

Seed development in *Phaseolus vulgaris* L. cv Seminole. 4. Embryo axis growth in late maturation seeds is constrained by lack of available water

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Abstract

The water status of embryo structures at the late maturation phase of seed development was studied by nuclear magnetic resonance (NMR) imaging of water molecules and by measurement of relative water content (RWC) and water potential (Ψ). NMR results and RWC data show the axis tissues have a lower water content than the cotyledons. Gradients of water concentration were detected in cotyledonary tissues by NMR imaging. Psychrometric measurement of water potential shows axes are maintained at a higher (less negative) Ψ relative to the cotyledons. Gradients in Ψ values were also found within cotyledon tissues. The vasculature of the cotyledonary node is differentiated but not lignified. The importance of the water status of pod tissues may also contribute to embryo and seed balances in late development. The results support the hypothesis that a balance of water relations within the embryo prevents precocious germination *in vivo* in late development.

Additional key words: axis, bean, cotyledon, ethylene, nuclear magnetic resonance imaging, precocious germination, water imaging, water redistribution

Introduction

Currently an extensive literature holds that abscisic acid (ABA), a plant hormone which accumulates in seed tissues during seed development, is the primary control of embryo axis growth and processes leading to tolerance toward desiccation (Chandler and Robertson, 1994; Kermode, 1997). Thus the embryo axis is inhibited from continuing growth for much of the later phase of seed development while reserve structures continue to grow. In late maturation, seeds are physiologically mature, of maximum size and are moist. A seed desiccation phase follows for orthodox seeds which results in dry seeds.

The late maturation phase of seed development in *Phaseolus vulgaris* cv. Seminole is characterised by reduced control over axis inhibition such that seeds and embryos are easily manipulated to precociously germinate if supplied with water. Further, in the absence of free water, seeds (but not embryos isolated from them) will germinate. Treatment with ethylene however induces embryo germination (Fountain and Outred,

1990). We have shown recently by NMR proton imaging of water, that changes in internal reserves of water in cotyledon and axis tissues precede this ethylene-induced germination in embryos by many hours (Fountain *et al.*, 1998).

We are working on the hypothesis that the normal control which inhibits axis growth at this stage is one of a water status balance which constrains available cotyledonary water from entry to the axis. Physiological evidence for this possibility rather than the commonly-accepted endogenous abscisic acid induced suppression of axis activity has been presented previously (Fountain and Outred, 1990), and includes the observation of immediate elongation growth of radicles excised from isolated embryos when supplied with water. In this water-status model of axis inhibition, induction and maintenance of the quiescent state results from developmental processes such as reserve deposition in embryo cotyledons, which contribute to the overall water relations of the embryo, restricting access of mobile water to the axis growth locus. A full understanding of

water status of embryo tissues is critical to developing a model for this developmental stage. Here we describe the system in terms of NMR proton imaging of water molecule concentration and mobility; psychrometric water potential (Ψ) data, and relative water content (RWC) measurement of embryo parts in late maturation stage seeds. Results from these three approaches agree in showing the axis is restricted in accessing water from the cotyledons.

Material and Methods

Late maturation phase seeds (35-45 d after anthesis (DAA)) of *Phaseolus vulgaris* L. cv. Seminole maturing on glasshouse grown plants were used. Pods were harvested and were kept briefly in plastic bags to minimise water losses *en route* to the NMR imaging laboratory. Seeds were removed and inserted into a custom-milled teflon cylinder containing a niche which supported the seed. The cylinder was inserted into a radio frequency coil which was positioned in the magnet. In all, three experiments were conducted and the reported distribution of proton relaxation signals was observed in each case. No germination was observed, although embryos were later shown to be capable of germination when the seeds were supplied with water.

