Original Research Article

Impact of Prolonged Hypoxia Stress on *Medicago truncatula* Growth

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Abstract	Keywords
Flooding is an important environmental factor restricting plant growth and productivity throughout the world. Growth, organic solute accumulation and proteolytic activity of *Medicago truncatula* were determined in hydroponically grown plants exposed to hypoxia during 30, 45 and 60 days. Obtained results showed that hypoxia until 45 days haven't a significant impact on the dry matter production. However, after 60 days of treatment, it was observed a decrease in the shoot and root growth. In addition, a development of adventitious roots is shown in the hypocotyle of treated plant. In fact, the water content varied in response to the hypoxia. The soluble carbohydrates content still stable on the shoot of treated plant until 45 day. Whereas, it was registered that the content of non-reducing soluble sugar present almost all of total soluble sugars, and that the accumulation of this sugars fraction in more important in the roots of hypoxia plants in comparison to the aerial organs. The proline content measured in stressed plants was increased with time over control plants. Contrary, hypoxia decreased the total endopeptidase activity in *M. truncatula* roots. It could be concluded that *M. truncatula* can be support the prolonged hypoxic stress by developing adventitious roots and accumulating proline.	Adventitious roots Endopeptidase activity Hypoxia *Medicago truncatula* Proline Soluble sugars

Introduction

The lack or the excess of the water in the rhizosphere present a critical factor on plant growth. Indeed, drought or waterlogging affect plant characteristics (Iqbal et al., 2009; Ibraheem et al., 2011; Promkhambut et al., 2011). Flooding is one of the most significant abiotic stresses, it causes a slow transfer of dissolved oxygen in the water filled pore space of the soil, and plants can have to face to the lack of oxygen during all stage of its growth. Seed imbibitions, flood irrigation, floods and excess of rainfall are examples of natural conditions leading to root hypoxia or anoxia. These conditions can severely disturb plants development (Voesenek and Bailey-Serres, 2013). The oxygen deficiency is thought to be a major determinant in the adverse effects of flooding on crops and other plant species (Mommer et al., 2004).

Not all plant species can survive to the waterlogging conditions, only some species are able to adapt to this stress by developing some physiological and morphological mechanisms like adventitious roots and

aerenchyma (Vartapetian and Jackson, 1997; Vignolio et al., 1999). Nevertheless, this adaptive capacity depends on the plant species (Poot and Lambers, 2003). Among species, legumes represent the third largest group of angiosperms and the second largest group of food and feed crops grown globally. They include 670 to 750 genera and 18000 to 19000 species. It regroups varied food and feed crop species, such as clover, chickpea, soybean, pea, alfalfa and mungbean. 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, some varieties 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). However, Leguminosae are able to evolve a symbiotic relationship with specific soil bacteria, called Rhizobia (de Faria et al., 1989). As a result, many legumes are sensitive to waterlogging, showing reductions in root growth, nodulation process, nitrogen fixation and photosynthesis activity (Linkemer et al., 1998; Bacanamwo and Parcell, 1999).

To develop more knowledge on legumes, *Medicago truncatula* get more attention because of its short growth cycle and its small genome (500-550 Mpb) (Young et al., 2005). It is a model plant and presents the subject of numerous researches that studying 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). In this work, we will try to investigate the impact of the prolonged time of hypoxia on the growth of *M. truncatula*. We follow the production of dry matter, the water content, the sugar content, the shoot proline, and the total endopeptidase activity after 30, 45 and 60 days of hypoxia.

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 days, the seedlings were grown hydroponically into glass container. The nutrient solution contained macronutrients: 2.25 mM KNO_3; 0.25 mM Ca $(NO_3)_2$; 0.35 mM KH_2PO_4 and 0.075 mM $MgSO_4$, micronutrients: 268.6 μM EDTA-Fe; 8,9 μM $MnSO_4$;

24.1 μM H_3BO_3; 1.7 μM $ZnSO_4$ and 3.9 μM $CuSO_4$ (Saglio and Pradet, 1980).

