ETHYLENE SYNTHESIS, AERENCHYMA FORMATION AND EXPRESSION OF XYLOGLUCAN ENDOTRANSGLYCOSYLASE IN ROOTS OF *VIGNA SPS.* UNDER WATERLOGGING CONDITION

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SUMMARY

The objective of this study was to examine the role of ethylene, aerenchyma formation and expression of xyloglucan endotransglycosylase (XET) in the waterlogging tolerance of contrasting mung bean (*Vigna radiata*) genotypes viz., T 44 (tolerant) and Pusa Baisakhi (susceptible), and a highly tolerant wild *Vigna* species *Vigna luteola* under pot-culture condition. Waterlogging resulted in decrease in relative water content (RWC) and chlorophyll (Chl) content in leaves, and membrane stability index (MSI) in root and leaf tissues. Waterlogging induced decline in RWC, MSI, and Chl was greater in Pusa Baisakhi (PB) than *V. luteola* and T 44. Ethylene production in the roots increased in all the genotypes, however, the concentration was higher in *V. luteola and* T 44 than Pusa Baisakhi. Though the waterlogging induced *XET* expression in the roots was observed in case of *V. luteola and* T 44, aerenchyma formation was observed only in the roots of *V. luteola*. PCR band products were cloned and sequenced, and partial cDNAs of 455 and 456 bp were obtained for *V. luteola* and T 44, respectively. The partial cDNA sequences of cloned *XET* genes showed 98% homology in *V. luteola* and T 44. T 44 and *V. luteola* showed 100 and 98 % homology with maize *XET* (Genbank Acc. No. ZMU15781), while in case of rice (Genbank Acc. No. Os06g0696600) the similarity was in the range of 87 and 86 %, respectively. The results suggest that one of the reasons of the waterlogging tolerance of *V. luteola* is its ability to develop aerenchyma. Further, besides XET some other factor may also be essential for aerenchyma formation in the roots, which might be absent in T 44.

***Key words*: Aerenchyma, ethylene, gene expression, mung bean, waterlogging, xyloglucan endotransglycosylase**

INTRODUCTION

Waterlogging is a serious problem affecting crop growth and productivity. Waterlogging blocks the oxygen supply to the roots thus inhibiting root respiration, resulting in a severe decline in energy status of root cells affecting important metabolic processes of plants. Plants react to an absence of oxygen by switching from an oxidative to a solely substrate-level phosphorylation predominantly involve glycolysis and fermentation. Many of the adaptive growth responses in hypoxic roots and shoots occur in response to ethylene. Ethylene accumulates in flooded soils and in submerged plant parts to concentrations of 10 ml dm^{-3} (Smith and Russell 1969, Musgrave *et al.* 1972). The immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), is

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synthesized to a large extent in roots (Bradford and Yang 1980). The rate of ACC and ethylene synthesis by root tips are strongly enhanced by hypoxia, but ethylene production is completely arrested by anoxia, which also halts further aerenchyma formation (Jackson *et al*. 1985, Hatwell *et al*. 1988). It has been reported that flooding resistant species such as *Rumex palustris* responds to flooding with rapid growth stimulation of the shoot, especially of the petioles of the youngest leaves. This induction of cell elongation requires ethylene action and is increased in low oxygen concentrations (Voesenek *et al*. 1997). In *R. palustris*, the endogenous ethylene concentration increases from 0.05 to 1 µl l^{-1} within 1 h of submergence (Banga *et al*. 1997) due to physical entrapment.

Plants respond to flooding by undergoing changes at the molecular, biochemical and tissue structural levels. Restoration of aerobic respiration in waterlogged root tissues is key to ensuring the continued growth and function of plants under flooding (Kludze *et al*. 1994, Topa and McLeod 1986). Flooded plants also undergo significant structural modification leading to visible changes in cell and organ structure to restore O_2 in root tissues (Drew *et al*. 1979, Grineva *et al*. 1988). Of these, aerenchyma plays a significant role (He *et al*. 1992, 1994, Brailsford *et al*. 1993). Aerenchyma provide a low-resistance internal pathway for the exchange of gases between aerobic shoot to anaerobic roots (Jackson 1989). It has been found that in hypoxic conditions, the accumulation of the plant hormone ethylene is necessary for aerenchyma formation. Aerenchyma formation in the monocot roots was stimulated by exogenous application of ethylene at rates as low as 0.1 ml l^{1} and was inhibited in the presence of Ag^{+} ions, an inhibitor of ethylene action (Drew *et al*. 1981, Jackson, 1985). Huang *et al*. (1997) reported hypoxia enhanced ethylene production and formation of crown roots and aerenchyma formation in wheat genotypes.

