http://dx.doi.org/10.22161/ijeab/2.2.7 *ISSN: 2456-1878*

Regulation of Seed Germination and the Role of Aquaporins under Abiotic Stress

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***Abstract*—** *Aquaporins play a major role in governing the movement of water between neighboring cells during seed germination and are major players in response to abiotic stress conditions that affect water availability. Seeds of pea (Pisum sativum L. cv. Arkel) were used for studying cell growth, expression and function of aquaporins during seed imbibition, radicle emergence and growth.Water channel functioning checked by inhibitory test with mercuric chloride showed closed water channels prior to growth initiation. Addition of mercury scavenging agents dithiothreitol and β-mercaptoethanol along with the $HgCl_2$ overcame the observed inhibitory effects in terms of moisture content. The presence of aquaporin inhibitors ($HgCl_2$ and $ZnCl_2$) and NaCl reduced seedling growth. Here we studied expression of a plasma membrane intrinsic protein (PsPIP1;2) and a tonoplast intrinsic protein (PsTIP1;1) by using the semi quantitative RT-PCR in the germinated seedlings exposed to different abiotic stresses. Treatment with NaCl, $HgCl_2$ and $ZnCl_2$ differentially regulated gene expression in radicle, cotyledon and plumule. NaCl and Hg, upregulated expression of PsPIP1;2 and PsTIP1;1 in radicle and expression of PsTIP1;1 was significantly upregulated in radicle and suppressed in cotyledon by Zn. A possible role for aquaporins in germinating seeds and seedling response to abiotic stresses is discussed.*

***Keywords*—Seed germination, Aquaporins, Pisum sativum L., Heavy metals, dithiothreitol (DTT), β-mercaptoethanol (ME).**

I. INTRODUCTION

Seed germination determines successful crop production and is usually the most critical stage in seedling establishment (Almansouri et al. 2001; Bhattacharjee 2008). The germination of seeds requires great adeptness since the process is very complex, a seed from its stillness is woken up to its active state (Dow & Schwintzer 1999). Imbibition of water marks the onset of germination of seeds and triggers arrested metabolic activities which culminates in the emission of a radicle and finalization of germination (Nanogaki et al. 2010). Water moves through the plant tissue via three different pathways: the apoplastic pathway through cell walls and intercellular spaces, the symplastic path from cell to cell either through cytoplasm and plasmodesmata, the transcellular path traversing through cell membranes. The movement of water via the transcellular path involves aquaporins, which are water selective channels (Preston et al. 1992; Agre et al. 1998).

Plant aquaporins fall into five subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin26-like intrinsic proteins (NIPs), the small basic intrinsic proteins (SIPs), and the uncategorized X intrinsic proteins (XIPs) (Maurel et al. 2015). Aquaporins are concentrated in zones of cell division and enlargement, play a major role in governing the movement of water between neighboring cells during seed germination (Tyerman et al. 2002; Jain et al. 2008). Recent studies have implicated the importance of aquaporins in seed imbibition and subsequent germination (Schuurmans et al. 2003; Liu et al. 2007; Liu et al. 2013; Cardoso et al. 2015). The function of seed aquaporins may be related to the water imbibition and activation of the metabolic system in the seed, which results in higher germination (Liu et al. 2007).

Seed germination occurs only when conditions become favorable. Seeds in their germinating stage are highly sensitive and germination is arrested by various heavy metals like mercury, cadmium and zinc etc. (Du et al. 2004; Chugh & Sawhney 1996; Rout & Das 2009). Mercury is widely believed to block aquaporins by binding to a cysteine residue located in close proximity to the aqueous pore of the protein (Daniels et al. 1994; Agre et al. 1998). Due to its aquaporins blocking activity (Przedpelska-Wasowicz & Wierzbicka 2011), mercurial compounds have been used to assess the contributions of aquaporins in water transport at various growth stages of plant. A reversibility of mercury effects by reducing agents like dithiothreitol (DTT) and β-mercaptoethanol (ME), have also been used in conjuction with $HgCl_2$in current studies that seek to evaluate the activity of aquaporins (Przedpelska-Wasowicz & Wierzbicka 2011; Obroucheva et al. 2012; Liu et al. 2014).