Hypoxic treatment was applied at the second leaf stage by stopping air bubbling, whereas control plants were continuously aerated. 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 were evaluated after 30, 45 and 60 days of hypoxia treatment. Plants were harvested, divided into roots and shoots. Roots were washed in distilled water. Fresh weights (FW) were immediately determined for roots and shoots. Dry weights (DW) were obtained by weighing the plant material after drying at 80°C until a constant mass was reached. Water content (WC) was calculated as (FW-DW) / DW.

Soluble carbohydrates and proline determination

Soluble carbohydrates, total and reduced, were determined as described by Aschi-Smiti et al. (2003). Free proline was quantified spectrophotometrically by the ninhydrin method according to Bates et al. (1973). The plant material was homogenized in 3 % (w/v) aqueous sulfosalicylic acid and the homogenate was centrifuged at 14000 rpm. The supernatant was used for the estimation of the proline concentration. The reaction mixture consisted of 2 ml of ninhydrin acid and 2 ml of glacial acetic acid, which was boiled at 100 °C for 1h. After termination of reaction in ice bath, the reaction mixture was extracted with 4 ml of toluene, and absorbance was read at 520 nm.

Proteolytic activity

Proteolytic activity in shoot crude extracts was determined spectrophotometrically by following the digestion of azocasein at 440 nm (Brouquisse et al., 1998).

Statistical analysis

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 samples per treatment. The significance of differences between the control and the treatment mean values was determined at the significance level of $p<0.05$.

Results

Vegetative growth analysis

In the present study, hypoxia was applied at the second leaf stage of *Medicago truncatula* growth. In function of time, the biomass production of *M. truncatula* was increased and the differences between control and treated plant were not significant up to 45 days of treatment. At 60 days of hypoxia decreased the growth of shoot and root respectively by 44 and 52% in comparison to control plants (Fig. 1).

Fig. 1: Shoot (A) and root (B) dry weight (DW) of control (grey) and hypoxia treatment (black) of *M. truncatula* during 30, 45 and 60 days. Values are the mean ± S.E. from 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* in comparison to the control.
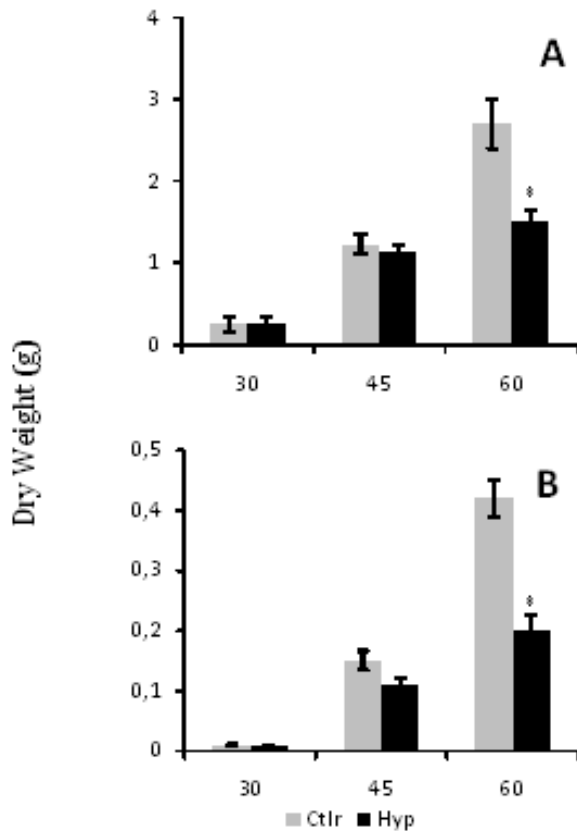
In fact, hypoxia decreased the water content in shoot respectively by 14, 36 and 33% after 30, 45 and 60 days of treatment (Fig. 2A). While, the water content in roots was decreased after 45 and 60 days of hypoxia and an augmentation of the water content was noticed after 30 days estimated by 34% (Fig. 2B).