The dissolution of protoplasm and cell wall during lyzigenous aerenchyma formation involves activity of various enzymes especially those involved in cell wall loosening and related enzymes like cellulase, xyloglucan endotransglycosylase (*XET*) etc. A homolog of *XET* was reported in maize during flooding-induced aerenchyma development (Saab and Sachs 1996). Treatment with ethylene synthesis inhibitor, amino oxyacetic acid (AOA), which prevented aerenchyma formation under flooding, almost completely inhibited XET accumulation. Zhang *et al.* (2004) concluded that *XET* is a novel water logging tolerance gene related to structural adaptation, and can be induced in roots of sesame and wheat under anoxia stress when endogenous ethylene production and formation of aerenchyma have been observed.

MATERIALS AND METHODS

Plant material and growth conditions: An experiment was conducted with two cultivated mung bean [*Vigna radiata* (L.) Wilczek] genotypes, viz., T 44 (tolerant) and Pusa Baisakhi (susceptible), and *V. luteola* (a highly tolerant wild *Vigna* sps.) under pot-culture to study their response to waterlogging stress. Plant material was procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi, India, Indian Institute of Pulse Research, Kanpur, (UP), India and National Bureau of Plant Genetic Resources, New Delhi, India. Sowing was done in 30 x 30 cm (h x dia) 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 60 kg ha^{-1} each of phosphorus and potassium. Before sowing seed were treated with the required *Rhizobium* culture. Initially four plants were sown in each pot, which were thinned to 2 plants per pot after 20 d. Waterlogging treatment was given by placing pots with 25 d old plants in plastic troughs measuring 100 x 70 x 35 cm (L x B x H), and filled with water to a height just 1-2 cm below the soil level in pots. Treatments consisted of control, 2, 4, 6 and 8 d of waterlogging, and recovery after 4 d of termination of treatment. As the 8-d waterlogged plants of susceptible genotypes Pusa Baisakhi showed more than 75 % mortality during recovery, therefore, recovery was uniformly studied in all the genotypes for 6 d waterlogged plants only. Two samples were collected from each of the 4 replicates (n = 8) for the estimation of relative water content (RWC), membrane stability index (MSI), chlorophyll (Chl) content and rate of ethylene production. Aerenchyma development and *XET* gene expression were studied in the roots of control, and 6 days and 24 h waterlogged plants, respectively. The design of the experiment was complete randomized and data was analyzed by factorial RBD.

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 was estimated by taking 2 set of 200-mg of leaf or root material in test tubes containing 10 ml of double distilled water (Sairam *et al.* 1997). One set was heated at 40 °C for 30 min in a water bath, and the electrical conductivity of the solution was recorded on a conductivity meter (C_1). Second set was boiled at 100 °C on a boiling water bath for 10 min, and its conductivity (C_2) was measured as above. Membrane stability index (MSI) was 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 dimethyl sulfoxide (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 665 and 645 nm.

Ethylene estimation: Measurement of rate of ethylene production, based on accumulation in sealed culture tubes over 24 h periods was made using the method of Wilson *et al.* (1994). Culture tubes containing the weighed amount of root samples were sealed with suba-seal rubber stoppers 24 h prior to taking gas samples. Perkin Elmer gas chromatograph fitted with FID detector and N – 200 poropak column was used for this purpose. Column temperature was maintained at 60°C and that of injector and detector at 200°C. N_2 was used as the carrier gas. Exactly 1 ml of the gas sample was injected into gas chromatograph from each culture tube. For calibration standard ethylene was obtained from EDJ Research Company, London, UK. For measurement of ethylene, four replicates were used each time and the experiment was repeated thrice. Rate of ethylene production was expressed as nmol per gram fresh mass per hour.

Anatomical Studies: Anatomical studies were done to observe aerenchyma formation in the roots of 6 d waterlogged and control plants of 3 genotypes. The root tip segments of 1 cm length were fixed in FPA (formaldehyde: propionic acid: ethanol: H_2O at volume ratios of 2: 1: 10: 7, respectively) for 24 h. The fixed root segments were subsequently dehydrated through a N-tertiary butyl alcohol (TBA) series (Lefebvre 1985). Then samples were embedded in paraffin wax as given below:

The root samples were placed in 70 % TBA for overnight, thereafter the samples were transferred to 85 % TBA for 2-3 h. Next change was given with 95 % TBA for 2-3 h. Then segments were kept in 100 % TBA for overnight. Next day three changes were given at 2 h interval with 100 % TBA. The samples were then transferred to solution containing equal (v/v) proportion of paraffin wax and 100 % TBA for overnight. Next the contents were transferred to another tube containing paraffin wax (semisolid) at 62-65°C and left for overnight at 62-65°C. Three changes were given with pure paraffin wax for next 2 days while maintaining at 62°C. Finally the paraffin blocks possessing the root segments were prepared.

