

## 豌豆种子吸胀过程中脱水耐性变化的时间模式

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**摘要:** 研究了豌豆种子吸胀过程中脱水耐性的变化模式。种子在吸胀初期迅速吸收水分, 然后缓慢吸收直到平台期。电解质渗漏速率在吸胀初期增加直到 11 h, 然后随着吸胀下降。在吸胀过程中, 种子的萌发率逐渐增加, 种子和胚轴的脱水耐性逐渐丧失, 10% 和 50% 的种子和胚轴被脱水致死的含水量明显增加。赤霉素和脱落酸处理改变豌豆种子的萌发特性, 提高胚轴的脱水耐性。研究结果表明, 吸胀的豌豆种子脱水耐性的丧失是一种数量性状, 正常性种子吸胀后脱水耐性的变化能够作为种子顽拗性研究的模式系统。

**关键词:** 豌豆; 脱落酸; 脱水; 脱水耐性; 吸胀作用; 萌发; 正常性; 顽拗性

中图分类号: Q 945

文献标识码: A

文章编号: 0253-2700 (2009) 03-239-08

## Temporal Pattern of Changes in Desiccation Tolerance during Imbibition of *Pisum sativum* Seeds

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**Abstract:** Changes in desiccation tolerance of pea (*Pisum sativum*) seeds during imbibition were studied in this paper. Water uptake by seeds was initially rapid, and then slowly increased until a plateau was reached. Rate of electrolyte leakage increased initially until 11 h, and then declined with imbibition. During imbibition, germination percentage of seeds gradually increased, and water content at which 10% and 50% of seeds and axes were killed by subsequent dehydration significantly increased while desiccation tolerance of seeds and axes was progressively lost. Gibberellin and abscisic acid altered seed germination characteristics and improved desiccation tolerance of axes. These results showed that the loss of desiccation tolerance of imbibed pea seeds was a quantitative feature, and the changes in desiccation tolerance of imbibing orthodox seeds could serve as a model system for the study of seed recalcitrance.

**Key words:** Abscisic acid; Dehydration; Desiccation tolerance; Imbibition; Germination; Orthodox; *Pisum sativum*; Recalcitrant

Orthodox seeds acquire desiccation tolerance during development, and those of most species undergo substantial drying as the final developmental phase. All orthodox seeds may be further dehydrated after they have been shed and will survive in this state for a considerable time. Recalcitrant seeds, however, are shed at high water contents and are intolerant of dehydration (Berjak, 2006; Berjak and Pammenter, 2004). There

is considerable variability in the post-harvest physiology of recalcitrant seeds, within as well as among species. Within a species, variation may occur from harvest to harvest, or within a single harvest (Berjak *et al.*, 1996; Finch-Savage and Blake, 1994). Water content and extent of embryo development at shedding may vary among seasons (e.g. for *Quercus robur*; Finch-Savage, 1996; *Camellia sinensis*; Berjak *et al.*, 1996).

There are also marked differences in the rate of water loss from recalcitrant material, which varies both from species to species, and with the stage of seed development (Berjak and Pammenter, 1997). A further characteristic of recalcitrant seeds (and one that probably varies among species) is that they are actively metabolic at shedding, be this a manifestation of ongoing development, or of the onset of germination (Berjak, 2006; Berjak and Pammenter, 2004). These considerable differences among recalcitrant seeds make comparative studies extremely difficult.

It has been suggested that a suit of mechanisms and processes, under complex genetic control which is still not fully understood, has been implicated in the acquisition and maintenance of desiccation tolerance in orthodox seeds. These mechanisms and processes include intracellular physical characteristics, intracellular de-differentiation and metabolic 'switch off', reactive oxygen species and antioxidants and protectants (late embryogenic accumulating abundant proteins, carbohydrates) (Pammenter and Berjak, 1999; Berjak and Pammenter, 2008). In fact, the mechanisms on desiccation tolerance of seeds are unknown. Berjak and Pammenter (2008) recently suggested that whatever the post-harvest responses of seeds of individual species may be, they are the outcome of the properties of pre-shedding development, and a full understanding of the subtleties of various degrees of non-orthodox behavior must await the identification of, and interaction among, all the factors conferring extreme orthodoxy.

Imbibition of water by orthodox seeds initiates a series of metabolic steps that lead to germination, and during this sequence of events, desiccation tolerance within the seed is lost. During the early stages of germination and prior to radicle emergence in most species, the seeds can be dried to its original moisture content without causing injury. The same degree of drying, however, imposed at progressively later stages of germination, dramatically reduces seed vigour and, if imposed after radicle elongation has commenced, usually results in seedling death (McKersie *et al.*, 1988). Senaratna and McKersie (1983) studied germinating soybean (*Glycine max* L. Merr cv. Maple Arrow) seeds and found that seeds germinated for 6 h were tolerant of severe drying, while those germinated for 36 h were not.

