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NON-SYMBIOTIC HEMOGLOBIN AND NITRATE REDUCTASE CONSTITUTE AN ALTERNATIVE TO FERMENTATION IN WATERLOGGING TOLERANCE OF MUNG BEAN [*VIGNA RADIATA* (L.) WILCZEK]

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SUMMARY

The objective of this study was to examine the role of nitrate reductase, nitric oxide and non-symbiotic hemoglobin in imparting waterlogging tolerance in mung bean genotypes. Experiment was conducted with five cultivated mung bean [*Vigna radiata* (L.) Wilczek] genotypes viz., T 44, MH 96-1 (relatively tolerant), and Pusa Baisakhi, MH 1K-24 and PS 10 (susceptible) under pot-culture condition. Waterlogging induced reduction in relative water content, chlorophyll content and membrane stability index was comparatively less in tolerant genotypes T 44, MH 96-1 than in Pusa Baisakhi, MH 1K-24 and PS 10. The nitric oxide (NO) production activity increased up to 6 days of waterlogging treatment in all the genotypes, however T 44, MH 96-1 maintained more than double NO content than Pusa Baisakhi, MH 1K-24 and PS 10. Increase in nitrate reductase (NR) activity under waterlogging was observed up to 6-days of treatment in all the genotypes, and T 44, MH 96-1 maintained significantly higher NR activity than Pusa Baisakhi, MH 1K-24 and PS 10. *Non-symbiotic hemoglobin* (*NSHb*) and *cNR* mRNA expressions were studied only in the roots of control and waterlogging treated plants of T 44, MH 96-1, and PS 10. Waterlogging induced increase in expression was observed only in tolerant genotypes T 44 and MH 96-1, while little expression was observed in PS 10. In this study we have reported the involvement of non-symbiotic hemoglobin-nitric oxide homeostasis in the waterlogging tolerance of mung bean.

Key words: **Nitric oxide, non-symbiotic-hemoglobin, *Vigna radiata*, waterlogging**

***Abbreviations*:** cNR - cytosolic nitrate reductase; DTT - dithiothreitol; EDTA - ethylene diamine tetra-acetic acid (di-sodium salt); GC – guanine-cytosine; HEPES - N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid; Ni-NOR – nitrite-nitric oxide reductase; NO - nitric oxide; NSHb - non-symbiotic hemoglobin; PB – Pusa Baisakhi; RT-PCR - reverse transcriptase polymerase chain reaction; Tm – melting temperature

INTRODUCTION

Waterlogging results in complete blockage of air (O_2) supply to the roots by clogging the soil air spaces, leading to hypoxic and subsequently anoxic conditions, and thus inhibiting root respiration. Under limited O_2 supply mitochondrial electron transport chain becomes inhibited and the intracellular level of ATP decreases as fermentation yields only 2 mol of ATP per mol of glucose. One of the major problems faced by plants/tissues

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experiencing hypoxia/anoxia is an increase in NAD(P)H/ NAD(P) ratio, which adversely affects the glycolytic pathway, the only pathway which provides energy under anaerobiosis. To ameliorate this situation, plants use fermentative pathway employing alcohol dehydrogenase and lactic dehydrogenase for recycling NAD(P)H to NAD(P).