Fig. 2: Shoot (A) and root (B) water content of control (grey) and hypoxia treatment (black) of *M. truncatula* for 30, 45 and 60 days. Values are the mean ± S.E. from 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* in comparison to the control.
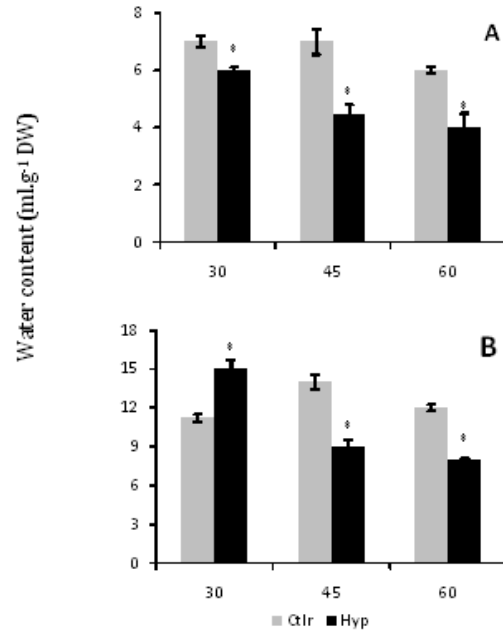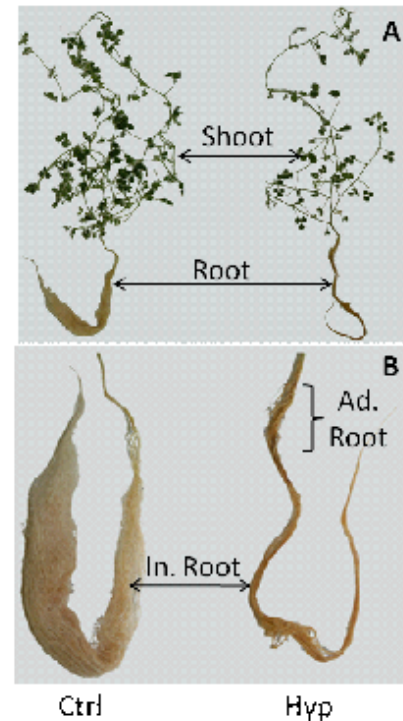

Fig. 3: Morphological aspect of *Medicago truncatula* (A) and roots (B) after 60 days of hypoxia treatment. Ad: Adventitious roots, In: initial.
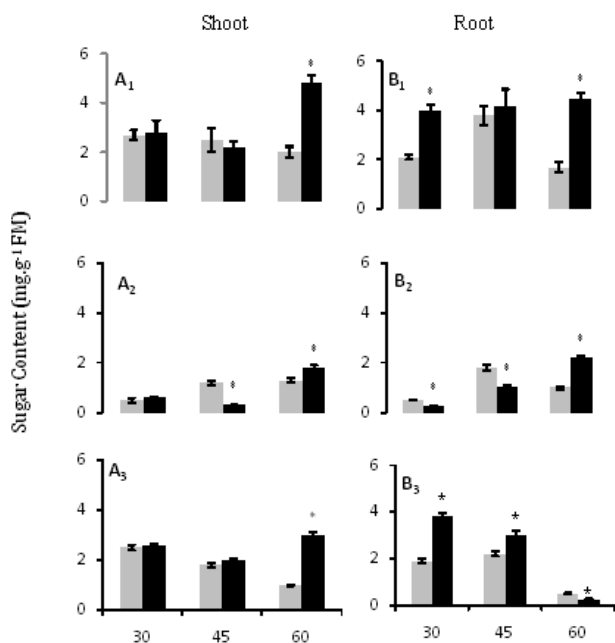
Nevertheless, prolonged root hypoxia (60 days) decreased the development of the whole plant. The picture presents the development of adventitious roots on the hypocotyle of *M. truncatula*. In the present study, compared with the control plant that had no adventitious root, it was observed the petrifaction of the initial root system (Fig. 3).

Soluble carbohydrates

Data presented in Figure 4 revealed that the content of total soluble sugars (TSS) in shoots still stable up to 45 days of treatment. After 60 days of hypoxic treatment, an augmentation of TSS was observed (Fig. $4A_1$). For the underground organ, the sugar content was increased by 50% between 30 and 45 days in control plants, while it decreased at 60 days by 55%. On the other hand, in treated plants, hypoxia increased total root sugar (Fig. $4A_2$).

