peat et *al.,* 1981).

The same phenomenon is observed in pea, where the need of the plant during the fruiting is characterized by an increasing of the number of nitrogen-fixing nodules and the specific activity of each nodule (Fischinger and Schulze, 2010). These results can explain the elevated amount of nitrates unregistered in *M. truncatula* shoot at 10 wpi.

However, *M. truncatula* present during the vegetative stage an elevated quantity of calcium. This could be related to the role of calcium in the structural rigidity of the cell walls (Jones and Lunt, 1967). Further than this period, lucerne present a decrease of the shoot calcium, how can be the result of: the cell elongation and its parietal plasticity, and it can explain the port truncated of this species. Or to the antagonist effect of potassium, indeed, it is known that increased potassium levels may decrease the absorption and translocation of calcium and magnesium from roots to leaves (Classen and Wilcon, 1974; Ohno and Grunes, 1985; Song and Fujiyama, 1996).

In plus, the elevated quantity of aerial organs potassium can be the result of the symbiotic interaction with *S. meliloti*. This suggestion is supported by the fact that the symbiotic association stimulates the water and the nutrient uptake by roots (Kloepper et *al.,* 1991).

On the other hand, the increase of the shoot nitrate in parallel to the decrease of the amount of nitrites and ammonium between flowering (8 wpi) and fructification (10 wpi) phases can be the result of the inhibition of nitrate reductase activity. Indeed, the over-expression of the gene coding for this enzyme in plants causes reduces of their levels of nitrate (Foyer et *al.,* 1994; Quilleré et al., 1994)

In conclusion, the growth of inoculated *M. truncatula* is characterized by a change of the carbon and nitrogen content. Every physiological stage of this legume presents a specific protein, chlorophyll, sugar and nitrogen compound status. The following of the growth of *M. truncatula* from a vegetative stage to fruiting present a general description of the growth of the model plant.

ACKNOWLEDGEMENT

CONCLUSION

The technique discussed in this paper provides an interactive approach in which the decision maker can search for an acceptable solution of the multi-objective optimization problem. The proposed method to solve multiobjective linear programming problem is better than many existing methods as the concept of bound is used in the iteration.

If we substitute some values to a_i, α_i in multi-objective linear programming problem (3.1), it reduces into single objective LPP. This discussion also holds in the case as given by by Kanniappan and Thangavel (1998). The same problem for integer solution was studied by Bhargava and Sharma (2003).

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