Another important metabolic process, which may recycle NAD(P)H to NAD(P) is hypoxia induced nitrate reductase leading to formation of nitric oxide (NO). In this context role of non symbiotic hemoglobin and NO has also been highlighted in many plant species (Anderson *et al.* 1996). Constitutive expression of barley class 1 non-symbiotic hemoglobin in wild-type and transformed maize cells lines has been reported to maintain cell adenine nucleotide levels and energy charge under hypoxic conditions, whereas wild-type cells and cells in which hemoglobin expression is suppressed had lowered adenine nucleotide levels and energy charge (Sowa *et al.* 1998). Transformed alfalfa root cultures lines constitutively expressing barley hemoglobin maintained root growth during hypoxic treatment, whereas wild-type and lines with suppressed stress induced hemoglobin expression had slower root growth (Dordas *et al.* 2004). NO is highly reactive and toxic to cells, and its reaction with hemoglobin is considered to be a major route for detoxification (Wennmalm *et al.* 1992). NO reacts rapidly with oxyhemoglobin forming nitrate and methemoglobin [Hb(Fe^{+3})]. This route for metabolism of NO, with nitrate being recycled, would be advantageous to the hypoxic plant cell, exposed to conditions of prolonged soil water logging, where nitrates would be severely depleted. Methemoglobin [Hb(Fe^{+3})] can be reduced to Hb (Fe^{2+}) via NADH- dependent reductases (Poole 1994) and this reaction would provide an additional NAD^+ for glycolysis.

Corpas *et al.* (2004) have reported at least nine routes for the synthesis of NO in plants. One of the mechanism is through the involvement of an enzyme variously mentioned as NO synthase (Barroso *et al.* 1999), nitric oxide like synthase enzyme (Kim *et al.* 2006) or NO associated protein1 (Gas *et al.* 2009). Stöhr *et al.* (2001) suggested the involvement of a plasma membrane bound nitrite-nitric oxide reductase (Ni-NOR) as well as cytosolic-nitrate reductase (cNR) (Yamasaki *et al.* 1999). Nitrate reductase is activated upon exposure of plant roots to hypoxia and nitrate (Botrel and Kaiser 1997), and it consumes 2 mol of NADH, without oxygen consumption, per mol of NO produced. NO produced in these reactions does not accumulate due to hypoxia induced non-symbiotic hemoglobins, which are reported to remain in the oxyhemoglobin form, even at oxygen concentration two times lower than necessary to saturate cytochrome-C oxidase. They act as NO dioxygenases converting NO back to nitrate, consuming NAD(P)H in the process (Igamberdiev *et al.* 2005).

Comprehensive studies on physiological and biochemical aspects of water logging tolerance have been done in cereal crops like maize, rice and some aquatic grasses and weeds. However, little work has been done in pulses, and more specifically on the significance of alternate routes for the regeneration of NAD. We have earlier reported that under waterlogging stress comparatively tolerant genotypes *Vigna luteola* and T 44 were able to retain greater contents of total-, reducing- and non-reducing-sugars and activity and gene expression of sucrose synthase and alcohol dehydrogenase than the susceptible genotype Pusa Baisakhi (Sairam *et al.* 2009). Mung bean, being a rainy season pulse crop, experiences large scale waterlogging during the early vegetative phase of its growth. The present investigation, therefore, has been planned to validate the role of non-symbiotic hemoglobin-NO interaction, and the underlying molecular mechanism in imparting hypoxia tolerance by using two tolerant and three susceptible mung bean genotypes at early vegetative stage.

MATERIALS AND METHODS

Plant material and growth conditions: The experiment was conducted with five cultivated mung bean [*Vigna radiata* (L.) Wilczek] genotypes, viz., T 44, MH 96-1 (tolerant) and Pusa Baisakhi, MH 1 K-24 and PS 10 (susceptible). Plant material was procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi and Indian Institute of Pulse Research, Kanpur. Sowing was done in 30 x 30 cm (height x diameter) earthen pots filled with clay-loam soil and farm yard manure in 3:1 ratio during the summer-rainy season. Pots were supplied with basal dose of 25, 60 and 60 kg ha^{-1} of N, P and K, respectively. Before sowing, seeds were treated with the required *Rhizobium* culture. Four plants

were sown in each pot, which were thinned to 2 plants per pot after 20 days. Waterlogging treatment was given by placing pots with 25 days old plants in plastic troughs measuring 100 x 70 x 35 cm (length x breadth x height), and filled with water to a height just 1-2 cm above the soil level in the pots. Treatments consisted of control, 2, 4, 6 and 8 days of waterlogging, and recovery after 4 days of termination of treatment. Because 8-days waterlogged plants of susceptible genotypes Pusa Baisakhi, MH 1 K-24 and PS 10 showed more than 75 % mortality during recovery, therefore, recovery was uniformly studied in all the genotypes for 6 days waterlogged plants only. NO production activity and *in-vitro* NR activity were assayed in 8 replicates (n = 8). The design of the experiment was complete randomized (CRD) and data was analyzed by factorial CRD.