Koster and Leopold (1988) found that germinating soybean (*G. max* L. Merr. cv. Williams) seeds lost desiccation tolerance between 12 and 18 h of imbibition, while germinating pea (*Pisum sativum* L. cv. Alaska) seeds lost tolerance between 18 and 24 h and maize (*Zea mays* L. cv. Merit) seeds by 48 h imbibition. Koster and Leopold (1988) correlated the loss of desiccation tolerance with changes in embryo sugar composition. Leprince *et al.* (1995) used electrolyte leakage to determine 'critical moisture contents' for germinating bean (*Phaseolus vulgaris* L. cv. Pole Kentucky Wonder) and maize (*Z. mays* L. cv. Kelvedon Glory) seeds. Below these water contents, these seeds were damaged by desiccation, and the injury was believed to be caused by oxygen radicals that resulted from respiratory metabolism. All of the studies described above used whole seeds, but the moisture content of embryonic axes is usually higher than that of the whole seeds during imbibition. In some studies germinating seeds were dried back to a single water content for the determination of desiccation tolerance. In other studies, only electrolyte leakage was used to assess desiccation tolerance of seeds, without survival data. The criterion for determining survival also differed among the studies.

Pea seeds imbibed for different times can provide convenient and reliable experimental materials with differing desiccation tolerance for the study of desiccation-tolerance and -sensitivity. In the present study, we attempted to better define the time course of the loss of desiccation tolerance in pea seeds and to determine how this quantitative trait changes during imbibition. To this end, we looked separately at survival of seeds and axes, at the changes of electrolyte leakage, and at the growth rate of seedlings produced by surviving seeds or axes. The effects of abscisic acid (ABA) and gibberellin (GA) on the desiccation tolerance of axes were also investigated. The results will provide a framework for the studies of loss of desiccation tolerance in germinating orthodox seeds and for the lack of this characteristic in recalcitrant seeds.

## Materials and Methods

### Plant material

Current harvest of pea (*Pisum sativum* cv. Greenfeast) seeds were obtained from McDonalds Seed Company (Pietermaritzburg, South Africa) and were kept at 16 °C until used. The

seeds were surface-sterilized in a solution of 1% hypochlorite, and rinsed three times in sterile water, and then imbibed by placing the seeds in a shallow layer of distilled water or treatment solution (GA and ABA) such that half the seed was immersed. Axes were excised from the seeds that had been imbibed for different times and were then treated as indicated below. All manipulations were conducted at room temperature (22 - 25 °C).

#### Water content determinations

Water content of 20 individual seeds or axes was determined gravimetrically (80 °C for 48 h). Water contents are expressed on a dry mass basis [ $\text{g H}_2\text{O} (\text{g dry weight})^{-1}$ ,  $\text{g g}^{-1}$ ].

#### Desiccation treatments

Dehydration of the differentially pre-imbibed seeds was achieved by burying them in activated silica gel within closed plastic buckets for different periods of time. Excised axes were dehydrated by placing them in a small boat on activated silica gel in a closed jar for different periods of time.

#### Assessments of germination and survival

Batches of 40 treated seeds or axes were germinated on moist filter paper in Petri dishes at 25 °C for 5 days. Seeds were placed in the dark and the axes in alternating light and dark with 16 h photoperiod (light intensity,  $66.25 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Seeds showing radicle emergence for 2 mm were scored as germinated, and axes showing a marked increase in length and volume were scored as survived. Although dried seeds and axes take up water during the early stages of re-imbibition, those seeds and axes that were injured by dehydration progressively deteriorated during continued re-imbibition.

#### Conductivity tests

Electrolyte leakage from 7 - 20 replicate seed or axis was measured individually for 12 h in 2.5 ml distilled water using a CM100 multi-cell conductivity meter (Reid and Associates, Durban, South Africa). The conductivity of the leachates was measured immediately after the drying treatments and leakage rate was expressed as  $\mu\text{S cm}^{-1} (\text{g dry weight})^{-1} \text{h}^{-1}$ .

#### Statistical analysis

All data were analysed using a one-way ANOVA model from the SPSS 11.0 package for Windows (SPSS Inc., 2006).

## Results

### Changes occurring during imbibition of seeds

Germination commences with the uptake of water by the dry seed and is completed when a part of the embryo, usually the radicle, extends to penetrate the structures that surround it. Water content of pea seeds increased from 0.13 g g in the dry seeds to 1.82 g g at 12 h of imbibition, and then slowly increased to 2.62 g g after 96 h of imbibition (Fig. 1a). Water content of

axes, however, exhibited a typical triphasic pattern of water uptake with a marked increase during the initial phase, and then a slow increase until 64 h of imbibition, followed by a second substantial increase (Fig. 1a). The water uptake by axis was greater than that by the whole seed; for example, when seeds had imbibed for 12 h, the water contents of seeds and axes were 1.82 and 2.24 g g, respectively, and for 96 h, 2.62 and 5.55 g g, respectively.