The root blocks were sectioned at 10 µm on rotary microtome. The sections were obtained as wax ribbons, which were placed on the glass slides .The glass slides were kept on hot plate till the paraffin wax melted leaving only the root tissue behind. The root tissue on the slides were stained with safranin and observed under light microscope.

Gene expression by RT-PCR: For gene induction studies 25 d old plants were subjected to waterlogging treatment for 24 h, as 48 h waterlogging treatment resulted in decline in gene expression of *XET* in all the genotypes. 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 the manufacturer. DNA contamination was removed from the RNA samples using DNase I (Qiagen Science, Maryland, USA). One µg of total RNA was reverse transcribed using gene specific 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 volts 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).

Primer sequences for XET and tubulin are as follows:

Primer Name	Sequence	GC content (%)	Tm (°C)*	Amplicon size (bp)
XET–F	GGGCAACACCAGCGG	49.8	55.2	460
XET–R	GAGCCATCGGCTAACAAA	47.3	54.1	
Tubulin-F	CTTGACTGCATCTGCTATG TTCAG	45.8	55.5	422
Tubulin-R	CCAGCTAATGCTCGGCAT ACTG	54.5	58.4	

Gene sequencing: 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 DH5á) cells. DH5á cells transformed with recombinant plasmid were selected based on antibiotic resistance as well as á-complementation method. Ampicillin resistant putative recombinants were selected for further analysis. Plasmid 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.

DNA PCR: DNA PCR was done to confirm the presence of XET in different genotypes**.** DNA was isolated from the roots of *Vigna luteola*, T 44 and Pusa Baisakhi genotypes using DNeasy plant mini kit (Qiagen Inc,USA). RNA contamination was removed from the DNA samples using RNase A (Qiagen Science, Maryland, USA). PCR amplification was done with genomic DNA of three genotypes using primers for XET. PCR reaction was carried out in Genie 32 Thermal Block PCR (Bioneer, Korea) under following conditions: 1: Denaturation at 95°C for 5 min, 2: 35 cycles of denaturation at 94 °C for 1 min, 3: primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min, 3: final extention at 72°C for 10 min.

After completion of PCR amplification reactions, 0.0025 ml of loading dye 10x was added to each PCR tube. A 1.0 % agarose gel was prepared in 1x TBE buffer and the contents of PCR tubes were loaded on to the gel. Known marker (lamda DNA/ Hind 111) was also loaded in one lane. Electrophoresis was carried out at 60 v for 1 h, i.e., till the bromophenol blue dye traveled less than 2/3 the length of gel. The resolved amplification products were visualized under UV –transilluminator. The gel was photographed using gel documentation system (UviTec Cambridge, U.K.).

RESULTS

Physiological parameters: Relative water content decreased under waterlogging condition in all the genotype and the decline was greater in Pusa Baisakhi (PB) than in *V. luteola* and T 44. By 8th d PB has suffered 54.0 % decline, while the decline in *V. luteola* and T 44 was only 16.4 and 24.28 %, respectively (Fig.1A). Recovery studied in 6 d waterlogged plants was better in *V. luteola* and T 44.

Total chlorophyll content decreased under waterlogging condition in all the genotypes. In case of *V. luteola* and T 44 the decline was 14.13 and 18.00; 7.93 and 12.50 % at 6th and 8th d, while in case of PB the decline was 46.48 and 59.24 % at 6th and 8th d, respectively (Fig.1B). Recovery was also quick and better in *V. luteola* and T 44.

Membrane stability index (MSI) in both leaves and roots decreased with duration of waterlogging, and the lowest values were observed on the 8th d. The decline