Physiological parameters: Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant weight is achieved (Weatherley 1950).

RWC = [(Fresh wt. – Dry wt.) / (Turgid wt. – Dry wt.)] X 100

Membrane stability index (MSI) was estimated as per Sairam *et al.* (1997). For estimation of membrane stability index 100 mg leaf material, in two sets, is taken in test tubes containing 10 ml of double distilled water. One set is heated at 40°C for 30 min in a metabolic water bath, and the electrical conductivity of the solution is recorded on a conductivity bridge (C_1). Second set is boiled at 100°C on a boiling water bath for 10 min, and its conductivity is measured on a conductivity bridge (C_2). Membrane stability index (MSI) is calculated as:

$$MSI = [1 - (C_1/C_2)] \times 100$$

Chlorophyll content was estimated by extracting 0.05 g of the leaf material in 10 ml dimethylsulfoxide (DMSO) (Hiscox and Israelstam 1979). Samples were heated in an incubator at 65°C for 4 h and than after cooling to room temperature, the absorbance of extracts were recorded at 663 and 645. Chlorophyll content was calculated as per Arnon (1949).

Chl a: [12.7 x A_{663} – 2.69 x A_{645}]; Chl b: [22.9 x A_{645} – 4.68 x A_{663}]

The values thus obtained were in µg/ml of extract (solvent). Values in mg/g fresh weight were obtained by multiplying the above values with "V/W x 1000", where V is volume of extract; W is fresh weight of sample.

In-vitro nitrate reductase activity assay: For the *in-vitro* nitrate reductase activity assay the procedure of Hageman and Flesher (1960) was followed with little modification. Enzyme extraction was done by pulverizing weighed amount of the root material in liquid nitrogen in a pre-chilled mortar-pestle, followed by grinding with 100 mM phosphate buffer (pH 7.5), 4 mM cysteine, 5 mM EDTA and 3% bovine serum albumin. Homogenate was centrifuged in a Sigma refrigerated centrifuge (model 3K 30, Osterode, Germany) at 4°C for 20 min at 12,000g. Supernatant was used as the source of enzyme. Assay mixture consisted of 0.5 ml enzyme extract, 0.2 mM phosphate buffer (pH 7.5), 50 mM potassium nitrate, 0.68 µM NADH. Incubation was done at 30°C. Reaction was stopped by adding 0.1 ml zinc acetate (1.0 M) solution and 1.9 cm^3 90% ethanol. In blank 0.1 ml zinc acetate (1.0 M) solution and 1.9 ml 90% ethanol were added prior to the addition of enzyme. The contents were centrifuged at 5,000 g for 10 min to remove the precipitate. Supernatant was used for colour development with 1 ml each of sulfanilamide (1% in 1N HCl) and naphthylethylene diamino dihydrochloride (0.02% aqueous solution). After 15 minutes absorbance was recorded spectrophotometrically at 540 nm wavelength against blank.

Nitric oxide production assay: NO production assay was done by a semi-quantitative method based on the nitrite-nitric oxide reductase assay given by Stohr *et al.* (2001) and Moller and Palmer (1982). The assay is based on the reduction of NO_2^- to NO by plasma membrane fraction, possibly caused by the enzyme nitrite-nitric oxide reductase. For preparation of crude membrane fraction, fresh leaf tissue was cut in to pieces and ground in pre-chilled mortar and pestle with 5 times its weight in ice cold extraction buffer consisting of 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 3 mm Na_2EDTA, 10 mM ascorbic acid and 5 mM diethyl dithiocarbamate. Homogenate was filtered through 4 layers of nylon/cheese cloth and centrifuged at 10,000g for 10 min at 4°C. For the isolation of crude membrane fraction the supernatant was twice