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Several studies have shown that abiotic stresses such as water deficit, salinity and heavy metals differentially regulate expression of aquaporins in various plant organs (Sakurai et al. 2005; Tyerman et al. 2002; Suga et al. 2001; Alexandersson et al. 2005; Jang et al. 2004). In Arabidopsis, the expression of MIP genes in different organs in response to cold, salt, drought and ABA treatment has been determined by using a semi-quantitative slot blot analysis and quantitative real-time RT-PCR (Alexandersson et al. 2005;Weig et al. 1997). Most of the studies showed that several aquaporins were predominantly expressed in one organ and that many were markedly up or downregulated under the different stress conditions. Analysis of 33 rice MIP gene expression at different growth stages and in different plant organs also showed that gene expression varied with plant organ and growth stage (Sakurai et al. 2005). Heavy metals such as Cd, Cu and Hg are known to modulate gene expression and function of MIPs in various plant species. For example, in *Pisum sativum* reduction of root hydraulic conductivity (Lpr) by $HgCl_2$ treatment was accompanied by an increase in the expression of *PsPIP2;1*, suggesting that the increase in *PsPIP2;1* might compensate for the AQPs blocked by Hg (Beaudette et al. 2007), whereas in *Populus deltoides* roots subjected to copper stress genes encoding plasmalemma (PIP) and tonoplast (TIP) AQPs were downregulated under Cu application (Guerra et al. 2009).

The present study was undertaken to assess the role of heavy metals (Hg, Zn), salinity (NaCl) known aquaporin inhibitors, along with several chemical agents known to reverse the inhibitory effects of mercuric chloride in regulating the water relations via aquaporins during pea seed germination and investigated the expression and function of plasma membrane and tonoplast aquaporins in seedling tissues during seed imbibition, radicle emergence and growth.

II. MATERIALS AND METHODS

1. Effect of $HgCl_2$ on seed germination

Seeds of pea(*Pisum sativum* L. cv.Arkel) were procured from Manipur Seeds Corporation, Manipur. Theyhad no visible signs of injury and were uniform in size and weight.The seeds were placed in petri dishes (90 mm in diameter) lined with two layers of Whatman no.1 filter paper, moistened with 3 ml of distilled water and $HgCl_2$ solutions at concentrations of 100, 300, 500 and 1000 µM. The petri dishes were incubated at 25 ± 2^0C in dark for 72 h, and daily evaluations of germination were performed.

2. Effect of $HgCl_2$, ME and DTT on activity of aquaporins

To study the involvement of aquaporins on seed germination, known aquaporin inhibitor*$HgCl_2$*and mercury scavenging agents (ME and DTT) were tested during germination as per the method of Jain et al. (2008). The seeds were placed in petridishes lined with two layers of Whatman no.1 filter paper, moistened either with 3 ml of the test solution i.e., $HgCl_2$ (0, 100, 300, or 500 µM) or in combination with ME (250 µM) or DTT (500 µM). The petri dishes were incubated at 25 ± 2^0C in dark for 72 h. Five seedlings were used to calculate the percent moisture. The fresh and dry weights of seedlings were recorded, and the percentage moisture content was calculated as {(W_1-W_2)/W_1} x 100, where W_1 and W_2 represent fresh and dry weights of five seedlings, respectively. All the experiments were repeated thrice.

