Impact of Hypoxia on Nodulation and Growth of the Legume Plant *Medicago truncatula* in Symbiosis with *Sinorhizobium meliloti*

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Abstract

Legumes form a symbiotic interaction with bacteria of the *Rhizobiaceae* family to develop nitrogen-fixing root nodules under nitrogen-limiting conditions. The present study aimed at analysing the impact of two- and four-week long periods of hypoxia on the nodulation and the growth of the legume plant *Medicago truncatula* (var Jemalong A17) in symbiosis with *Sinorhizobium meliloti*. Results showed that the nodulation was not affected by moderate hypoxia (4.5% O_2), but the number of nodules decreases for high hypoxia (0.1% O_2) and anoxia. The analysis of growth parameters (fresh and dry weight, leaf area and thickness, chlorophyll contents) and of various metabolite contents (total sugars, chlorophylls, inorganic nitrogen compounds) in the leaves, roots and nodules showed that nodulated *Medicago truncatula* tolerates periods of 4 weeks hypoxia and may be considered as a hypoxia-tolerant legume plant.

Introduction

Flooding is one of the most significant abiotic stresses (Blom and Voeseneck, 1996). It causes a slow transfer of dissolved oxygen in the water-filled pore space of the soil, and plants can have to face with the lack of oxygen during all stages of its growth. Seed imbibition, flood irrigation, floods and excess of rainfall are examples of natural conditions leading to root hypoxia or anoxia. The oxygen deficiency is thought to be a major determinant in the adverse effects of flooding on crops and growth of plants (Mommer et al., 2004). The most important effects of flooding are the reduction of water and nutrient uptake and perturbation of plant metabolism (Gibbs and Greenway, 2003; Greenway and Gibbs, 2003; Ricard

et al., 2006; Horchani et al., 2010). To survive these conditions, plant cells need to reduce their energy requirements for maintenance, and also direct the limited amount of energy produced during anaerobic catabolism to the energy-consuming processes that are critical to survival (Greenway and Gibbs, 2003). The switch from respiration to fermentative metabolism has been demonstrated as a main process that allows regeneration of NAD^+ in the absence of oxygen dependent-respiration, thereby maintaining glycolysis and the generation of ATP under anaerobic conditions (Ricard et al., 2006). The symbiotic interaction between legumes and soil bacteria of *Rhizobium* type leads to the development of a new organ, the root nodule, wherein the differentiated *Rhizobia* reduce atmospheric nitrogen (N_2) into ammonia

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(NH_3) which readily assimilated by legume plants. As a result, many legumes are sensitive to waterlogging, showing reductions in root growth, nodulation process, nitrogen fixation and photosynthesis activity (Minchin and Pate, 1975; Minchin and Summerfield, 1976; Scott et al., 1989; Sung, 1993; Linkmer et al., 1998; Bacanamwo and Purcell, 1999). Legume root nodules are particularly sensitive to hypoxia, and legumes relying on N_2-fixation are generally more sensitive to flooding than legumes grown on alternative nitrogen sources, such as nitrate (Michin and Pate, 1975; Bacanamwo and Purcell, 1999; Roberts et al., 2009).

In a previous study, we analysed the physiological response of non-nodulated *Medicago truncatula* plants grown under prolonged hypoxia stress in the presence of nitrate (El Msehli et al., 2015). However, in most if not in all cases, crop legumes in the field grow in symbiosis with soil rhizobia and have different sensitivity to environmental stress from that of legumes grown in non-symbiotic conditions. In the present work, we investigated the impact of two-and four-week periods of hypoxia on nodulation and growth of *Medicago truncatula* in symbiosis with *Sinorhizobium meliloti*.

Materials and methods

A total of 125 samples were collected from infants and other age groups between zero to 60 years suffering from diarrhoea (more than 3 loose stools per day for 2-3 days) admitted to a private hospital in around Namakkal area. These patients presented varying kinds of symptoms ranging from nausea, vomiting and had not taken any antimicrobial agents during the week of preceding sampling. Patients with concomitant infections were excluded from the study.