centrifuged at 25,000g for 20 min at 4°C. The pellet was suspended in suspension buffer made up of 5 mM K-phosphate buffer, pH 7.8, 250 mM sucrose and 3 mM KCl. The crude membranes extract was stored at -70°C. For NO assay 100 µg protein was incubated at 30°C in 0.45 ml of 0.1 M Hepes – KOH (pH 6.0). The reaction was started by addition of $NaNO_2$ (1mM) and reduced cytochrome c (0.3 mg ml^{-1}) reduced with ascorbate (40 mM) to give a final volume of 0.5 ml. The assay mixture was flushed with nitrogen before adding protein, and incubated by shaking at 30°C for 3 h in glass vials with screw tops. For NO detection, pre- cut filters (3 mm, Whatman No. 10) were soaked in a solution of 5% sulfanilamide and 0.2 % N- (1-naphthyl) ethylene-diamine dihydrochloride in methanol (Nims *et al.* 1996), air-dried and used immediately for the assay. The filters were placed into the screw tops of the vials so that only gaseous NO had access and interference by NO_2^- was avoided. After incubation, each filter was extracted with 2 ml methanol for 30 min. at room temperature and the absorbance of the solution was determined at 463 nm against methanol. The genotypic variation was also on expected lines. Protein estimation was done as per the Bradford (1976).

Gene Expression by RT-PCR: For gene induction studies 25 days old plants of T 44, MH 96-1 and PS 10 were subjected to waterlogging for 24 h, and root samples were harvested from control and treated plants. Total RNA from root tissue was extracted using Trizol reagent (GibcoBRL), as per the recommendations of manufacturer. DNA contamination was removed from the RNA samples using DNase I (Qiagen Science, Maryland, USA). One microgram of total RNA was reverse transcribed using gene specific degenerate primers and Qiagen one step RT-PCR kit. PCR conditions were standardized using gene-specific primers for tubulin. Linear amplification for semi-quantitative RT-PCR was obtained with 35 cycles. Reactions were conducted using My Genie 32 Thermal Block PCR (Bioneer, Korea) under the following conditions: initial PCR activation step: 15 min at 95°C, reverse transcription: 30 min at 50°C, denaturation: 1 min at 94°C, annealing: 1 min at 60°C, extension: 1 min at 72°C, final extension: 10 min at 72°C. The amplification products were electrophoresed on 1.2% agarose gel at 120 V in TBE buffer (0.4M Tris – borate, 0.001 M EDTA, pH 8.0) using known concentration DNA ladders. Gels were stained with ethidium bromide and visualized on Uvi Pro Gel Documentation system (Uvitec, England).

Nucleotide sequences for *class 1 non-symbiotic hemoglobin* and *cNR* genes were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The Basic Local Alignment Search Tool (Altschul *et al.* 1997; http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify the homologs of candidate genes. For RT-PCR expression analysis and cloning of cDNAs, the following oligonucleotide primers were designed manually, and oligo quality (to avoid primer dimmer, self dimer etc.), Guanine-cytosine (GC) % and melting temperature (Tm) were analyzed by using Oligoanalyzer 3.0 tool (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/, Integrated DNA Technologies, Coralville, IA 52241, USA). Primers for various genes were designed using the data base; *non-symbiotic-Hb - Arabidopsis thaliana* (Trevaskis *et al.* 1997), *Medicago sativa* (Seregelyes *et al.* 2000) and *Glycine max* (Anderson *et al.* 1996), cNR - *Glycine max* (Wu *et al.* 1995) and tubulin - *Vigna radiata* (Chen *et al.* 2003). Sequences for various genes are as follows:

Primer Name	Sequence	GC content (%)	Tm* (°C)	Expected amplicon size (bp)
cNR –F	TGAACATCACCACGAGAGGT	45.8	60.4	486
cNR –R	CAAGTGTGCCATCCATGTTC	52.8	60.4	
NSHb-F	GTTTCWCKGAAGARCAAGAAGCTC	41.6	60.3	428
NSHb-R	GGCAYYGACCARCTGATCATAAGC	45.8	57.8	
Tubulin-F	CTTGACTGCATCTGCTATGTTCAG	45.8	55.5	422
Tubulin-R	CCAGCTAATGCTCGGCATACTG	54.5	58.4	

*Oligo concentration 1.0 µM, Na^+ concentration 50 mM.