NMR microscopy experiments were carried out using a Bruker AMX300 spectrometer. All images were obtained using a spin echo (Callaghan, 1991) pulse sequence with an echo time, TE, of 15 milliseconds (ms). The NMR signal arises from hydrogen nuclei (protons), in the sample. These nuclei may occur as isolated H⁺ ions or in the hydrogen atoms of any hydrogen-bearing molecule, such as water, lipids or carbohydrates. However, because of the finite echo time, the images arise from molecules whose proton relaxation times are of the order of, or longer than, TE (echo time). Because of the extreme sensitivity of proton relaxation to molecular mobility, the signals detected here are due to molecules in a highly mobile liquid state, i.e., rapidly tumbling water molecules. The images can be regarded as a map of the free water: that is, water which is not bound to the surface of proteins or carbohydrates.

The acquisition time for each image was 34 min with 8 signal averaging scans, repetition time (TR) of 1 s and acquisition bandwidth, 40 kHz. Each image comprised 256x256 pixels in-plane with a field of view of 25 mm and slice thickness of 2000 microns. This slice was non-invasively selected using a tailored radio-frequency

pulse and the seed remained intact during the experiment. Image data were extrapolated to 0 ms time to yield water activity (concentration) maps and the T2 relaxation data yielded water mobility maps relative to a pure water standard imaged in an adjacent capillary tube. Water mobility refers to rotational mobility of water molecules and 'mobile water' is water undergoing rapid rotational re-orientation. The larger the T2 (in ms), the more mobile the water. Full description of the NMR microscopy method can be found in Callaghan (1991) and a non-technical description is given in Callaghan (1992). The colour scales shown in Figures 1 and 2 indicate differing grades of water signal. In Figures 1a and 2a, the colour scale refers to the intensity of the proton NMR signal which suffers attenuation due to relaxation at the echo time of 15 ms. This primary image is less useful for interpretation of water status. In Figures 1b and 2b, the colour scale refers to the intensity of the proton NMR signal extrapolated to echo time of zero so that relaxation effects are removed. The image is then a map of the total free water and indicates water concentration differences ranging from lower water concentration (black > purple > blue) to higher water concentration (white > red > yellow > green). In Figures 1c and 2c, the colour scale shown refers to the proton relaxation time where the maximum is 60 ms and the minimum is 0 ms. The result is a map of water mobility ranging from relatively bound or immobile water (black > purple > blue) to free mobile and unconstrained water (white > red > yellow > green).

For anatomical study, embryos were embedded in paraffin wax following fixation in formalin-acetic acid-alcohol 50:30:20 v/v and dehydration through an ethanol: water series. Sections of 8 μ m were taken from the cotyledonary node region of the axis and cotyledons, and from the axis and stained with safranin and fast green.

Water potential measurements were made on tissue pieces approximately 3 mm³ taken quickly from cotyledons or on whole axes, from embryos isolated from late maturation-stage seeds. For cotyledons, eight transverse sections were cut from the long axis of the cotyledon and each was further cut into four pieces. The sections were carefully stored in humid conditions whilst awaiting determination of water potential (Ψ). This was estimated by the dew point method using a Wescor C-52 psychrometric dew point microvoltmeter after between 10 to 30 min equilibration in the sample chamber, using a temperature corrected calibration curve relating microvoltmeter output to Ψ of NaCl solution standards.