The germination percentage of seeds increased with imbibition time ( $P$  value 0.001); the first seeds germinated after about 40 h, and full germination was achieved by 96 h. The time taken for 50% seeds to germinate ( $T_{50}$ ) was about 74 h (Fig. 1b).

The rate of electrolyte leakage from seeds dramatically increased until 11 h of imbibition, from about  $5 \mu\text{S cm}^{-1} (\text{g DW})^{-1} \text{h}^{-1}$  at 1 h to  $94 \mu\text{S cm}^{-1} (\text{g DW})^{-1} \text{h}^{-1}$  at 11 h, and then obviously declined to  $29 \mu\text{S cm}^{-1} (\text{g DW})^{-1} \text{h}^{-1}$  at 49 h ( $P$  value 0.001, Fig. 1c). The leakage of seed, however, increased with imbibition up to 60 h ( $P$  value 0.001, Fig. 1d).

### Effect of pre-imbibition on desiccation tolerance of seeds and axes

Dehydration of partially imbibed seeds was achieved by burying them in silica gel for 24 h to a water content of approximately 0.1 g g; axes were dehydrated over silica gel for 12 h to approximately 0.05 g g. Seeds and axes of pea were tolerant on dehydration during the first 24 h of pre-imbibition (data not shown). But the tolerance was progressively lost with pre-imbibition times longer than 28 h ( $P$  value 0.001, Fig. 2a). The pre-imbibition times at which 10% and 50% of seeds were killed by subsequent dehydration were about 32 h and 55 h, respectively; and 10% and 50% of axes were killed about 36 h and 42 h, respectively (Fig. 2a). Pea seeds and axes lost completely desiccation tolerance after imbibition for 96 h and 60 h, respectively. The desiccation tolerance of the epicotyl was greater than that of the radicle (data not shown).

The fresh weight of seedlings integrates the desiccation tolerance of the seed or axis producing the seedling, and the subsequent metabolism associated with germination and growth. The fresh weight of seedlings produced by surviving seeds or axes that had been dried after pre-imbibition declined when the pre-imbibition periods of