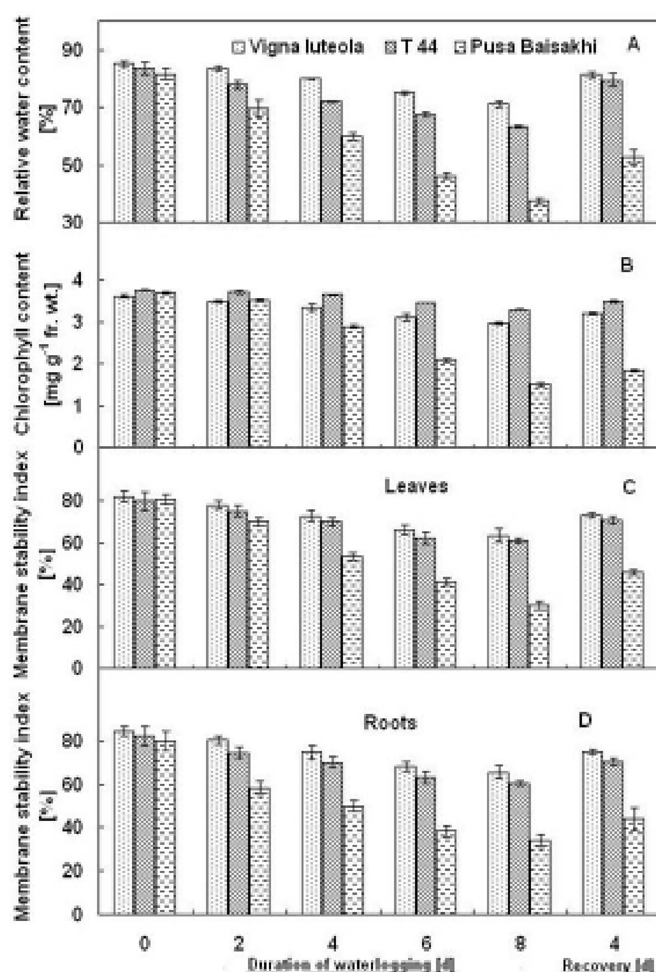

Fig. 1. Effect of waterlogging on relative water content (A), chlorophyll content (B), and membrane stability index of leaf (C) and root (D) tissues in *V. luteola*, and tolerant (T 44) and susceptible (PB) genotypes of *V. radiata*. LSD significant ($P \leq 0.05$). Vertical bars show ± SE of mean.

in MSI was greater in PB, i.e., 43.31 and 45.11 % in leaves and roots, respectively, than *V. luteola* and T 44, which suffered 10.84 and 11.31; 11.37 and 14.15 % decline in leaves and roots, respectively (Fig.1C, D). Recovery was faster in *V. luteola* and T 44 than PB.

Rate of ethylene production: Shifting of plants to waterlogged condition increased the rate of ethylene production in all the genotypes up to 6 days of treatment, except PB, which showed rapid decline in ethylene production from 6th day onward (Fig. 2). The rate of ethylene production was very slow and contents were less in PB, while T 44 and *V. luteola* recorded very high

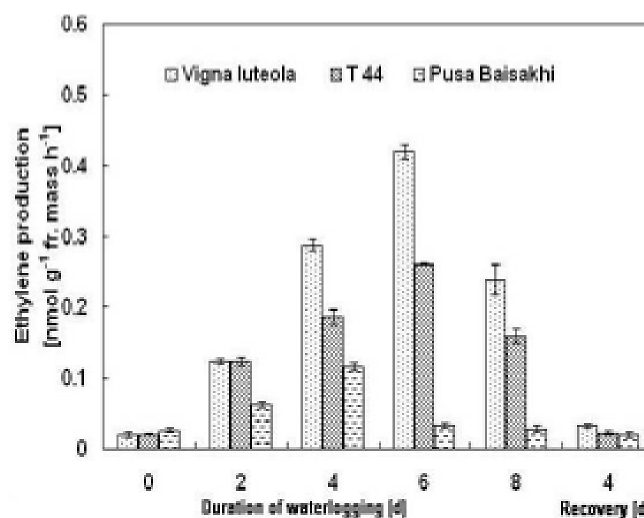

Fig. 2. Effect of waterlogging on rate of ethylene production in the root tissues in *V. luteola*, and tolerant (T 44) and susceptible (PB) genotypes of *V. radiata*. LSD significant ($P \leq 0.05$). Vertical bars show ± SE of mean.

ethylene production, and even on the 8th day of waterlogging the ethylene production was twelve times higher than untreated control plants in *V. luteola* and eight times higher than untreated control plants in T 44. Recovery of 6 days waterlogged plants recorded on 4th day of release of stress showed uniform decline in ethylene production in all the genotypes.

Anatomical studies: Aerenchyma formation was studied in green gram genotypes T 44 (tolerant), Pusa Baisakhi (susceptible) and *V. luteola* (Fig. 3). The results showed a very well defined aerenchyma type spaces in the cortex region of the root sections only in the waterlogging treated wild species *V. luteola*, while there was no such structures in control plants and in both treated and control plants of T 44 and PB.

Xyloglucan endotransglycosylase gene expression study: RT-PCR analysis Xyloglucan endotrans glycosylase (*XET*) gene yield an amplicon of 460 bp only in case of waterlogging treated *V. luteola* and T 44, and expression was not seen in PB (Fig. 4A). However, slight expression was also seen in control plants of *V. luteola*.

