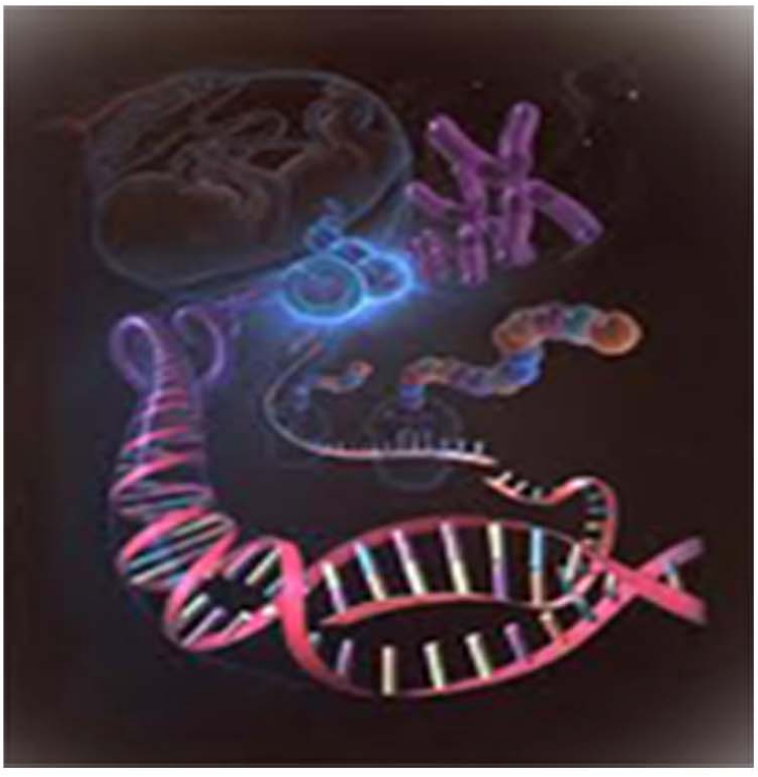
IJLBPR

ISSN 2250 - 3137
Vo1. 2, No. 3, July 2013

International Journal of

Life Sciences Biotechnology and Pharma Research



International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA

www.ijlbpr.com

Email : editorijlbpr@gmail.com or editor@ijlbpr.com

International Journal of Life Sciences Biotechnology and Pharma Research

ISSN 2250-3137 www.ijlbpr.com
Vol. 2, No. 3, July 2013

Research Paper

IMPACT OF WATERLOGING-INDUCED HYPOXIA ON NITROGEN METABOLISM IN THE LEGUME *MEDICAGO TRUNCATULA*

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Flooding and waterlogging due to the induced oxygen limitation in the root zone is harmful for plant development. This study examines short term modulation of nitrogen metabolism in *Medicafo truncatula* submitted to waterlogging. The objective was to evaluate whether and how nitrogen metabolism contributes to the mitigation of damaging effects of hypoxia. The processes that were affected early after the onset of stress were nitrate reduction and amino acids synthesis. NADH-dependent nitrate reductase activity increased dramatically in the root. It is suggested that nitrate reductase contributes to cellular acclimation to hypoxia by regenerating NAD^+ from NADH. The regeneration of NAD^+ is a crucial issue in hypoxic cells because it is necessary for supporting increasing rates of glycolysis. Amino acids metabolism shifted from the ATP consuming pathway leading to asparagines, the most accumulated amino acid in *Medicago truncatula*, to pathways leading to alanine and GABA accumulation. Synthesis of alanine is not dependent on ATP and allows for storage of carbon used in glycolysis (pyruvate) in a form readily utilizable at the return to normoxic condition. GABA synthesis through the GABA shunt starts by decarboxylation of glutamate by glutamate decarboxylase (GDC) a proton consuming enzyme that helps maintaining cytosolic pH homeostasis.

Keywords: Alanine, GABA, Nitrogen Metabolism, Hypoxia, Waterloging, *Medicago truncatula*

INTRODUCTION

Plants are subject to numerous environmental events that impact growth and survival either in cultivated areas or natural ecosystems. In the context of climatic change, heavy rains associated to transient flooding and waterlogging are predicted to become more frequent jeopardizing plants development and survival essentially because crops were not selected to tolerate hypoxia/anoxia stress. Consequently an increasing interest is paid by the scientific community to the adaptation of plants to low oxygen limitation at both cellular and whole plant levels.

