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Effects of water stress on: mycorrhizal root colonization, chlorophyll pigment, number of spore, mycorrhizal dependency and nodules of two mycorrhizal leguminous plants (*Tephrosia vogelii* and *Vigna subterranea*) at an early stage of growth

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Abstract

The present study carried out in a randomized blocks design, aimed at evaluating the effects of water stress on root colonization, mycorrhizal dependency, number of spores and number of root nodules of mycorrhizal *T. vogelii and V. subterranea*. The four treatments applied were: 90, 60, 30 and 15 for control, low, average, severe stress respectively, with or without AMF and for 31days. Results obtained showed that, with respect to stress severity, the harmful effects of water stress were more marked with *T. vogelii* compaired to *V. subterranea*. However, the mycorhization alleviated the negative impacts of the water stress thus favored root nodule installation. The AMF increased significantly the number of root nodules for the two leguminous plants used in this study for both unmycorrhizal or mycorrhizal plants. One could thus conclude that, the mycorrhizal inoculum used could serve as an effective microbial material for alleviating the harmful impacts of water stress on leguminous plants growth at an early stage of development.

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Introduction

Leguminous plants are valuable in many domains. In agriculture they are used as green manure (Westphal *et al.,* 1985; Wu *et al*., 2011; Tsoata *et al*., 2015), and thus serve as pioneer plants on degraded land (Baligar et Fageria, 2007). They are mainly used for erosion control, soil building and ground cover (Richardson and Diseker, 1965; Baligar et Fageria, 2007). Leguminous crops belong to group of major plant in human nutrition because of the high proteins content, both quantitatively and qualitatively; their seeds are valued both for their nutritional and economic importance (Owonubi *et al*., 2011). Leguminous seeds contribute tremendously in animal and human nutrition especially in area where diets are mainly based on cereals (Westphal *et al*., 1985; Tsoata *et al*., 2015). They constitute an important source of human dietary proteins (Fivawo and Msolla, 2011 ; De La Fuente *et al*., 2012). Seeds and haulm provide by some leguminous plants are used to feed livestock and poultry (Anchirina *et al*., 2001).

Extracts from leguminous plants are used to cure some human diseases (Baligar et Fagera, 2007; Aune *et al*., 2009; Fivawo and Msolla, 2011) and effective control of some skin desease of cattle (Kalume *et al*., 2012; Mshana *et al*., 2000; Gadzirayi, 2009). Furthermore some products of leguminous plants exhibited pesticidal property and can contribute in insect pest management by farmers (Belmain *et al*., 2012; Magreta *et al*., 2011; Nkenda *et al*., 2014). Leaves extracts from *Tephrosia vogelii* are used as fish poison by fisherman (Obomanu, 2007; Solomon *et al*., 2014). Climate change is an anthropogenic factor influencing reduction in crop productivity (Ziska, 2011); for abiotic factors, water stress is one of the primary constraints militating against crop development and yield worldwide and leguminous plant in particular (Lacroix *et al*., 2003; Kim *et al*., 2012). Abiotic stresses are the principal cause of crop failure, decreasing average yields for major crops by more than 50% (Buchanan *et al*., 2000) and threatening the sustainability of agricultural industry (Mahajan and Tuteja, 2005). Strengthening of drought during growth phase lead to inhibition of photosynthesis, damage to photosynthetic pigments (Jain *et al*., 2013).

Leguminous plants are frequently prone to drought stress depending on their geographical distribution. In order to face this environmental constraint some of them established symbiosis with arbuscular mycorrhizal fungi (AMF). Many beneficial effects of AMF for drought stress plants has been reported (Chaussod et Nouaim, 1996; Ruiz-Lozano *et al*., 2001; Abdelmoneim *et al*., 2014; Tsoata *et al*., 2015), but those concerning early phase of growth are scare. The aim of the present study was to evaluate the effects of water stress on roots colonization, chlorophyll pigments, mycorrhizal dependency, number of spores and number of nodules for mycorrhizal *T. vogelii* and *V. subterranea* at an early growth phase.

Materials and methods

Plant material, conditions of growth and experimental design

Selected uniform shape and healthy seeds of each species were surface sterilized and germinated. Seedlings obtained were grown in plastic pots on sterilized substrate already inoculated at level of seedlings holes with AMF mixture, except controls pots which receive bacterial filtrate; in randomized block design according to Tsoata *et al.* (2015).