3. Effect of $HgCl_2$, $ZnCl_2$ and NaCl on seedling growth

In order to verify the significance of aquaporins in germinating pea seeds $HgCl_2$, $ZnCl_2$and NaCl was administered in germinating pea seeds. The germinated seeds were treated with different concentrations of $HgCl_2$(100, 300, 500 and 1000 µM), $ZnCl_2$(10, 25, 50 and 100 µM) and NaCl (100, 300, 500 and 1000 mM). Growth of the seedlings was determined by measuring the length of the radicle and plumule. Measurements were done inthree replicates using five plants and expressed as cm.The data were recorded after every 24 h upto 72 h.

4. Expression study of aquaporins

To study the effect of salt (NaCl) and heavy metals (Hg and Zn) on gene expression, germinated seeds were treated with NaCl (100 mM), $HgCl_2$(100 and 300µM),and $ZnCl_2$ (50 and 100µM). After 24 h and 48 h, radicle, plumule and cotyledon samples were taken from each treatment for RNA extraction.100 mg of the tissue samples were homogenized using liquid nitrogen and the total RNA was isolated using Total RNA purification kit (Nucleopore's) following the manufacturer's instructions. The RNA isolated were quantified spectrophotometrically at 260/280 nm. Out of total RNA isolated from the seed tissues, approximately 1000 ng was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit #K1622 following the manufacturer's instruction. The cDNA thus obtained was kept at -20^0C till further use.The gene specific primers were designed from the nucleotide sequences of *PsPIP1;2* and *PsTIP1;1* (Gene bank accession number: AJ548795.1 and AJ243309.1 respectively). The gene specific forward primer 5'-TGATGCAGGTTCTTGGTG-3', reverse primer 5'-CGTGCTGGGTTGATACCA-3' were used for

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amplifying *PsPIP1;2*, and forward primer 5'-TGGCTGAGTTCATCTCCA-3', reverse primer 5'-CACTCCAACTCCTGCGGA-3' were used for amplifying *PsTIP1;1*.The PCR conditions were in house validated and reconfirmed for each aquaporin gene. Each reaction system contained 2.5 µl of 10x PCR buffer with $MgCl_2$, 1.0 µl of 10 pmole/µl each primer, 0.5 µl Taq DNA polymerase (5 U/µl), 2 µl cDNA and the volume was made up to 25 µl with deionized water.The thermal cycle used for *PsPIP1;2* was as follows: 96^0C for 2 min; 30 cycles of 96^0C for 30 s, 56^0C for 1min, 72^0C 30 s, and a final 72^0C for 5 min. The PCR reaction conditions for amplifying *PsTIP1;1*were the same as those for *PsPIP1;2* gene, but the annealing temperature was changed into 56^0C.The amplicons so generated were resolved on 1.2% agarose (Sigma-Aldrich,) gel electrophoresis. Then the gel was examined in Gel Doc (KODAK) and the photographs were taken.

III. RESULTS

1. Effect of $HgCl_2$ concentration on seed germination

Initial experiments were carried out to determine the effect of various concentrations of $HgCl_2$(0, 100, 300, 500 and 1000 µM) on seed germination. The percentage seed germination decreased gradually up to 1000 µM (Fig. 1). The seeds soaked in 100 µM $HgCl_2$ showed similar trend in germination as the seeds soaked in water and germination percentage were not significantly different from the control. However, 1000 µM $HgCl_2$ caused drastic reduction in germinationpercentage of the seeds.

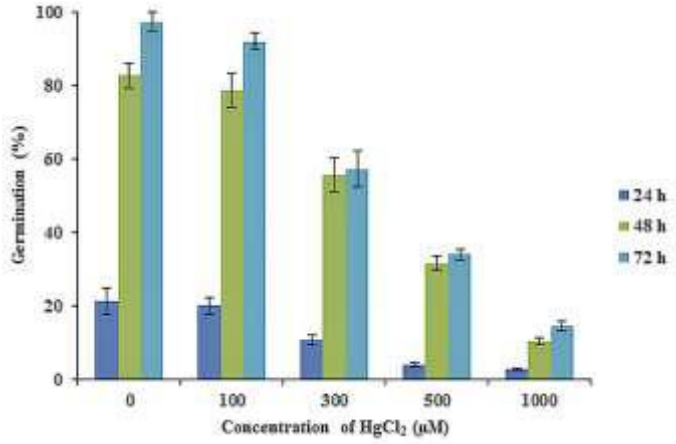

Fig. 1: Effect of different concentrations of $HgCl_2$ upon germination percentage of pea seeds. Values are means ± S.E. based on three independent experimental data.