Biological material and growth conditions

Seeds of *Medicago truncatula* (Var. Jemalong A17) were sterilized with sodium hypochlorite (0.6 %), rinsed with distilled, imbibed for 2 hrs in sterilized water, set on moist filter paper in a Petri dish and incubated at 25°C. After 5 days, the seedlings were transferred and grown hydroponically in glass tanks. The nutrient solution was continuously aerated with pumps, and contained macronutrients: $CaCl_2$ H_2O (1mM); $MgSO_4 7H_2O$ (25mM); K_2SO_4 (0.51mM); $KH_2PO/$ K_2HPO_4 (5.5mM); Fe EDTA (0.05mM), NH_4NO_3 (5mM) and micronutrients (for 1L): H_3BO_3 (2g); $MnSO_4$ $4H_2O$ (2.23g); $ZnSO_4 7H_2O$ (0.287g); $CuSO_4 5H_2O$ (0.125g);

$CoCl_2 6H_2O$ (0.065g), $NaMoO_4 2H_2O$ (0.12g) (Frendo et al., 1999). Seedlings were grown in a growth room at 25°C/20°C (day/night) with 18h/6h (day/night) photoperiod and 75% relative humidity. Nutrient solution was weekly changed. After 3 weeks, plants were transferred on nutrient solution without nitrogen (the same composition described previously without NH_4NO_3). After 3 days, plants were treated for either nodulation, or growth analysis. For nodulation analysis, tanks were flushed with mixed O_2/N_2 gas containing either 21, 4.5, 0.1 or 0% O_2. After 3 days, *Medicago* plants were inoculated with *Sinorhizobium meliloti* (strain RCR2011) as described in Frendo et al. (2005). One week post-inoculation (wpi), plants were harvested and nodules were counted. For growth analysis, plants were inoculated with *Sinorhizobium meliloti* (strain RCR2011) as mentioned above, and subdivided into three groups. The first group (control) was maintained under air bubbling for 4 weeks. For the second group (2 weeks of hypoxia; 2WH) air bubbling was maintained for 2 weeks, and then stopped for 2 additional weeks; For the third group (4 weeks of hypoxia; 4WH), air bubbling was stopped and the plant were kept without aeration for 4 weeks. At the end of the experiment, plants were sampled into roots, shoots and nodules, and immediately analysed.

Vegetative growth analysis

Roots were washed in distilled water, blotted on filter paper to remove excess of water, and denodulated. Fresh weights (FW) were immediately determined for nodules, roots and shoots. Dry weights (DW) were obtained by weighing the plant material after drying at 80°C until a constant mass was reached (48 to 72 hrs). Leaf area (LA) was measured as in Horchani et al. (2008). Leaf mass per area (LMA) was calculated by dividing total leaf FW by total leaf area for each plant.

Chlorophyll and total soluble carbohydrates

Chlorophyll measurement was performed according to Wintermans and De Mots (1965), and total chlorophyll concentration was calculated as in Horchani et al. (2008). Total soluble carbohydrates were determined in roots and leaves as in Horchani et al. (2009).

Nitrogen compounds

Fresh root, nodules and leaf samples were ground thoroughly with mortar and pestle in Tris-HCl (pH 7.5),

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and centrifuged at 20 000 g for 10 min. The supernatant was analysed for nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) contents. 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. NH_4^+ was determined by the phenol-hypochlorite method (Brouquisse et al., 1991).

Statistical treatments

Three biological replications were used in this study. The results are given as means with standard deviation of at least 10 samples per treatment. Statistical data analysis was made using Student *t*-test. The significance of differences between the control and the treatment mean values was determined at the significance level of *p*<0.05.