In order to confirm whether the gene is present in all genotypes or not, DNA-PCR was performed using

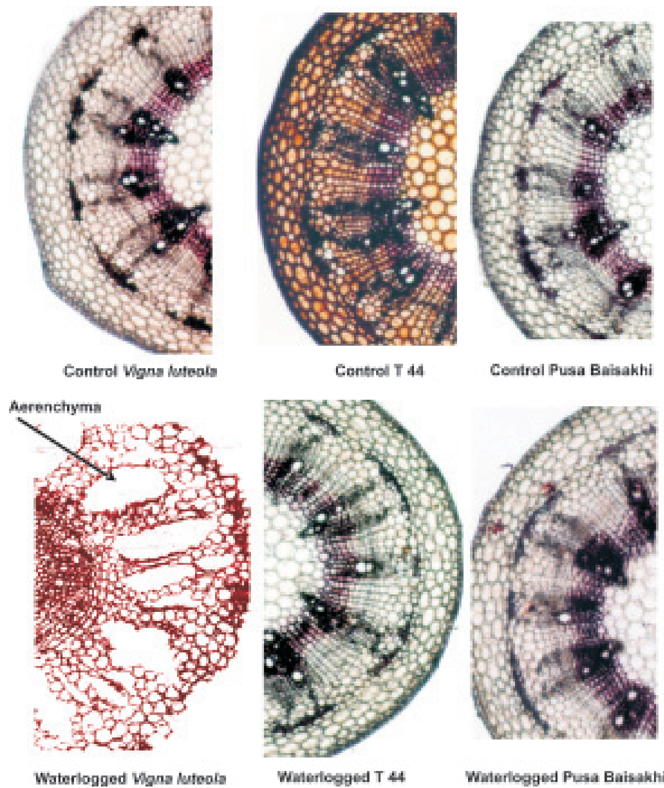

Fig. 3. Effect of waterlogging treatment on aerenchyma formation in the roots of green gram genotypes

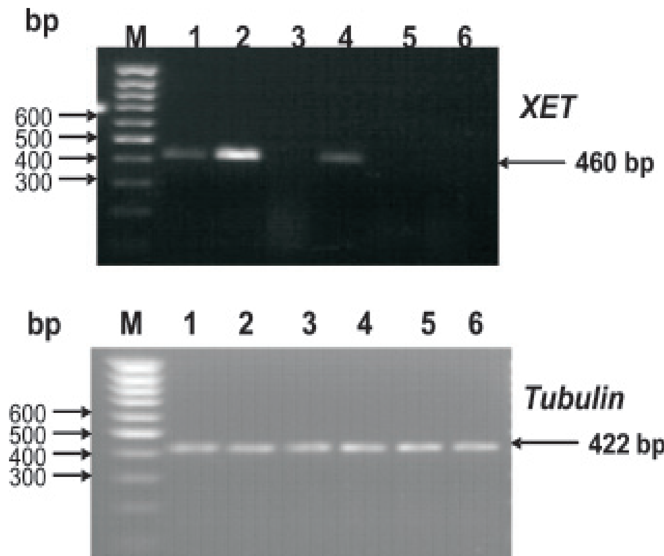

Fig. 4A. RT-PCR expression analysis and DNA-PCR of *XET* and tubulin genes under waterlogging stress and control conditions (M - 1 Kb ladder, 1 - Control *V. luteola,* 2 - Treated *V. luteola,* 3 - Control T 44, 4 - Treated T 44, 5 - Control Pusa Baisakhi, 6 - Treated Pusa Baisakhi)

genomic DNA of all the three genotypes using the same primers. The DNA bands corresponding to XET primers were observed in all the three genotypes (Fig. 4B).

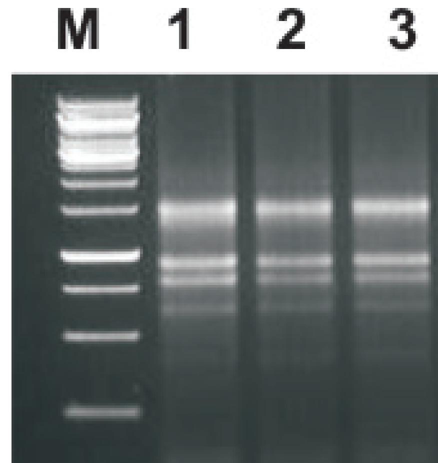

Fig. 4B. DNA-PCR of *XET* gene under waterlogging condition (M: marker, 1: *V. luteola*, 2: T 44, 3: Pusa Baisakhi)