Evaluation of root colonization

Slices of root samples of 1 to 2cm were collected in each treatment 45days after sowing. They were then introduced in test tubes and treated according to Kormanik and Mc Graw (1982) method. The procedure was as follows: the collected root samples were washed with tap water, immersed in 10 % KOH for 30mn in hot water (90°C). After this they were washed again 3 times in tap water, acidified with 1 % HCl in a 0.05 % acid fuchsine solution added in the solution made up of acid lactic-glycerol-water (4-1-1) V/V for at least 24hours. The colored root samples (30 samples/treatment),were mounted in parallel on blades and covered by slide covers arranged in groups

of 10, observed under a microscope at 40X and 100X three repetitions were necessary. The presence or the absence of the structures characteristic of the mycorrhizae (mycelia filaments, spores and vesicles) made it possible to evaluate the root colonization frequency.

RC (%) = n / N X 100.

With (RC) root colonization, (n) the number of root samples observed having one or more mycorrhizal structure and (N) the total number.

Evaluation of Chlorophyll a, b and a+b

The chlorophyllic pigments were extracted and titrated according to Lichtenthaler and Buschmann's (2001) method. 0.5g of each sample of fresh leaf was crushed in pure methanol and after filtration; the optical density of filtrat is read on spectrophotometer at 666 and 653nm. The chlorophylls a, b and chlorophylls a + b ($mg.g^{-1}FW$) content are calculated according to the following formulas:

(1): Chlorophyll a = 16.72 X A666 – 9.16 X A653

(2): Chlorophyll b = 34.09 X A653 – 15.28 X A666

Chlorophyll a + b and ratio chlorophyll a/b are deducted.

Evaluation of the mycorrhizal dependency

The mycorrhizal dependency (MD in %) of the plants was calculated according to Plenchette *et al.* (1983) formula:

MD (%) = DM - Dm / DM X 100

With DM = dry matter of mycorrhizal plant, Dm = dry matter of unmycorrhizal plant

Evaluation of spore's number

After harvesting the plants, a quantity of substrate used was kept safe from rain. Samples were made by mixing substrates from 5pots of the same treatment. The number of spores contained in the substrate of each sachet was evaluated. The extraction of the spores was carried out according to Gerdemann and Nicolson (1963) method. 100g of substrate were introduced into one liter Erlenmeyer plus 300ml of tap water. The mixture was homogenized, left for 15 seconds before successive sieving through two sieves of mesh 80 µm and 40 µm. Washing and decantation was repeated three times, and the residues in the second sieve was transferred separately by washing in Petri dishes of 9.4cm diameter. The observation and counting of the spores was made at 10X to 40X on ZEISS stereo microscope.

The spores were counted in 10 contiguous squares divided in 2 sets of 5 squares of $2.5cm^2$. If "N" is the number of the spores counted in the 10 squares, then the number of spores counted is 69.4/2.5 X N. The total number of spores obtained for one Petri dish is used to calculate the total number of spores in 100g of sample.

Evaluation of nodules number

The nodule number of was calculated according to the method of Mohamed *et al.* (2000): the roots were collected, washed with distilled water and the nodules of each plant were counted.

Statistical analysis

Data recorded were analyzed statistically using SPSS 18.0 software for ANOVA and Pearson's correlation between parameter, at probability level of 5%. The differences between means were compared by Duncan multiple range test at 5% level of probability. Comparison of means and trends were considered significant when the value of the compared sets were different at probability level of 5% or1%.

Results

Effects on mycorrhizal root colonization

Root of *T. vogelii* and *V. subterranea* plants were observed after the period of application of water stress (Fig. 1a). Uninoculated plants did not present any mycorrhizal infection, whereas inoculated plants presented mycorrhizal root infection. The root colonization is significantly (Table 1) impaired

according to the severity of the water stress: 30, 50 and 70 % for *T. vogelii* and 46, 54 and 70 % for *V. subterranea*, for low, average and severe water stress respectively (Fig. 1a). Our findings showed that for low and average water stress root colonization inhibition is slightly low for arbustive *T.vogelii* compared to herbaceous *V.subterranea*. The inhibitory effect is the same for the severe water stress for both species (Fig. 1a).

Table 1. ANOVA of number of nodules, spores and root colonization for *T. vogelii* and *V. subterranea* submitted to various levels of water stress with or without AMF.