Results

Effects of hypoxia on nodulation

We first analysed the effects of two levels of hypoxia (4.5 and 0.1% O_2), compared to normoxia (21% O_2) and anoxia (0% O_2), on nodulation events after inoculation of *Medicago truncatula* with *Sinorhizobium meliloti*. As shown in Fig. 1, a moderate hypoxia (4.5% O_2) did not change significantly the nodule number per plant when compared to control normoxia (21% O_2), but a strong hypoxia (0.1% O_2) triggered a 55% reduction of nodule number, whereas anoxia (0% O_2), nodulation was almost totally abolished (Fig. 1).
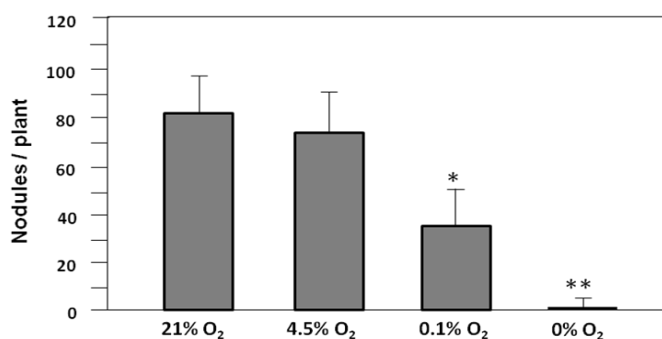
Fig. 1: Effect of hypoxia on nodulation of *Medicago truncatula*. Four weeks-old plants were submitted to hypoxia for 3 days before inoculation with *Sinorhizobium meliloti*. Nodules were numbered one week post-inoculation. The results are the mean ± SE of four independent experiments. * and **, the significance of differences between the control and the treatment mean values were determined by the Student's t-test at the significance level of *p*<0.05 and <0.01, respectively.

Vegetative growth analysis

In a second hand, we aimed at analysing growth parameters on inoculated *M. truncatula* plants submitted to a progressive hypoxia. As already reported, the interruption of air bubbling in hydroponic culture mimics the establishment of hypoxia in flooded soils (Gibbs and Greenway, 2003), and within 24 to 48 h the O_2 pressure decreases to 2-5% O_2 before stabilisation (Gharbi et al., 2007; Horchani et al., 2008). In the present study, considering that a moderate hypoxia (4.5% O_2) does not impair the nodulation process (Fig. 1), it was expected that the interruption of air bubbling would not impact the final nodule number of plants submitted to 2 and 4 weeks of progressive hypoxia (2WH and 4WH, respectively) compared to the normoxia treated plants (Control). Indeed, no significant difference was found between control, 2WH and 4WH treated plants that contained 210 to 360 nodules per plant at 4 wpi whatever the treatment (data not shown).

At 4 wpi, control and hypoxia treated plants were analysed for morphological aspects, biomass production (fresh and dry weights, FW and DW), leaf area (LA) and mass per area (LMA), and some metabolite contents (chlorophylls a and b, soluble sugars and nitrogen mineral compounds: nitrate, nitrite and ammonium).

Table 1. Leaf area (LA) and leaf mass per area (LMA) of *Medicago truncatula* submitted to two and four weeks of hypoxia. The results are the mean ± SE of three replicates. * The significance of differences between the control and the treatment mean values was determined by the Student's t-test at the significance level of *p*<0.05.

	C	2WH	4WH
LA (cm^2. plant)	90 ±12	60±7 *	40 ±5 *
LMA ($g.cm^{-2}$)	0.24 ±0.027	0.58±0.01 *	0.57 ±0.014 *

As shown in Fig. 2 and 3, hypoxia treatments affected moderately the morphological aspect and the biomass of *M. truncatula* plants. If total shoot FW was not significantly modified after 2 and 4 WH treatment (Fig. 3), some modifications were observed at leaf level. Hypoxic treatment affected the total leaf area (LA) per plant which decreased by 33 and 56% for 2WH and 4WH plants compared to the control, respectively (Table 1).