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Sensitivity or tolerance of plants to flooding and waterlogging is dependent on inducible metabolic mechanisms that allow counteracting deleterious symptoms of hypoxia at the cellular level. Under hypoxia glycolytic enzymes are more active as a consequence of the need for increased glycolysis to compensate for the lower ATP yield due to the inactivation of oxidative phosphorylation (Sato *et al*., 2002). The regeneration of NAD^+ is a crucial issue for the adaptation to hypoxia because in the absence of NAD^+ glycolysis ceases (Ismond *et al*., 2003; Kürsteiner *et al*., 2003). For this aim under hypoxia it was observed that de novo synthesis of fermentative enzymes pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) is boosted up (Sachs *et al*., 1996). As a result fermentative products derived from pyruvate, i.e., acetaldehyde, ethanol and lactate accumulate thus allowing for the regeneration of NAD^+ from NADH. The importance of ethanol fermentation in tolerance of low oxygen stress is supported by numerous studies on several species; it has been shown that null mutants affected in the expression of alcohol dehydrogenase thus deprived of ethanol fermentation exhibited reduced tolerance to oxygen deficiency compared to wild types (Harberd and Edwards, 1982; Jacobs *et al*., 1998; Matsumura *et al*., 1995). This strategy however contains its own limit, the regeneration of NAD^+ via ethanol fermentation induces the production of acetaldehyde by PDC a highly reactive molecule that induces damages by forming acetaldehyde-protein adducts and ethanol production by ADH induces a faster depletion of sugar stores and thus carbon starvation stress and ultimately cellular death(Ismond *et al*., 2003). The regeneration of NAD^+ via LDH worsens cytoplasm acidification due to lactic acid synthesis (Davies, 1987; Davies and Patil, 1974).

Nitrogen metabolism and its involvement in the adaptive response of plants to hypoxia received fewer attentions than carbon metabolism. Nitrate fertilization was shown to improve tolerance of plants to root hypoxia suggesting an ameliorating effect of nitrate on deleterious effects of hypoxia (Allègre *et al*., 2004; Horchani *et al*., 2010; Morard *et al*., 2004). Although exposure of plants to hypoxia or anoxia increased gene expression and de novo synthesis of alanine aminotransferase (*AlaAT*) in various animal and plant species (Sachs *et al*., 1996) the fermentative pathway of AlaAT received less attention than PDC-LDH / ADH. Elevated levels of alanine are currently observed in waterlogged roots and expression of AlaAT encoding gene increased under hypoxia in roots of barley (*Hordeum vulgare*), corn (*Zea mays*), *Panicum miliaceum* and *Lotus japonicus* (Good and Muench, 1992 and 1993; Muench and Good, 1994; Ricoult *et al*., 2005 and 2006; Limami *et al*., 2008; Rocha *et al*., 2010).

In the present article modulation of primary nitrogen metabolism by short term waterlogging-induced hypoxia was studied in the model legume *Medicago truncatula*.

MATERIALS AND METHODS

Plant Material and Stress Application

Medicago truncatula (line A17) were grown in pots on neutral substrate and watered each other day with a half Murashige and Skoog nutrient solution. Plants were randomly distributed in a culture chamber at temperature 22 ± 0.2° C and relative humidity of 70% ± 2. Circadian cycle consisted of 16 hours light and 8 hours darkness. Three weeks after germination young plants were constituted into 2 batches; one batch of control plants and one batch of treated plants were submitted to waterlogging-induced hypoxia by

filling the pots with the nutrient solution for a maximum duration of 72 h. The shoots were not flooded. The establishment of hypoxia was controlled by measuring oxygen content in the pots by an electrochemical oxygen meter (GMH 3630, Greisinger, Regenstauf, Germany). Control plants were watered by the same nutrient solution. Root and shoot were collected separately, weighed and frozen in liquid nitrogen and maintained in –80°C freezer for further analyses.

RNA Extraction and Reverse Transcription

Total RNA was extracted using TRIzol Reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's protocol. A 2 mg sample of total RNA was retro transcribed for 1 h at 37°C, using 200 units of M-MLV Reverse Transcriptase (Promega, Madison, WI) and 2 mg of pd (N)6 Random Hexamer (Amersham Biosciences, Freiburg, Germany) in the presence of 40 units of Recombinant Rnasin Ribonuclease Inhibitor (Promega), and in a final volume of 50 ml. Genomic DNA was removed by purifying the first strands using a QIAquick PCR Purification Kit (Qiagen, German Town, MD).