2. Effect of mercury- scavenging agents on activity of aquaporins

It is well known that the inhibitory effect of $HgCl_2$ on aquaporins can be reversed by mercury-scavenging agents such as DTT and ME. The moisture content of the seedlings wasmeasured after treatment with distilled water, 250 µM ME and 500µM DTT. Their presence had no inhibitory effect on seedling moisture content (Fig. 2). Application of these agents individually with 100, 300 or 500µM of $HgCl_2$ overcame the inhibitory effect of $HgCl_2$in terms of moisture content. The seedling moisture content was significantly lower than the control with 500 µM of$HgCl_2$. This inhibitory effect of $HgCl_2$ was reversed by using DTT or ME (Fig. 2).

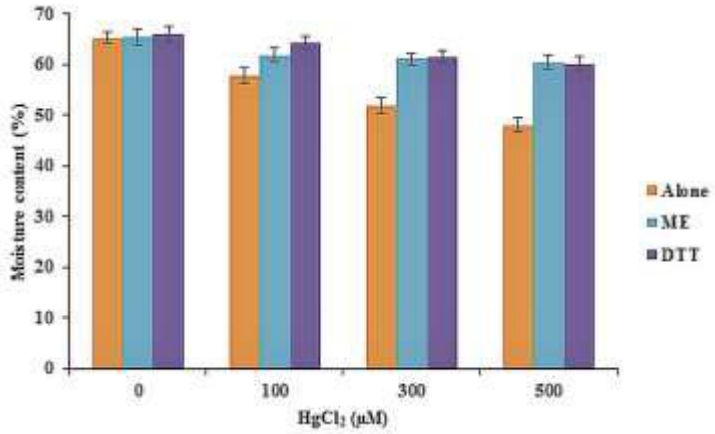

Fig. 2: Moisture content of pea seedlings germinated in the presence of different concentrations of $HgCl_2$ (0, 100, 300, or 500 µM) either alone or in combination with reversal agents ME (250 µM) or DTT (500 µM). Values are means ± S.E. based on three independent experimental data.

3. Effect of $HgCl_2$, $ZnCl_2$ and NaCl on seedling growth

Radicle and plumule length were measured to illustrate the impact of $HgCl_2$, $ZnCl_2$ and NaCl upon the growth of pea seedlings. Different concentrations of $HgCl_2$ (100, 300, 500 and 1000 µM) showed marked impact upon the measured growth parameters in pea seedlings. Both radicle and plumule length significantly inhibited at high concentration (1000 µM) as compared with the control (Fig. 3). Seed germination in $ZnCl_2$ also had reduced radicle and plumule length at a concentration of 100 µM compared to the control (Fig. 4). In the present study, radicle and plumule length decreased progressively with increasing NaCl concentration (Fig. 5).