Fig. 4: Shoot (A) and root (B) sugar content of control (grey) and hypoxia treatment (black) of *M. truncatula* after 30, 45 and 60 days. A_1 and A_2: Total sugar; B_1 and B_2: Reducing sugar and A_3 and B_3: Non-reducing sugar. Values are the mean ± S.E. from 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 $p<0.05$ in comparison to the control.

oxygen presents an elevated content on NRS in their shoots, whereas it was decreased in the root with the treatment. However, at 45 and 60 days the NRS was decreased by 21 and 95% in comparison to the content at 30 days. In parallel to this decreases, it showed an augmentation of RS on stressed roots by 75 and 55 % at 30 days.

Proline and endopeptidase activity

Proline is uncharged amino acid, it characterize by a neutral pH and a highly water solubility. The results presented in Fig. 5A revealed that the proline content of control plant shoots is stable up to 45 days, at 60 days; we showed an augmentation by 40%. However, hypoxia induced synthesise of proline on function of time. The increase estimated by 25 and 30% respectively at 45 and 60 days in comparison to control plant.

Fig. 5: Figure 5: Shoot's proline (A) and root's proteolytic activity (B) of control (grey) and hypoxia treatment (black) of M. truncatula after 30, 45 and 60 days. Values are the mean ± S.E. from 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 $p<0.05$ in comparison to the control.
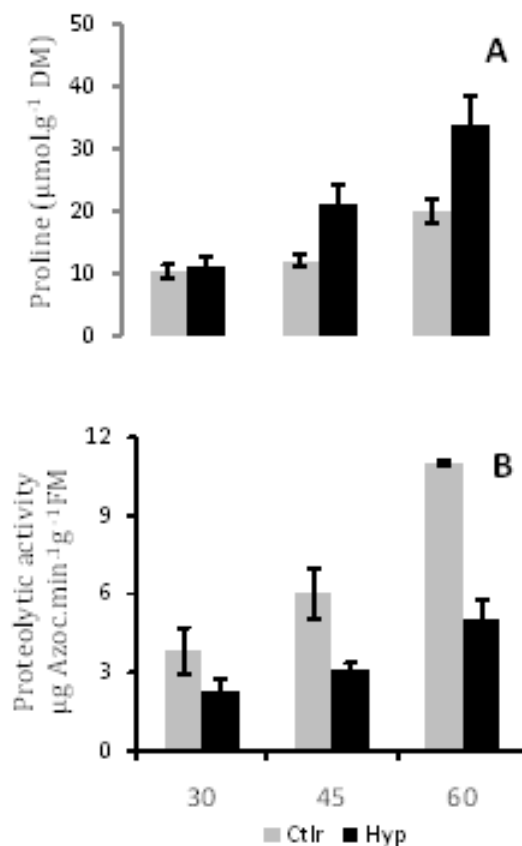

The results of two fractions of soluble sugar: reducing (RS) and non-reducing sugar (NRS) showed that the content on NRS (Fig. 4C) is more important in *M. truncatula* organs (Fig. 4B). In fact, deficient plant on

The variation of the total endopeptidase activity (Fig. 5B) in roots of control plant was increased by 37 and 45% at 45 and 60 days, in comparison to the content obtained at 30 days. On contrary, hypoxia decreased the endopeptidase activity in roots. This reduction estimated by 39, 48 and 55% at 30, 45 and 60 days in comparison to control.

Discussion

Waterlogging stress is known to cause a number of morphological and anatomical changes in plants (Sairam et al., 2008). For example, the apparition of hypertrophied lenticels on roots, the formation of adventitious roots (Malik et al., 2001), the development of aerenchyma (Evans, 2004, Abiko et al., 2012) were observed under flooding stress. In this work, the application of prolonged hypoxia treatment to *M. truncatula* caused the apparition of adventitious roots on the hypocotyle. In spite this adaptation to the hypoxia; it was noticed a diminution of the plant water content. This decrease in the water status can be the result of the reduction of the stomata conductance. This phenomenon is considered one of the first plant responses to waterlogging (Folzer et al., 2006). Indeed, plants exposed to flooding stress exhibit increased stomata resistance as well as, limited water uptake leading to internal water deficit (Parent et al., 2008). Moreover the lack of oxygen can reduce hydraulic conductivity due to hampered root permeability (Else et al., 2001). Furthermore, *M. truncatula* present decline of its hall growth. This decrease can be the result of the petrifaction on the principal root that can caused perturbation of the element uptake's (Horchani et al., 2010). In plus, the oxygen deficiency in the growth medium can cause a decline of net photosynthetic rate (Ashraf et al., 2011).

