Differential water uptake kinetics in axes and cotyledons during seed germination of *Vigna radiata* under chilling temperature and cycloheximide treatment

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Water uptake kinetics of axes and cotyledons of *Vigna radiata* seeds has been studied during incubation at chilling temperature (4°C) and under cycloheximide treatment. Germination rate of scarified seeds was faster than intact seeds, and this happens in correlation with their comparative water uptake kinetics. Chilling temperature during incubation significantly slowed down water uptake by both intact and scarified seeds. Treatment with cycloheximide was also somewhat effective in retarding water uptake, but only in scarified seeds. Water uptake by axes isolated from intact seeds (attached or detached from the cotyledons) was inhibited completely by chilling temperature as well as by cycloheximide treatment while these treatments were ineffective in preventing water uptake by cotyledons. In the case of scarified seeds, such treatments again inhibited water uptake by axes only, the effect being more prominent in attached ones. Preincubation of intact seeds with cycloheximide for 6 h also inhibited water uptake by isolated axes, but not by cotyledons, during subsequent incubation in water.

Key Words: Chilling treatment, cycloheximide, germination, water uptake, *Vigna radiata* seeds.

INTRODUCTION

The earliest event of seed germination is characterized by imbibitional uptake of water to saturate (physical hydration) the system (Osborne et al., 2002). A second spell of water uptake seems to be critical for germination, since turgidity developed as a consequence of such water uptake initiates the elongation growth of embryonic axes (Mohr and Schopfer, 1995). Cell growth, in general, is initiated by local cell wall loosening (Cosgrove, 1996) and osmotic driving forces, which are activated by proton pump-powered ion fluxes (Philippart et al., 1999). Although water uptake in case of germinating embryo has been proposed to be controlled by cell wall loosening rather than by changes of osmotic pressure or hydraulic conductance (Schopfer and Plachy, 1985, 1993), in some species solute accumulation is claimed to be associated with increased growth potential of

embryonic axes (Black et al., 2006). More recently, regulation of water uptake by germinating tobacco seeds was studied spatially and temporally by nuclear magnetic resonance (NMR) microimaging and NMR spectroscopy (Manz et al., 2005). Results of this study indicate that water distribution within the seed in the water uptake phases II and III is non-homogeneous and the micropylar endosperm and the radicle show the highest hydration. However, the exact metabolic regulation of the water uptake phenomenon is not yet clear, though primary observations indicate the membrane system as major locus of germination stimulus (Enríquez-Arredondo et al., 2005).

In the present investigation water uptake kinetics were studied separately for embryonic axes, cotyledons and whole seeds, either intact or scarified, of *Vigna radiata* under the effects of chilling temperature and cycloheximide, a protein synthesis inhibitor. The study was performed in order to

understand the differential regulation of water uptake (by axes and cotyledons, former only having elongation growth upon imbibition) involved in the process of germination.

MATERIALS AND METHODS

Seeds of mung bean [*Vigna radiata* (L.) Wilczek var. 'B1'], collected from Pulses and Oilseeds Research Station, Berhampur, Murshidabad, West Bengal, India, were used as experimental material. Germination time-courses of the seeds, intact or scarified (punctured on the side opposite to the micropylar end by a needle or forceps), were studied by determining percentage germination (as considered by emergence of the radicle by at least 2 mm) at hourly intervals up to 24 h of incubation on moist filter paper in covered Petri dishes under controlled temperature ($30 \pm 2^\circ\text{C}$) in a Seed Germinator. For water uptake kinetics studies, we relied upon the fresh weight changes of the seeds or embryonic axes and cotyledons separately since dry weight of these tissues does not change significantly during Phase I germination (data not shown) and, as a result, fresh weight of the tissues reflects the water content. Fresh weight changes (mg) of seeds (intact or scarified) were recorded at hourly intervals up to 12 h of incubation in distilled water or cycloheximide solution (CHI; 10^{-4} M) at room temperature (30°C) or chilling temperature (4°C).

For studying water uptake kinetics for embryo parts, seeds (intact or scarified) were first imbibed in water at room temperature. As the intact seeds of mung bean start absorbing water after a lag period of 4 h due to hard seeds coat, seeds were allowed to imbibe sufficient water (6 h preincubation) that made it possible to dissect out the embryo. As there was no such lag period in case of scarified seeds, these seeds were preincubated for 1 h only. For attached axes and cotyledons these preincubated seeds were again incubated as whole seeds under prescribed conditions (under chilling temperature or with CHI) and at 2 h intervals fresh weight of the axes and cotyledons was determined separately after dissecting the seeds from respective sets. In case of detached axes and cotyledons, embryos were dissected from seeds at the end of preincubation followed by incubation of isolated axes and cotyledons separately under conditions mentioned above. Water contents of isolated axes and cotyledons were also determined at 2 h intervals.