Real-time Polymerase Chain Reaction (RT–PCR) and SYBR Green Detection

PCR was performed on the light cycler ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA) with the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Each reaction was performed on 5 ml of a 1: 2 (vv) dilution of the first cDNA strands, synthesized as described above with 0.3 mM of each primer in a total reaction of 20 ml. The reactions were incubated for 2 min at 50°C and 10 min at 95°C followed by 60 cycles of 15 s at 95°C and 1 min at 60°C. The specificity of the PCR amplification procedures was checked with a heat-dissociation protocol (from 65 to 95°C) after the final cycle of the PCR. Each reaction was done in triplicate and the corresponding Ct values were determined. The amplified fragment (100 bp) was cloned into pGEM T Easy vector (Promega). The plasmids were diluted several times to generate templates ranging from 105 to 103 copies used for standard curves for the estimation of copy numbers. The results are expressed as copy number of cDNA in 5 ml of first strands.

Metabolites Extraction and Analysis

The total amino acids and sugars were extracted in 96% ethanol for 1 h at 4°C. After centrifugation, the ethanol fraction was removed and the same process was then repeated with de-ionized water. The ethanol and water fractions were combined and stored at –20°C. After evaporation of the extract under vacuum, organic residues were dissolved in de-ionized water and extracted with the same volume of chloroform. After centrifugation, an aqueous phase containing amino acids and sugars was vacuum dried and resuspended in de-ionized water for High Performance Liquid Chromatography (HPLC) analysis.

Amino acids were determined by the Waters (MA, USA) AccQTag method. The AccQTag method uses Waters AccQFluor Reagent (patent pending) to derivatize the amino acids. The reagent is a highly reactive compound, 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC), which forms stable derivatives with primary and secondary amino acids. Derivatives are separated by reversed-phase (C18 column) HPLC and quantified by fluorescence detection.

Carbohydrates were determined straight forward in the extract by HPLC on a Carbopac PA-1 column (Dionex Corp., Sunnyvale, CA, USA).

Nitrate was extracted in boiling water and determined by the colorimetric method of Cataldo (1975).

Nitrate Reductase Extraction and Assay

Extraction was performed on frozen plant material in 1.5 mL of mM Hepes-KOH, pH 7.6, 15 mM magnesium chloride, 1 mM DTT, 10 µM FAD, 10 µM leupeptin and 0.2 mM pefabloc. The homogenate was centrifuged and the supernatant was used as the crude extract. Actual nitrate reductase activity was assayed in the following medium, 50 mM Hepes-KOH, pH 7.6, 10 mM magnesium chloride, 200 µM NADH, 5 mM potassium nitrate for 15 min at 27°C. The reaction was initiated by adding 500 µl of crude extract. Activity of the enzyme was estimated by determination of nitrite formed during the enzymatic reaction. Nitrite was determined by colorimetric reaction by incubation an aliquot of the reaction medium, 30 min in a mixture of sulfanilamide diluted HCl and N-(1-naphtyl) ethylenediamine dihydrochloride. Optical density was measures at 540 nm.

RESULTS AND DISCUSSION

Although carbon metabolism was not thoroughly investigated we found that glucose and fructose contents increased significantly in the shoot and the root (Figure 1) while starch and sucrose contents decreased to the point that sucrose was not detected by HPLC (data not shown) indicating probably that carbon reserves were thoroughly mobilized to support glycolysis. This result is in agreement with the commonly observed increase in the rate of glycolysis and subsequent increase in substrate level ATP production to ameliorate the hypoxia – induced energy crisis (Greenway and Gibbs, 2003).

The contribution of nitrogen metabolism to the short term – adaptive response of plants to waterlogging was more thoroughly studied. For this aim several aspects of nitrogen metabolism under hypoxic condition were analyzed in *Medicago truncatula* seedlings during the first hours of stress. In the present article we are reporting only the most significant changes *i.e.* changes pertaining to nitrate reduction and assimilation into amino acids.

