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BRIEF COMMUNICATION

NADPH oxidase as the source of ROS produced under waterlogging in roots of mung bean

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Abstract

The objective of this study was to examine the role of NADPH oxidase on superoxide radical production under waterlogging in mung bean (*Vigna radiata*) cvs. T 44 (tolerant) and Pusa Baisakhi (PB) (susceptible), and wild species *Vigna luteola.* Two days of waterlogging caused decline in superoxide radical ($O_2^{\bullet-}$) contents in all the genotypes, however, further waterlogging up to 8 d caused significant increase in $O_2^{\bullet-}$ contents. In control and revived plants $O_2^{\bullet-}$ contents were higher in PB, while under waterlogging stress T 44 and *V. luteola* showed greater increases in the $O_2^{\bullet-}$ contents. During waterlogging the increase in $O_2^{\bullet-}$ content was found to be due to the diphenylene iodonium chloride-sensitive NADPH oxidase (NOX). This was further confirmed by the waterlogging induced increase in NOX activity, which was higher in tolerant genotypes T 44 and *V. luteola* compared with PB. Gene expression studies showed enhanced expression of *NOX* in the roots of waterlogged *V. luteola* and T 44, while little expression was observed in control or treated plants of PB. PCR band products were cloned and sequenced, and partial cDNAs of *NOX* was obtained. Results suggest that increase in $O_2^{\bullet-}$ content during waterlogging could be due to the induction of membrane linked *NOX*.

Additional key words: anoxia, gene expression, reactive oxygen species, *Vigna luteola*, *Vigna radiata*.

Lack of oxygen or anoxia is a common environmental challenge, which plants have to face throughout their life. Seed imbibitions, flood irrigation, floods and excess of rainfall are examples of natural conditions leading to root hypoxia or anoxia. A decrease in adenylate energy charge (ATP/ATP+ADP+AMP), cytoplasmic acidification, anaerobic fermentation, elevation in cytosolic Ca^{2+} content, changes in the redox state [NAD(P)H/NAD(P)] and a decrease in the membrane barrier function, are the main features caused by lack of oxygen (Crawford and Braendle 1996, Drew 1997, Tadege *et al.* 1999, Sairam *et al.* 2008). Regulation of anoxic/hypoxic metabolism is complex and not all the features are well elucidated.

Excessive generation of reactive oxygen species (ROS), or oxidative stress, is an integral part of many stress situations, including hypoxia. Hydrogen peroxide accumulation under hypoxic conditions has been shown in the roots and leaves of *Hordeum vulgare* (Kalashnikov *et al.*1994) and in wheat roots (Biemelt *et al.* 2000). The presence of H_2O_2 in the apoplast and in association with the plasma membrane has been visualized by transmission electron microscopy under hypoxic conditions (Blokhina *et al.* 2001). Indirect evidence of ROS formation such as lipid peroxidation under low oxygen content has also been reported (Chirkova *et al.* 1998, Blokhina *et al.*1999). Earlier we have reported waterlogging induced superoxide radical and H_2O_2 production (Kumutha *et al.* 2009) and *NADPH oxidase* (*NOX*) gene expression (Sairam *et al.* 2009a) in pigeon pea. In this communication we further confirm that hypoxia

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Abbreviations: DEDC - diethyldithiocarbamate; DPI - diphenyleneiodonium chloride; NOX - NADPH oxidase; PB - Pusa Baisakhi; ROS - reactive oxygen species.
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induced ROS production is mediated by induction of *NOX* gene and synthesis of membrane linked NADPH oxidase in case of mung bean.

An experiment was conducted with two cultivated mung bean [*Vigna radiata* (L.) Wilczek] cvs. T 44 (tolerant) and Pusa Baisakhi (PB; susceptible), and highly tolerant wild genotype *V. luteola* (Jacq.) Benth. under pot-culture condition to study their response to waterlogging stress. Sowing, treatments and interculture operations were done as described in Sairam *et al.* (2009b). Samples for biochemical estimations were collected in quadruplicate from 4 pots. Observations were recorded on superoxide radical, activity of NADPH oxidase, and expression and cloning of *NOX* gene. The values are means of 4 replicates assayed twice (n = 8). The design of the experiment was complete randomized and data was analyzed by factorial RBD (Gomez and Gomez 1984).