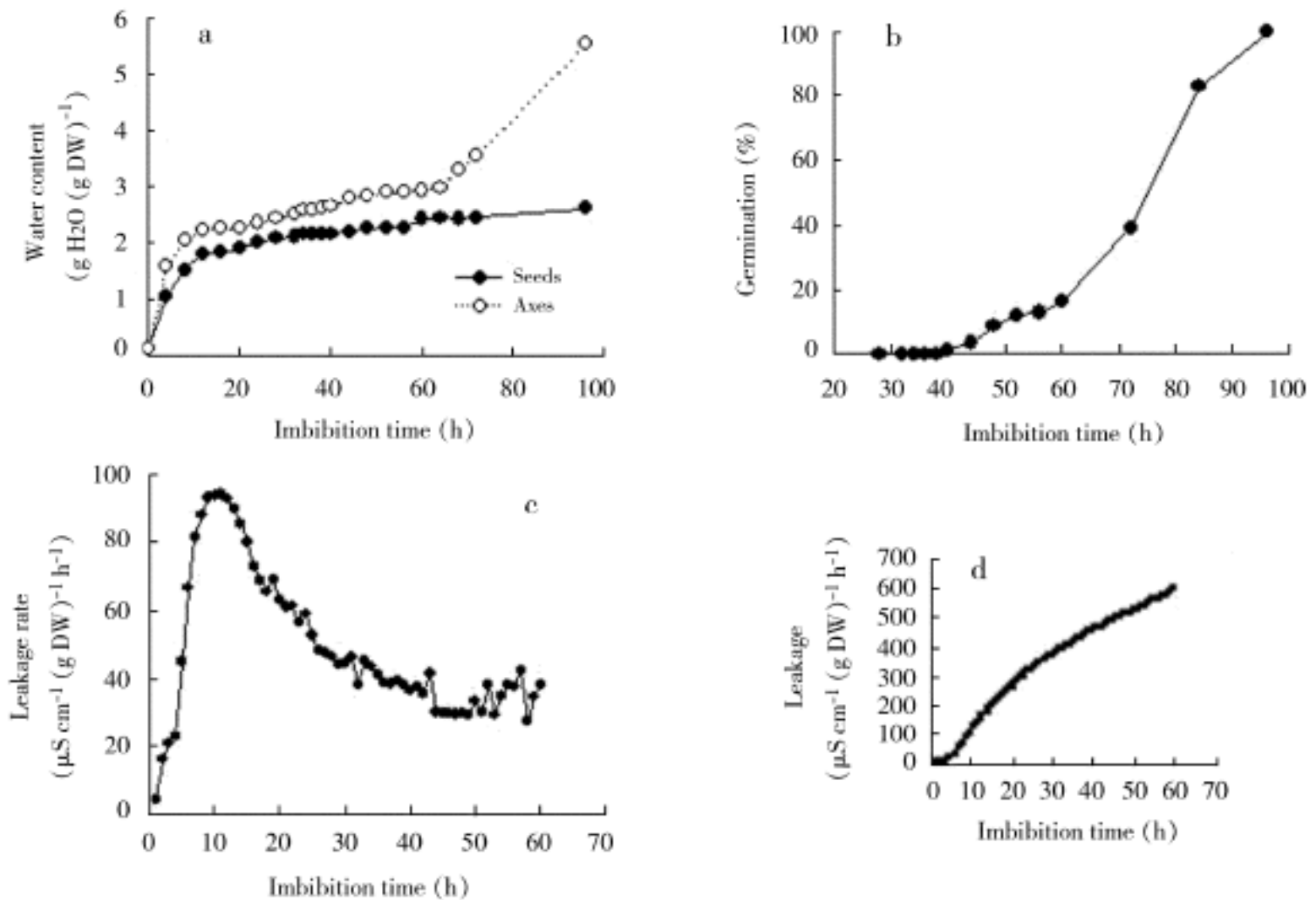


Fig. 1 Changes during imbibition of pea seeds. (a) Time courses of water content in seeds and axes. Values are means  $\pm$  SD of three replicates of 20 seeds or 20 axes each and expressed on a dry mass basis. (b) Changes in germination. Seeds showing radicle emergence for 2 mm were counted as germinated. All values are means  $\pm$  SD of three replicates of 40 seeds each. (c) Changes in rates of electrolyte leakage. (d) Changes in leakage. Individual seed was placed in 2.5 ml distilled water and conductivity of the leachate was measured. All values are means  $\pm$  SD of 20 replicates of individual seed each.

time increased ( $P$  value 0.001, Fig.2b). Following 36 h pre-imbibition and dehydration, fresh weight of seedlings produced by surviving seeds and axes decreased by 32 and 53%, respectively, relative to controls.

Rate of electrolyte leakage from pea seeds and axes after dehydration markedly increased with increasing pre-imbibition time ( $P$  value 0.001), but the leakage rate of axes was much higher than that of seeds (Fig.2c). Leakage rate of axes increased from an initial value of  $141 \mu\text{S cm}^{-1} (\text{g DW})^{-1} \text{h}^{-1}$  to  $316 \mu\text{S cm}^{-1} (\text{g DW})^{-1} \text{h}^{-1}$  at 48 h, and to  $785 \mu\text{S cm}^{-1} (\text{g DW})^{-1} \text{h}^{-1}$  at 96 h of imbibition (Fig.2c).

#### Relationship among pre-imbibition time, water content and desiccation tolerance of axes

To assess and quantify the loss of desiccation tolerance during imbibition, pea seeds were pre-imbibed for various times, the axes were excised and then dried for increasing periods of time. Water contents of axes from seeds imbibed for 40, 56 and 64 h were 3.04, 3.24 and 3.77 g g, respectively, and declined rapidly with dehydration over silica gel, all have been de-

creased in water content to 0.05 g g after 12 h dehydration ( $P$  value 0.001, Fig.3a).

For axes from seeds imbibed for 40, 56 and 64 h, respectively, their survival and seedling fresh weight produced decreased obviously, and their leakage rate increased notably, with dehydration ( $P$  value 0.001, Figs.3b-d). With increasing pre-imbibition time, dehydration damage of axes, as measured by survival, seedling fresh weight produced and leakage rate, did become apparent; and the water contents at which 10% of axes ( $W_{10}$ ) or 50% of axes ( $W_{50}$ ) were killed by dehydration increased (Fig.3e).

#### Effects of GA and ABA on germination of seeds and desiccation tolerance of axes

Germinating pea seeds in 1 and 10  $\mu\text{mol L}$  GA marginally enhanced germination from an initially high value, but a GA concentration of 100  $\mu\text{mol L}$  partially inhibited, and a concentration of 1000  $\mu\text{M}$  completely inhibited germination (Fig.4). Treatment of seeds with 1 - 100  $\mu\text{mol L}$  ABA increasingly inhibited, and a concentration of 1000  $\mu\text{mol L}$  ABA totally inhibited germination (Fig.4).