RESULTS

Physiological parameters: Waterlogging of 25 d old plants results in decrease in relative water content (RWC), membrane stability index (MSI) and chlorophyll (Chl) content in all the 5 genotypes (Fig. 1A,B,C). The magnitude of decline increased with the increase in duration of waterlogging. Minimum decline in RWC under waterlogging was observed in T 44 and MH 96-1, even on the 8th day of treatment, while highest per cent decline in RWC was observed in Pusa Baisakhi, PS 10 and MH 1K-24. Recovery recorded 4 days after termination of treatment was also highest in T 44 and MH 96-1, while Pusa Baisakhi, MH 1K-24, PS 10 should less recovery.

MSI and Chl content also showed similar trend. The waterlogging induced decline in MSI and Chl content was highest in Pusa Baisakhi, PS 10 and MH 1K-24. Highest MSI and Chl content as well as least decline under waterlogging condition was observed in T 44 and MH 96-1. Recovery for MSI and Chl content after termination of waterlogging was also highest in T 44 and MH 96-1 and minimum in case of Pusa Baisakhi, PS 10 and MH 1K-24.

Biochemical estimations: Shifting of plants to waterlogging condition increased the NO production activity up to 6 days in the roots of all the genotypes and decline was observed on the 8th day of waterlogging (Fig, 2A). Water logging induced increase in NO production activity was more than 100 % higher in T 44 and MH 96-1 than Pusa Baisakhi, PS 10 and MH 1K-24. Even on 8th day T 44 and MH 96-1 maintained significantly higher NO production activity. Recovery recorded after 4 day of release of stress showed uniform decline in NO production activity in all the five genotypes,

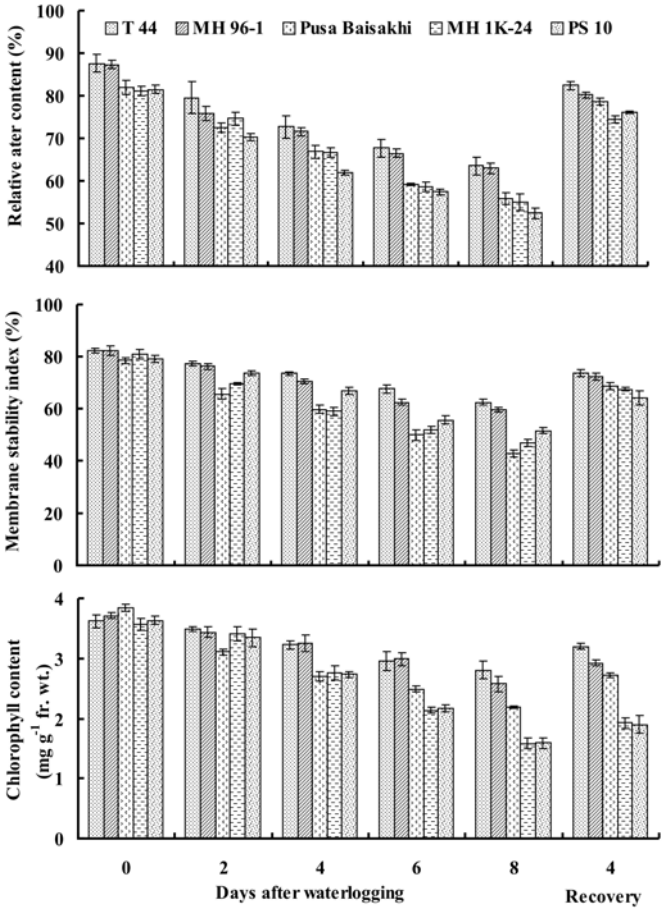

Fig. 1. Effect of waterlogging on relative water content (A), membrane stability index (B) and chlorophyll content (C) in mung bean genotypes. Vertical bars shows ± SE of mean.