Superoxide radical content was quantified by its capacity to reduce nitroblue tetrazolium chloride (NBT) and the absorbance of end product was measured at 540 nm by UV-Vis spectrophotometer (*Specord-200*, *Analytic Jena*, Jena, Germany) in the presence of 1 mM diethyldithiocarbamate (DEDC, to inhibit SOD activity), and 10 μM diphenyleneiodonium chloride (DPI, to inhibit NADPH oxidase dependent $O_2^{\bullet-}$ generation) (Chaitanya and Naithani 1994). NADPH oxidase activity in mixed microsomal + plasma membrane (PM) fraction was determined as the rate of SOD-inhibitable (by adding 5 mM diethyldithio-carbamate in extraction buffer) reduction of NBT using NADPH as electron donor as per the method of Quartacci *et al*. (2001). For RT-PCR expression analysis and cloning of cDNAs, the oligo-nucleotide primers were designed manually, and oligo quality (to avoid primer dimer, self dimer, *etc*.), GC and Tm were analyzed by using *Oligoanalyzer 3.0* (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/, *Integrated DNA Technologies*, Coralville, IA, USA).

For gene induction studies 25 d old plants were subjected to waterlogging treatment for 24 h in all the genotypes. Root samples were harvested from control and treated plants. Total RNA from the root tissue was extracted using *Trizol* reagent (*GibcoBRL*, *Invitrogen*, CA, USA) as per the recommendations of the manufacturer. DNA contamination was removed from the RNA samples using DNase I (*Qiagen Science*, Maryland, USA). Total RNA (1 μg) was reverse transcribed using gene specific degenerate primers and *Qiagen* one step RT-PCR kit. PCR conditions were standardized using gene-specific primers for β-tubulin. Linear amplification for semi-quantitative RT-PCR was obtained with 35 cycles. Reactions were conducted using *QB 96* thermal cycler *(Quanta Biotech,* Byfleet, *UK*). The amplification products were electrophoresed on 1.2 % agarose gel at 120 V in TBE buffer (0.4 M 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*, Cambridge, UK). RT-PCR amplified cDNAs were fractionated on agarose gel and purified. The purified cDNAs for each gene were cloned into pTz57R/T vector and transformed into *E. coli* (strain DH5α) cells. DH5α cells transformed with recombinant plasmids were selected based on antibiotic resistance as well as α-complementation method. Ampicillin resistant putative recombinants were selected for further analysis. Plasmids were isolated from the confirmed colonies and restriction analysis was carried out by using *Kpn I* and *Hind III* enzymes flanking the cloning site of the vector pTz57R/T, to confirm the presence of cloned insert cDNA. Cloned insert cDNA in the pTz57R/T vector was sequenced by dideoxy chain termination method (Sanger *et al.* 1977) using T7 and SP6 primers.

Table 1. Primer sequence and GC [%] and Tm [°C].

Primer	Sequence	GC	Tm
Tubulin-F	CTTGACTGCATCTGCTATGTTCAG	45.8	55.5
Tubulin-R	CCAGCTAATGCTCGGCATACTG	54.5	58.4
NOX-F	TGGCAATAGCANTTGGTGTTG	45.2	55.1
NOX-R	GAGANACAGCAGCACAATTGAC	47.7	55.4

A very striking observation under waterlogging is the production of various ROS, especially superoxide radical and hydrogen peroxide (Yan *et al.* 1996). Two days after waterlogging $O_2^{\bullet-}$ content declined over control plants in all the genotypes (Fig. 1*A*). However, continuous waterlogging up to 8 d resulted in significant increase in total $O_2^{\bullet-}$ production over 2-d waterlogged plants in the 3 genotypes, though remaining less than the control plants. The increase was greater in *V. luteola* and T 44 than in PB. Recovery recorded after 4 d of termination of treatment resulted in a drastic increase in $O_2^{\bullet-}$ content over that observed in waterlogged and control plants of the 3 genotypes, and the increase in $O_2^{\bullet-}$ production was highest in PB compared to *V. luteola* and T 44. Superoxide radical content in the presence of DPI decreased under waterlogging, recording only a marginal variation in the 3 genotypes (Fig. 1*B*). However, after the recovery the DPI-insensitive $O_2^{\bullet-}$ production increased, and PB recorded higher DPI-insensitive $O_2^{\bullet-}$ production. DPI sensitive $O_2^{\bullet-}$ generation was significantly greater in *V. luteola* and T 44 reaching a peak on 8th day of waterlogging, while only a marginal increase was observed in case of PB (Fig. 1*C*). With the onset of recovery the DPI-sensitive $O_2^{\bullet-}$ content declined in all the genotypes. The increase in ROS production after recovery is probably due to the resumption of aerobic respiration, and higher ROS in PB indicate its vulnerability to oxidative stress. Similar results were observed in waterlogging-susceptible pigeon pea genotypes ICP 7035 and Pusa 207, which showed