In a different experiment, intact or scarified seeds of *V. radiata* were preincubated for 6- or 1 h, respectively, in CHI solution at room temperature along with control seeds (preincubated in distilled water) and then dissected to isolate the embryos after several wash in water. These were then separated into the axes and the cotyledons and incubated in distilled water at room temperature and at 2 h intervals assessed for water contents.

Experiments were carried out with three replicates (each containing 50 seeds for germination studies and 10 seeds or axes or pairs of cotyledons for water uptake studies) for each treatment and mean values were presented. Data were statistically analyzed and the standard error for each mean value was depicted as bars in the figures. For assessing the significance of difference among means at time intervals of incubation period data were subjected to ANOVA.

RESULTS

Germination time-course studies of *V. radiata* at 30°C (Figure 1A) showed that the intact seeds started to complete germination after seven hours of imbibition while scarified seeds showed radicle protrusion earlier i.e. after 3 h of imbibition. Water uptake kinetics of such seeds also showed a similar trend. Thus, water uptake by intact seeds, as reflected by changes in fresh weight (Figure 1B), was delayed until 4h of incubation at 30°C followed by a rapid rate up to 8 h beyond which it became slow. Scarified seeds started absorbing water very rapidly from the beginning and attained a level of moisture content equivalent to 47.5 mg fresh weight within 1 h whereas intact seeds reached this moisture content only after 7 h. Similar sets of seeds incubated at chilling temperature (4°C) showed a considerable retardation of water uptake (compared to control seeds incubated at 30°C), particularly in the case of intact seeds where a very slow water uptake was noticed up to 12 h of incubation (Figure 1C). In scarified seeds incubated at 4°C , however, in spite of a retarded water uptake compared to control seeds (at 30°C), their water content ultimately was the same as that of intact seeds incubated at 30°C . In the case of CHI treatment, both intact and scarified seeds showed an initial water uptake identical to the control (untreated) seeds (Figure 1D). After 2 h of incubation CHI retarded the rate of water uptake at 30°C , in the case of scarified seeds, while it somewhat accelerated uptake in intact seeds.

Figure 1. Germination time course (percentage germination) (A) and water uptake kinetics (fresh weight changes) (B) of intact and scarified seeds of *Vigna radiata*. Effect of chilling temperature (4°C) (C) and cycloheximide (CHI 10^{-4} M) (D) on water uptake [along with control (30°C and CON)] by intact and scarified seeds of *V. radiata*. SE indicated by bars.

In the next series of experiments, water uptake kinetics have been studied separately for embryonic axes or cotyledons (attached or detached) during incubation of both intact or scarified seeds at 30°C or 4°C, on water or in the presence of CHI. The increase in fresh weight of the attached axes, isolated from intact seeds, was very high at 30°C, almost doubled in the 6 h between 6 h and 12 h of incubation (Figure 2A). Chilling treatment (4°C) prevented such increase in fresh weight almost completely. On the other hand, cotyledons (attached) showed a very slow increase in fresh weight during this period (6 h to 12 h of incubation) regardless of imbibition temperature (Figure 2B). Similarly, CHI treatment at 30°C also prevented increase in fresh weight by attached axes (isolated from intact seeds) (Figure 2C) while having no effect on that of cotyledons (Figure 2D). In the case of detached axes (isolated from intact seeds) also fresh weight increased at 30°C (Figure 3A) and chilling temperature again inhibited such increase almost completely. On the other hand, detached cotyledons showed no effect of chilling temperature on water uptake as reflected in fresh weight changes (Figure 3B). CHI treatment on detached axes (from intact seeds) was not very effective

in retarding water uptake until 12 h of incubation (Figure 3C) while no inhibition was noted in CHI-treated detached cotyledons (Figure 3D).