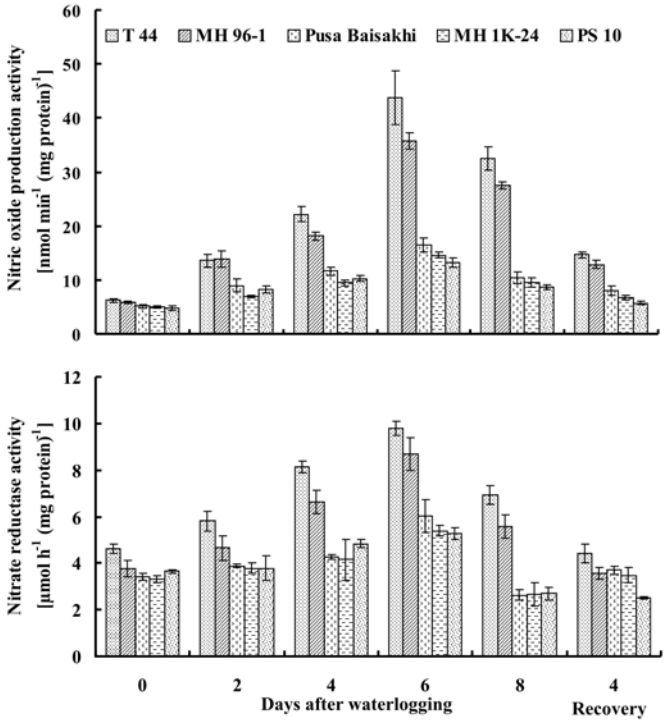

Fig. 2. Effect of waterlogging on nitric oxide production activity (A) and, nitrate reductase activity (B) in mung bean genotypes. Vertical bars shows ± SE of mean.

though T 44 and MH 96-1 still maintained higher NO production activity.

In-vitro nitrate reductase (NR) activity in the roots increased under waterlogging stress in all the genotypes (Fig. 2B). NR activity increased up to 6th days of waterlogging in all the genotypes. Significantly higher NR activity was observed in T 44 and MH 96-1, compared to susceptible genotypes Pusa Baisakhi, PS 10 and MH 1K-24. Under all the waterlogging treatments highest NR activity was observed in by T 44 and MH 96-1. Eight days of waterlogging caused decline in NR activity in all the genotypes. Recovery of 6 days waterlogged plants showed further decrease in activity in all the genotypes.

Gene expression: The results of RT-PCR analysis of *cNR* gene yielded an amplicon of about 480 bp in all the three genotypes (Fig. 3A). *cNR* mRNA expression was greater in waterlogged roots of T 44 and MH 96-1, while little expression was observed in waterlogged plants of PS 10.

In the case of *NSHb* gene expression, amplicons of size of 430 bp were observed in the waterlogging treated plants of all the genotypes. Significantly higher *NSHb* expression was observed in T 44 and MH 96-1 (Fig. 3B). Very slight expression was observed in control plants of all the three genotypes and waterlogged roots of PS 10.

The tubulin expression was almost constant in all the genotypes, and did not change under control and waterlogging conditions (Fig. 3C).