significantly higher $O_2^{\cdot-}$ and H_2O_2 content on recovery from waterlogging than tolerant genotypes ICPL 84035 and ICP 301 (Kumutha *et al.* 2009, Sairam *et al.* 2009a).

Waterlogging caused significant and gradual increase in the activity of NOX in *V. luteola* and T 44 up to 8th d of stress, while in case of PB the waterlogging induced increase in NOX was significantly less than that of T 44 and *V. luteola*. The NOX activity in T 44 and *V. luteola* was 123 and 131 %, respectively of that in PB on 8th day of waterlogging (Fig. 1*D*). During recovery, the NOX activity drastically decreased in all the genotypes.

The results obtained in this study clearly shows that increase in ROS during waterlogging is primarily due to an increase in DPI-sensitive and NOX-dependent $O_2^{\cdot-}$ production. Blokhina *et al.* (2001) reported H_2O_2 formation during anoxia as evidenced by electron dense

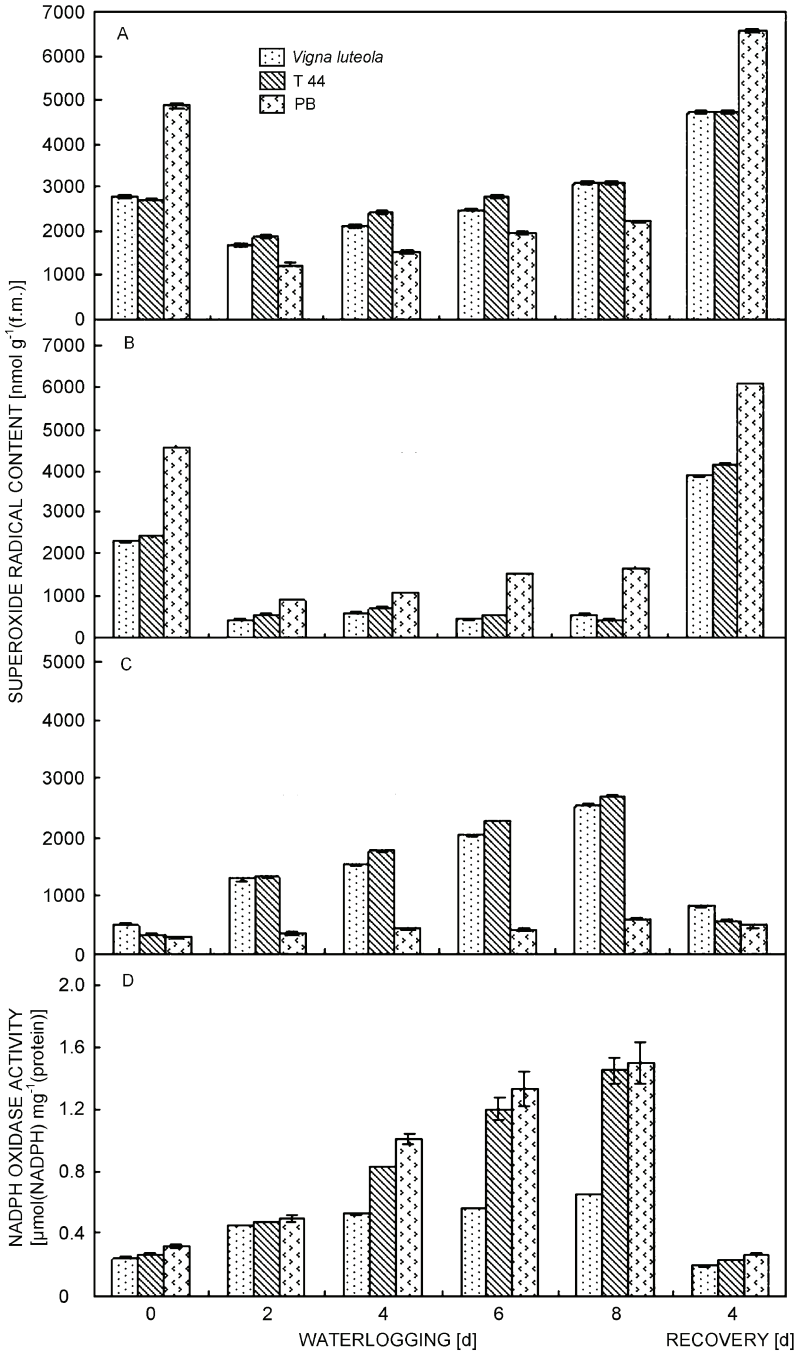
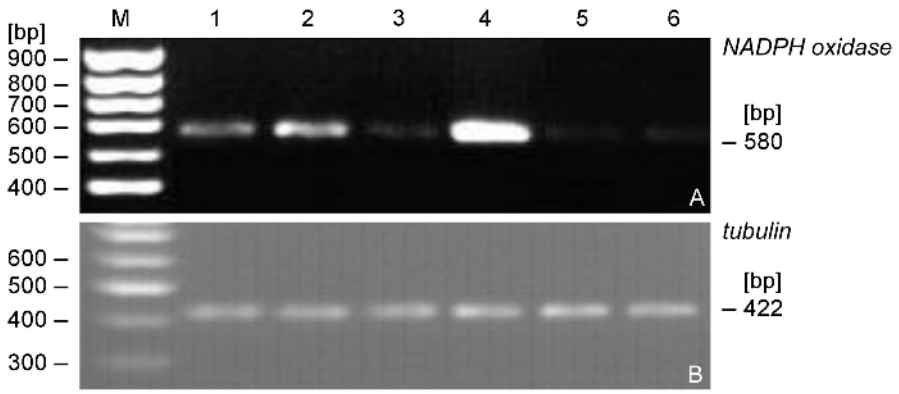