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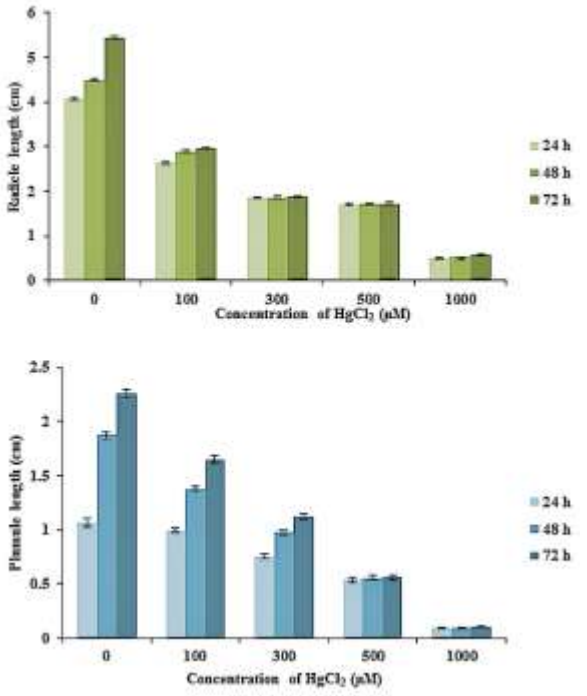

Fig. 3: Effect of different concentrations of $HgCl_2$ upon radicle length, plumule length of pea seedlings. Values are means ± S.E. based on three independent experimental data.

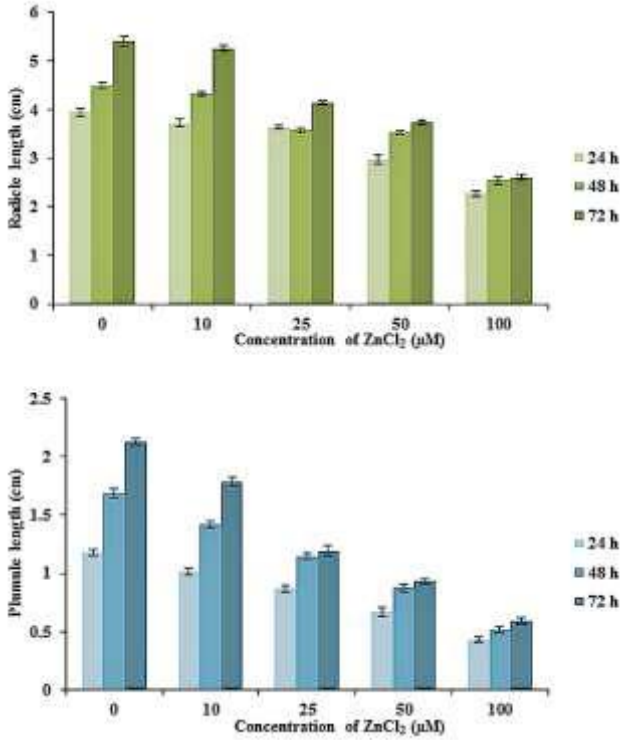

Fig. 4: Effect of different concentrations of $ZnCl_2$ upon radicle length, plumule length of pea seedlings. Values are means ± S.E. based on three independent experimental data.

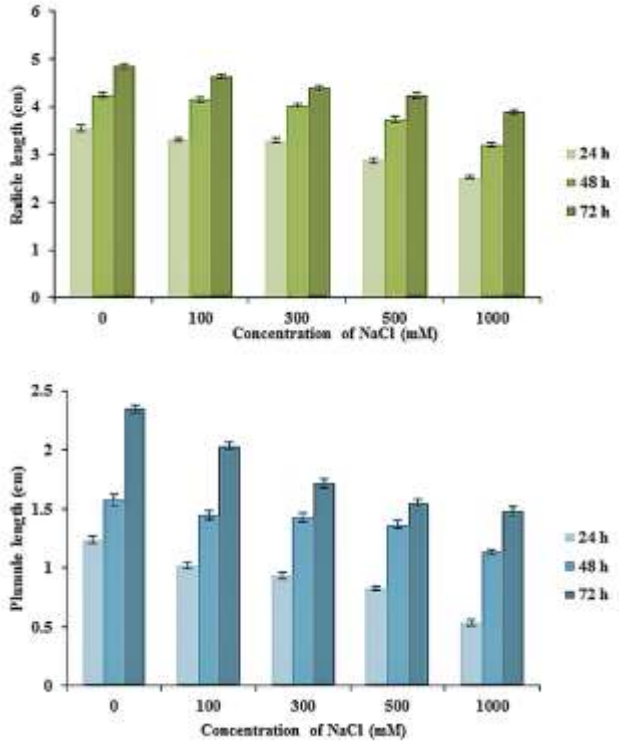

Fig. 5: Effect of different concentrations of NaCl upon radicle length, plumule length of pea seedlings. Values are means ± S.E. based on three independent experimental data.