Nitrate Metabolism

Major changes in nitrate metabolism occurred in the root. Nitrate Reductase Activity (NRA) increased dramatically in hypoxic roots to represent two to three times NRA in the control while it remained fairly similar in the shoot of the control and waterlogged plants (Figure 2). This result agrees with the observation that hypoxia-induced acidification of cytosolic pH may increase NR activity because of the low pH optimum of this enzyme (Botrel and Kaiser, 1997; Stoimenova *et al*., 2007). It was shown that NR was highly phosphorylated, thus inactive in aerated roots of hydroponically grown barley while it was partly dephosphorylated and active in hypoxic roots (Shi *et al*., 2008) (Botrel and Kaiser, 1997). Changes in NRA impacted nitrate and ammonium contents in the root. As expected the content of nitrate was lower and the content of ammonium, the product of nitrate reduction was higher in hypoxic roots than that in the control (Figures 2 and 3). It is important to note that the increase in ammonium content under hypoxia strongly suggest a discrepancy between nitrate reduction and

Figure 1: Sugars Quantified in *Medicago truncatula* Seedlings by HPLC in Roots and Shoot, Results are the Mean ± SE of Three Replicates

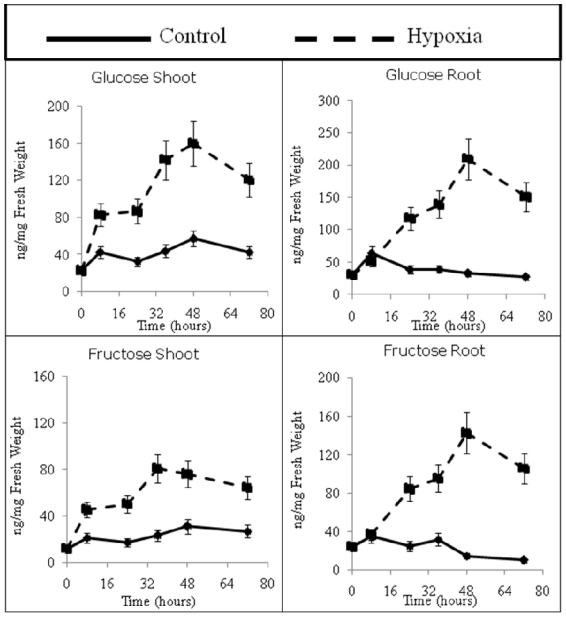


Figure 2: Nitrate Reductase Activity and Nitrate Content Quantified in *Medicago truncatula* Seedlings in Roots aqnd Shoot, Results are the Mean ± SE of Three Replicates

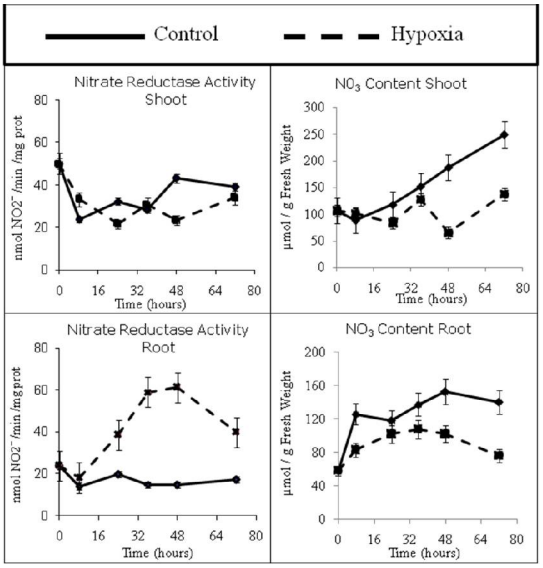
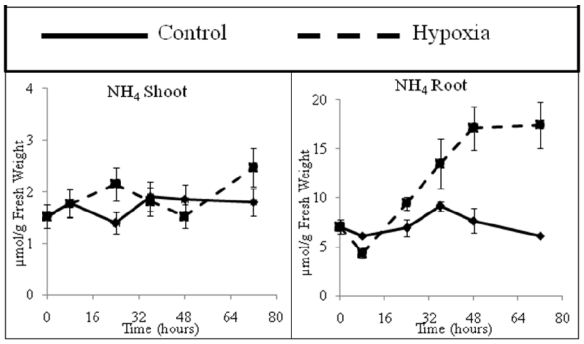