	T. vogelii			*V. subterranea*		
	Stress intensity (I)	Mycorrhizae (M)	Interaction (I x M)	Stress intensity (I)	Mycorrhizae (M)	Interaction (I x M)
Nnd	*	*	*	**	**	**
Nsp	* *	nd	nd	**	nd	nd
RC	* *	nd	nd	**	nd	nd

Note: * significant effect at 5 %; ** significant effect at 1 %; Nnd: Number of nodules; Nsp: Number of spores; RC: Root colonization; nd: not determine.

Effects on spore number

The spore's number of fungi evaluated in the substrate used to grow *T. vogelii and V. subterranea* (Fig. 1b), at various level of water stress showed that, contrary to inoculated substrate uninoculated substrate is deprived of spores. According to the severity of water stress for *T. vogelii* a non-significant increase in the number of spores by 7 % at the level average water stress was recorded as well as a significant improvement of 59 % for the severe water stress (Table 1). For *V. subterranea*, a significant increase in the number of spores by 20 % and 24 % were noted for average and severe water stress respectively. At the level of the low water stress for both leguminous plants, no improvement in spore number was observed.

Table 2. Pearson correlation between number of nodules, spores and root colonization.

	T. vogelii			*V. subterranea*		
	Nnd	Nsp	RC	Nnd	Nsp	RC
Nnd	1	- 0.510	0.789**	1	- 0.432	0.795**
Nsp		1	- 0.589*		1	- 0.638**
RC			1			1

Note: * significant at P=5% and **significant at P=1%. Nnd: number of nodules, Nsp: number of spores, RC: root colonization.

Effects on nodules number

The number of nodules declined for both leguminous plants with increasing water stress level (Fig. 1c).This decline when compared with control is significant (Table 1) except for mycorrhizal plants of *V.subterranea* subjected to low water stress. With increasing level of water stress, for unmycorrhizal plants, the nodules number declined. This decline is in order 61-89% and 10 – 66% for *T.vogelii* and *V.subterranea* respectively. A similar drop of 50-87% and 35-42% was observed for *T.vogelii* and *V.subterranea* for mycorrhizal plants. For both species of leguminous plants mycorhization significantly increased nodules number when the water stress level increased. This increase is in order 53, 63, 50 and 60 % for *T.vogelii* and 27, 39 and 23% for *V. subterranea* (Fig. 1c).

Mycorrhizal dependency for both leguminous species under water stress was not significantly affected by low stress (Fig. 1d). However, for average and severe stress, mycorrhizal dependency increased with the level of stress from average to severe stress for *V.subterranea*, whereas *T.vogelii* showed the contrary.

Pearson correlation for both species shows that root colonization significantly slowed down the number of spores formed in the substrate; whereas nodule formation was positively and significantly linked to root colonization by AMF (Table 2).

Effects on Chlorophylls a, b and a + b

Chlorophyll a content has decreased with increase in water stress level; this decrement is significant only for severe stress and for all treatments (Fig. 2a). This content for mycorrhizal plant is always higher than for unmycorrhizal. But mycorhization allowed a significant increase in this pigment of: 69, 43 and 17% for not stressed *T. vogelii*, not stressed *V. subterranea* and *V. subterranea* under low water stress respectively (Fig. 2a).

Mycorhization and various water stress levels (Fig. 2b) do not have significant effects on the chlorophyll b content for the two species.