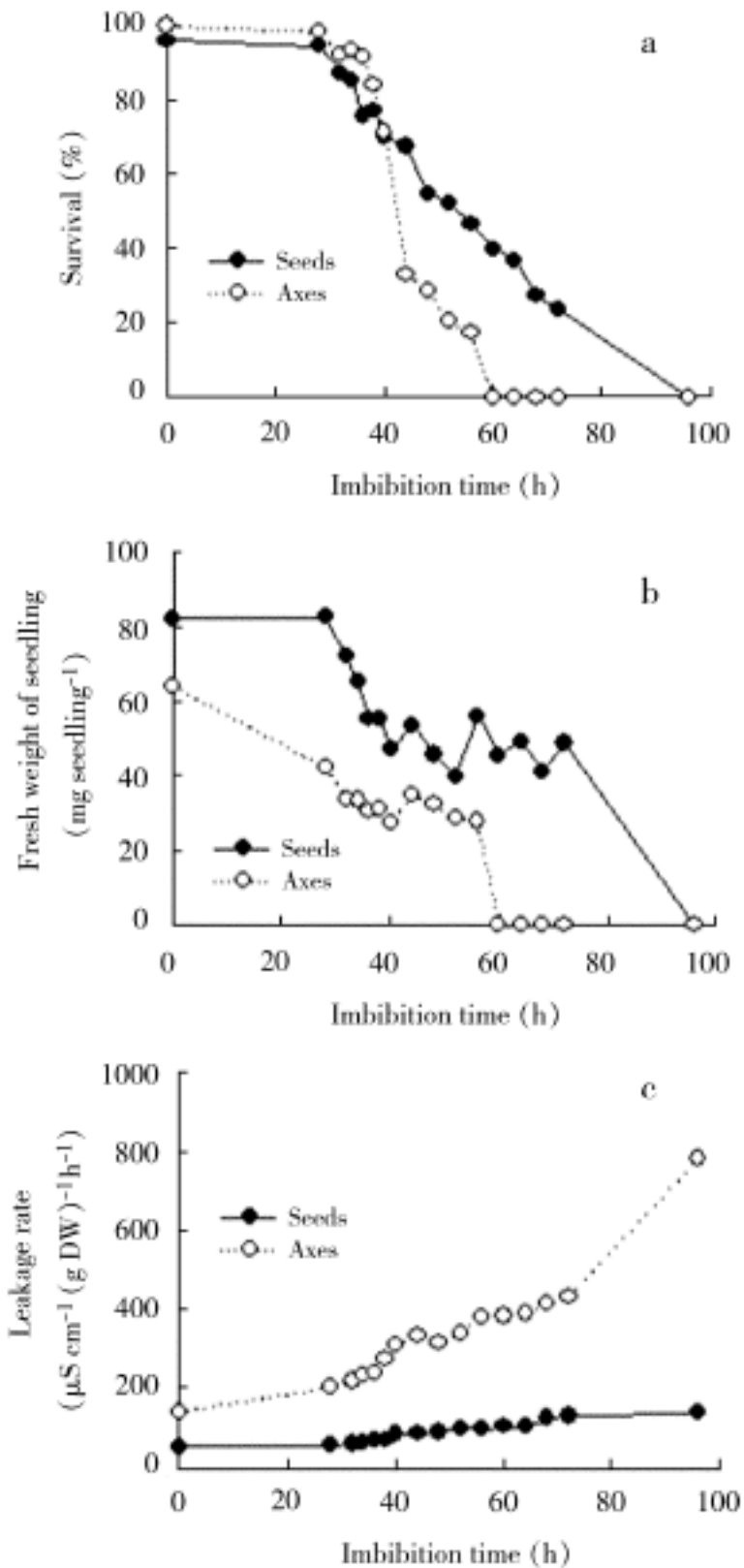


Fig. 2 Changes in desiccation tolerance of seeds and axes during imbibition. After imbibition for various periods of time, seeds and excised axes were dehydrated (seeds were buried in silica gel for 24 h, water content was approximately 0.1 g g; axes were placed above silica gel for 12 h, water content was approximately 0.05 g g) and then re-imbibed on moist filter paper for 5 days. (a) Survival; seeds showing radicle emergence for 2 mm were counted as germinated, and axes showing increase in length and volume were counted as survived. All values are means  $\pm$  SD of three replicates of 40 seeds or 40 axes each. (b) Fresh weight of seedlings produced by surviving seeds or axes. Seedling fresh weight does not include cotyledons. (c) Rates of electrolyte leakage. Individual seed or axis was placed in 2.5 ml distilled water and conductivity of leachate was measured. All values are means  $\pm$  SD of seven replicates of individual seed or axis each.

Axes from seeds treated in 10 and 100  $\mu\text{mol L}$  GA for 44 h showed improved desiccation tolerance, in that survival and seedling fresh weight increased, relative to

water-treated controls, but the rate of electrolyte leakage also increased (Table 1). ABA at 1 and 10  $\mu\text{mol L}$  also increased desiccation tolerance of axes, the axes showing a decreased rate of electrolyte leakage as well as increased survival and seedling fresh weight. For example, survival and seedling fresh weight of axes from seeds treated in 10  $\mu\text{mol L}$  ABA increased by 36% and 12%, respectively, and leakage rate decreased by 21%, relative to controls (Table 1).