Axes (attached) from scarified seeds showed a faster increase in fresh weight that became almost double at the end of 6 h of incubation (Figure 4A). Again chilling treatment inhibited such an increase in fresh weight. Cotyledons showed a slow increase in fresh weight and chilling treatment could not inhibit this increase significantly (Figure 4B). CHI also inhibited water uptake by attached axes from scarified seeds completely (Figure 4C) while it was without effect on cotyledons (Figure 4D). Increase in fresh weight by detached axes (isolated from scarified seeds, Figure 5A) at 30°C was lower than attached ones (Figure 4A) and low imbibition temperature (4°C) prevented such rise. Detached cotyledons from scarified seeds absorbed water at a similar rate at both 30°C and 4°C (Figure 5B). The effect of CHI in preventing water uptake was less remarkable for detached axes isolated from scarified seeds (Figure 5C) and CHI was ineffective in preventing water uptake for cotyledons (Figure 5D).

Figure 2. Effect of chilling temperature (**A, B**) and cycloheximide (CHI 10^{-4} M) (**C, D**) on water uptake kinetics (fresh weight changes) by attached axes (**A, C**) and cotyledons (**B, D**) isolated from intact seeds of *V. radiata*. SE indicated by bars.

Figure 3. Effect of chilling temperature (**A, B**) and cycloheximide (CHI 10^{-4} M) (**C, D**) on water uptake kinetics (fresh weight changes) by detached axes (**A, C**) and cotyledons (**B, D**) isolated from intact seeds of *V. radiata*. SE indicated by bars.

Figure 4. Effect of chilling temperature (**A, B**) and cycloheximide (CHI 10^{-4} M) (**C, D**) on water uptake kinetics (fresh weight changes) by attached axes (**A, C**) and cotyledons (**B, D**) isolated from scarified seeds of *V. radiata*. SE indicated by bars.

Figure 5. Effect of chilling temperature (**A, B**) and cycloheximide (CHI 10^{-4} M) (**C, D**) on water uptake kinetics (fresh weight changes) by detached axes (**A, C**) and cotyledons (**B, D**) isolated from scarified seeds of *V. radiata*. SE indicated by bars.

Axes isolated from intact seeds pretreated with CHI for 6 h, showed an inhibition of water uptake during subsequent incubation (6 h to 12 h) in distilled water (Figure 6A) while isolated cotyledons revealed no such inhibition by CHI on

subsequent water uptake (Figure 6B). Similar results were obtained for scarified seeds (Figure 6C and 6D for axes and cotyledons, respectively) where seeds were pretreated with CHI for 1 h only.

Figure 6. Effect of cycloheximide (CHI 10^{-4} M) during preincubation of intact seeds (6 h) (**A, B**) or scarified seeds (1 h) (**C, D**) on subsequent water uptake kinetics (fresh weight changes) by detached axes (**A, C**) and cotyledons (**B, D**) of *V. radiata*. SE indicated by bars.

DISCUSSION

Hydration of seeds during germination shows three distinct phases, namely rapid hydration (imbibition; Phase I), a lag phase (Phase II) and a steady hydration phase (embryo elongation; Phase III), in accordance with the kinetics of water uptake (Bewley and Black, 1985). Hydration starts initially as a consequence of matric forces of cell walls and cell contents of the seed (Mayer and Poljakoff-Mayber, 1989). Such initial uptake, however, may be delayed or prevented by hard seed coats as is often found in the case of leguminous seeds. In the present study with seeds of *V. radiata*, water uptake by intact seeds was delayed until 4 h of incubation at

30°C, as was observed earlier also (De and Kar, 1995), while scarified seeds started absorbing water without any delay indicating the degree of hardness of the seed coat in intact seeds. Early germination in scarified seeds may therefore be explained by rapid water uptake (as revealed by fresh weight increase) compared to intact seeds. During incubation at 4°C intact seeds could absorb very little water, apparently because of possible difficulty in the entry of water through the micropylar region due to increased viscosity of water at low temperature, although other effects of temperature like slow down of metabolic reactions and inhibition of oxygen influx to embryo can not be ruled out either. Edelstein et al (1995) ascribed seed coat imposed dormancy in melon seeds at