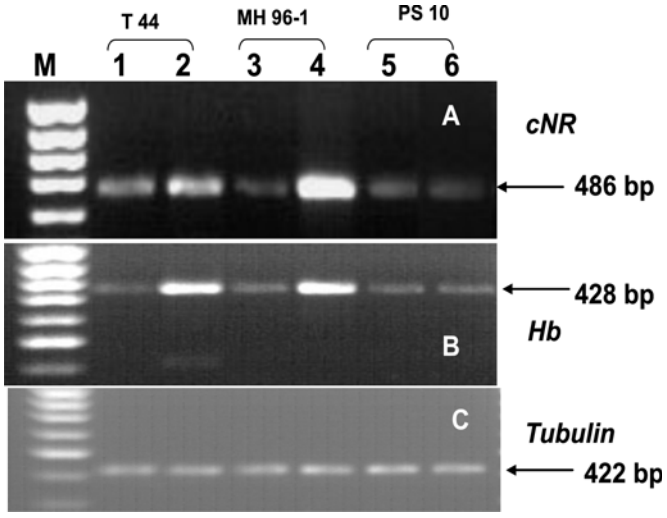

Fig. 3. RT-PCR expression analysis of cytosolic *nitrate reductase* (*cNR)* (A), *non-symbiotic hemoglobin (NS-Hb)* (B) and tubulin (C) genes under waterlogging stress and control conditions in roots of mung bean (M - 1 Kb ladder, 1 - Control T 44, 2 -Treated T 44, 3 - Control MH 96-1, 4 - Treated MH 96-1, 5 - Control PS 10, 6 - Treated PS 10).

DISCUSSION

As a result of 8 d of waterlogging mung bean (*V. radiata*) genotypes exhibited loss in RWC, MSI and Chl content. The decline in RWC, MSI and Chl was less in tolerant genotypes T 44 and MH 96-1 than in susceptible genotypes Pusa Baisakhi, MH 1K-24 and PS 10. Min and Bartholomew (2005) reported decrease in RWC during flooding, which further declined with the duration of flooding stress. Various workers have also reported waterlogging induced reduction in leaf water potential (Naidoo 1983, Else *et al.* 1995). Wilting under excess water stress is due to the higher resistance to mass flow of water through the roots (Jackson and Drew 1984). Membrane disintegration is one of the consequences of oxygen deprivation (Rawyler *et al.* 2002); resulting in more than 40 times increase in solute leakage from 4 d waterlogged pea plants (Jackson *et al.* 1982). Decrease in chlorophyll content under waterlogging has also been reported in wheat (Huang *et al.* 1994, Collaku and Harrison 2002), maize (Younis *et al.* 2003, Prasad *et al.* 2004) and *V. sinensis* (Younis *et al.* 2003).

One of the problems faced by plants/tissues experiencing hypoxia/anoxia is an increase in NAD(P)H/ NAD(P) ratio, which adversely affects the glycolytic pathway, the only pathway which provides energy under anaerobiosis. To ameliorate this situation, plants use fermentative pathway employing lactic dehydrogenase and alcohol dehydrogenase for recycling of NAD(P)H to NAD(P). Earlier we have reported that roots of tolerant genotypes of pigeon pea (*Cajanus cajan*) and mung bean have higher contents of total-, reducing- and non-reducing sugars; and greater activity and level of genes expression of sucrose synthase and alcohol dehydrogenase (Kumutha *et al.* 2008, Sairam *et al.* 2009). However, glycolysis, followed by fermentation will most possibly lead to accumulation of toxic lactic

acid, ethanol and CO_2 in the root tissues. Therefore for long term survival of hypoxia a tolerant species/genotype must have some alternative to fermentation. Involvement of hypoxia induced non-symbiotic hemoglobin and nitric oxide (NO) have been suggested as possible route for recycling of NADH to NAD in waterlogged plants (Ohwaki *et al.* 2005).

Waterlogging induced increase in both NO production activity as well as NR activity was highest in mung bean genotypes T 44 and MH 96-1as compared to Pusa Baisakhi, MH 1K-24 and PS 10. Dordas *et al.* (2003a) reported NO production by alfalfa root culture under hypoxia stress and suggested that it has role in waterlogging tolerance of plants, a mechanism, which also involve hypoxia induced non-symbiotic hemoglobin (Dordas *et al.* 2003b).