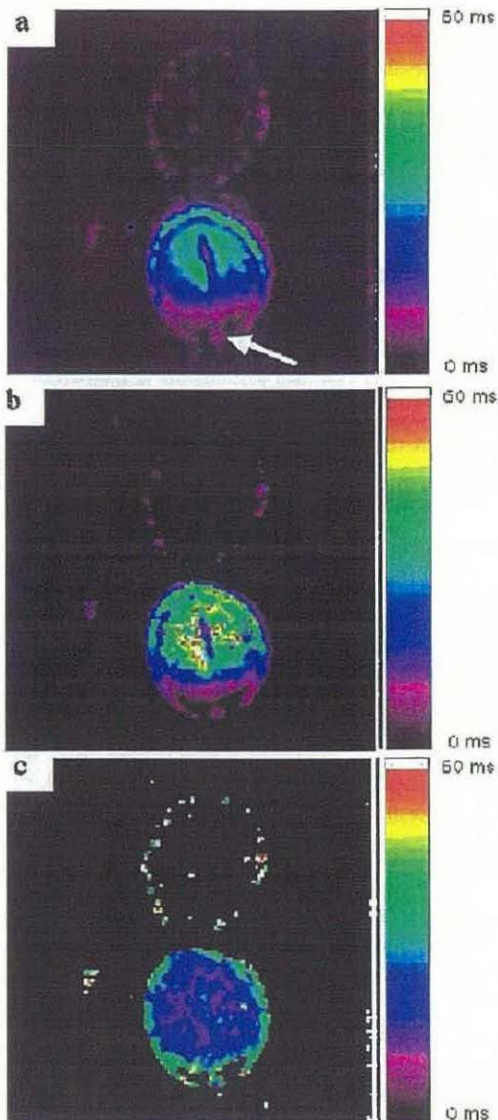


Figure 1. NMR imaging of water in a transverse section of a late maturation stage of *Phaseolus vulgaris* seed: a) Primary image; b) Water concentration map; c) Water mobility map. For interpretation of the colour scale see text.

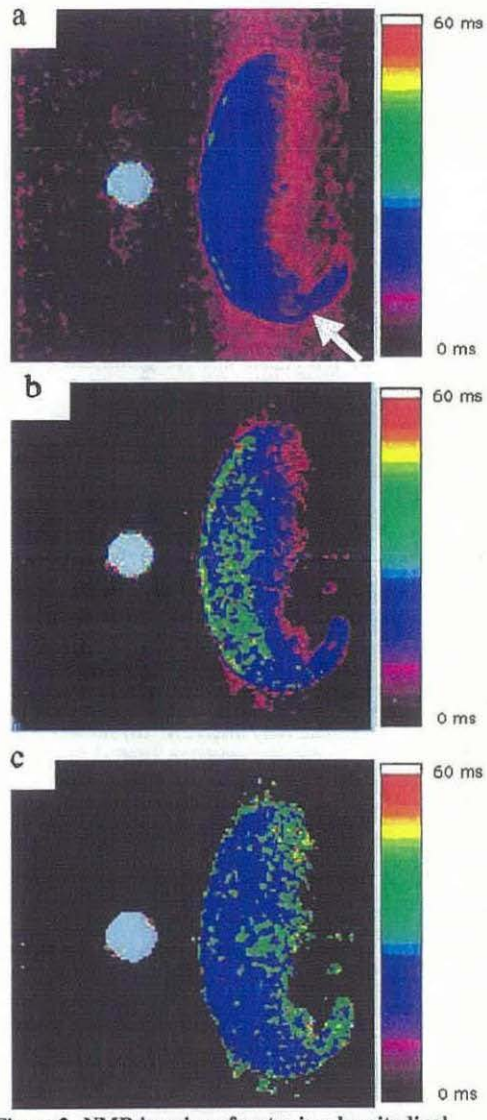


Figure 2. NMR imaging of water in a longitudinal section of a late maturation stage of *Phaseolus vulgaris* seed. Other detail as in Figure 1. A pure water standard (maximum water concentration; maximum water mobility) is shown at the right of both diagrams

Relative Water Content (RWC) was measured as $(FW - DW)/(FW2 - DW) \times 100$ where FW is fresh weight, DW is dry weight and FW2 is the fresh weight following overnight incubation of the cotyledons or axes in distilled water at 4°C.

Results and Discussion

Late maturation phase seeds are a distinct developmental stage 35-45 days after anthesis (in the glasshouse grown conditions used here)(Fountain *et al.*, 1989), where the normal inhibition of axis growth is sufficiently relaxed to allow precocious embryo germination to be induced by intervention (Fountain and Outred, 1990). At this stage, the pod is achlorophyllous and moist. A moisture-filled testa, purple and white in colour, encloses a moist fully-filled embryo. Water relations in embryo tissues of seeds at this stage were examined by three complementary methods.