On the other hand, the leaf mass per area (LMA) of hypoxia treated plants was increased by about 230% for both 2WH and 4WH treated plants compared to control ones (Table 1). Such modifications in LA and LMA were

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already observed in tomato plants affected by root hypoxia (Horchani et al., 2008). This means that when the *M. truncatula* root system is subjected to hypoxia, the plant reduces leaf expansion, but maintains leaf biomass production by increasing the thickness of the leaves. After 4 wpi, root FW and DW were unaffected by hypoxic treatment, whereas nodule number and FW slightly increased in hypoxia treated plants (Fig. 3). These data are in agreement with previous observations made with N-supplied non-inoculated *M. truncatula* plants submitted to hypoxic treatments (El Msehli et al., 2015), indicating that the adaptive growth response of *M. truncatula* to hypoxia is similar whatever the either symbiotic, or non-symbiotic, status of the plant.
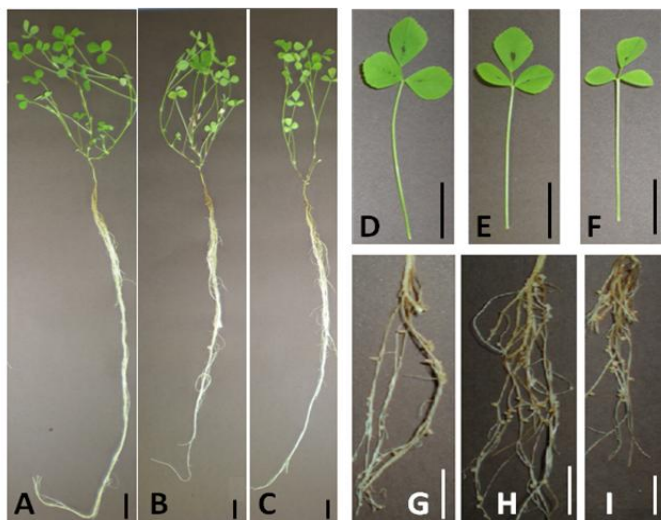

Fig. 2: Morphological aspect of *Medicago truncatula* plants submitted to hypoxia. Respectively allure of whole plants (A, B, C), third stage leaves (D, E, F) and nodule roots (G, H, I). 2WH and 4WH, two and four weeks of hypoxia. Control (A, D, G), 2WH (B, E, H) and 4WH (C, F, I). Bars represent 1 cm of length.