XET gene sequences: *XET* gene specific primers yielded partial sequences of 455 and 456 bp in *V. luteola,* and T 44, respectively. Partial nucleotide sequences for *XET* of the two genotypes are given below:

V. luteola

CGTGGACATCACCTGGGGCGACGGCCGCGGCAA
GATCCTCGACAACGGCCAGCTCCTGACGCTGTC
CATGGACAGGTCCTCCGGCTCGGGCTTCCAGTC
CAAGGCCCAGTACCTCTACGGCCGCTTCGACATG
CAGCTCAAGCTCGTCCCGGGGGACTCCGCCGGCA
CCGTCGCCACCTTCTATCTTTCGTCGCAGGGTT
CGCAGCACGACGAGATCGACTTCGAGTTCCTGG
GGAACGCGAGCGGGGAGCCGTACACGGTGCAC
ACGAACGTGTACAGCCAGGGGAAGGGCGGGC
GGGAGCAGCAGTTCCGGATGTGGTTCGACCCC
ACGGCGGCCTTCCACGCCTACTCCGTGCTGTGG
AACCCCGCCCACGTCGTCTTCTACGTGGACGGCG
TCCCCATCCGGGAGTTCCGGCGCCGCGGCGAC
GGGACCGTGCCGTTCCCGACGTCGCAGCC

T 44

GTGTGACATCACCTGGGGCGACGGCCGCGGCAAGATCC
TCGACAACGGCCAGCTCCTGACGCTGTCCATGGACAGGT

CCTCCGGCTCGGGCTTCCAGTCCAAGGCCCAGTACCTCT
ACGGCCGCTTCGACATGCAGCTCAAGATCGTCCCGGG
GGACTCCGCCGGCACCGTCGCCACCTTCTATCTTT
CGTCGCAGGGTTCGCAGCACGACAAGATCGACTTCG
AGTTCCTGGGGAACGCGAGCGGGGAGCCGTACACGGTG
CACACGAACGTGTACAGCCAGGGGAAGGGCGGG
CGGGAGCAGCAGTTCCGGATGTGGTTCGACCCCAC
GGCGGCCTTCCACGCCTACTCCGTGCTGTGGAA
CCCCGCCCACGTCGTCTTCTACGTGGACGGGGTCCC
CATCCGGGAGTTCCGGCGCCGCGGCGACGGGACCGT
GCCGTTCCCGACGTCGCAAC

The two genotypes showed 98 % similarity with each other. *V. luteola* and T 44 showed 98 % and 100 % similarity with *XET* CDS of *Zea mays* (Genbank Acc. No. ZMU15781), while the similarity was 86 and 87 % when compared with *XET* CDS of *Oryza sativa* (Genbank Acc. No. Os06g0696600) (Table 1).

DISCUSSION

Though water scarcity is one of the major constraints limiting crop productivity, excess water in the root environment of land plants can be injurious or can be lethal because it blocks transfer of oxygen and other gases between atmosphere and soil. Crop plants require free exchange of atmospheric gases for photosynthesis and respiration.

As a result of 8 d of waterlogging mung bean genotypes T 44 and PB, and wild species *V. luteola* suffered decline in RWC, Chl, and MSI both in roots and leaves. The decline in RWC, Chl, and MSI both in roots and leaves was less in *V. luteola* and T 44 than PB, which also suffered about 75 % mortality during recovery (data not reported). Min and Bartholomew (2005) reported decrease in RWC during flooding, which further

Table 1. Similarity between nucleotide sequence (A) and amino acid sequence (B) of mung bean genotype (T 44) and wild sp. (*Vigna luteola*) for xyloglucan endo transglycosylase gene with *Zea mays* (Genbank Acc. No. ZMU15781 (AAC 49011) and *Oryza sativa* (Genbank Acc. No. Os06g0696600).

Seq A	Name	Len (nt)	Seq B	Name	Len (nt)	Score
(A)						
1	T44 XET	456	2	VL XET	455	98
1	T44 XET	456	3	ZMU15781	843	100
1	T44 XET	456	4	Os06g0696600	867	87
2	VL XET	455	3	ZMU15781	843	98
2	VL XET	455	4	Os06g0696600	867	86
3	ZMU15781	843	4	Os06g0696600	867	81
Seq A	**Name**	**Len (aa)**	**Seq B**	**Name**	**Len (aa)**	**Score**
(B)						
1	T44 XET	150	2	VL XET	150	98
1	T44 XET	150	3	AAC49011	280	100
1	T44 XET	150	4	Os06g0696600	288	83
2	VL XET	150	3	AAC49011	280	98
2	VL XET	150	4	Os06g0696600	288	82
3	AAC49011	280	4	Os06g0696600	288	76

declined with the duration of flooding stress. Various workers have also reported waterlogging induced decrease in leaf water potential (Naidoo 1983, Else *et al.* 1995). Wilting under excess of water 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 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).