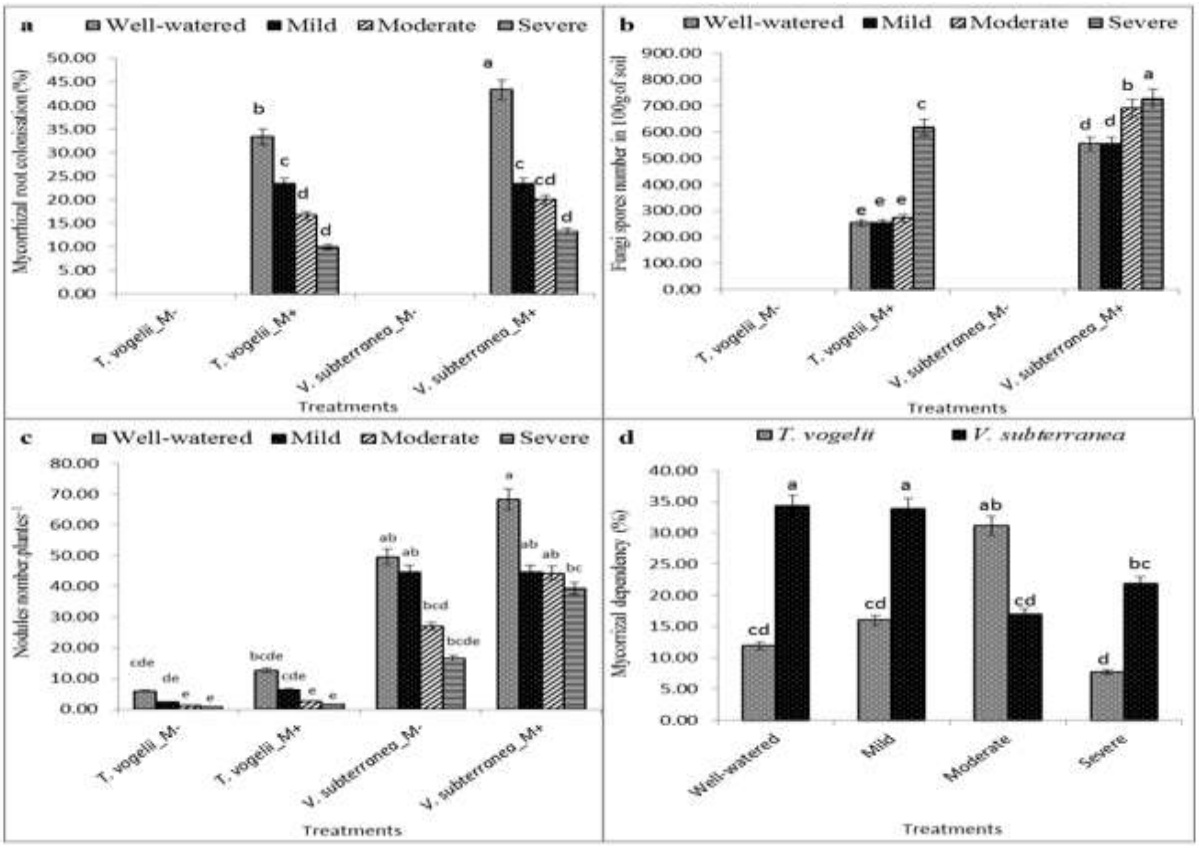

Fig. 1. Mycorrhizal root colonisation (a), fungi spores number in 100g of soil (b), nodules number.plante^{-1} (c), mycorrhizal dependency (d) in mycorrhizal and non-mycorrhizal *T. voglii* and *V. subterranea* plants under severe (15%), moderate (30%), mild (60%) and no drought stress (Well-watered = 90%) conditions.

Total chlorophyll (a + b) for the two species increases significantly of: 41 and 29% for mycorrhizal not stressed plants of *T.vogelii* and *V.subterranea* respectively (Fig. 2c).

The ratio chlorophyll a/chlorophyll b, for all treatment and water stress level, is always equal or higher than one for the two species (Fig. 2d). But for mycorrhized plant, it increases significantly of: 66, 40 and 23% for not stressed *T. vogelii*, not stressed *V.*

subterranea and under low stress *V. subterranea*, respectively. This ratio is higher for *V.subterranea* compared to *T. vogelii*.

Water stress inhibits the synthesis of chlorophyll a, this inhibition increased with stress level. Mycorhization strongly decreases these inhibitory effects, especially for mycorrhizal not stressed plant, with lesser extend for mycorrhizal stressed plant.