Chlorophyll and total soluble carbohydrates

Chlorophyll and soluble contents were then analysed to assess roughly the global impact of hypoxia on the capacity of symbiotic *M. truncatula* plants to main their photosynthesis. As compared to control plants, chlorophyll a and b contents were not modified in 2WH and 4WH leaves (Fig. 4), indicating that the photosynthetic machinery was not affected by hypoxia. In good agreement with this observation, shoot total soluble carbohydrate (TSC) content was not either modified significantly (Fig. 5). Root TSC, only moderately decreased by 22 and 29% after 2 and 4 WH, respectively (Fig. 5). These data are different to those
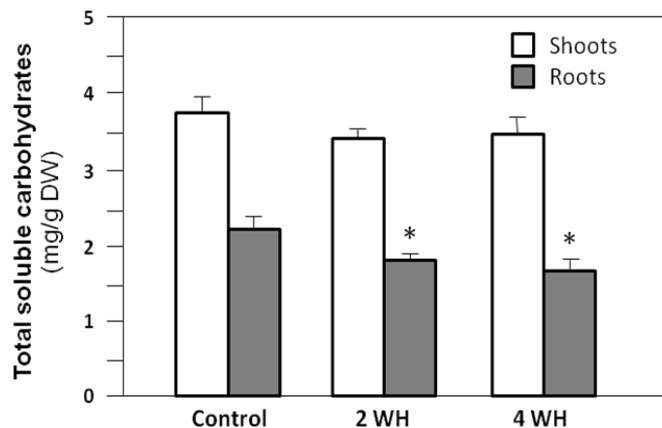
observed in the roots of non-symbiotic *M. truncatula* plants where TSC content increased by a factor 2 after 30 d of hypoxia (El Msehli et al., 2015). Such discrepancy may be related to the nitrogen status of plants which strongly differs between N-supplied (El Msehli et al., 2015) and symbiotic (this study) plants. Thus, we analysed the main nitrogen mineral compounds (nitrate, nitrite and ammonium) in shoots, roots and nodules of the symbiotic plants submitted to hypoxia.
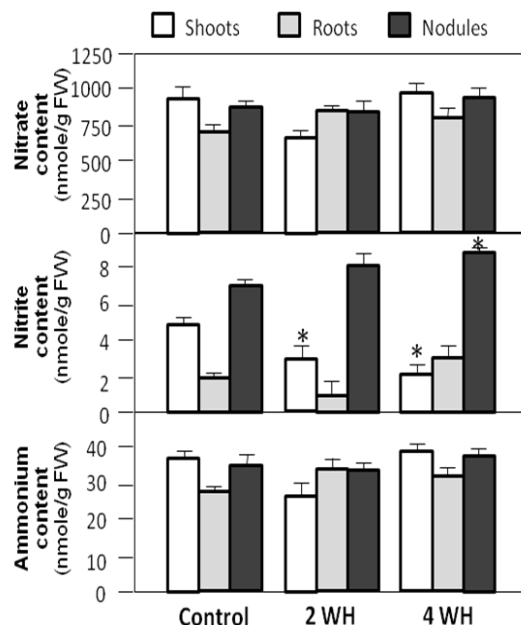
Fig. 3: Effects of hypoxia on shoot (A), root (B) and nodule (C) grow parameters. FW, fresh weight; DW, dry weight. Nodule number refers to the number of nodules per plant after four weeks of treatment. The results are the mean ± SE of ten plants. * The significance of differences between the control and the treatment mean values was determined by the Student's t-test at the significance level of *p*<0.05.
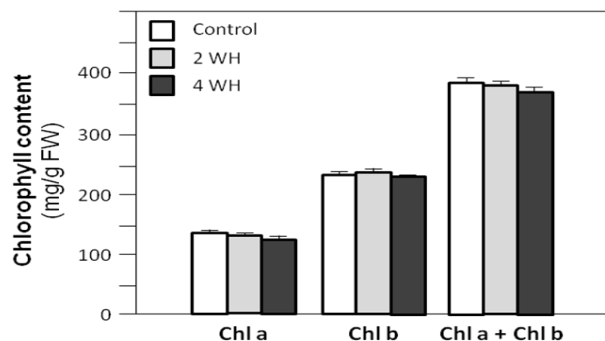
Fig. 4: Foliar Chlorophylls (A) and Ratio Chl a/Chl b; (B) of *Medicago truncatula* submitted to two and four weeks of hypoxia. The results are the mean ± SE of ten measurements. * The significance of differences between the control and the treatment mean values was determined by the Student's t-test at the significance level of *p*<0.05.

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Fig. 5: Total soluble sugar in shoot and root of *Medicago truncatula* submitted to hypoxia after two and four weeks of hypoxia. The results are the mean ±SE of ten plants. * The significance of differences between the control and the treatment mean values was determined by the Student's t-test at the significance level of *p*<0.05.