Another adaptation road of plant to survive under hypoxia is the shifting of the energy metabolism from aerobic to anaerobic mode since the energy requirements of the tissue is greatly restricted as very few ATPs are generated per molecule of glucose (Jackson and Drew, 1984). Thus, maintaining adequate levels of readily fermentable sugars under oxygen deficient in roots is one of the adaptive mechanisms to hypoxia (Xia and Saglio, 1992; Sairam et al., 2009). Moreover, waterlogging, in tolerant genotypes, induces to increase the content of reducing sugar through increased activity of sucrose synthase by increasing the level expression of its mRNA (Sairam et al., 2009). Therefore, the

availability of sufficient sugar reserves in the roots with the increased activity of TSS to provide reducing sugars for anaerobic respiration is one of the important mechanisms of waterlogging tolerance. In our results, *M. truncatula* present more non-reducing sugar than reducing form. Therefore, sucrose represents almost all of the total soluble sugars in this legume. The concentration of soluble carbohydrate in *M. truncatula* was increased during the prolonged long-term oxygen deficit, and we register an augmentation of the root and shoot content on reducing sugar on function of time (Kumutha et al., 2008). In addition the maintenance of elevated content of shoots non-reducing sugar on parallel to its spectacular diminution in root. These results are in agreement with those obtained by Germain and collaborators (1997) on tomato. Moreover, maintaining of elevated quantity of non-reducing sugar in root until 45 days car suggesting the eventual transport of sucrose from shoot to root (Yu et al., 2012).

Concentrations of free proline in the leaves of *M. truncatula* increased synchronously with time of hypoxia treatment. Proline accumulation is known to have multifunctional role on the defense mechanisms developed by plants grown under various stress (Nanjo et al., 2003). It's related also to the non-enzymatic detoxification of free radicals (superoxide, peroxide or hydroxyl) that are generated excessively under stress (Radyukina et al., 2008). It plays an important role of osmolyte for osmotic adjustment and contributes to the stabilization of cell structures, protection of membranes and proteins against reactive oxygen species (Steffens et al., 2012). The authors explain such ability of this amino acid by the presence of tertiary carbon which can form stable radical tearing off free radical reactions induced by ROS (Radyukina et al., 2008).

In roots of *M. truncatula* the proteolytic activity was increased with time but still fewer that obtained in roots of control plant. In fact, the increase of the proteolytic activity is explained by the nature and the development of tissues. Indeed, roots of control plants are much vacuolated and the endopeptidase activity quantified with the azocasein characterizes vacuole. These results are similar to those found by Aschi-Smiti and collaborators (2003) on *Trifolium subterraneum.* The lowest proteolytic activity was found in hypoxic's root can be the result of the purification and the necrosis of root under hypoxia. Since that adventitious roots ensure survive and nutrition of *M. truncatula*; this roots are physiologically young and haven't important vacuoles.

These results are consistent with those obtained on maize under dark stress by Brouquisse et al. (1998) and on *Trifolium subterraneum* grown in hypoxia by Aschi-Smiti et al. (2003).

Conclusion

M. truncatula seems supporting the prolonged hypoxia stress. Its capacity to develop adventitious roots, accumulating proline and getting an elevated content of sugar help it to achieve its biological cycle. Indeed, it is important to study the molecular and the genetic basis of this adaptation to oxygen deficiency of this model plant.

Acknowledgements

References

Abiko, T., Kotula, L., Shiono, K, Malik, A.I., Colmer, T.D., Nakazono, M., 2012. Enhanced formation of aerenchyma and induction of a barrier to radial oxygen loss in adventitious roots of *Zea nicaraguensis* contribute to its waterlogging tolerance as compared with maize (*Zea mays ssp. mays*). Plant Cell Environ. 35, 1618–1630.

Ameline-Torregrosa, C., Cazaux, M., Danesh, D., Chardon, F., Cannon, S.B., Esquerre-Tugaye, M.T., Dumas, B., Young, N.D., Samac, D.A., Huguet, T., Jacquet, C., 2008. Genetic dissection of resistance to anthracnose and powdery mildew in *Medicago truncatula.* Mol. Plant Microbe Interact. 21, 61–69.