4. Differential Expression of PsPIP1;2 and PsTIP1;1 during germination under stress conditions

The expression pattern of *PsPIP1;2 and PsTIP1;1* in pea seedling tissues under salt and heavy metal treatment was examined by semi-quantitative RT-PCR. The results showed that the transcripts of *PsPIP1;2* and *PsTIP1;1* were responsive to salt stress in radicle. Transcript level of *PsPIP1;2* accumulated 24 h after treatment of salt, then decreased as the same level as the control 48 h later. Transcript level of *PsTIP1;1* downregulated 24 h after treatment of salt and further decreased as the same level as the control 48 h after treatment (Fig. 6). Since heavy metals are known to block water movement by inhibiting the activity of aquaporins, we studied the effects of Hg and Zn on *PsPIP1;2* and *PsTIP1;1* expression. In radicle, Hg upregulated expression of *PsPIP1;2* and *PsTIP1;1* after 24 h and 48 h of treatment (Fig. 6).

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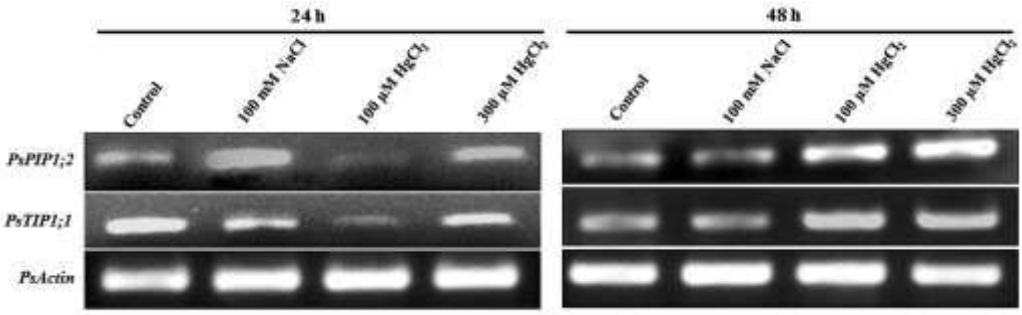

Fig. 6: Tissue Expression pattern of PsPIP1;2 and PsTIP1;1 in radicle of pea seedlings after 24 h and 48 h of treatment with 100 mM NaCl, 100 µM $HgCl_2$and 300 µM $HgCl_2$;PsActin primers were used as internal control.

Expression of *PsTIP1;1* was studied under different concentrations of Zn stress after 24 h in cotyledon, plumule and radicle.Zn downregulated expression of *PsTIP1;1*in cotyledon whereas upregulated in radicle. Expression of *PsTIP1;1* was not affected in plumule (Fig. 7).

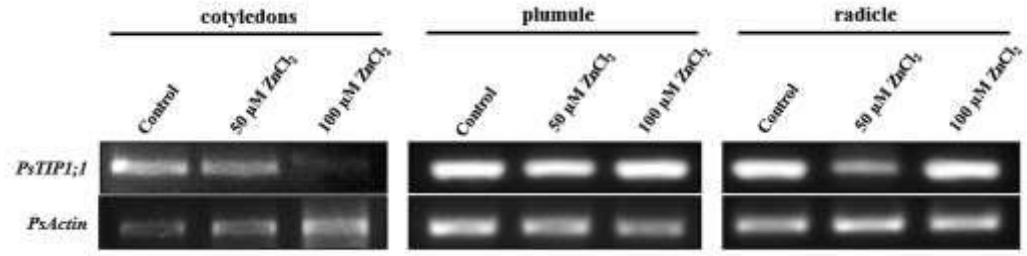

Fig.7: Tissue Expression pattern of PsTIP1;1 under $ZnCl_2$ stress in pea seedlings. PsTIP1;1 expression in cotyledons, plumule and radicle after 24 h treatment with 50 and 100 µM $ZnCl_2$;PsActin primers were used as internal control.