Fig. 1. Effect of waterlogging on total superoxide radicals (*A*), NADPH oxidase independent-superoxide radicals (*B*), NADPH oxidase dependent-superoxide radicals (*C*) and NADPH oxidase activity (*D*) in root tissues in *V. luteola* and *V. radiata* genotypes T 44 (tolerant) and PB (susceptible). LSD significant ($P \leq 0.05$). *Vertical bars* show ± SE of mean ($n = 8$).

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VL-NOX          ------------------------------------------------------LKAV 4
T44-NOX         ------------------------------------------------------------
PB-NOX          ------------------------------------------------------LKAV 4
AY821802        LHVSKPQGFKYHSGQYIFVNCADVSPFQWHPFSITSAPGDDYVSVHIRTAGDWTSQLKAV 660

VL-NOX          FAKACQPASGDQSGLLRADMLQGNNIPRMPKLLIDGPIGATPLISILKDVLNNMKQQYGA 64
T44-NOX         -------------------MLQGNNIPRMPKLLIDGPIGATPLISILKDVLNNMKQQYGA 41
PB-NOX          FAKACQPASGDQSGLLRADMLQGNNIPRMPKLLIDGPIGATPLISILKDVLNNMKQQYGA 64
AY821802        FAKACQPASGDQSGLLRADMLQGNNIPRMPKLLIDGPYGAPAQDYKDYEVILLVGLGIGA 720
                                  ****************** **..      :*:  :    **

VL-NOX          PAQDYKDYEVILLVGLGKDIEQGVVESGVKNNKRKPFATNRAYFYWVTREQGSFEWFKGV 124
T44-NOX         PAQGLQDYEVILLVGLGKDIEQGVVESGVKNNKRKPFATNRAYFYWVTREQGSFEWFKGV 101
PB-NOX          PAQDYKDYEVILLVGLGKDIEQGVVESGVKNNKRKPFATNRAYFYWVTREQGSFEWFKGV 124
AY821802        TPLISILKDVLNNMKQQKDIEQGVVESGVKNNKRKPFATNRAYFYWVTREQGSFEWFKGV 780
                ..      :*:  :   ****************************************