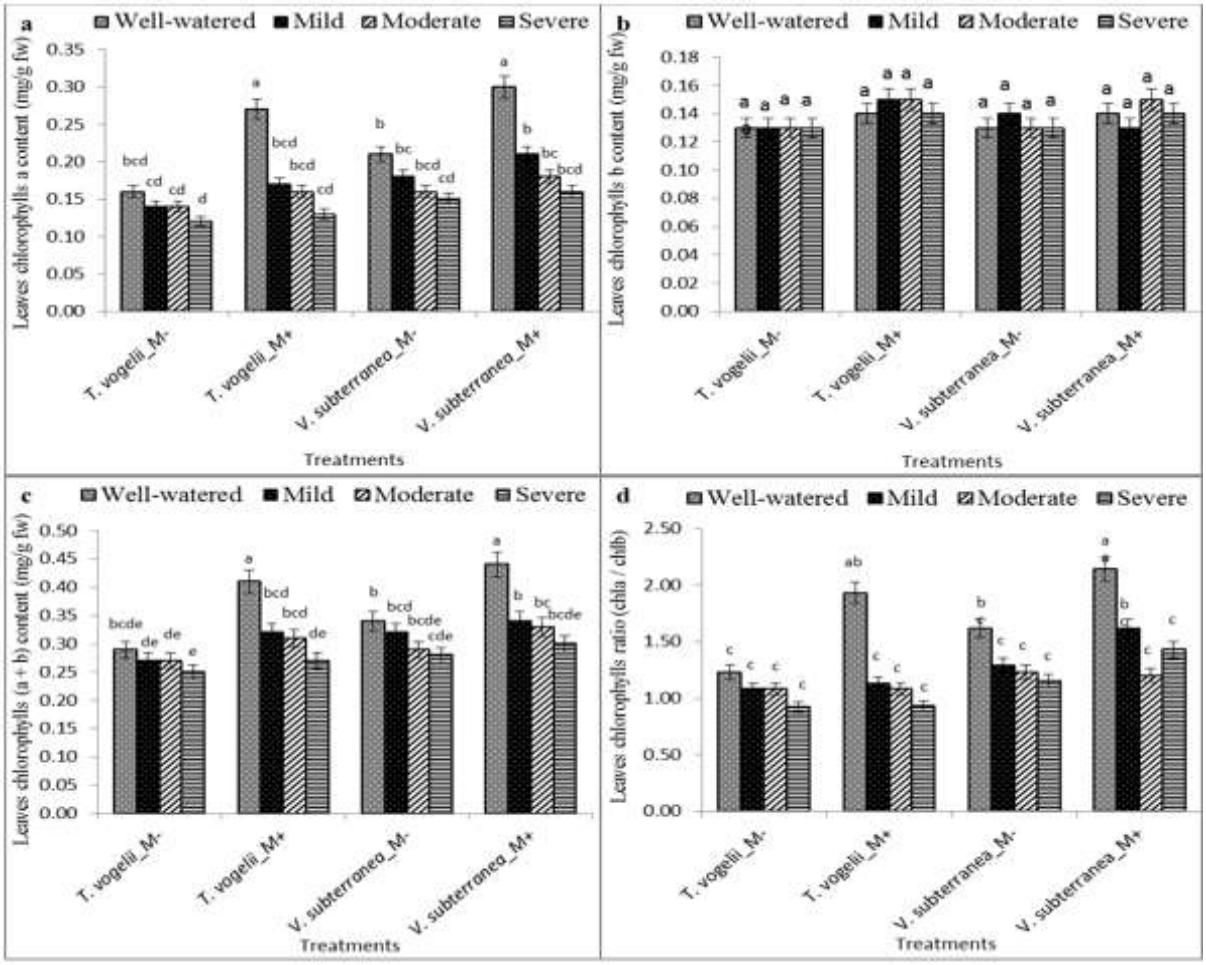

Fig. 2. Leaves chlorophylls a content (a), Leaves chlorophylls b content (b), Leaves chlorophylls (a + b) content (c), Leaves chlorophylls ratio (chla/chlb)(d) in mycorrhizal and non-mycorrhizal *T. voglii and V. subterranea* plants under severe (15%), moderate (30%), mild (60%) and no drought stress (Well-watered = 90%) conditions.

Discussion

The findings of the present experiment on *T.vogelii and V. subterranea* showed that uninoculated plants present no root colonization, contrary to inoculated ones, where mycorhization is effective. Over 80% of all land species in non sterilized substrate or soil forms mycorrhizae with AMF (Smith and Read, 2008). In this mutualistic symbiosis, fungi which is an obligate biotroph receives carbohydrates from the host (Parnicke, 2008; Smith and Read, 2008), whereas plants benefit several advantages such as: increasing growth and nutrients uptake (Gianinazzi *et al.,* 2010). Furthermore, mycorhization alleviates detrimental effects of drought stress on plant (Auge, 2001; Miransari *et al.,* 2010; Sochacki *et al.,* 2013). Water stress negatively affects the development of the AMF and consequently root colonization. According to the severity of water stress, the decrease of root colonization would be related to a progressive reduction of the availability and the restitution of the biogenic salts of the ground by AMF to the plants. In response to the behavior of the AMF, the plants would

produce fewer carbohydrates necessary for symbiosis, which would lead to a reduction of root colonization. This result does not corroborate that of Abdelmoneim *et al.* (2014).