NMR imaging

The primary NMR image of water molecules within a 1 mm transverse slice of a late maturation bean seed is shown in Figure 1a. The two cotyledons, the enclosed space between them, the testa and hilar region are distinguishable. The axis (arrowed), 'sectioned' through the hypocotyl or upper radicle region is also clearly evident. The water activity map (Fig. 1b) shows the axis to have a lower water concentration relative to the bulk of cotyledonary tissue. There is evidence of a gradient in water activity within the cotyledons, the tissue adjacent to the hilar region and hence nearest the radicle, exhibiting a lower water activity. The water mobility map (Fig. 1c), shows water molecules within the axis tissues to have a greater mobility than those in the bulk of the cotyledonary tissues. The testa is seen to show a relatively low water activity but high mobility status.

Image data from within a 1 mm longitudinal slice of a seed are shown in Figure 2. The calibration signal from pure water is seen to the left. The slice includes cotyledonary and axis tissue. The axis (arrowed) is 'sectioned' very close to, or through the cotyledonary node. Figure 2b shows a water activity map of the tissues and shows the axis contains water molecules at a lower concentration than the bulk of cotyledonary water. The water molecules in the radicle and hypocotyl however are shown to have a higher mobility than those in the cotyledons (Fig. 2c). There is evidence of a gradient in both water activity and mobility within

cotyledonary tissue. The 'section' orientation includes the space between the cotyledons to the right hand side of the image and this may account for much of this although the gradient pattern is consistent with that seen in transverse section: a lower water activity and higher mobility being shown in tissue near the axis.

We conclude that generally, water is contained within cotyledonary tissues at higher concentrations than in axis tissue. The water molecules however have lower mobility and are presumed to be constrained within cells and apoplast structures by association with storage reserves and other matric materials. This suggests that in normal development, the water content of the cotyledons is not available to the axis, and germination is suppressed. In the ethylene-promoted germination described previously (Fountain and Outred, 1990), it is hypothesised that ethylene acts on this compartmentation to alter it sufficiently to allow water flow to the axis. Evidence of such a 'flow' was shown recently (Fountain *et al.*, 1998).

Water potential

A representative set of water potential values for one longitudinal set of sections taken from the mid-region of the cotyledon is shown in Figure 3. The location of each value corresponds to the positions of individual tissue pieces excised for analysis. Values obtained in cotyledons were generally lower (more negative) than axis values. Cotyledon tissue adjacent to the axis-cotyledonary node region exhibited lowest values, with distal tissues having higher values. The arrows drawn show the hypothetical transport pathways dictated by the values shown. The greatest $\Delta\Psi$ in adjacent tissues was across the cotyledonary node ($\Delta\Psi = 0.78$ MPa) where a driving force for water exists from axis to cotyledon.

We conclude from these data that the axis is maintained at a relatively high compared to adjacent cotyledonary tissue. The equilibrium lies in favour of water withdrawal from the axis. Spontaneous precocious germination would thus not be expected to be observed.

We speculate that ethylene disrupts this equilibrium and allows water to flow to the axis from the cotyledons. The process is likely to involve ethylene-induced changes in the compartmentation of water molecules in cotyledons by a mechanism unknown at present but perhaps similar to internal redistributions of water described in other 'closed' systems (Matyssek *et al.*, 1991a; Matyssek *et al.*, 1991b).

Water potential gradients have been described by Yeung and Brown (1982) within the developing embryo

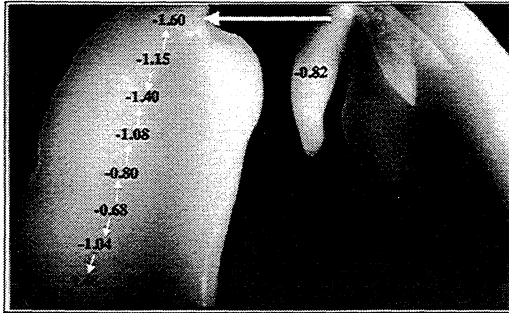


Figure 3. Water potential (ψ) values for a representative set of serial sections of tissue from axis and cotyledon of late maturation stage *Phaseolus vulgaris* seeds. The locations of the values correspond to tissue pieces excised from approximately the positions shown. The arrows drawn show hypothetical water transport pathways dictated by the values shown. The embryo has been pulled apart to reveal the junction of the axis with the left hand (detached) cotyledon. The cotyledonary node of the right hand cotyledon is intact and behind the axis.