We have earlier reported that waterlogging tolerance of *Vigna luteola* and T 44 was associated with higher root sugar accumulation, specifically reducing sugars and activity and gene expression of sucrose synthase and alcohol dehydrogenase (Sairam *et al.* 2009).

Many terrestrial plants respond to flooding with enhanced ethylene production. Ethylene synthesis during waterlogging is an adaptive response (Mattoo and Sattle 1991, Abeles *et al.* 1992, Bragina *et al.* 2003). The results on ethylene production showed very significant effect of waterlogging in all the genotypes. Tolerant genotypes T 44 and *V. luteola* showed more increase in ethylene production under waterlogging, with *V. luteola* showing highest ethylene production, while the susceptible cv. Pusa Baisakhi showed drastic decline. The drastic decrease in ethylene production in susceptible genotypes PB could be due to the complete breakdown of its metabolic system and also O_2 supply, required for the last step of oxidation of ACC to ethylene. This hypothesis seems plausible because in susceptible genotype PB more than 75 % of plants failed to recover when they were put for recovery after 6 days of waterlogging, suggesting that its metabolic systems were damaged beyond the point of any repair (Sairam *et al.* 2009). Tolerant genotypes also suffered decline in ethylene production at 8 days waterlogging, which could again be due to partial decline in metabolic activities as well as limited O_2 supply. Ethylene is associated with regulation and induction of enzymes and genes associated with hypoxia tolerance viz., *ADH* (Peng *et al.* 2001) and possibly *sucrose synthase*, and development of aerenchyma (Visser *et al.* 1997). Many of the adaptive growth responses in hypoxic and anoxic roots occur in response to ethylene.

Aerenchyma formation under hypoxia is reported to be mediated by ethylene induced mRNA and protein synthesis, viz., cellulase (He *et al*. 1994) and XET (Saab and Sachs 1996). Aerenchyma development is one of the very important adaptive responses observed under waterlogging condition, which allow exchange of gases between shoot and root, and it is formed in response to hypoxic condition in roots. In wild *Vigna* sp. *V. luteola*, there was formation of cortical aerenchyma by 6 days waterlogging condition, most probably due to breakdown of cell wall and membranes of adjacent cortex cells. In contrast, in control plants of *V. luteola*, and control and waterlogging treated plants of tolerant genotype T 44 and susceptible genotype PB there was no 'aerenchyma' like structure in the roots. Less amount of ethylene synthesis in these genotypes could be attributed to their failure to develop aerenchyma or even rudimentary aerenchyma, which helps in diffusion of gases including oxygen required for conversion of ACC to ethylene. This was also correlated with more *XET* gene expression in wild *V. luteola*, relatively less expression in T 44, and complete lack of expression in Pusa Baisakhi. DNA PCR using genomic DNA from the three genotypes confirmed the presence of *XET* gene in all three genotypes. This proved that the transcription of the gene is waterlogging induced and also only in tolerant genotypes. Very little synthesis of hypoxia induced ethylene, coupled with absence of *XET* expression could be the reason for absence of aerenchyma in susceptible genotype PB. Both ethylene biosynthesis and aerenchyma development are co-dependent on each other. Ethylene has a role in aerenchyma formation, which helps in oxygen absorption and thus has a role in plant survival (Visser *et al.* 1996, Mergemann and Sauter 2000). A flooding induced maize gene encoding a homolog of *XET* was reported during flooding induced aerenchyma development (Saab and Sachs 1996).

RT-PCR amplified cDNAs of *V. luteola* and T 44 for *XET* gene were sequenced and about 460 bp of partial

coding sequences and corresponding deduced amino acid sequences were compared with *Zea mays* and *Oryza sativa XET* gene coding sequences using CLUSTAL W(1.83) multiple sequence alignment database of BLAST (Figs. 5A and 5B). *V. luteola* and T 44 showed about 98 % similarity in both nucleotide and amino acid sequence comparison. Similarly, T 44 showed about 100% sequence similarity with maize *XET* gene sequence, while *Vigna luteola* showed 98 % similarity with maize. Both the genotypes showed approximately 86 and 87 % similarity with *Oryza sativa XET* gene coding sequence (Table 1). The partial amino acid sequence of *XET* showed a conserved domain, Glycosyl_Hydrol_F16 (PS01034) (amino acid residues 70-80), which is a catalytic domain of *XET* and the 2Es (glutamate) are the active site residues (Fig. 6B). Mung bean genotype T 44 showed an amino acid replacement at the place of E (glutamate) by K (lysine) at the 70th active site residue position. This may be one of the reasons for the less expression of *XET* in T 44 under waterlogged condition than *V. luteola* or the translated protein was not catalytically active as enzyme due to the replacement of glutamate by lysine in the catalytic domain.