In roots, two distinct types of NR have been reported, one located in the cytosol (cNR) and the other attached to the plasma membrane and facing the apoplast (PM-NR) (Stöhr and Ullrich 1997, Stöhr and Mäck 2001). A 2.5-fold activation of cNR during exposure of plant roots to hypoxia have been reported by Botrel and Kaiser (1997), with nitrite reduction being suppressed at the nitrite reductase step (Botrel *et al.* 1996). It has been suggested that a side-reaction of cNR is the reduction of nitrite to NO with NADH as an electron donor, probably catalyzed by the same molybdenum cofactor-containing domain as in nitrate reduction (Yamasaki *et al.* 1999). Our results revealed a parallel increase in NR activity and NO production activity (Stöhr *et al.* 2001) under various treatments and genotypes. Thus it seems plausible that NR may be involved in NO synthesis under hypoxic conditions, particularly in T 44 and MH 96-1.

Waterlogging induced *cNR*-mRNA expression was greater in the roots of MH 96-1, followed by T 44, while little expression was observed in susceptible genotype PS 10. This correlates with the *in-vitro* activity of NR in root tissue. Hoff *et al.* (1991) has also reported a root specific *NR* gene from *Phaseolus vulgaris*. In the present study, the gene expression of *non-symbiotic hemoglobin* (*NS-Hb*) was observed only in the waterlogged roots of T 44 and MH 96-1, whereas very little expression was observed in the control plants of all the three genotypes as well as in waterlogged roots of PS 10. Watts *et al.* (2001) cloned *Arabidopsis NS-Hb* cDNA under stress induction. Stress-induced hemoglobins have also been implicated in regeneration of NAD^+ during hypoxia (Hill 1998) based on the observations that alcohol dehydrogenase activity and CO_2 production is reduced under hypoxia in maize cells constitutively expressing barley hemoglobin (Sowa *et al.* 1998). Similarly transformed alfalfa root culture lines constitutively expressing barley hemoglobin maintained root growth during hypoxic treatment, whereas wild-type and lines with suppressed stress-induced hemoglobin expression had slower root growth (Dordas *et al.* 2003a; 2004).

Regeneration of nitrate is essential under nitrate limiting conditions of anaerobic roots for the continuation of NSHb-NO cycle. It has been suggested that oxyhemoglobin ($HbFe^{2+}$-O_2) can donate negatively charged dioxygen to NO forming NO_3^- and methemoglobin, a known reaction of oxyhemoglobin (Di Iorio 1981). The reduction of methemoglobin to hemoglobin can occur by a methemoglobin reductase demonstrated in the nodules of leguminous plants (Topunov *et al.* 1980), or by endoplasmic reticulum cytochrome b_5 reductase (Hagler *et al.* 1979) or dihydrolipoamide dehydrogenase (Igamberdiev and Hill, 2004).

Oxygen deficiency induced class 1 non-symbiotic hemoglobin and similarly induced NO, via cNR and/or some other mechanism, may serve as an alternative to fermentation pathway by facilitating consumption of reducing potential and regeneration of NAD, essential for the continuance of glycolytic pathway, the only energy generating pathway during the oxygen deficiency. Both cNR and NO production activity require reducing potentials in the form of NADH or other reduced intermediates, which may further require NADH for their reduction, for the synthesis of NO_2^- and NO. Further reduction of methemoglobin ($HbFe^{3+}$) to hemoglobin ($HbFe^{2+}$), which is essential for the formation of oxy-hemoglobin complex and regeneration of NO_3^- from NO, requires reducing potential in the form of NADH. It is, thus evident that Hb-NO interaction may

acts as an alternative pathway for oxidation of NADH to NAD, and consequently of fermentation pathway, thus lowering the possibility of accumulation of toxic CO_2, ethanol and lactic acid, which are otherwise produced during fermentation pathways, and thus could be one of the physiological mechanism providing tolerance to mung bean genotypes T 44 and MH 96-1 under waterlogging stress.

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