of *Phaseolus vulgaris* (cv. Taylors Horticultural). At early stages, lower values for the axis, relative to the cotyledons were found. However at the stage closest to the maturation stage studied here, closely similar values for axis and cotyledon tissue were found. They concluded that throughout development, the axis was a sink for water, supporting axis elongation. The fact of axis growth termination in late maturation phase is not

consistent with a continued water sink situation. Our data, showing a reversed gradient situation in late maturation stage embryos, are consistent with the notion of cotyledon tissues acting to hold available water away from the growth locus of the axis. The perturbation induced by ethylene which results in precocious germination may thus be a small one in this system, acting to reverse the gradient in water potential. This however remains to be tested.

Relative water content

Table 1 shows RWC measured in cotyledons and axes at three stages of late development. Mean values for testa and pods are also shown for comparison. In each case, the late maturation phase situation is compared to subsequent mid-desiccation and dry states. At late maturation, the RWC of axes is lower than subtending cotyledons. The cotyledonary tissues have a relative water content of 92.1 % at this stage of development (later falling to 17.6 % after *in vivo* seed desiccation), indicating the cotyledons are still very much moist structures. The axis is lower at 84.6 %. Thus a gradient in RWC exists which would support water flow to the axis. For this to occur, a supply of unconstrained (high mobility) water and a transport pathway is necessary. These conditions are not met during the normal course of seed development when germination at this stage is suppressed, but are hypothesised to be met following ethylene exposure which allows precocious germination. Pod tissues appressed to the seeds are still lower. The pod may act as a further regulator of seed water relations as a large RWC sink for available water and further prevent free water gaining access to the axis.

Anatomy of cotyledonary node

The vascular trace passing through the cotyledonary node region was investigated in thin sections (result not

Table 1. Relative water content of pods and parts of developing seeds of *Phaseolus vulgaris*.

	Late maturation (35-45 DAA) ¹	Desiccating (46-50 DAA)	Dry (51-60 DAA)
cotyledons	92.12 ± 0.23 ²	92.30 ± 0.52(n=4)	17.56 ± 0.68(n=3)
axes	84.60 ± 0.92	79.19 ± 3.74(n=4)	12.52 ± 4.03(n=3)
pod	76.92 ± 2.40	76.89 ± 0.44	5.34 ± 0.22(n=3)

¹DAA = days after anthesis.

²Unless otherwise indicated, n = 5; values are ± standard error of mean.

shown). The cells of the trace were elongate and relatively undifferentiated. No safranin positive staining was evident indicating absence of lignification. The vascular trace however is assumed to be capable of water transport from cotyledons to axis following ethylene treatment.

Conclusions

The axis of late maturation seeds is maintained in a water relations status which prevents water gaining access to the growth locus of the radicle. The cotyledonary water status is seen to play a controlling role. NMR imaging of water status shows that water, although at a higher concentration, is of lower mobility, probably held as hydration shell water associated with the storage role of the cotyledons. Water potential measurements show the prevailing gradient to favour water withdrawal from the axis. Relative water content estimation shows the potential for the axis to attract water is present. The physiological status of the axis is thus of a potentially water-attracting sink but one that is unable to access the water available in the cotyledons. Cytological study showed the axis-cotyledon vascular trace is present but in the relatively undifferentiated state observed earlier in this species by Opik (1965). It is clear that cotyledonary water is sufficient to support precocious germination but is not available unless ethylene is supplied. The water in the cells of the cotyledons would be expected to be proportioned between a bulk phase fraction and a relatively immobile phase associated with the storage organelles and their contents. It is possible ethylene perturbs this balance to provide sufficient bulk water to be drawn to the axis.

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