The results obtained show no colonization for control; confirming that the sterilized substrate used was effective and no contamination occurred during the experimental period. Mycorhization of both species confirmed the effectiveness of the infection by fungi of the inoculum used. This result is in accordance with those of Parniske (2008), Smith and Read (2008) which revealed that over 80% of all land plant species form ancient mutualistic interactions with AMF. AMF are considered to be obligate biotrophs which complete their life cycle by obtaining carbohydrates from the host (Parnicke, 2008; Smith and Read, 2008). In symbiotic association with *T. vogelii* and *V. subteranea*, AMF play key roles such as: increasing plant growth, nutrient uptake and ecosystem functioning for sustainable agriculture (Smith and Read, 2008; Gianinazzi *et al.,* 2010) as well as enhancing plant tolerance to abiotic stress (Smith and Read, 2008; Finlay, 2008; Gianinazzi, 2010; Chen *et al.*, 2014) and mitigating the negative effects of water stress on plant growth (Sochacki *et al.*, 2013). The significant impairing of AMF development and consequently root colonization according to the severity of the water stress observed in this experiment could be related to a progressive reduction of the availability and the restitution of biogenic salt of the ground plant in one hand, and to the slowdown of photosynthetic activities and decrease in activities of enzymes of Calvin cycle (Monakhova and Chernyadev, 2002; Allahverdiyev, 2015) on the other hand. Consequently this leads to reduction of the amount of carbohydrate received by fungi for its growth and development, provoking a reduction of root colonization. This inhibition increases and culminates for severe water stress probably because plants subjected to water stress first pass throw a dynamic phase to adjust their water content before entering a survival phase where stomata are completely closed and no photosynthetic activity takes place (Tardieu, 2005). This result is similar to those of Gholamhoseini *et al.* (2013) on sunflower inoculated with *Glomus hoi* and *G. mosseae* which after 90 days showed that root colonization was reduced by 22 % for an average stress (40 % of FC) and 27 % for a severe stress (20 % of FC) after 90 days. Moreover the work of Augé (2001) supported the fact that drought affected levels of root colonization, about half of the reports providing of data, showed increasing root colonization more often than a decrease.

The findings of the present experiment revealed that the number of fungi spores formed under water stress significantly increased with the severity of the stress, are similar to result obtained by Abdelmoneim *et al.* (2014). The increment was high for *T. vogelii* under severe stress compared to *V. subterranea* under average or severe stress. The exposure of AMF mycelia to ground water shortage causes formation of spores in response to the stress (Jacobson, 1997). As resistant and reproductive form of AMF, spores enable them to survive in the ground in case of an environmental constraint such as water stress. Therefore, by using adequate level of water stress on soil where mycorrhizal plants grow one can accumulate AMF spores in order to increase second year plant productivity or produce mycorrhizal biofertilizer that are mainly made of soil, spore, mycelia and infected plant root slices. In this experiment for the severe water stress, the woody plant (*T. vogelii*) produced more spores then the herbaceous plant (*V. subterranea*) indicating that the fungi reaction may depend on plant type or species.

The number of nodules for both Leguminous plants (*T. vogelii* and *V. subterranea*) was significantly impaired according to the severity of water stress. This could be related to a progressive reduction in plants water supply. Water is an essential component of photosynthesis involved in the production of organic compounds which are partly translocated to nodules to maintain *Rhizobium*-leguminous plant symbiosis. This result is similar to that of Goicoechea

et al. (2004) which showed that *Anthyllis cytisoides* subjected to water stress on sterilized substrate and in the presence of *Glomus fasciculatum*, showed significant reduction of 27 % in the number of nodules. Irigoyen *et al.* (1992) specified that the limitation of the availability of the carbohydrates intended for the nodules can induce their senescence. Moreover the nodules are very sensitive to dehydration. However, the significant improvement of the number of nodules within mycorrhizal plants could be related to the capacity of the AMF to mitigate the negative effects of water stress by improving the mineral-water nutrition of the plants which has a positive incidence on the improvement of the photosynthetic gaseous exchange. This result is similar to that of Osonubi *et al.* (1992) on *A. albida* grown on sterile ground watered every 2 weeks during 12 weeks, in the presence of *Glomus clarum*, showing that the mycorhization significantly increased by 87 % the number of nodules for plant.