Table 1 The effect of GA and ABA on desiccation tolerance of pea axes. Seeds were imbibed in GA or ABA solution for 44 h, the axes were excised and then desiccated to  $0.06 \pm 0.003 \text{ g H}_2\text{O (g DW)}^{-1}$ . For survival and seedling fresh weight, values are the means  $\pm$  SD of three replicates of 40 axes each. For electrolyte leakage, values are the means  $\pm$  SD of seven replicates in a single axis.

Treatment	Survival (%)	Leakage rate ( $\mu\text{S cm}^{-1} (\text{g DW})^{-1} \text{h}^{-1}$ )	Seedling fresh weight (mg seedling <sup>-1</sup> )
Water	19 $\pm$ 1	399 $\pm$ 28	41.6 $\pm$ 2.2
10 $\mu\text{M}$ GA	25 $\pm$ 2	464 $\pm$ 30	49.6 $\pm$ 2.9
100 $\mu\text{M}$ GA	36 $\pm$ 2	421 $\pm$ 28	48.2 $\pm$ 2.9
10 $\mu\text{M}$ ABA	55 $\pm$ 3	355 $\pm$ 26	46.6 $\pm$ 1.8
100 $\mu\text{M}$ ABA	77 $\pm$ 3	304 $\pm$ 12	50.6 $\pm$ 2.3

## Discussion

Seed germination incorporates those events that commence with the uptake of water by the quiescent seed and terminate with the elongation of the embryonic axis (Bewley, 1997). Water uptake by pea seeds occurred rapidly during the initial phase of imbibition, and then more slowly with a plateau phase (Fig. 1a). The initial rapid hydration phase (phase I) was related to the matric potential of dry seeds; the second slow hydration phase (phase II) corresponded to the period of germination. The length of phase II is affected by imbibition temperature and the water potential of the medium in which the seeds are imbibed (Bewley, 1997; Bradford, 1995). A pea axis is located near the surface of the seed and can imbibe water more rapidly than the whole seed. This, together with a different chemical composition, leads to a higher water content of the axis than of whole seed (Fig. 1a). By 64 h of imbibition over 20% of the seeds had germinated (Fig. 1b); that is, the cells of some axes had extended, taking up more water, and so water uptake by axes exhibited a typical triphasic pattern over 96 h of imbibition