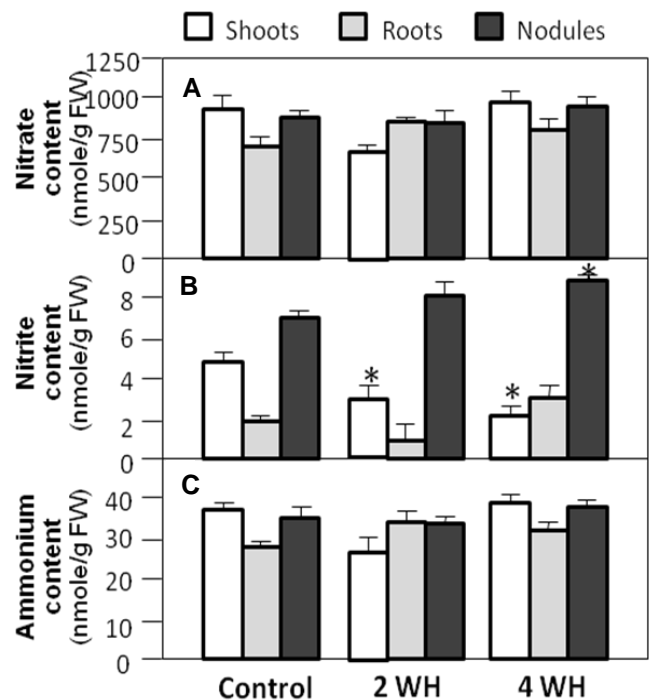### Nitrate, nitrite and ammonium contents

Nitrate content was of the same order in shoots, roots and nodules of symbiotic *M. truncatula* plants. It was found to moderately decrease (by 28%) in the leaves after 4 WH, whereas it conversely increased (by 24%) in the roots, and remained unchanged in the nodules (Fig. 6A).

Interestingly, nitrite accumulated more importantly in nodules than in shoots and roots. Hypoxia triggered a transient 32% and 86 % increase in nodule and root nitrite content after 2 WH, to decrease after 4 WH (Fig. 6B), whereas an opposite trend was observed in the shoots, with a transient decrease in nitrite content after 2 WH (Fig. 6B). Ammonium contents were of the same order in the three organs and did not change significantly during hypoxia treatment (Fig. 6C).

Discussion

Most of the studies investigating flooding or waterlogging effects on symbiotic nodulated legumes analysed nodule structure or functional properties (nitrogenase activity, nitrogen fixation), but only few of them investigated their impact on nodulation event, i.e. nodule number (Minchin and Pate, 1975; Sung, 1993). In these studies, plants were submitted to hypoxia after either waterlogging of soils or arrest of air bubbling in hydroponic cultures that both result in O_2 pressure generally ranging from 1 to 10 kPa in the rhizosphere (Sung, 1993; Gibbs and Greenway, 2003; Gharbi et al., 2007; Horchani et al., 2008). In the present study, we

first investigated the impact of various O_2 pressures, from normoxia to anoxia, on nodulation events in *M. truncatula plants* (Fig. 1). As compared to hypoxia-sensitive legumes, such as pea (Minchin and Pate, 1975) and soybean (Sung, 1993; Youn et al., 2008) which exhibit reduced nodule numbers when grown under hypoxic conditions, nodule numbers on *M. truncatula* roots were not modified under 4.5% O_2 (4.5 kPa), and only 45% decreased under 0.1 % O_2. In addition, whereas nodule fresh weight per plant was 50-70% reduced under hypoxia in alfalfa (Arrese-Igor et al., 1993), soybean (Sung, 1993; Youn et al., 2008) and cow pea (Minchin and Summerfield, 1976), it remained unchanged in *M. truncatula* after 4 WH (Fig. 3). Thus, when considering nodulation events and nodule growth, *M. truncatula* (Var. Jemalong A17) may be defined as a hypoxia tolerant legume.

Fig. 6: Nitrate (A), nitrites (B) and ammonium (C) contents in shoots, roots and nodules of *Medicago truncatula* submitted to two and four weeks hypoxia. The results are the mean ±SE of ten plants. * The significance of differences between the control and the treatment mean values was determined by the Student's t-test at the significance level of *p*<0.05.