Figure 3: Ammonium Content Quantified in *Medicago truncatula* Seedlings in Roots and Shoot, Results are the Mean ± SE of Three Replicates



ammonium assimilation into amino acids. Another source may contribute ammonium accumulation under hypoxia is proteolysis. Nitrate content in the shoot was lower in plants submitted to waterlogging condition (Figure 2). As NRA and ammonium content were similar in the control and stressed plants, it is suggested that the lower content of nitrate in the shoot of stressed plants is probably due to a lower transport of nitrate to the shoot. Nitrate beneficial effect is probably not exerted by nitrate per se but due to the action of nitrate reductase. The reduction of nitrate is NADH-dependent; it occurs in the cytosol, the same compartment as glycolysis and contributes to cellular acclimation to hypoxia by regenerating NAD^+ from NADH. Accordingly tolerant species to oxygen deprivation show higher nitrate reductase activity than sensitive species (Bailey-Serres and Voesenek, 2008). Tobacco as well as tomato deprived of NRA either by genetic manipulation or by inhibition by tungstate showed higher sensitivity to waterlogging than control plants (Horchani *et al*., 2010; Stoimenova *et al*., 2003). Conversely it was observed that maize seedlings supplied with nitrate during anoxia showed better tolerance to hypoxia and control of cytosolic pH (Libourel *et al*., 2006).

ACTIVATION OF ALANINE AND GABA SHUNT

It has been suggested that one of the aspects of tolerance to hypoxia is the decrease in the activity of ATP consuming processes. Ammonium assimilation via glutamine synthetase / glutamate synthase (GS / GOGAT) cycle and asparagine synthesis by asparagine sythetase are ATP consuming processes. In plants submitted to long term hypoxia stress it was observed that contents of glutamine and asparagine decrease as a consequence of ATP shortage (Bailey-Serres *et al*., 2012; Bailey-Serres and Voesenek, 2008). It is interesting to note that the pathway of ammonium assimilation at the whole plant level was affected very early after the onset of hypoxia stress as suggested by the accumulation of ammonium content in the root and the decrease in asparagine content in the shoot of stressed plants as early as 8 hours after the onset of stress (Figure 4). Alternatively two pathways of amino acids syntheses were promoted *i.e.* alanine via alanine aminotransferase (AlaAT) and GABA via GABA transaminase (GABA-T) (Figure 5). The activation of these pathways occurred at the transcriptional level as shown by the activation of

Figure 4: Amino Acid, Asparagine Content Quantified in *Medicago truncatula* Seedlings in Roots and Shoot, Results are the Mean ± SE of Three Replicates

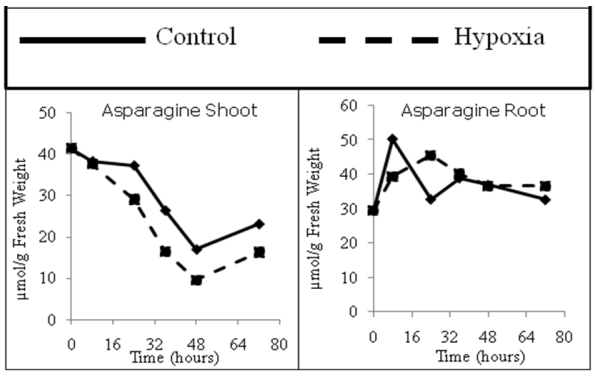
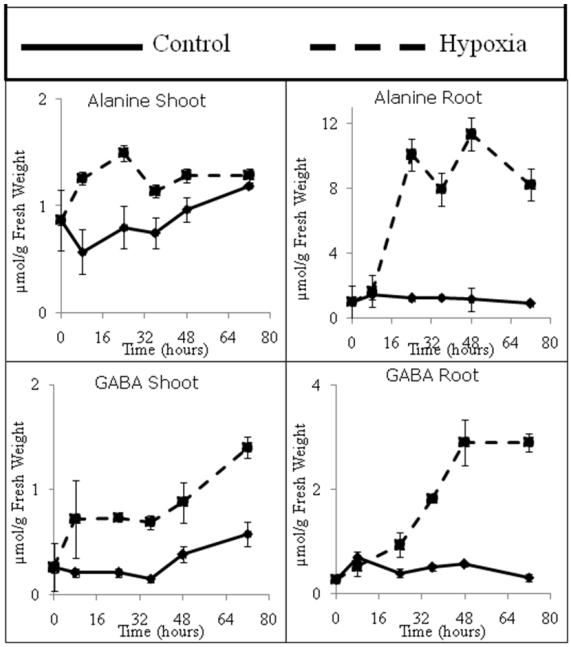
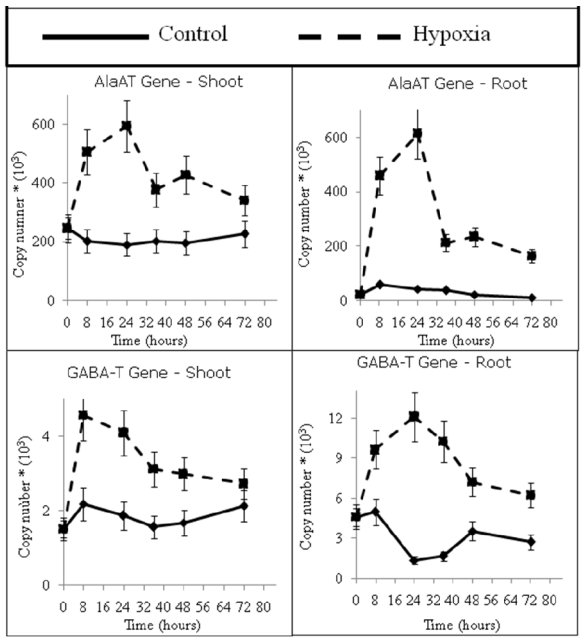