Ané, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E.D., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kaló, P., Rosenberg, C., Roe, B.A., Long, S.R., Denarié, J., Cook, D.R., 2004. *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science. 303, 1364–1367.

Aschi-Smiti, S., Bizid, E., Hamza, M., 2003. Effet de l'hydromorphie sur la croissance de quatre variétés de trèfle (*Trifolium subterraneum* L.). Agronomie. 23, 97-104.

Ashraf, M.A., Ahmad, M.S.A., Ashraf, M., Al-Qurainy, F., Ashraf, M.Y., 2011. Alleviation of waterlogging stress in upland cotton (*Gossypium hirsutum* L.) by exogenous application of potassium in soil and as a foliar spray. Crop Pasture Sci. 62(1), 25-38.

Bacanamwo, M., Purcell, L.C., 1999. Soybean root morphological and anatomical traits associated with acclimation to flooding. Crop Sci. 39(1), 143-149.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil. 39(1), 205-207.

Brouquisse, R., Gaudillère, J.P., Raymond, P., 1998. Induction of a carbon-starvation-related proteolysis in whole maize plants submitted to light/dark cycles and to extended darkness. Plant Physiol. 117, 1281–1291.

de Faria, S.M., Lewis, G.P., Sprent, J.I., Sutherland, J.M., 1989. Occurrence of modulation in the Leguminosae. New Phyto. 111, 607–619.

Else, M.A., Coupland, D., Dutton, L., Jackson, M.B., 2001. Decreased root hydraulic conductivity reduces leaf water potential, initiates stomatal closure and slows leaf expansion in flooded plants of castor oil (*Riccinus communis*) despite diminished delivery of ABA from the roots to shoots in the xylem sap. Physiol. Plant. 111, 46-54.

Evans, D.E., 2004. Aerenchyma formation. New Phytol. 161, 35-49.

Folzer, H., Dat, J., Capelli, N., Rieffel, D., Badot, P.M., 2006. Response to flooding of sessile oak: An integrative study. Tree Physiol. 26, 759-766.

Germain, V., Raymond, P., Ricard, B., 1997. Differential expression of two tomato lactate dehydrogenase genes in response to oxygen deficit. Plant Mol. Biol. 35, 949-954.

Graham, P.H., Vance, C.P., 2003. Legumes: Importance and constraints to greater use. Plant Physiol. 131, 872–877.

Horchani, F., Hajri, R., Khayati, H., Aschi-Smiti, S., 2010. Physiological responses of tomato plant to the combined effect of root hypoxia and salinity. J. Phytol. 2(11), 36–46.

Ibraheem, O., Dealtry, G., Roux, S., Bradley, G., 2011. The effect of drought and salinity on the expressional levels of sucrose transporters in rice (*Oryza sativa* Nipponbare) cultivar plants. Plant Omics J. 4(2), 68-74.

Iqbal, M., Amjad Ali, M., Abbas, A., Zulkiffal, M., Zeeshan, M., Sadaqat, H.A., 2009. Genetic behavior and impact of various quantitative traits on oil contents in sunflower under waters stress conditions at productive phase. Plant Omics J. 2(2), 70-77.

Jackson, M.B., Drew, M.C., 1984. Effect of flooding on growth and metabolism of herbaceous plant. In: Flooding and Plant Growth (Ed.: Kozlowski, T.T.). Academic Press, Orlando. pp.47-128.

Kumutha, D., Sairam, R.K., Meena, R.C., 2008. Role of root carbohydrate reserves and their mobilization in imparting waterlogging tolerance in green gram (*Vigna radiata* (L.) Wilczek) genotypes. Ind. J. Plant Physiol. 13(4), 339–346.

Linkemer, G., Board, J.E., Musgrave, M.E., 1998. Waterlogging effects on growth and yield components in late-planted soybean. Crop Sci. 38(6), 1576-1584.

Malik, A.I., Colmer, T.D., Lamber, H., Schortemeyer, M., 2001. Changes in physiological and morphological traits of roots and shoots of wheat in response to different depths of waterlogging. Aust. J. Plant. Physiol. 28, 1121-1131.