Physiological processes like nitrate reduction, amino acid synthesis and nitrogen metabolism are early affected after the onset of waterlogging-induced hypoxia in legumes (Diab and Cukier, 2013), and the nitrogen status of the plant is an important parameter to take into account to assess the impact of hypoxia on it. Thus, in

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soybean plants, N_2 fixation was more sensitive to flooding than biomass accumulation, and the supplementation of soybean plants with nitrate may improve their tolerance to flooding relative to those relying on N_2 fixation (Bacanamwo and Purcell, 1999). Similarly, in nodulated pea plants grown in absence of nitrogen, waterlogging reduced drastically N_2 fixation and nitrogen content compared to control plants, whereas waterlogging effects were less severe in nitrate-fed plants (Minchin and Pate, 1975). More recently, the analysis of waterlogging effects on either wild type or supernodulating soybean mutants showed that supernodulated mutants possess a weaker waterlogging tolerance than their wild types (Youn et al., 2008). These data indicate that the more nitrogen supply is limited and the plant is nodulated, the more it is sensitive to hypoxia. Interestingly, when compared to nitrate-fed *M. truncatula* (El Msehli et al., 2015), nodulated *M. truncatula* plants grown in absence of nitrate (this study) showed similar tolerance to hypoxia after 4 WH in terms of leaf and root development and biomass accumulation (Figs. 2 and 3). This means that, contrary to hypoxia sensitive legumes such as cow pea (Minchin and Summerfield, 1976), lentil (Malik et al., 2015), *M. sativa* (Rogers et al., 2007), *Trifolium subterraneum* (Aschi-Smiti et al., 2002; Rogers et al., 2009), or soybean (Henshaw et al., 2007; Youn et al., 2008), *M. truncatula* maintained its growth and biomass production after one month of hypoxia, and may be considered as a hypoxia tolerant legume. It was reported that, under hypoxia, a close relationship exists between chlorophyll level and carbohydrate contents (Netto et al., 2005). In the present study, both the increase of leaf thickness that compensates the reduction of total leaf area (Table 1) and the maintenance of the total chlorophyll content (Fig. 4) indicate that the photosynthetic machinery was not structurally affected and remained probably functional after 4 WH. This interpretation is supported by the fact that TSC were roughly maintained booth in leaves and roots in hypoxia-treated and in control plants (Fig. 5). It has been demonstrated for many plant species that the assimilation of NH_4^+ and the root growth are competing for carbohydrates, and that the use of carbon skeletons for the assimilation of NH_4^+ may be at the expense of root growth (Cramer and Lewis, 1993; Schortemeyer et al., 1993). The maintenance of chlorophylls, sugars and NH_4^+ contents after 4 WH (Figs. 4, 5 and 6) clearly indicate that *M. truncatula* carbon and nitrogen metabolisms are unaffected by hypoxia treatment, and that photosynthesis products enough sugar to cover the needs in carbon skeletons for NH_4^+ assimilation and root

growth at that stage. In nitrate-fed *M. truncatula* roots (El Msehli et al., 2015), sugar contents were shown to increase after one month of hypoxia. Considering that the plant metabolic response to hypoxia strongly depends on the availability and mobilization of carbohydrates (Drew, 1997; Bailey-Serres and Voesenek, 2008), these observations suggest that nitrate-fed *M. truncatula* plants are probably better adapted to face long-term root hypoxia than nodulated once grown in the absence of nitrate.

Nitrogen source and metabolism were reported to regulate the response of plants, including legumes, to hypoxia (Horchani et al., 2010; Horchani and Aschi-Smiti, 2010). The use of nitrate through a metabolic process named "hemoglobin-nitric oxide respiration" was shown to improve the energy status of roots, but not leaves, under oxygen-limited conditions (Igamberdiev et al., 2010; Gupta et al., 2011). In this process, NO_2^- substitute to O_2 as a terminal acceptor of the mitochondrial electron transfer chain, allowing the functioning of the mitochondrial respiration and the regeneration of ATP. This process was found to occur in *M. truncatula* nodules in hypoxic conditions (Horchani et al., 2011). Interestingly, in the present study, NO_2^- content was found to increase in roots and nodules, but not in leaves, of *M. truncatula* plants after 2 and 4 WH, which fits with the set-up of a Hb-NO respiration process during hypoxia, the balance of the energy state in roots and nodules, and finally the tolerance of *M. truncatula* to hypoxia. This hypothesis is presently under investigation in our laboratory.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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