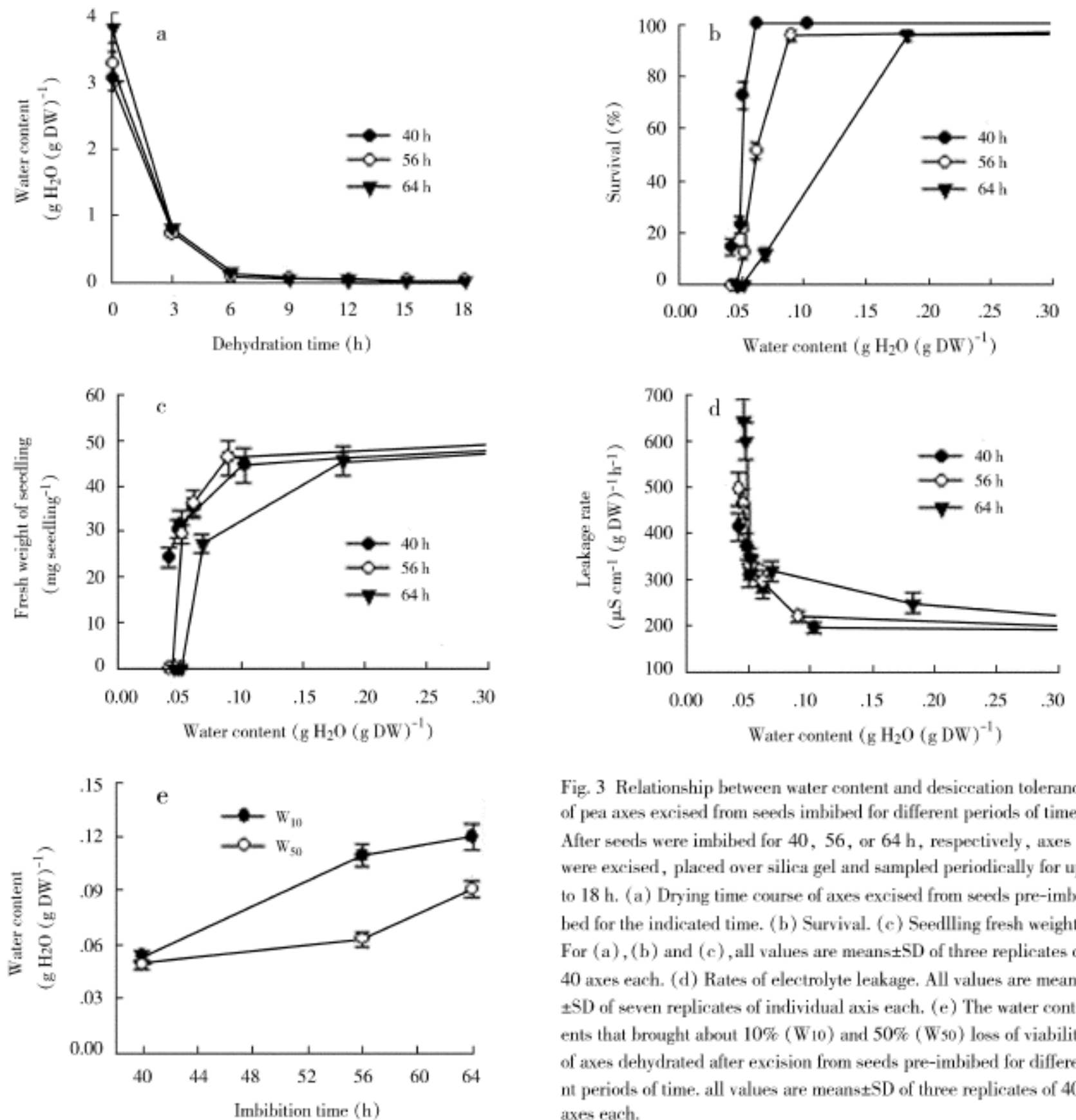


Fig. 3 Relationship between water content and desiccation tolerance of pea axes excised from seeds imbibed for different periods of time. After seeds were imbibed for 40, 56, or 64 h, respectively, axes were excised, placed over silica gel and sampled periodically for up to 18 h. (a) Drying time course of axes excised from seeds pre-imbibed for the indicated time. (b) Survival. (c) Seedling fresh weight. For (a), (b) and (c), all values are means  $\pm$  SD of three replicates of 40 axes each. (d) Rates of electrolyte leakage. All values are means  $\pm$  SD of seven replicates of individual axis each. (e) The water contents that brought about 10% (W<sub>10</sub>) and 50% (W<sub>50</sub>) loss of viability of axes dehydrated after excision from seeds pre-imbibed for different periods of time. all values are means  $\pm$  SD of three replicates of 40 axes each.

(Fig. 1a), as reviewed by Bewley (1997), Bewley and Black (1994), and Bradford (1995). The axes comprised only about 3% of the dry mass of the seeds, and so the pattern of water uptake by axes would not influence the pattern observed in whole seeds.

Rate of electrolyte leakage from pea seeds increased during the first 11 h of imbibition, and then slowly declined (Fig. 1c). The influx of water into the cells of dry seeds during phase I results in temporary structural perturbations, particularly to membranes, which lead to an immediate and rapid leakage of solute and low molecular weight metabolites into the sur-

rounding imbibition solution. This is a consequence of the transition of the membrane phospholipid components from the gel phase formed during maturation drying to the normal, hydrated liquid-crystalline state. Within a short time of rehydration, the membranes return to their more stable configuration, at which time solute leakage is curtailed (reviewed by Bewley, 1997).

Increasing periods of pre-imbibition of seeds prior to the dehydration of whole seeds and axes, lead to declining survival and fresh weight of seedlings (Fig. 2a, b) and progressively increasing rates of electrolyte leakage (Fig. 2c), showing that desiccation tolerance

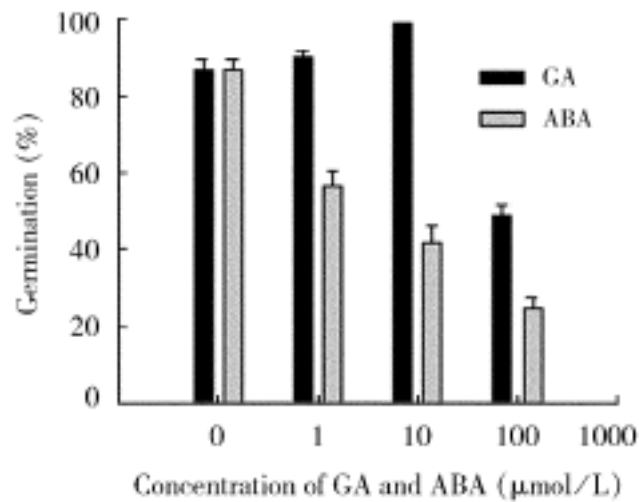


Fig. 4 Effects of different concentrations of GA and ABA on germination of pea seeds. Seeds were germinated at 25 °C for 5 days. All values are means  $\pm$  SD of three replicates of 40 seeds each.