VL-NOX          MDEIADYDKDGLIELHNYCTSVYEEGDARSALITMLQSLH-------------------- 164
T44-NOX         MDEIADYDKDGLIELHNYCTSVYEEGDARSALITMLQS---------------------- 139
PB-NOX          MDEIADYDKDGLIELHNYCTSVYEEGDARSALI-------------------------- 157
AY821802        MDEIADYDKDGLIELHNYCTSVYEEGDARSALITMLQSLHHAKSGVDIVSGTRVKTHFAR 840
                ********************************

VL-NOX          ------------------------------------------------------
T44-NOX         ------------------------------------------------------
PB-NOX          ------------------------------------------------------
AY821802        PNWRTVFKHTALKHPGKRVGVFYCGAAGLVGQLKSLSLDFSRKTNTKFEFHKENF 895
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Fig. 2. Expression analysis of *NADPH oxidase (NOX)* gene (*A*) and *β-tubulin* gene used as an internal control (*B*) under 24 h waterlogging stress and in control conditions. Gene expression was determined by RT-PCR utilizing gene specific primer sets for each gene (M - 1 kb ladder, 1 - control *V. luteola,* 2 - treated *V. luteola,* 3 - control T 44, 4 - treated T 44, 5 - control PB, 6 - treated PB). *Below* - *Clustal W* (*1.83*) multiple sequence alignment and comparison of deduced amino acid sequence of *NOX* in root tissues in *V. luteola* and *V. radiata* genotypes T 44 (tolerant) and PB (susceptible) with *Medicago sativa* complete CDS (GeneBank Acc. No. 821802) (*shows conserved nucleotides; dark/bold letters show nucleotide polymorphisms).

insoluble precipitate of cerium perhydroxide, visualized through electron microscope. The enzymatic origin of ROS production during waterlogging is confirmed due to its inhibition by diphenylene iodonium chloride, a specific inhibitor of membrane linked NOX, and further confirmed by waterlogging induced increase in NOX activity. These findings further confirm our earlier results in pigeon pea that waterlogging induced ROS production is mainly due to the induction of membrane linked NADPH oxidase (Kumutha *et al.* 2009, Sairam *et al.* 2009a).

Gene expression studies for *NOX* by RT-PCR elicited bands of about 580 bp. *NOX* gene showed constitutive as well as induced expression in the case of *V. luteola*, however, the induced expression was much higher than constitutive expression in *V. luteola* and T 44. In case of PB there was little constitutive or induced expression of *NOX* gene (Fig. 2*A*). Waterlogging induced ROS production, and NOX activity and gene expression in tolerant genotypes *V. luteola* and T 44 could have a role in signaling anaerobiosis tolerance mechanism, as H_2O_2 signaling has been reported in the induction of *ADH* gene

(Fukao and Bailey-Serres 2004) and SOD and APX (Agarwal *et al.* 2005). The little amount of DPI-insensitive $O_2^{\bullet-}$ production during waterlogging could be due to the extremely reducing conditions in the rhizosphere, which might have facilitated reduction of dissolved oxygen to superoxide radical (Ponnamperuma 1972). Tubulin expression (Fig 2*B*) did not show variations in control and waterlogged plants of the three genotypes.

Sequencing of partial *NOX* cDNA obtained in this study yielded amplicons of 505, 498 and 484 bp in *V. luteola,* T 44 and PB, respectively. Partial nucleotide sequences for *NOX* of the three genotypes are given below:

V. luteola

GGACATCACAATTGAAGGCTGTTTTCGCCAAGGCATGTCAGCCAGCAAGCGGTGACCAAAGTGGTCTTCTAAGAGCTG
ATATGTTACAAGGCAACAACATACCAAGGATGCCAAAGCTATTGATTGATGGACCTATTGGTGCCACCCCATTGATTA
GCATACTCAAAGATGTACTAAACAACATGAAACAACAATATGGAGCACCAGCACAAGACTACAAAGACTATGAAGTG
ATCCTTCTAGTAGGTTTAGGAAAAGACATAGAACAAGGAGTGGTAGAAAGTGGAGTTAAAAACAACAAAAGAAAGC
CATTTGCCACGAACAGAGCCTATTTCTATTGGGTTACTCGTGAACAAGGTTCCTTTGAATGGTTTAAAGGTGTGATGGA
TGAAATCGCAGATTATGACAAGGATGGACTGATTGAACTTCATAATTATTGCACAAGTGTGTATGAAGAAGGAGATGC
TAGATCAGCTTTGATCACTATGCTTCAATCACTTCATCA