Mommer, L., Pederson, O., Visser, E.J.W., 2004. Acclimation of a terrestrial plant to submergence facilitates gas exchange under water. Plant Cell Environ. 27, 1281-1287.

Nanjo, D., Paleg, L.G., Wiskich, J.T., 2003. Effect of praline, betaine and some other solutes on the heat stability of mitochondrial enzymes. Aust. J. Plant Physiol. 9, 47-57.

Narasimhamoorthy, B., Bouton, J.H., Olsen, K.M., Sledge, M.K., 2007. Quantitative trait loci and candidate gene mapping of aluminum tolerance in diploid alfalfa. Theor. Appl. Genet. 114, 901–913.

Parent, C., Berger, A., Folzer, H., Dat, J., Crevecoeur, M., Badot, P.M., Capelli, N., 2008. A novel nonsymbiotic hemoglobin from oak: Cellular and tissue specificity of gene expression. New Phytol. 177, 142-154.

Poot, P., Lambers, H., 2003. Growth responses to waterlogging and drainage of woody *Hakeae* (Proteaceae) seedlings, originating from contrasting habitats in South-western Australia. Plant Soil 253, 57–70.

Promkhambut, A., Polthanee, A., Akkasaeng, C., Younger, A., 2011. Growth, yield and aerenchyma formation of sweet and multipurpose sorghum (*Sorghum bicolor* L. Moench) as affected by flooding at different growth stages. Aust. J. Crop Sci. 5(8), 954-965.

Radyukina, N.L., Shashukova, A.V., Shevyakova, N.I., Kuznetsov, V.V., 2008. Proline involvement in the common sage antioxidant system in the presence of NaCl and paraquat. Russian J. Plant Physiol. 55, 649-656.

Saglio, P.H., Pradet, A., 1980. Soluble sugars, respiration and energy charge during aging of excised maize root tips. Plant Physiol. 66, 516-519.

Sairam, R.K., Kumutha, D., Chinnusamy, V., Meena, R.C., 2009. Waterlogging- induced increase in sugar mobilization, fermentation and related gene expression in the roots of mung bean (*Vigna radiata*). J. Plant Physiol. 166, 602-616.

Sairam, R.K., Kumutha, D., Ezhilmathi, K., Deshmukh, P.S., Srivastava, G.C., 2008. Physiology and biochemistry of waterlogging tolerance in plants. Biol. Plant. 52(3), 401–412.

Scott, P., Pregelj, L., Chen, N., Hadler, J., Djordjevic, M., Gresshoff, P., 2008: *Pongamia pinnata*: An untapped resource for the biofuels industry of the future. Bio. Energy Res. 1, 2–11.

Steffens, B., Kovalev, A., Gorb, S.N., Sauter, M., 2012. Emerging roots alter epidermal cell fate through mechanical and reactive oxygen species signaling. Plant Cell. 24, 3296–3306.

Vartapetian, B.B., Jackson, M.B., 1997. Plant adaptations to anaerobic stress. Ann. Bot.79, 3–20.

Vignolio, O., Fernandez, O., Maceira, N., 1999. Flooding tolerance in five populations of *Lotus glaber* Mill. (*Syn. Lotus tenuis* Waldst. Et. Kit.). Aust. J. Agric. Res. 50, 555–559.

Voesenek, L.A.C.J., Bailey-Serres, J., 2013. Flooding tolerance: O_2 sensing and survival strategies. Curr. Opin. Plant Biol. 16(5), 647–653.

Xia, J.H., Saglio, P.H., 1992. Lactic acid efflux as a mechanism of hypoxic acclimation of maize root tips to anoxia. Plant Physiol. 100, 40-46.

Young, N.D., Cannon, S.B., Sato, S., Kim, D., Cook, D.R., Town, C.D., Roe, B.A., Tabata, S., 2005. Sequencing the gene spaces of *Medicago truncatula* and *Lotus japonicus*. Plant Physiol. 137, 1174–1181.

Yu, X., Luo, N., Yan, J., Tang, J., Liu, S., Jiang, Y., 2012. Differential growth response and carbohydrate metabolism of global collection of perennial ryegrass accessions to submergence and recovery following de-submergence. J. Plant Physiol. 169, 1040–1049.