IV. DISCUSSION

Mercury at different concentrations affect seed germination differently.Higher concentrations of mercury cause death of the seed embryo by exerting oxidative stress and damaging cellular components. Mercury at low concentrations affectsseed germination by changing the hydration pattern (Munzuroglu & Geckil 2002; Jain et al. 2008).In previous studies mercurials reduced the speed of seed imbibition and seed germination in pea and Arabidopsis, respectively (Veselova et al. 2003; Vander Willigen et al. 2006). Since mercury blocks aquaporin activity and aquaporins play major role in transmembrane transport of water (Aroca et al. 2012), high concentration of $HgCl_2$ might have suppressed aquaporin activity and subsequent reduction in hydration and germination.

In this study, addition of DTT and ME to $HgCl_2$ treated seeds reduced the inhibition of imbibition. Similar results were obtained by Jain et al. (2008) in which addition of DTT and ME overcame the inhibitory effect of $HgCl_2$on tomato seed germination and moisture content. Veselova and Veselovsky (2006) reported that DTT restored reduced rate of water uptake by mercury-containing compound in pea.

Excessive accumulation of heavy metals in the soil environment due to rapid industrialization, urbanization and intensive agriculture adversely affects the germination of seeds, plant growth, alters the level of biomolecules in the cells and interferes with the activities of many key enzymes related to normal metabolic and developmental processes (Parmar & Chanda 2005; Jayakumaret al. 2008; Ogundiran2007). Diminution in plant growth is one of the clearest symptoms induced by heavy metals.Reduced root and shoot length in response to heavy metal has been reported by a number of investigators (Nag et al. 1989; Zhenguo et al. 1998; Tomulescuet al. 2004; Zhanget al. 2009; John etal. 2009).The visible symptoms of mercuric chloride toxicity were decrease in germination percentage, decrease in radicle length and plumule length. Root growth inhibition and lateral roots development are symptoms of mercury toxicity which can be attributed to the inhibition of mitosis, reduced synthesis of cell wall components and changes in photosynthetic activity. Similar observations with $HgCl_2$ treatment to *Arachis hyposea* seeds were

http://dx.doi.org/10.22161/ijeab/2.2.7 *ISSN: 2456-1878*

noticed by Abraham and Damodaran (2012).To further substantiate the results, $ZnCl_2$, another inhibitor of aquaporins, was used. $ZnCl_2$ inhibits water transport in a permanent manner as it reacts with sulfhydryl groups of a cysteine in the vicinity of the conserved NPA motif, blocking the constriction region of the channel (Niemietz & Tyerman, 2002). According to Flowers et al.(2010) salinity (as NaCl) reduces seed germination and growth of pea seedlings. There is reduction in both seedling growth and root growth. It may be due to the fact that the root cannot balance the nutrient uptake due to osmosis. The osmotic effect takes place during salt stress which affects seed germination (Welbaum et al. 1990) which in turn slows down the water uptake by the plant. Neuman (1995) also observed that salinity inhibits root growth rapidly and hence the capacity of water uptake. Similar results were observed by Demir and Arif (2003) in safflower. This osmotic effect in the plant cell cause negative pressure in the pore of water channel affecting hydraulic conductivity (Ye et al. 2004). The hydraulic conductivity when lower also lowers the aquaporins activity. The reduction in root hydraulic conductivity is also correlated with a dynamic change in the post-translational modifications such as phosphorylation and amidation that affect aquaporin function(Khan et al. 2015). Aquaporin is also altered by ROS which leads to channel closure through a direct oxidative mechanism (Kourie 1998), and induces internalization of PIPs and reduces hydraulic conductivity through cell signaling mechanisms (Boursiac et al. 2008a, Boursiac et al. 2008b).