T 44

AAGATTCCTACCAGAAGGCTGATTTCGCCAAGGCATGTCAGCCAGCAAGCGGAGACCAAAGTGGTCTTCTAAGAGCT
GATATGTTACAAGGCAACAACATACCAAGGATGCCAAAGCTATTGATTGATGGACCTATTGGTGCCACCCCATTGATT
AGCATACTCAAAGATGTACTAAACAACATGAAACAACAATATGGAGCACCAGCACAAGGGCTACAAGACTATGAAGT
GATCCTTCTAGTAGGTTTAGGAAAAGACATAGAACAAGGAGTGGTAGAAAGTGGAGTTAAAAACAACAAAAGAAAG
CCATTTGCCACGAACAGAGCCTATTTCTATTGGGTTACTCGTGAACAAGGTTCCTTTGAATGGTTTAAAGGTGTGATGG
ATGAAATCGCAGATTATGACAAGGATGGACTGATTGAACTTCATAATTATTGCACAAGTGTGTATGAAGAAGGAGATG
CTAGATCAGCTTTGATCACTATGCTTCAATCTT

PB

GGGAAACCTAATTGAAGGCTGTTTTCGCCAAGGCATGTCAGCCAGCAAGCGGTGACCAAAGTGGTCTTCTAAGAGCTG
ATATGTTACAAGGCAACAACATACCAAGGATGCCAAAGCTATTGATTGATGGACCTATTGGTGCCACCCCATTGATTA
GCATACTCAAAGATGTACTAAACAACATGAAACAACAATATGGAGCACCAGCACAAGACTACAAAGACTATGAAGTG
ATCCTTCTAGTAGGTTTAGGAAAAGACATAGAACAAGGAGTGGTAGAAAGTGGAGTTAAAAACAACAAAAGAAAGC
CATTTGCCACGAACAGAGCCTATTTCTATTGGGTTACTCGTGAACAAGGTTCCTTTGAATGGTTTAAAGGTGTGATGGA
TGAAATCGCAGATTATGACAAGGATGGACTGATTGAACTTCATAATTATTGCACAAGTGTGTATGAAGAAGGAGATGC
TAGATCAGCTTTGATCAC

V. luteola, T 44 and PB showed 87, 83 and 85 % similarity with the *NOX* complete CDS of *Medicago sativa* (Genbank Acc. No. AY821802). *V. luteola* showed 96 and 98 % similarity with T 44 and PB, and the similarity between T 44 and PB was 97 %.

The partial deduced amino acid sequences of NOX were also compared with *Medicago sativa* (Gene Bank Acc. No. AY821802) using *BLAST* tool and *CLUSTAL W* (1.83) multiple alignment (Fig. 2). All the three genotypes showed approximately 83 to 87 % similarities with *Medicago sativa*, and among the genotypes, the similarity varied from 96 to 98 %. Search for conserved domain using *BLAST* tool revealed that the deduced protein sequences belonged to cd06186: NOX_Duox _like_FAD_NADP, NOX, which catalyzes the generation of ROS, such as superoxide and hydrogen peroxide. The expression of *β-tubulin* did not vary under control or waterlogged condition in all the three genotypes. The sequences of β-tubulin is highly conserved throughout the eukaryotic kingdom, and therefore, β-tubulin was used as an internal control.

From the results it is obvious that in spite of apparent oxygen deficiency during waterlogging, *V. luteola* and *V. radiata* genotype T 44 experienced increase in ROS production, though less than the control and recovered plants, and this oxidative stress were primarily due to the increase in the gene expression and activity of DPI-sensitive-NOX. Role of hypoxia induced $O_2^{\bullet-}/H_2O_2$ production as a positive signaling molecule responsible for the induction of defense genes is confirmed by the observation that both productions of $O_2^{\bullet-}/H_2O_2$ as well as activity and gene expression of NOX was significantly more in tolerant genotypes *V. luteola* and T 44. This suggests that induction of NOX and consequent production of $O_2^{\bullet-}$ (and H_2O_2) during waterlogging could be factors contributing to the overall tolerance of the *Vigna luteola* and T 44 against waterlogging stress.

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