in imbibing pea seeds was gradually lost. The data in Fig. 2 suggest that excised axes are more sensitive to dehydration than whole seeds. However, the drying treatments reduced the water content of the axes to levels lower than that of whole seeds (0.05 g/g and 0.1 g/g, respectively). The progressive loss of desiccation tolerance during imbibition of pea seeds is similar to findings for bean and maize by Leprince *et al.* (1995) and for mung bean (*Vigna radiata*) by Sun (1999). Senaratna and McKersie (1983) showed that soybean seeds imbibed for 36 h were able to tolerate drying to 20% but not to 10% moisture content (fresh weight basis), and Vertucci and Farrant (1995) have suggested that desiccation tolerance is a quantitative trait. Figs. 3b-d indicate that desiccation sensitivity of pea axes (on the basis of the influence of water content on survival, seedling fresh weight and rate of electrolyte leakage) gradually increased with increasing imbibition time of seeds. The water contents corresponding to 10% and 50% loss of viability more precisely express the changes in desiccation sensitivity of imbibed seeds (Fig. 3e). This indicates that the change in the desiccation tolerance of imbibed pea seeds is not an "all-or-nothing" response. These data are in agreement with the concept of Vertucci and Farrant (1995) concerning the quantitative nature of desiccation tolerance.

One of the earliest symptoms of injury following dehydration is the loss of function or structure of either the plasmalemma or organelle membranes (McKersie *et al.*, 1988). Consequently, sensitivity to desiccation of

both orthodox and recalcitrant seed tissues has been quantitatively expressed in terms of a 'critical water content' determined by a leakage assay (Berjak *et al.*, 1993; Vertucci *et al.*, 1993; Leprince *et al.*, 1995). However, each of these laboratories quantified electrolyte leakage differently, making direct comparisons difficult. The growth rate of seedlings, reflecting both desiccation tolerance and subsequent activities of germination and or growth, could also be used a parameter to assess desiccation tolerance. Additionally, Pammenter *et al.* (1998) have pointed out that the response to desiccation can depend on the rate of drying, and consequently have questioned the concept of a 'critical water content'. Therefore, it seems important to include survival, leakage rate and growth rate of seedling when discussing desiccation tolerance.

The marginal enhancement of germination of pea seeds by 1 - 10  $\mu\text{mol/L}$  of GA (Fig. 4) might be because GA promoted the *de novo* synthesis of  $\alpha$ -amylase mRNA and  $\alpha$ -amylase (Bewley and Black, 1994). The mechanism by which GA improved desiccation tolerance of imbibing pea axes (Table 1) may be indirectly via  $\alpha$ -amylase production, which would result in sucrose being translocated from the cotyledons to the axis. However, this aspect requires further study. Exogenous ABA treatment markedly inhibited germination of pea seeds (Fig. 4), and increased desiccation tolerance of axes (Table 1). Prevention of embryo radicle extension can be achieved by incubating mature seeds in a solution of ABA. This inhibition can occur even when ABA is introduced late during germination, an hour or so before radicle extension. ABA prevention of radicle extension is caused by inhibition cell wall loosening (reviewed by Bewley, 1997). The mechanism by which ABA increased desiccation tolerance of imbibing pea axes is not clear. It is known that ABA can change the desiccation tolerance of developing immature seeds through late embryogenic abundant (LEA) protein synthesis and prevention of precocious germination (Kermode and Finch-Savage, 2002).

Pea seeds progressively lost desiccation tolerance with imbibition; this desiccation tolerance is a quantitative feature which is mainly dependent on the germination activity of the seed or axis. Seeds germinated at

28 - 30 are more desiccation sensitive than at 22 - 23 (Sun, 1999). The expression of desiccation-induced damage is dependent on metabolism, which is a function of oxygen concentration and temperature (Leprince *et al.*, 1995). Many characteristics of germinating orthodox seeds are similar to those of recalcitrant seeds. These include desiccation sensitivity, high degree of subcellular development and metabolic activity, the degree of sensitivity being affected by developmental stage and drying environment, and desiccation injury possibly being associated with free radical-mediated oxidative damage (Song *et al.*, 2004; Berjak, 2006; Berjak and Pammenter, 2008). Germinating seeds of different desiccation sensitivity and developmental stage can be prepared using the same seed lot, and germination activity and desiccation sensitivity of seeds can be controlled by altering temperature, oxygen and water content during imbibition. Consequently, desiccation sensitive imbibing orthodox seeds can serve as a model system for the studies of seed recalcitrance.

**Acknowledgements:** We are grateful to the National Research Foundation of South Africa for providing postdoctoral fellowship to Songquan Song.

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