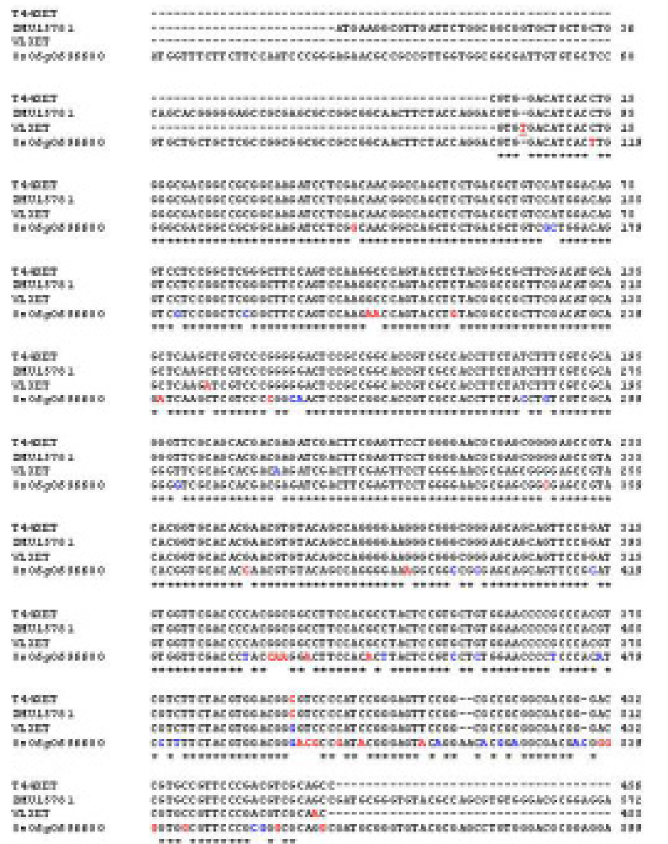

Fig. 5 A. Comparison of *XET* nucleotide sequence by CLUSTAL W(1.83) multiple sequence alignment (* shows conserved nucleotides; colored letters show nucleotide polymorphisms)

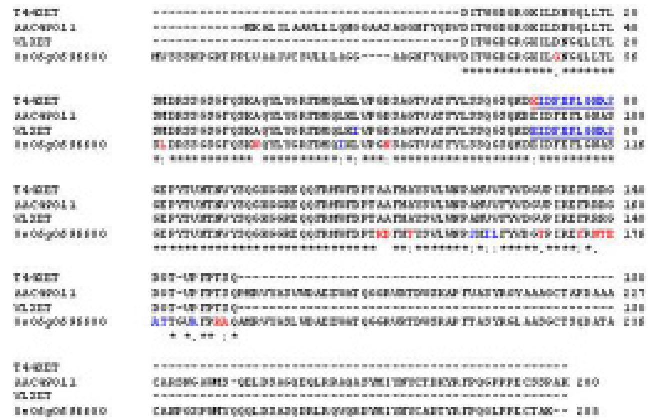

Fig. 5B. Comparison of deduced amino acid sequence of *XET* by CLUSTAL W(1.83) multiple sequence alignment (* shows conserved amino acid; colored letters show amino acid polymorphisms; blue, bold and underlined amino acid residues indicate the conserved catalytic domain, Glycosyl_Hydrol_F16 of *XET*)

V. luteola is a wild species, well adapted to growing under waterlogged/marshy lands, and therefore, it has well developed aerenchyma tissues as an adaptive mechanism. The cultivated mung bean cultivars are grown under arid-water limiting conditions, and therefore, these may not require aerenchyma. However, our earlier results showed that T 44 was comparatively tolerant to waterlogging condition. Though in case of highly susceptible genotype Pusa Baisakhi both aerenchyma tissue and *XET* expression were completely absent, indicating the essentiality of *XET* for aerenchyma development, however, the absence of aerenchyma in T 44 in spite of waterlogging induced expression of *XET* suggest that either the *XET* gene in T 44 was unable to transcribe active protein due to replacement of glutamate by lysine in the conserved catalytic domain, or some additional factor, other than the XET, is also required for aerenchyma development, which may be missing in T 44.

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