The results obtained show a mycorrhizal dependency (MD) which is more significant for *T. vogelii* for an average water stress. For *V. subterranea* it is more significant for the control and the low water stress. These results are not in agreement with those presented by Subramanian *et al.* (2006) on *Lycopersicum esculentum* (tomato) which showed that 88 days after sowing, under different level of water stress: low stress (80 % FC); average stress (60 % of FC) and severe stress (40 % of FC), the MD increased for approximately 19 %, 43 % and 46 % respectively for the three above level of water stress, compared to the control (100 % of FC). Moreover Osonubi *et al.* (1992) on *Acacia nilotica* and *A. albida* under water stress obtained a significant reduction in MD by 41%. The differences in the MD observed for these two leguminous plants would be related to their genetic predisposition. High ground moisture reduces the output of the Vouandzou (Ameyaw and Doku, 1983). *V. subterranea* behaves well on semi-arid soil, thus, for great amount of water in the soil it would largely need the AMF which, would thus make it possible for the plant to take only the minimal quantity of water necessary for its growth and development. For *T. vogelii*, the higher MD observed for the average water stress (30% Of FC) could be explain by the fact that well established symbiosis plant-AMF have succeeded in establishing a thermodynamic equilibrium, being able to allow this plant to better tolerate the water stress.

The positive and highly significant correlation obtained confirms that the presence of AMF, the increase in the mycorrhizal infection and consequently root colonization have contributed to facilitate root nodulation.

Chlorophyll content of plants is often performed to assess the impact of most environmental stresses, as the pigment content is linked to visual symptoms and photosynthetic plant productivity (Jain *et al.*, 2013); drought stress produced changes in photosynthetic pigments and components (Anjum *et al.,* 2003). Reduction in chlorophyll content by water stress observed in the present experiment has been shown in a few systems (Albert and Thornber, 1977; Tomati *et al.,* 1978; Zayed and Zeid, 1998; Jeyaramraja *et al.,* 2005; Yadav *et al.*, 2013; Allahverdiyev, 2015). This reduction correlated with water stress level has been previously observed by Zhang *et al.* (2004) on drought stressed soybean plants, Allahverdiyev (2015) on durum and bread wheat subjected to water stress, could be related to the activity of the glutamate synthetase. Indeed, studies showed that the application of water stress impaired the activity of the glutamate synthetase, which is an enzyme involved in the biosynthesis of glutamate which is a precursor of chlorophyllic pigments (Tahri *et al.,* 1998).The decrement of chlorophyll content to a significant level at higher water deficits observed here, has been previously reported by Nayyar and Gupta (2006) on maize and wheat plants. In addition, the significant increase in the chlorophyll content of the inoculated plants compared to nonmycorrhized control could be explained by both better water up take and biogenic salts such as Magnesium necessary for the renewal of chlorophyll. The Mg allows the activation of enzymes

such as ATPases, ribulose -1.5-bisphosphate carboxylase (RUBISCO), RNA polymerase and proteins kinases (Shaul, 2002) which are vital enzymes for the plant.

These results are similar to those of Wu and Xia (2006) which showed that after 80 days of water stress application (corresponding to 55% of FC), and in the presence of *Glomus versiforme*, mycorhization significantly increased the chlorophyll content by 25%. Photosynthetic pigments determine the physiological status of plants; changes in chlorophyll a/b ratio provides further information about modification process taking place in the photosynthetic apparatus (Jain *et al.*,2013; Yadav *et al.*, 2013) and can be used as a good indicator of threshold value of water stress tolerance. Guettouche (1990) reports that higher value of this ratio is associated to high tolerance to water deficit. We can thus say that *V. subterranea* with relatively high ratio of chlorophyll a/b can adapt well to water stress then *T. vogelii*.

Conclusion

Water is one of the most significant environmental factors that limits plant performance, growth and productivity, and can compromise their installation in their biotope at an early stage of development. In the present experiment, mycorhization of young leguminous plants improves positively most of the parameters studied under water stress. Thus AMF can be considered as one powerful means that can be used to alleviate negative effects of water stress on plant at an early stage of grow.

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