Since abiotic stresses such as salt and heavy metals induce osmotic stress to plants and disturb plant water balance, we tried to investigate the molecular mechanisms involved in maintaining osmotic homeostasis by studying gene expression and water transport activity of one PIP and one TIP isoforms in pea. Transcripts of *PsPIP1;2 and PsTIP1;1* studied were detected in both radicle and plumule at different levels. Our results showed that transcript levels responded differently to 100 mM NaCl treatment depending on the type of gene. In radicle, salt stress significantly upregulated transcript of *PsPIP1;2*during the initial 24 h of treatment but reached to same level as of unstressed after 48 h of treatment. The transient induction of *PsPIP1;2* by salt stress might confer the membrane permeability to water transport in water-deficient condition (Yamada et al. 1997). Our study is in accordance with a real-time PCR analysis of *PIP* gene expression (Jang et al. 2004), which revealed in salt-treated roots an increase in abundance more pronounced for PIP genes. Salt stress downregulated the expression of*PsTIP1;1*after 24 and 48 h of treatment. At the gene level, expression of TIPs and PIPs was co-ordinately reduced. This could mean that TIPs also contribute to control transcellular water transport in radicle. Mercury, having inhibitory effect on water transport activity (Carvajal et al. 1996; Maggio & Joly 1995) stimulated gene expression as in the current study and as reported earlier in pea(Beaudette et al. 2007). Mercury enhancing transcript accumulation of *PsPIP1;2* and *PsTIP1;1*, suggests that seedlings tend to overcome water shortage by compensating the water channels blocked by Hg.

Being the aquaporins of the tonoplast, TIPs are thought to permit a more rapid transcellular water flow by increasing the effective cross-section of the cytoplasm, and to facilitate osmotic adjustment between cytosol and vacuole (Barrieu et al. 1998). Previously Schuurmans et al. (2003) found *PsTIP1;1*and/or its close homologues and their abundant expression in cotyledons of developing and germinating pea seeds, and in roots and shoots of seedlingsand concluded that TIP1 members play a role in the rehydration of the dry seed during imbibition and subsequent germination. In our case, expression of *PsTIP1;1* was significantly high in plumule and radicle which wasconsistent with the expression pattern of TIP1s in *Arabidopsis* (Alexandersson et al. 2005). The expression of *PsTIP1;1* upregulated by Zn in radicle and suppressed in cotyledon after 24 h of treatment and was not affected in plumule. High expression of *PsTIP1;1* might contribute to transcellular water transport in germinating seeds and facilitate water supply to expanding tissues.

V. CONCLUSION

Based on the results of our study, higher concentration of mercuric chloride suppressed aquaporin activity and subsequent reduction in hydration and germination of pea seeds.Addition of mercury scavenging agents along with $HgCl_2$ overcame the inhibitory effects. It concludes a putative role for aquaporins in controlling pea seed germination, by possibly acting in the initial phases of germination. The presence of aquaporin inhibitors ($HgCl_2$ and $ZnCl_2$) and NaCl reduced seedling growth and differentially regulated expression of plasma membrane intrinsic protein (*PsPIP1;2*) and tonoplast intrinsic protein (*PsTIP1;1*) in different parts of the seedlings, suggesting that the isoforms have a distinct role under these stress conditions.

ACKNOWLEDGEMENTS

We acknowledge Manipur Seeds Corporation, Manipur, India for providing pea seeds.

http://dx.doi.org/10.22161/ijeab/2.2.7 *ISSN: 2456-1878*

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