low temperature as due to restricted oxygen availability to the embryo. Interestingly, in the case of scarified seeds too, chilling temperature retarded water uptake (increase in fresh weight) significantly though not to the extent as in intact seeds. Once again viscosity might be a factor for crossing the membranes through aqueous channels at cold temperature resulting in impeded water uptake. Our observation (unpublished) on increasing water uptake by isolated axes of germinating *V. radiata* seeds with increasing temperature along with increase in germination percentage may support this notion. However, even the scarified seeds could not complete germination at 4°C (data not shown) despite attaining a water status comparable to intact seeds incubated at room temperature, which did complete germination. This could be explained by the fact that the water uptake by embryonic axes, though inhibited by cold temperature (as also revealed from data on isolated axes), was not reflected in the fresh weight change by whole seed, since water uptake by bulky cotyledons, the major contributor for fresh weight increase of seeds upon imbibition, overshadowed the changes in minute axes. On the other hand, CHI treatment that inhibits seed germination (data not shown), could not affect early imbibitional water uptake by scarified seeds but retarded only in later phase (after 2 h). Protein synthesis is probably essential for radicle protrusion (Brooker et al., 1977) and such synthesis may occur either from stored mRNA or from newly transcribed mRNA (Downie, 2001). Rajjou et al (2004) demonstrated germination inhibition in *Arabidopsis* by cycloheximide but not by α -amanitin (transcription inhibitor). We have also shown earlier that treatment with cycloheximide inhibited radicle protrusion indicating the essentiality of protein synthesis during early incubation for seed germination (Kar and Chakraborty, 2003). Whether such proteins are connected with water entry through membrane or cell wall loosening resulting in water uptake is not clear at present. Indeed, there is a paucity of information available on genes functionally related to the initial processes of germination (Bradford et al., 2000). The reason for the slight promotion of water uptake by CHI in intact seeds is not clear, though CHI clearly inhibited water uptake by embryonic axes (which is a prerequisite for turgor driven initiation of axis growth completing germination process) as revealed from experiment with isolated axes.

Because seed germination usually culminates with extension growth of the embryonic axes (mainly radicle), water uptake by the axes is critical for the completion of germination. In the subsequent experiments water uptake analysis was

done separately for embryonic axes and cotyledons after a 6 h (intact seeds) or 1 h (scarified seeds) preincubation (when imbibitional uptake is expected to be complete). Water uptake by the axes attached to cotyledons was always somewhat higher than for detached axes, difference being more prominent in the case of scarified seeds. This is possibly because cotyledons supplemented the water supply to the axes possibly through vascular connection that might have differentiated by that time (Sundås et al., 1992) or through diffusion. Chilling treatment prevented water uptake by both attached and detached axes, again suggesting some problem in entry of water through membranes. However, for cotyledons there was no effect of chilling incubation since such water uptake is not related to growth, rather it is imbibitional that depends on the presence of matric substances. Scarified seeds also showed similar behaviour except that here water uptake by axes during germination started earlier (after 1 h preincubation). Inhibition of seed germination by low temperature may thus be explained by impediment of water uptake by embryonic axes that may result either due to difficulty in passing through aqueous channels because of increased viscosity of water or as a consequence of chilling-induced slow down of metabolic reactions and/or inhibition of oxygen influx as mentioned earlier. Also, low temperature may affect early tissue-specific gene expression leading to differential water uptake responses in axes and cotyledons. Treatment with CHI also prevented water uptake by axes in a similar fashion without any effect on the cotyledons. Treatment with CHI was even effective in inhibiting water uptake by the axes when given during preincubation of intact- (6 h) or scarified- seeds (1 h). This indicates that some early protein(s) associated with the water uptake process of embryonic axes is (are) synthesized that prepares axis cells to absorb water and initiates turgor-driven cell elongation eventually leading to the completion of germination (radicle protrusion).

Finally, it can be concluded that the water uptake by the embryonic axes in the lag phase (after the completion of imbibitional uptake of water), which leads into radicle elongation, is a point of regulation during germination involving early synthesis of proteins (may be expansins and/or cell wall hydrolases) possibly related to the water uptake mechanism, which is also thermo-sensitive. Radicle emergence depends on cell elongation and such elongation is most likely brought about by cell wall relaxation. Plasma membrane localized H^+ -ATPase has been ascribed for acid-induced cell wall

loosening and subsequent water uptake by germinating seeds (Antipova et al., 2003; Obroucheva and Antipova, 2004). Besides, expression of aquaporins has also been correlated with water uptake by seeds during germination (Gao et al., 1999; Schuurmans et al., 2003). Thus, it may be speculated that certain proteins responsible directly or indirectly for cell wall relaxation and water uptake are synthesized *de novo* and initiates cell elongation in axes of *Vigna radiata* seeds during germination. Our future experiments on the effects of different germination influencing agents like phytohormones and their inhibitors on water uptake kinetics and cell wall/membrane properties in the embryonic axes will lead to a better understanding of the regulation of seed germination. Such understanding may have far-reaching implications on manipulating the process of germination for quicker field establishment or preservation of seeds.

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