Figure 5: Amino Acid, Alanine GABA Content Quantified in *Medicago truncatula* Seedlings in Roots and Shoot, Results are the Mean ± SE of Three Replicates



the expressions of genes encoding two AlaAT and GABA-T (Figure 6). This observation is supported by several studies on legume plants like *Glycine max*, *Lotus japonicus* and *Medicago truncatula* (Oliveira *et al*., 2013; Ricoult *et al*., 2005; Ricoult *et al*., 2006; Rocha *et al*., 2010). Alanine and GABA syntheses are proposed to contribute to the mitigation of damaging effects of hypoxia. Alanine constitutes a carbon and nitrogen store readily utilizable during post-stress recovery. By accumulating alanine plants save carbon (pyruvate) that otherwise would be lost because alcohol fermentation pathway leads to ethanol a dead end product that either accumulates or leaks out of the tissue representing in both cases a net loss of carbon (Albrecht *et al*., 2004; Ismond *et al*., 2003). Furthermore through alanine shunt, synthesis of alanine generates 2-oxoglutarate which can be further metabolized to succinate, via the TCA cycle enzyme succinate CoA ligase,

Figure 6: Expression of *Medicago truncatula AlaAT* and *GABA-T* Measured by Q-RT-PCR, Results are the Mean ± SE of Three Replicates



thus providing additional ATP per molecule of sucrose metabolized (Rocha *et al*., 2010). The GABA shunt starts by the decarboxylation of glutamate by glutamate decarboxylase (GDC). This first step contributes to cellular pH homeostasis as glutamate decarboxylation is a proton consuming reaction that increases pH (Drew, 1997; Greenway and Gibbs, 2003).

CONCLUSION

In the present study we have shown that nitrogen metabolism contributes to the adaptive response of plants to hypoxia very early after the onset stress. Nitrate reduction as well as amino acids syntheses were involved in the mitigation of hypoxia damaging consequences, i.e., energy crisis and cytosolic pH acidification. Our results together with the increasing amounts of results published in similar areas dealing with low oxygen sensing, signaling and adaptation of plants to oxygen limitation at both cellular and whole plant levels should be taken into account in breeding programs and agronomical practices for saving plant fitness, growth and development in a world threatened by climatic change and increase of frequency of heavy rains, flooding and waterlogging (Licausi, 2013).

ACKNOWLEDGMENT

The author HD is indebted to French ministry of research for Ph.D. fellowship. Research was funded by QUALISEM contract with 'Region des pays de Loire'.

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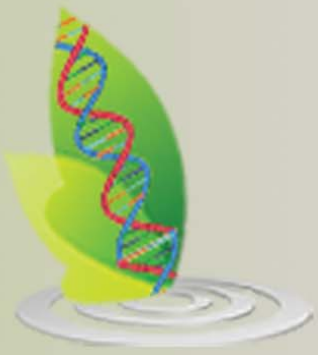

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ISSN 2250-3137